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Epidemiology of *Toxoplasma gondii* in the pig industry of Yucatan, Mexico.

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DECLARATION

The candidate declares that this thesis which is submitted to the University of Salford has been composed solely by her own, and that no portion of it has been submitted in support of an application for another degree or qualification of this or another university or institution. Except where states otherwise by appropriate credits, the work presented is entirely her own and effort has been made to indicate with references to the literature or acknowledgements of collaborative research.

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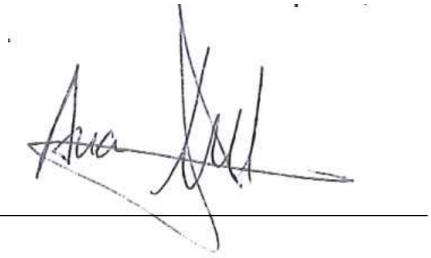

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ABBREVIATIONS

A. niger	*Aspergillus niger*
AF	Amniotic fluid
AG	*Toxoplasma* Agglutination Test
AIDS	Acquired immune deficiency syndrome
Alt-SAG2	Alternative SAG2
AUC	Area under the curve
BALB	Bagg's albinos
BLAST	Basic Local Alignment Search Tool
bp	base pair
BR	Binding ratios
BSA	Bovine serum albumin
BTUB	Beta-tubulin
C-PCR	Classic PCR
C. albicans	*Candida albicans*
CDC	Center for Disease Control and Prevention
CI	Confidence interval
CO_2	Carbon dioxide
COFEPRIS	Federal Commission for the Protection against Sanitary Risk (regulatory body of the *Mexican* government)
Cp. abortus,	*Chlamydophila abortus*
CSF	Cerebrospinal fluid
CV	Coefficient of variance
DALY	Disability Adjusted Life Year
d.p.i.	days post inoculation
df	degrees of freedom
dntp	deoxynucleotide triphosphates
DT	Dye Test

ECACC	European Collection of Authenticated Cell Cultures
EDTA	Ethylene diamine tetra-acetic acid
EFSA	European Food Safety Authority
EF1	Elongation Factor 1
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
FAO	Food and Agriculture Organization of the United Nations
FCS	Fetal calf serum
fg	femtogram
FIV	Feline immunodeficiency virus
g	gravity (force) known also as Relative Centrifugal Force (RCF)
G	Gauge
GRA6	Dense Granule Protein 6
HCI	Hydrochloric acid
HeLa	Henrietta Lacks' cell line
HIV	Human immunodeficiency virus
HP2	Hypothetical protein 2
HRP	Horse radish peroxidase
IFAT	Indirect Fluorescent Antibody Test
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHAT	Indirect Haemagglutination test
IL-10	Interleukin 10
IL-4	Interleukin 4
IQ	Intelligence Quotient
ITS	Internal transcribed spacer
IU	International Units

IUPAC	International Union of Pure and Applied Chemists
k	kappa agreement
kb	kilo base
KO	knock-out
LAMP	Loop-Mediated Isothermal Amplification
LAT	Latex Agglutination Test
LQAS	Lot Quality Assurance Sampling
M. musculus	*Mus musculus*
MAT	Modified Agglutination Test
MC-PCR	Magnetic Capture PCR
MDBK	Madin-Darby Bovine Kidney
MEGA	Molecular Evolutionary Genetics Analysis
MLEE	Multilocus enzyme electrophoresis
MLST	Multi-locus sequencing typing
MS	Microsatellite
N-PCR	Nested PCR
N. caninum	*Neospora caninum*
NaCl	Sodium Chloride
NAHMS	National Animal Heath Monitoring System
NEAA	Non-Essential Amino acids
NHANES	National Health and Nutrition Examination Survey
INEGI	National Institute of Statistics and Geography of Mexico
ng	nanogram
NHNS	National Health and Nutrition Survey
NHS	National Health Survey
NSS	National Seroepidemiological Survey
OCD	Obsessive–compulsive disorder
OD	Optical density
OIE	World Organization for Animal Health
OR	Odds Ratio

P. carinii,	*Pneumocystis carinii*
P. manuculatus	*Peromyscus maniculatus*
p.i.	post inoculation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	picogram
PK1	Pyruvate kinase 1
RBC	Red blood cells
RFLP	Restriction Fragment Length Polymorphism
ROC	Receiver Operating Characteristic
RT-PCR	Real time PCR
S. gigantea	*Sarcocystis gigantea*
SAG1-3	Surface antigen 1-3
SAGARPA	Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (Mexico's agriculture ministry)
SC	Sodium Chloride
SD	Standard deviation
SNP	Single nucleotide polymorphism
T. gondii	*Toxoplasma gondii*
TBE	Tris-borate-EDTA
UPRT	Uracil Phosphoribosyl Transferase
UADY	Autonomous University of Yucatan
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
WB	Western blot
WBC	White blood cells
WHO	World Health Organization
w.p.i.	weeks post infection
3' SAG2	3' end of SAG2 gene
5' SAG2	5' end of SAG2 gene

ABSTRACT

Toxoplasmosis is a worldwide distributed parasitic disease caused by the zoonotic pathogen *Toxoplasma gondii*. Serological studies have estimated that more than 30% of the human population has been exposed to this protozoan. *T. gondii* is considered a leading cause of death attributed to foodborne illness. The consumption of infected pork meat is suggested to be an important source for human infection. However, the prevalence of *T. gondii* in pigs vary greatly between countries, the reasons for this heterogeneity has been addressed to the differences in climate distribution, environments, husbandry systems and fam management. The geographical location of this study is Yucatan, a state located in the south-east of Mexico. Yucatan is considered an endemic area of toxoplasmosis; the last National Seroepidemiological Health Survey (NSHS) revealed more than 70% of prevalence among the human population. Numerous studies suggested that the consumption of pork in this geographical area may be a major source of *T. gondii.* The aims of this study were to investigate the disease levels in the pig industry of Yucatan, assess an in-home ELISA widely used in this area (ELISA kit Human-GmbH, WB), study the risk factors associated with the disease in theses pig farms and evaluate the contamination with *T. gondii* in pork meat intended for human consumption. To do that, swine blood samples were collected through a cross-sectional age stratified opportunistic sampling of 12 farms across the state during the year 2014. Farm management and characteristics were obtained by interviewing farmers. In addition, meat and blood samples were collected from a local abattoir from 2013 to 2015. Anti - *T. gondii* IgG antibody levels were investigated with the well validated MAT (Modified Agglutination Test), with an ELISA (Enzyme-Linked Immunosorbent Assay) for use on pig serum (ID Screen ®, IDVet) and with the gold standard Dye test. The overall seroprevalence was 1.4% (95%CI: 0.6%-2.7%) among 632 pigs sampled. This seroprevalence increased with age ($p < 0.05$), reaching the 11.5% (95% CI: 2.5%-30%) in pigs older than 20 weeks. The seroprevalence was even higher, 17.8% (95% CI: 6%-37%), in slaughtered animals (n=34). In addition, *T. gondii* prevalence was investigated using a highly sensitive nested PCR protocol targeting the SAG1 gene. PCR diagnosis revealed the high frequency of 21.2% (CI: 18%-24.6%) of *T. gondii* DNA circulating in the blood of these pigs (n=632). Furthermore, MLST (Multi-Locus Sequence Typing) of four alleles (SAG1, SAG2, SAG3 and GRA6) allowed, for first time in Yucatan, genetic diversity to be assessed.

Data revealed the presence of high genetic diversity among *T. gondii* strains of this geographical area with shared alleles to strains from both North and South/Central America origin. Moreover, a relatively high number of pigs presented multiple infections with different *T. gondii* strains suggesting high levels of *T. gondii* transmission on the intensive pig farms sampled. The frequency of *T. gondii* DNA was also investigated in pig tongues sampled at the abattoir of which 38.2% (95% CI: 22%-56.4%) were shown to harbour *T. gondii* DNA in their tissue. The viability of the parasite was also investigated in the tongues intended for human consumption and a total of 8.8% (95% CI: 1.8%-23%) were shown to have viable *T. gondii* using a mouse bioassay. However, the agreement between serology, PCR and mouse bioassay was low (k=0.12-0.29). Due to the risk of underestimating *T. gondii* infection by using solely one diagnostic method, a combination of indirect (serology) and direct methods (PCR or/and bioassay) is preferred for a robust diagnosis. This study was pioneering in using a serological test validated in pigs in the state of Yucatan and the data revealed a much lower prevalence than previously reported (95.8%-100%) in market age pigs (Ortega-Pacheco *et al.,* 2013, Hernández-Cortazar *et al.,* 2016a). Although a more optimistic view is obtained; due to the potential of *T. gondii* to lead to severe illness, measures to control the disease should still be taken. Furthermore, questionnaire results suggested the need for an improvement to the sanitary management of the pig farms of this geographical area not only to prevent *T. gondii* transmission but also that of other pathogens of zoonotic significance.

CHAPTER 1

General introduction

1.1 Overview

The present work contains a detailed study of the epidemiology of the parasite *Toxoplasma gondii* in the swine host in an endemic area of Mexico, Yucatan. Yucatan was chosen because a high seroprevalence of 70% was reported among the human population in this geographical area in last Mexican national health survey (Caballero-Ortega *et al.,* 2012). Recent studies suggested that the pork produced in this area could have important public health implications due to the high seroprevalence of 95% -100% of anti-*T. gondii* antibodies found in pigs (Ortega-Pacheco *et al.,* 2011, Ortega-Pacheco *et al.,* 2013). Therefore, a cross-sectional survey in pig farms alongside collecting samples from a local abattoir were considered in order to investigate the disease levels of this parasite in pigs intended for human consumption.

1.2 Historical perspective

Toxoplasma gondii was first discovered more than 100 years ago by Nicolle and Manceaux, (1908) in the tissues of a rodent known as the gundi (*Ctenodactylus gundi),* in the Pasteur Institute in Tunisia. They were doing investigations on the intracellular parasite *Leishmania* and initially they erroneously thought that the infection stages were *Leishmania*. The name *Toxoplasma gondii* was proposed by Nicolle and Manceaux, (1909) based on the shape, in modern Latin *toxo* means arc and *plasma* means creature, and the gundi host which was identified by mistake or misspelled as the gondi. From its discovery until the late 1920s toxoplasmosis remained as an obscure disease of laboratory animals (Levaditi *et al.,* 1928). However, between 1930 and 1950, several studies associated the new organism with animal and human infection. The infection was mostly seen as congenital toxoplasmosis (Wolf and Cowen, 1937) but ocular infection and the disseminated form of the disease were also reported (Janku, 1923, Pinkerton and Weinman, 1940, Wilder, 1952). By 1939, Wolf, *et al*., had already defined the typical symptoms associated with human congenital toxoplasmosis which were

hydrocephalus, retinochoroiditis and encephalitis. Special mention must be made of the development of the Dye Test, which was the first serological tool that allowed intensive diagnosis of the disease in animals and humans (Sabin and Feldman, 1948). Thanks to this approach, it was possible to generate epidemiological data on the prevalence of *Toxoplasma* infection and it was revealed that a high proportion of animals and humans had anti *T. gondii* antibodies. This finding changed the picture of the parasite from being a rare exotic disease to the most common parasitic infection. This fact was initially perplexing as the only known transmission at the time was congenital and it seemed unlikely that the greater prevalence of the parasite relied solely on this type of transmission. Moreover, vertical transmission could not easily explain how adults became infected. This fascinating parasite awoke the interest of the researchers to undertake further investigations. Because of its widespread distribution, the initial association with *Leishmania* and the fact that the only known form at the time was the tachyzoite which circulates in blood; it was suspected that transmission occurred through arthropods. However, all attempts to demonstrate this transmission were fruitless (Woke *et al.,* 1953, Frenkel, 1970). Sabin and Olitsky, (1937) noted that mice became infected by feeding on recently dead animals and suggested for the first time that one of the methods of natural dissemination was by carnivorism. One of the earliest descriptions of an encysted form of the parasite was made in 1951 by Frenkel and Frielander. These early conjectures were later reinforced when Jacobs *et al.,* (1960) demonstrated that unlike tachyzoites, tissue cysts had the capacity to survive acid and trypsin digestion and could thus survive passage through the gut. The transmission of *T. gondii* through consumption of contaminated meat was confirmed when Desmonts *et al.,* (1965) experimented by feeding raw meat to children in a sanatorium hospital in Paris. Desmonts *et al.,* (1965) observed that the prevalence of anti *T. gondii* antibodies rose from 10% before consumption to 50% after feeding raw beef or horse and up to 100% when lamb was fed instead. However, the fact that herbivores and strict vegetarians had a similar incidence of the disease still remained unexplainable. Researchers suspected the existence of a faecal-oral route. The dog was the first species in which faecal samples were investigated because of its role in herding sheep. The work undertaken with dogs was unsuccessful (Jacobs *et al.,* 1955). The first clue of this mysterious transmission was revealed by Hutchison (1965) who continued Jacobs *et al.,* (1955) experiments but this time using feline faecal samples. The reason why cats were chosen was simply because in his laboratory there was no place to house

dogs (Dubey, 2009a). Hutchison (1965) collected faecal samples from cats following infection with the tissue cysts. Faeces induced toxoplasmosis in mice and parasites after being purified by flotation in 33% zinc sulphate solution and being stored in tap water for 12 months. When the faecal samples were observed under microscope only oocysts similar to *Isospora* and ova of the nematode *Toxocara cati* could be identified. Hutchison (1965) speculated that *T. gondii* was transmitted within the ova of *T. cati.* His incorrect conjectures were enhanced when cat faeces induced toxoplasmosis in mice after feeding *T. cati* infected cats (n=2) but failed when feeding *T. cati* free cats (n=2) (Hutchison, 1967). This theory of the transmission of *T. gondii* in association with *T. cati* was discarded when in 1969 it was demonstrated that cat faeces from nematode free cats induced *T. gondii* in mice (Frenkel *et al.,* 1969, Sheffield and Melton, 1969). The existence of the *T. gondii* sexual cycle in the intestine of the cat and other felids was demonstrated in 1970 (Frenkel *et al*., 1970). In 1970 Ben Rachid induced a fatal toxoplasmosis to gundis after feeding *T. gondii* oocysts. The knowledge of the sexual cycle permitted the classification of *T. gondii* in the suborder Eimeriina (Frenkel, 1970). This understanding of the *T. gondii* life cycle could explain how the gundis probably became infected in 1908 as it has been speculated that at least one cat was present in the laboratory of Nicolle and Manceaux (Frenkel, 1970, Dubey, 1977).

1.3 *T. gondii* stages and life cycle

Four different stages have been described in *T. gondii:* the rapidly multiplying stage, named tachyzoite, derived from Greek where *tachos* means speed and zoite means animal; the bradyzoite within tissue cysts surrounded by a well-defined membrane, derived also from Greek where *brady* means slow; the merozoite within the schizont which develops into sexual stages or gamonts in the cat intestine, and the resistant sporozoite stage where *sporo* means seed in Greek, which is found in oocysts outside the host (Dubey *et al.,* 1998a, Dubey, 2008, Dubey, 2009a).

The biological cycle of *T. gondii*, has two subcycles, sexual and asexual. The sexual can only take place in the carnivores of the Family Felidae, which are the definitive host of the parasite (Frenkel *et al*., 1970, Dubey 1998b). The sexual cycle of the parasite has been described in 17 different felid species (Dubey, 2010a). When a cat ingests a tissue cyst, the gastric acid

and the enzymes secreted by the gastrointestinal tract break the cyst membrane releasing the bradyzoites. Bradyzoites penetrate then in the epithelial cells of the small intestine where there is a limited period of asexual multiplication to produce 5 generation stages, named as A-E schizonts. Schizonts multiply by a specialized form of schizogony were the nucleus divides two or more times without cytoplasmic division. The daughter cell resulting from these divisions is the merozoite. The plasmalemma of the schizont invaginates around each merozoite, merozoites move towards the periphery of the schizont and eventually these separate from the schizont at their posterior end (Ferguson and Dubremetz, 2014). The number of merozoites per schizont varies from 2 to 80 (Dubey, 2010a). Merozoites derived from schizont types D and E initiate the formation of the gamonts (Dubey 1998b). The trigger for the conversion from asexual to sexual development is unknown. The male and female gamonts are named as microgametocytes and macrogametocytes respectively. It is unknown what is responsible for deciding whether an invading merozoite develops into either a microgametocyte or macrogametocyte. The male gamont swims with the help of its two flagella and penetrates the female gamont which produces the zygote (Dubey, 2010a). The nucleus of the zygote divides twice giving rise to four nuclei, which are situated at the periphery of the zygote. After the cytoplasm divides, two spherical sporoblasts are formed, each of which contains two nuclei (Ferguson and Dubremetz, 2014). The zygote is surrounded by one membrane with few micropores. To produce the oocyst, five layers are formed around the zygote membrane (Ferguson *et al.,* 1975). In contrast to other coccidians such as *Eimeria tenella*, the oocyst wall is characterized by the absence of micropores (Dumétre *et al.,* 2013). The infected epithelial cells eventually rupture and discharge the oocyst into the intestinal lumen where is ready to be shed with the cat faeces into the environment (Dubey and Frenkel, 1976). Once the oocyst is in the environment a process characterized by the internal formation of two elongated sporocysts, each of which contains four haploid sporozoites, is necessary to become an infective stage. This process called sporulation usually happens within 1-5 days after oocyst shedding (Dubey *et al*., 1998a). The oocyst wall is a highly robust multilayer which protects the parasite from the environment to ensure the survival for long periods outside the host (Dumètre *et al*., 2013). The preceding stages to the gamont formation have not been found when cats ingest oocyst or tachyzoites. It has been hypothesized that in this case sporozoites first transform into tachyzoites and these into bradyzoites or by directly transformation to bradyzoites in the case of tachyzoite ingestion.

Some of these bradyzoites return to the intestinal epithelia where the enteroepithelial cycle is then initiated in the cat intestine. The time from the cat becoming infected until the oocysts are shed is known as the prepatent period. The prepatent period varies accordingly to the ingested stage of *T. gondii*, being from 3 to 10 days after ingesting tissue cysts and more than 18 days after ingesting oocysts or tachyzoites, independently of the dose (Dubey and Frenkel, 1976, Dubey, 2006, Dubey, 2005).

In contrast, the asexual cycle, can take place in both intermediate and definitive hosts. All warm blooded vertebrates are considered to be intermediate hosts of *T. gondii*. The broad number of species include marine mammals such dolphins, seals, whales, otters; rodents, squirrels, bats, dogs, wild canids, primates (human and non-human), marsupials, bears, buffaloes, deer, marmots, antelopes, hares, rabbits and livestock such pigs, ruminants, chickens, etc. Intermediate hosts can become infected by either ingestion of oocysts spread in the environment or from tissue cysts. Subsequent to ingestion, either sporozoites or bradyzoites penetrate into the gut epithelium and in both the parasite converts to the rapidly dividing tachyzoite stage to invade and spread via the vascular system to all organs of the body. Tachyzoites can travel as free parasites in blood and tissues, by intracellular dissemination as 'Trojan horses' within migrating leucocytes or by attachment to the exterior of the migrating leucocytes (Santos *et al.,* 2009, Unno *et al*., 2008). Tachyzoites can infect any nucleated cell of the body by either active penetration through the membrane or by phagocytosis. This stage divides by a specialized progress called endodyogeny in which two daughter cells form from the parent parasite (Dubey *et al*., 1998a). Endodyogeny occurs within the parasitophorous vacuole and when this division is synchronous, lead to the typical arrangement of tachyzoites in rosettes (figure 1.1).

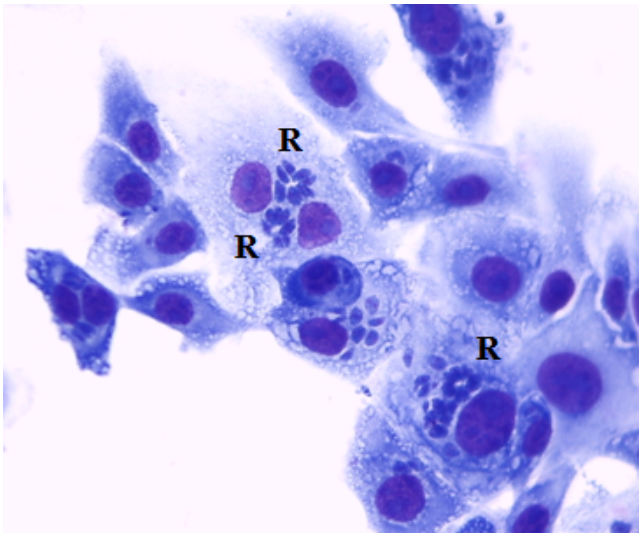

Figure 1.1 Formation of rosettes (R) by tachyzoites of the virulent RH strain. Cultures were fixed at 24 hours post-inoculation and stained with 5% of Giemsa.

However, division can also be asynchronous leading in this case to a more random arrangement of the tachyzoites. Ferguson and Hutchison (1981) observed that virulent strains have synchronous division while in non-virulent strains, which have a slower growth, an asynchronous division is more common. Tachyzoites are released from the host cells by either active egress or due to the rupture of the infected cell. Extracellular tachyzoites invade nearby cells to continue the dissemination through the system. By one to two weeks after oral infection, tachyzoites will have colonized lungs, spleen and brain. Tachyzoites can also invade the placenta in the pregnant dam transmitting the infection to the foetus. Around the third week of infection the host immune system suppresses the growth of tachyzoites and the conversion to bradyzoites occurs to start the formation of early tissue cysts (Black and Boothroyd, 2000; Dubey and Beattie, 1988). The conversion is transitional and not an all-or-nothing transformation. Thus, frequently these stages are found simultaneously. Bradyzoites grow very slowly by asynchronous division. Bradyzoites are the chronic stage of the parasite and therefore, well adapted to long term survival (Lyons *et al.,* 2002). There is a notable tissue tropism and the majority of cysts are found in striated muscle and central nervous system, in which the immune system is less efficient. However, the distribution varies between species of hosts and between strains of *T. gondii* (Dubey *et al*., 1998a, Dubey, 1997b). Several studies in rodents have shown a preference for brain over other tissues, while in livestock *T. gondii* has been found

more frequently in muscular or visceral tissues (Dubey, 1997a, Dubey, 1997b). This preference for distribution in muscle rather than brain has been demonstrated in experimentally infected sheep, pigs, goats, cattle and dogs (Dubey *et al*., 1986a, Dubey *et al*., 1996, Dubey *et al*., 1998a) and in naturally infected cats, sheep and pigs (Jacobs *et al*., 1963, Dubey *et al*., 1986a, Dubey *et al*., 2004a). Interestingly, in one study, when mice were fed with the Type III VEG strain (non virulent) and killed 21, 29, 64 or 237 days after infection cysts were found in the brain, skeletal muscle, heart and kidneys of mice at each time point; in the lungs, intestines and mesenteric lymph nodes in 3 of the 4 instances; in the tongue, liver, and eyes in 2 instances and in the spleen only in one instance. When mice were infected with the type II ME49, type I GT1 or P89 strains (moderate virulence and virulent), tissues cyst were found consistently only in the brain (Dubey, 1997b).

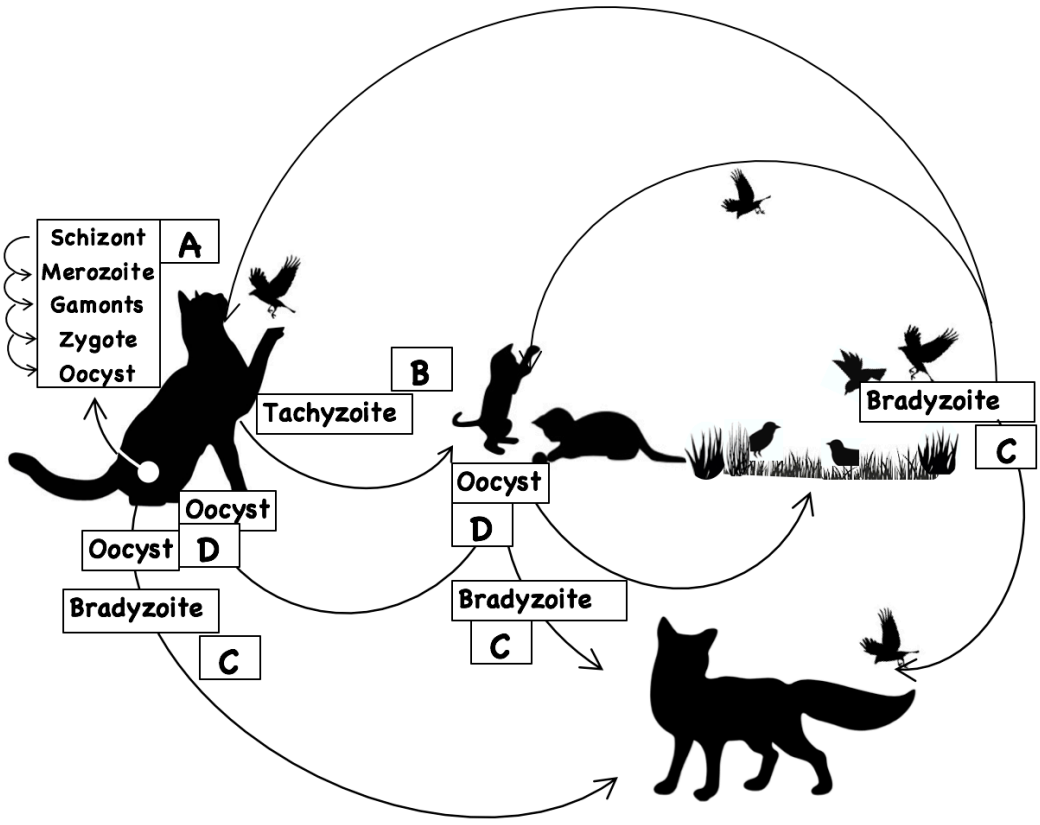

Figure 1.2 Life cycle of *T. gondii.* A: sexual cycle in the definitive host. B, C and D: asexual cycle in the definitive and intermediate hosts which can be transmited through B- tachyzoites by vertical transmission, C- brazydoites within the tissue cysts by carnivorism and D- ingestion of oocyts spread in the environment.

1.4 *T. gondii* transmission

Tachyzoite, bradyzoite and oocyst stages are naturally infective for both intermediate and definitive host (figure 1.2). Merozoites are not naturally infective as they are transitional stages wich invade exclusively feline enterocites (Dubey, 2010a). Experimental infections of mice and cats with these enteroepithelial stages have shown to be infective for cats but not for mice (Dubey, 2010a). Transmission through tachyzoites is more relevant in congenital or vertical toxoplasmosis. Congenital toxoplasmosis occurs when *T. gondii* is transmitted from the pregnant or lactating dam to the offspring. In contrast, oocysts and cysts are more important in horizontal transmission. Horizontal transmission happens when a host acquires toxoplasmosis by ingestion of oocysts spread in the environment or bradyzoites within tissue cysts of an infected host (figure 1.2).

1.4.1 Transmission through oocysts

Faecal surveys have estimated that at a given time only 1% of cats are shedding oocysts (Dubey, 2010a). Moreover, cats shed oocysts for a short period of time (up to 20 days), and commonly, only once in their life. These findings may lead to the false perception of a limited probability of transmission through this stage; however, the risk is rather high as cats can shed more than 100 million oocysts and these are highly resistant and survive in the environment (Dubey and Beattie, 1988, Jones and Dubey, 2010). It is not clear whether naturally infected cats can excrete oocysts more than once, but experimentally infected cats have re-shed oocysts (Dubey, 1995). In addition, cats have re-excreted oocysts after being immunosuppressed with corticosteroids (Lappin *et al*., 1991, Dubey and Frenkel, 1974), after being fed with deficient diets (Ruiz and Frenkel, 1980) and after being challenged with *Isospora felis* (not *Isospora rivolta*) (Chessum, 1972). However, cats did not re-shed oocysts after being challenged with the feline immunodeficiency virus (FIV), which is the feline virus related to HIV (human immunodeficiency virus) (Lappin *et al*., 1992). Oocysts can survive more than 4 years in freshwater (4°C), 2 years in sea water and soil (20°C-35°C) (Dubey, 1998a, Dubey and Beattie, 1988, Frenkel *et al*., 1975) and one month in frozen water (-21°C) (Frenkel and Dubey, 1973). Oocysts are resistant to most disinfectants (Katlin *et al*., 2007, Dubey *et al.,* 1970). However, they are highly susceptible to temperatures above 60°C and to dry environments (Dubey, 1998a,

Lélu *et al*., 2012). Oocysts can also be mechanically spread to other areas through invertebrates such flies, worms and beetles (Saitoh and Itagaki, 1990, Graczyk *et al.,* 2005), water sources such as rivers or drainages (Miller *et al*., 2002), they survive climate conditions such as snow, wind and rain (Simon *et al*., 2013), and can be disseminated in crops such hay, straw, and grains (Tenter *et al*., 2000), shoe soles (Dubey, 2010a) or dog fur (Frenkel and Parker, 1996). People and animals can become infected by oocysts either through ingestion of soil, water, harvested food, shellfish and more rarely but possible, by ingestion of insects and dog petting (specially children) (Etheredge *et al*., 2004, Chinchilla *et al*., 1994). The risk of infection through contaminated soil can occur through contaminating crops, gardening activities, cleaning the cat litter or by direct ingestion; for example, children in school play-grounds and animals which root around or peck the soil like pigs and chickens respectively (Tenter *et al*., 2000). In addition, insects can not only carry oocysts on their surface, but also internally. Oocysts remained infective to mice after being 19 days in the gut of two cockroaches (Chinchilla *et al*., 1994).

1.4.2 Transmission through tissue cysts

Carnivores and omnivores, in addition to ingestion of oocysts, can become infected through eating the tissues of prey carrying bradyzoites. This is considered one of the main routes of infection in humans; in fact, foodborne toxoplasmosis has been reported as the third leading cause of mortality due to foodborne illness in the American population (Mead *et al*, 1999). Humans can become infected through consumption of raw or undercooked meat loaded with tissue cysts. Additionally, some meat treatments such as smoking, curing and drying do not necessarily kill the parasite. In addition, tissue cysts are relatively resistant; these can remain infective after being refrigerated for three weeks (1-4°C), after light freezing from one week (-1°C to -8°C) to three weeks (-1 to -4°C) and after deep freezing for three days (-20°C) (Djurkovic-Djakovic and Milenkovic, 2000, Kotula *et al*., 1991). Nevertheless, tissues cysts are killed at an internal cooking temperature of 67°C. Of the meat-producing animals, pigs, sheep and goats are considered the main source of tissue cysts for humans. In contrast, in descending order; poultry, rabbits, horses, cattle and buffaloes are less important from the public health point of view. This is related to the frequency of tissue cysts that has been found in these species,

but any edible animal can harbour the parasite in their tissues (Dubey, 1991, Hill and Dubey, 2002). Game animals such hares, wild boars, cervids, birds, bears or kangaroos also represent an important source of tissue cysts. It has been suggested that the risk of harbouring tissue cysts in these animals might be very high because of the accumulative effect of age (Tenter *et al*., 2000). Tissue cysts may develop as promptly as 7 days post infection and persist for the entire life of their hosts. In addition, tissue cysts can be found in any edible part (Dubey *et al*., 1998a). Although less relevant, *T. gondii* can also be transmitted by skinning, eviscerating and handling animals. This risk is especially important for hunters, hunters' families, abattoir workers and butchers. For example, an outbreak of congenital toxoplasmosis in Inuits in Canada was associated with skinning animals (foxes, wolves, martens) for fur (McDonald *et al*., 1990). In a case-control study, infrequent washing of kitchen knives after chopping raw meat was associated with acquiring toxoplasmosis during pregnancy (Kapperud *et al*., 1996). In addition, there is an odd report from 1986 in which a woman developed fatal toxoplasmosis after being stabbed with a butcher's knife (Ferre *et al.,* 1986). Tissue cysts may be also transmitted after organ transplantation from a positive donor (Hill and Dubey, 2002).

1.4.3 Transmission through tachyzoites

Congenital or vertical transmission is exclusively due to tachyzoites. When a primary infection is acquired by a pregnant female, tachyzoites can colonize the foetus through the placenta. In addition, if the infection occurs when the female is breastfeeding tachyzoites can be transmitted within the milk (Bonametti *et al*., 1997, Prandota, 2013). This means the parasites can survive transit in the gut, the phenomenon was not totally unexpected as Dubey, (1998c) re-evaluated Jacobs (1960) experiment and demonstrated that some tachyzoites could survive in 0.5% trypsin solution for one hour and up to 2 hours in pepsin solution without losing infectivity. Congenital toxoplasmosis can occur several times for a given dam in some animal species such mice or hamsters. This phenomenon as a rule does not happen in humans. Humans, sheep and goats are especially susceptible to congenital toxoplasmosis (Dubey, 2004). This route of infection can happen in cats, and kittens infected congenitally or lactogenically can shed oocysts (Hill and Dubey, 2002). It has been previously mentioned that tachyzoites are not relevant in horizontal transmission, nevertheless this can occasionally occur. For example, tachyzoites can be transmitted by blood transfusion and organ transplantation from positive

donors (in this case, bradyzoites are also involved) (Singh and Sehgal, 2010, Hill and Dubey, 2002). Accidental injections, scratching and splashing of the parasite when manipulating in laboratory practise are a possible way to acquire the disease. It has been suggested that tachyzoites can invade the body entering through wounds, mucosal tissue and cornea (Dubey, 2010a, Skorich *et al*., 1988, Tenter *et al*., 2000). Viable tachyzoites have been found in unpasteurized milk and cheese of sheep, goat and cows. Nevertheless, *T. gondii* infection has only been associated with ingestion of unpasteurized goat milk (Sacks *et al*., 1982, Riemann *et al*., 1975, De Andrade *et al*., 1984). Tachyzoites are very susceptible to temperature and gastrointestinal enzymes in comparison with oocysts and tissue cysts (Dubey and Beattie, 1988, Dubey, 1991). It is interesting that in one British family, two children contracted toxoplasmosis drinking raw milk while the parents remained seronegative because they were only having small amounts of milk in hot drinks such tea and coffee (Skinner *et al*., 1990). Tachyzoites have been found in eggs of experimentally infected hens. However, to date, there is no evidence that tachyzoites can be found in eggs of naturally infected poultry (Dubey, 2009a). Tachyzoites have been also found in semen of pigs, goat, sheep and humans and in saliva, sputum, urine and tears of humans but again there is no evidence of *T. gondii* transmission through any of these additional routes (Tenter *et al*., 2000).

1.5 Toxoplasmosis in humans

1.5.1 Epidemiology

Toxoplasma gondii could be considered to be the most successful of all parasites. It has been reported in all continents, even in places were felids are virtually absent such as Antarctica (Rengifo-Herrera *et al*., 2012) or in remotes places near the North Pole (Prestrud *et al*., 2010). *T. gondii* has been estimated to be one of the most prevalent diseases infecting around 30%-50% of the human population (Flegr *et al*., 2014, Hill *et al*., 2005, Montoya and Liesenfeld, 2004, Luft and Remington, 1992). However, there is a considerable variation in prevalence between different geographical areas. (Joss, 1992, Dubey and Beattie, 1988, Dubey, 2010a). Prevalence data are more frequently reported as seroprevalence (detection of specific antibodies in serum) since this is a cumulative measure of exposure to the parasite during a person's lifetime. The epidemiological studies in humans are more frequently addressed to pregnant

women or women of childbearing age due to the risk of congenital transmission. Thus, prenatal screening programs are commonly developed for surveillance of this disease in order to monitor and prevent the possible fatal consequences that can develop in the infected foetus. In 1996, the WHO adopted a method to measure the disease burden based on morbidity and mortality, named as the Disability Adjusted Life Year (DALY) (Murray and Lopez, 1996). The DALYs for a disease are the potential years of life lost due to premature death, poor health or disability. One DALY can be thought of as one lost year of a 'healthy' life (Flegr *et al*., 2014). The annual global burden of congenital toxoplasmosis was calculated to be 1.20 million DALYs (95% CI: 0.76–1.90). However, the number of DALYs for congenital toxoplasmosis varies greatly between countries; European countries have the lowest DALYs in contrast to South American and African countries (Torguerson and Mastroiacovo, 2013). For example, in Europe, the Netherlands and France were the countries with the highest DALYs, having values of 2464 and 1691 respectively. These values obtained in Europe were much lower than the 73807 and 51082 obtained in Nigeria and Brazil respectively. A detailed list of the number of DALYs for congenital toxoplasmosis by contry is available at www. vetepi.uzh.ch/research/Diseaseburden/ Burden CT-Appendices.pdf.

In America, seroprevalence is monitored periodically through the National Health and Nutrition Examination Survey (NHANES). Seroprevalence to *T. gondii* IgG antibodies was estimated to be 12.4% in 2010 in the last NHANES survey among the healthy population (6-49 years old). The seroprevalence among healthy women of childbearing age (15-44 years old) was 9.1% (Jones *et al*., 2014). Data from Canada reflected a similar seroprevalence of 14.2%-18.6% among the healthy general population (Shuhaiber *et al*., 2003). In contrast, prevalences in South and Central America were, in general, much higher (figure 1.3). Prevalences in Brazil ranged from 7% to 51% in healthy women during child bearing years and this increased dramatically during pregnancy, ranging from 47% to 77.5%. Other countries have also shown high seroprevalence of IgG anti-*T. gondii* antibodies in pregnant women, oscillating from 48% to 53% in Argentina, from 48% to 64% in Colombia, from 45% to 61% in Cuba, 65% in Peru, 55% in Costa Rica, 57% in Grenada, 43% in Trinidad del Tobago (Tenter, *et al*., 2000, Flegr *et al*., 2014, Dubey, 2010a, Pappas *et al*., 2009). Mexico, although located in North America, has a high overall prevalence of 44% among the general population (1 to 98 years old) (Caballero-Ortega *et al*., 2012). Venezuela and Chile have lower prevalences of 38% among the general

population (20 to 40 years old). Interestingly, seroprevalence was higher in the United States among the Mexican-American and African-American groups who were born outside the USA (Jones *et al*., 2014).

In Europe, the lowest seroprevalences in women of childbearing age have been reported in Northern countries, where it ranged from 8% to 9% in the United Kingdom and Iceland and 11% in Norway (figure 1.3). In Central Europe, prevalence ranged from 19% in the Czech Republic to 63% in Germany in women of childbearing age. High prevalence has been reported in France (54%), Belgium (48.7%), Austria (42%) and Poland (35.5% to 43.7%). In Switzerland, the prevalence varied from 8.2% to 35% also in women of childbearing age. In contrast, South Europe has shown intermediate values in the same type of population, ranging from 17.5% to 34.4% in Italy, from 18.8% to 43.8% in Spain, from 20% to 36.4% in Greece and 24% in Portugal (Tenter, *et al*., 2000, Flegr *et al*., 2014, Dubey, 2010a, Pappas *et al*., 2009).

Even though most African countries have a high prevalence, seroepidemiological studies are limited in this continent. Studies in the general population have noted prevalence of anti-*T. gondii* IgG antibodies of 87.3% in Ethiopia, 58% in Egypt, 58.4% in Tunisia, 20.8% in Nigeria and 21% in Mali. The seroprevalence among pregnant women and women of childbearing age is also very high. Seroprevalence of 60% has been reported for the Ivory coast, 50.6% in Morocco, 75.25% in San Tome and Principe, and 34.1% in Sudan (Pappas *et al*., 2009, Dubey *et al.,* 2012a, Flegr *et al*., 2014).

In Asia, values ranged from 0.8%-3.7% in Korea to 70% in Indonesia. India has reported prevalences from 11.6% to 45%, Iran from 29.4% to 64%, Thailand from 5.3% to 21.5%, Malaysia from 45.7% to 49%. Japan, Singapore, China and Vietnam noted a much lower prevalence ranging from 10% to 17.2% (Gao *et al*., 2012). Finally, in Oceania, values of 23% in Australia, 35.4% in New Zealand and 56.7% in New Caledonia have been reported among pregnant women (Tenter, *et al*., 2000, Flegr *et al*., 2014, Dubey, 2010a, Pappas *et al*., 2009).

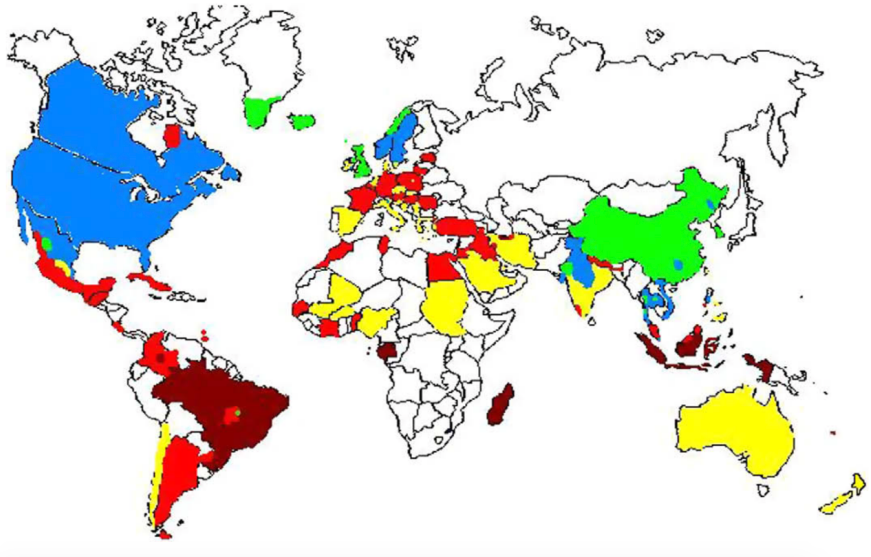

Figure 1.3 Global status of *T. gondii* seroprevalence in pregnant women and of childbearing age. Dark red equals prevalence above 60%, light red equals 40–60%, yellow 20–40%, blue 10–20%, green equals prevalence < 10% and white no data. Prevalence data were collected from 1999 to 2008. Map taken from Pappas *et al*., (2009). Licence number provided by Elsevier to reuse this figure: 3927140664052.

In general, there is a pattern in which prevalence is higher in humid tropical areas than in hot and dry and arctic areas (figure 1.3). This variation has been attributed in part to differences in the climatology as this is important for oocyst survival (Gangneux and Dardé, 2012, Tenter *et al*., 2000, Meerburg and Kijlstra., 2009). Interestingly, Meerburg and Kijlstra (2009) have suggested that global warming will influence the distribution of toxoplasmosis by an increase in prevalence in North-Western Europe. The prevalence may also depend on the economy, social habits, cultural habits, hygienic measures or public health situation for a given area. The higher prevalence found in central Europe has been suggested to be related in part to the French habit of eating undercooked meat products. Interestingly, a Canadian-Inuit population showed a seroprevalence of 60%, much higher than the maximum seroprevalence of 18.6% reported in the general Canadian population. This high seroprevalence was associated with the habit of skinning animals for fur and eating seal and caribou meat (Messier *et al.,* 2009). In contrast, the high level of *T. gondii* infection in developing countries has been related to higher levels of contamination of the environment with oocysts (Dubey and Beattie, 1988). It has been suggested that in less developed countries, where people live under conditions of poor-hygiene and drinking water is unfiltered, higher prevalences are seen (Bahia-Oliveira, *et*

al., 2003). Differences can be seen between countries, and across different regions in the same country (Pappas *et al*., 2009). Bahia-Oliveira *et al*., (2003) studied the variation of *T. gondii* seroprevalence between different socioeconomic groups in the population of Rio the Janeiro in Brazil. The seroprevalence in the upper socioeconomic level was 23% while this rose to 62% in the middle socioeconomic level and reached the 84% in the lowest socioeconomic level. These differences were put down to differences in the water supply, where drinking unfiltered water was a common practice among the economically disadvantaged populations (Bahia-Oliveira *et al*., 2003). A similar study was conducted in the USA and *T. gondii* infection was associated with people who were economically disadvantaged (Hotez, 2008). Alarmingly high seroprevalences of 73.5% to 95.7% have been reported in the Amazonian indigenous tribes attributed to the cultural habit of eating rodents and monkeys together with drinking unfiltered water (Bóia *et al*., 2008). Higher prevalences have also been reported among populations under poor health conditions such as patients with HIV compared to healthy individuals. For example, in Ethiopia, patients with HIV showed a seroprevalence of *T. gondii* antibodies of 93.3% while in non-HIV individuals this was 86.7% (Dubey *et al*., 2012a). In Nigeria, patients with HIV showed a seroprevalence of IgG antibodies of 54% while among the non HIV control group was 30% (Akanmu *et al*., 2010). In Mozambique, similar results were obtained, where the seroprevalence of IgG antibodies anti-*Toxoplasma* was 31.3% in HIV-positive patients while in HIV-negative patients of IgG antibodies anti-*Toxoplasma* was 10.9% (Sitoe *et al*., 2010).

1.5.1.1 Prevalence of *T. gondii* in Mexico in humans

Toxoplasmosis was first reported in Mexico in the year 1950 (Palomino *et al.,* 1950), however the overall pattern of human infection was established in 1987 when the first National Seroepidemiological Survey (NSS) was developed.

In Mexico, the number of DALYs has been stimated to be 8184 for congenital toxoplasmosis (Torgerson and Mostroiacovo, 2013). An overall prevalence of 43.9% of *T. gondii* infection among the Mexican general population was reported in the last National Seroepidemiological Health Survey (NSHS) of 2006 using IgG-ELISA test. However, the prevalence varied between states (figure 1.4), for example, northern states such as Nuevo Leon and Durango have shown prevalences between 5% and 22% of IgG antibodies by ELISA

(Alvarado-Esquivel *et al*., 2012a, Alvarado-Esquivel *et al*., 2012b, Kelso *et al*., 2000) while central areas such as the western-pacific state of Jalisco or the state of Mexico have prevalences between 18% and 43% using the same technique (Galván-Ramírez *et al*., 2007, Galván-Ramírez *et al*., 2010b, Cañedo-Solares *et al*., 2009). Finally, states near the Gulf of Mexico and at the south-eastern area of the country such as Yucatan had a prevalence over 70% in the latest studies (Caballero-Ortega *et al*., 2012).

This variation in prevalence might be explained by differences in climate and geographical conditions. Higher prevalence has been found in warm, rainy and humid tropical conditions than in dry, hot and artic areas (Jokelainen *et al*., 2010, Flegr *et al*., 2014, Galván-Ramírez *et al*., 2012). This has been ascribed to oocyst survival which is much longer in humid, low altitude regions, especially at mid-latitudes where freezing and thawing are rare events (Walton *et al*., 1966). Mexico is a heterogeneous country in terms of geographical conditions and climates.

Mexico is a country with heterogeneous sociodemographic and economic conditions, and these characteristics may also influence disease prevalence. For example, one study carried out in Jalisco compared three socioeconomic groups among women with high-risk pregnancies and the prevalence of IgG *Toxoplasma* antibodies was 41% for women in the low socioeconomic class, 32% for the middle and 13% for the high socio-economic level (Galván-Ramírez *et al*., 1995).

In addition, some occupational groups have been suggested to be risk groups for toxoplasmosis for example, people who are in contact with raw or undercooked meat such as abattoir workers. Seroprevalence reached the 72% when workers in slaughterhouse were tested in Jalisco (Galván-Ramírez, *et al*., 2008). This prevalence was much higher when compared with other groups of the population, for example, Galván-Ramírez *et al*., (2005) obtained a prevalence among blood donors of 29% in Jalisco. Similarly, cat owners and immunocompromised patients have been categorised as risk groups (Galván-Ramírez *et al*., 2012, Galván-Ramírez *et al*., 1999).

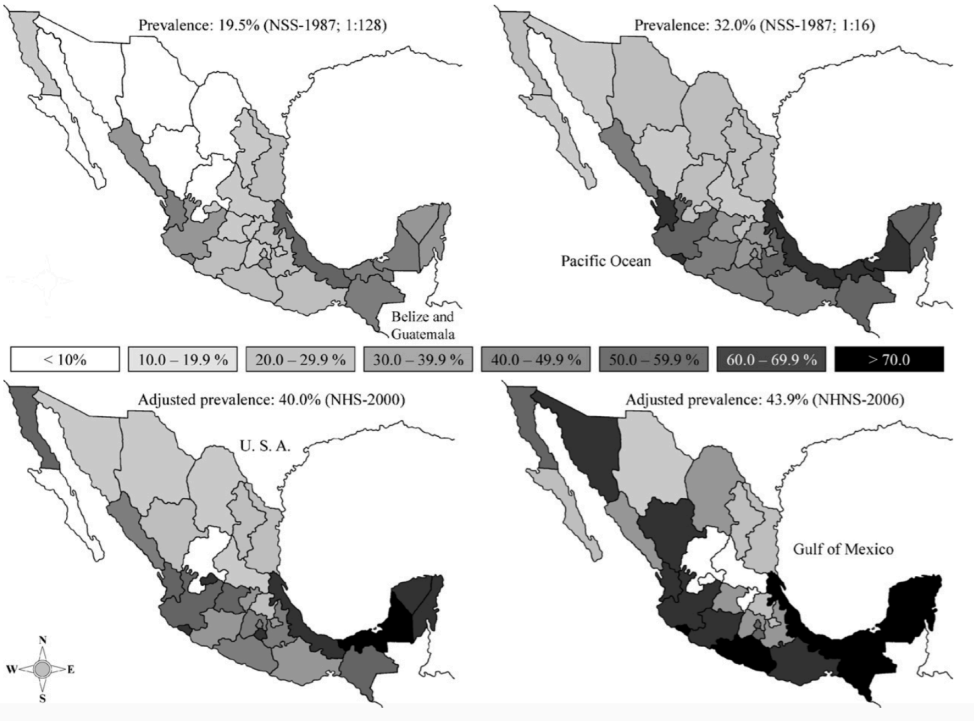

Figure 1.4 Prevalence ranges per state of *T. gondii* antibodies in Mexico in 1987, 2000 and 2006. Prevalence ranges are specified by different shades of grey. Prevalence data of 1986 were obtained by the National Seroepidemiological Survey (NSS-1987) using 1:128 and 1:16 serum dilutions cut offs in IFAT. Prevalence data of 2000 were obtained by National Health survey (NHS-2000) and of 2006 by the National Health Nutrition Survey (NHNS-2006). For the NHS-2000 and NHNS-2006, prevalence values were adjusted by Caballero-Ortega *et al*., 2012 considering the sensitivity and specificity of the ELISA employed. Licence number provided by Oxford University Press to reuse this figure: 3902390804827.

A noticeable general increase in *T. gondii* prevalence has been described in Mexico between 1987 and 2006 (figure 1.4). This increase is more noticeable in the south-eastern states, which include Yucatan, Campeche, Quintana Roo, Chiapas, Tabasco, Guerrero and Veracruz. For example, Góngora-Biachi, 1998 reported in Yucatan a prevalence of 47-69% of IgG antibodies by ELISA in 1998 and according to Caballero-Ortega *et al.,* (2012) the general prevalence in Yucatan in 1987 was 50-60% by IFAT and rose to 70% in 2006 by ELISA (figure 1.4). It has been suggested that the climate change could affect *Toxoplasma* prevalence (Meerburg and Kijlstra 2009) and a global increase in temperature of 1°C has been reported from 1987 to 2005 in Mexico (Caballero-Ortega *et al*., 2012). The reported prevalence of *Toxoplasma* in southeastern Mexico is alarming and the disease is present in many states,

however most of the investigations focus the work in the few states cited above. Further studies are necessary.

1.5.2 Clinical manifestations

T. gondii is probably one of the most highly robust parasites in the world being able to infect more than third of the human population and persist for the entire life of their hosts, and sometimes, without these noticing. Nevertheless, *T. gondii* infection can be very serious, causing a wide range of symptoms.

1.5.2.1 Toxoplasmosis in the immunocompetent individual

1.5.2.1.1 Acquired toxoplasmosis

T. gondii primary infection is asymptomatic in most immunocompetent children and adults (Remington, 1974). When the infection is symptomatic, it usually causes a self-limited illness that rarely needs treatment. The most characteristic symptom associated with the acute infection is lymphadenopathy. Lymphadenopathy is frequently accompanied by viral-like mild symptoms such lethargy, fatigue, headache, fever, myalgia and sweating (Ho-Yen, 1992, Bowie *et al*., 1997). Although lymphadenopathy can involve any lymph node, occipital and cervical nodes are the most frequently affected. Lymph nodes are enlarged, nonsuppurative, and rarely painful. Lymphadenopathy symptoms can fluctuate from four weeks to more than a year (Montoya and Liensenfeld, 2004, Dubey, 2010a). In addition, occasionally the disease maybe associated with encephalitis, meningitis, meningoencephalitis, myositis, myocarditis, pericarditis, pancreatitis, pneumonia and gastrointestinal symptoms in immunocompetent patients (Schreiner and Liesenfeld, 2009, Townsend *et al*., 1975, Demar *et al* 2012, Montoya *et al*., 1997). It had been suggested that symptomatic illness affects only about 10% of the infected patients (Montoya and Liensenfeld, 2004). On the other hand, studies of outbreaks have shown symptoms in more than 70% of the infected patients (Ho-Yen, 1992, Bowie *et al*., 1997). It has been suggested that because of the failure to recognize the disease due to the non-specific symptomology, most of cases of toxoplasmosis in immunocompetent individuals remain undiagnosed rather than being asymptomatic (Ho-Yen, 1992).

1.5.2.1.2 Congenital toxoplasmosis

The global incidence of congenital toxoplasmosis has been estimated to be 190,100 (95% CI: 179 300–206 300) cases per year (Torgerson and Mastroiacovo, 2013). The symptoms in the pregnant woman are identical to those in the immunocompetent patient. Although, the pregnant woman may be asymptomatic or may remain undiagnosed despite experiencing illness, the infection can be still transmitted to the foetus (Boyer *et al*., 2005). Most commonly, the infection is transmitted to the foetus when a seronegative woman is acutely infected. Nevertheless, there are exceptions, congenital infection may occur in seropositive women when a cryptic infection has been reactivated or although more rarely, when the pregnant woman become reinfected (Kodjikian *et al*., 2004, Andrade *et al*., 2012). However, not every maternal active infection leads to a vertical infection. The risk of transmission when maternal infection is acquired before conception is practically absent and it remains low (10%-25%) during the first trimester. During the second trimester, the transmission rate increases (30%-50%) to reach the highest for women infected during the third trimester (60%-90%). Daffos *et al*., (1994) studied cerebral development in 148 infected foetuses from mothers which seroconverted during pregnancy based on ultrasound examination and reported that 48%, 12% and 3% of the foetuses had ventricular dilations in mothers which became infected during the first, second and third trimester respectively. In addition, the severity of the infection is proportional to the transmission rate. Thus, congenital infection during the first and second trimesters can have disastrous outcomes for the foetus such as miscarriage, stillbirth and severe illness of the live infant. When the foetus survives, the most common clinical manifestations include retinochoroiditis, hydrocephalus, encephalitis strabismus, blindness, epilepsy, psychomotor or mental retardation, microcephaly, microcornea, microphthalmy, intracranial calcifications, anaemia, jaundice, rash and petechiae (Remington *et al*., 2001, Remington *et al*., 2011). On the other hand, infection during the third trimester usually results in asymptomatic newborns with subclinical infection. However, although they may seem asymptomatic, the child can develop a variety of symptoms in later life including chorioretinitis, growth delay, retinitis or meningitis (Montoya and Liesenfeld, 2004).

1.5.2.1.3 Ocular toxoplasmosis

The highest incidence of ocular complications occurs when the central nervous system is affected, and the lowest when lymphadenopathy is the only manifestation. Ocular disease can occur as a result of congenitally or postnatally acquired toxoplasmosis. Ocular disease can also occur during acute infection or as a result of a reactivation of a chronic infection. Either way, the eye lesions may be identical. Chorioretinitis, retinitis and uveitis are the most common presentations of the ocular disease. However, other manifestations such optic neuritis, iritis, retinal vasculitis, acute retinal necrosis, macular disease, iridocyclitis and vitritis may also occur alone or in combination (Su *et al.,* 2014). Clinical manifestations of the acute phase include pain, photophobia, epiphora, blurred vision, partial or complete blindness and glaucoma (Holland, 2004). Chorioretinitis has been associated with a high rate of 90% of congenital cases, sometimes being the only manifestation present (McAuley *et al*., 1994, Alford *et al*., 1974, Wilson *et al*., 1980). In contrast, the frequency of ocular involvement in postnatally acquired infection has been estimated to be 20% (Burnett *et al*., 1998, Delair *et al.,* 2008). Conversely, ocular toxoplasmosis is more commonly associated with acquired infection than congenital infection, probably because acquired toxoplasmosis is more common. Severe ocular disease has been associated with recurrence of the chronic stage under periods of stress such as school-entry, adolescence, elderly ages and pregnancy, but also under periods of reduced immunity (O'Connor, 1983, Su *et al.,* 2014).

1.5.2.1.4 The chronic infection and mental disorders

It is well known that during the chronic stage of infection *T. gondii* forms cysts in the brain infecting different kind of cells such as neurons and astrocytes. Alterations such as anatomical, immunological, pathological and changes in the levels of neurotransmitters which may occur during brain infection with this parasite, have been sugested to contribute to several mental and neurological disorders. For example, epidemiological studies have revealed an association between *T. gondii* infection and later development of schizophrenia (Torrey *et al*., 2007, Torrey and Yolken, 2003). Yolken *et al*., (2001) found higher prevalence of anti-*T. gondii* IgG antibodies in patients with schizophrenia (42.1%) than in a control group (11.1%). There is also evidence that maternal and prenatal *T. gondii* infection are risk factors for schizophrenia

and psychoses development in the later life of children (Torrey and Yolken, 2003, Brown *et al*., 2005, Mortensen *et al*., 2007). Likewise, other case-control studies have found a much higher prevalence of IgG anti *T. gondii* antibodies in patients with obsessive–compulsive disorder (OCD) (47.6%) than in the control group (19%), in patients with epilepsy (52%) than in the control group (18%), in patients with Parkinson's disease (42.3%) than in the control group (22.5%), in patients with Alzheimer's disease (44.1%) than in the control group (24.3%) and in patients with migraine (44.2%) than in the control group (26%) (Dalimi and Abdoli, 2012). In addition, studies also noted decreased psychomotor skills associated with chronic *T. gondii* infection (Havlíček *et al*., 2001, Flegr *et al*., 2003). This reduction of the psychomotor performance has been connected with the higher risk of traffic and work accidents in subjects with latent toxoplasmosis (Alvarado-Esquivel *et al*., 2012a; Flegr *et al*., 2003; Kocazeybek *et al*., 2009; Yereli *et al*., 2006). A lower intelligence quotient (IQ) has also been observed in children with subclinical congenital toxoplasmosis (Saxon *et al*., 1973, Alford *et al*., 1974) and in men with latent toxoplasmosis (Flegr *et al*., 1996, Flegr *et al*., 2003). In contrast women with latent toxoplasmosis showed higher IQ (Flegr and Havlíček,1999). More recent studies have also linked the latent infection with personality and behavior changes (Flegr *et al*., 1996). For example, infected men were more suspicious, jealous and bumptious than seronegative individuals. In contrast, infected women were more sociable, warm-hearted and easy-going than seronegative women who were more detached, critical and reserved (Flegr, 2013). Both men and women with latent toxoplasmosis reported a weak instinct for 'self-preservation', in other words, they were not afraid in situations that people might normally fear, when compared to *Toxoplasma*-free individuals (Flegr, 2013). In addition, a suicidal behavior has been associated with *T. gondii* latent and acute infection in men and women (Godwin, 2012, Okusaga and Postolache, 2012, Pedersen *et al*., 2012, Ling *et al*.2011).

1.5.2.2 Toxoplasmosis in the immunocompromised individual

In patients with AIDS or immunosuppressed by cancer, treatment, transplant or other illnesses, toxoplasmosis can become a life-threatening disease (Israelski and Remington, 1993). *T. gondii* is one of the most common opportunistic agents in patients with AIDS. Actually, around 10% of patients with AIDS in USA and 30% in Europe die of toxoplasmosis (Luft and Remington, 1992). In most of the cases of immunodeficiency (with AIDS or not) the disease is

a result of reactivation of the chronic infection (Ho-Yen, 2009). In patients with AIDS disseminated toxoplasmosis is the most common presentation of the disease in acute acquired infection while toxoplasmic encephalitis is more frequent in the case of reactivation of the disease. Symptoms of disseminated toxoplasmosis include fever, chills, malaise, coughing, vomiting, diarrhea, septic shock, multi-organ failure, confusion, intravascular disseminated coagulopathy or coma (Williams and Burton, 2009, Albrecht *et al*., 1995). Symptoms of toxoplasmic encephalitis include seizures, hemiparesis, cereberal signs, meningismus, alteration of the mental state, behavioural and psychomotor disturbances such psychosis, anxiety, dementia, drowsiness or coma (Luft and Remington, 1992). *Toxoplasma* infection has been reported after heart, kidney, liver, bone narrow and hematopoietic stem cell transplantations. The symptomatology is developed in most cases three months after the intervention and is very similar to the symptomatology present in patients with AIDS (systemic and cerebral toxoplasmosis). The patient becomes infected either by reactivation of the chronic infection or through transplant from a positive donor (Fernàndez-Sabé, 2012, Aubert, 1996, Remington, 1974).

1.5.3 *Toxoplasma* as a foodborne disease

It has been estimated that the incidence of prenatal infection ranges from 0.01% to 1.2% (Tenter *et al*., 2000). Thus, this suggests that horizontal transmission plays the more important role than vertical in human infection. *Toxoplasma gondii* has been considered the most common foodborne parasitic infection which requires hospitalization (Mead *et al*., 1999). *T. gondii* is also the third highest cause of death over all foodborne pathogens (Mead *et al*., 1999). Whether transmission through tissue cysts is more or less relevant than oocysts transmission is difficult to assess. Probably the source of transmission varies greatly among different populations. Despite that, epidemiological studies have been developed to investigate risk factors associated with the disease, from 14% to 49% of the cases remained unexplained (Cook *et al*., 2000, Jones *et al*., 2009). A multicentre case-control study (n=960) developed in six large European cities (Naples, Milan, Copenhagen, Oslo, Brussels, Lausanne) attributed 30% to 63% of the infections to consumption of undercooked or cured meat (Cook *et al*., 2000). In contrast, only 6% to 17% of the *Toxoplasma* positives individuals were associated with

infection through oocysts (Cook *et al*., 2000). Similarly, in a case-study in United States (n=561) 54% of *T. gondii* infected individuals were associated with meat consumption of which 22% of the cases were associated with cured, smoked and dried meat, 20% were associated with eating undercooked lamb, 7% with eating raw beef and 5% working with meat (Jones *et al*., 2009). In contrast, only 14% of the cases were associated with oocysts (10% live with > 3 kittens and 4% seafood consumption) and 4% with tachyzoites by unpasteurized milk consumption (Jones *et al*., 2009). In Europe and the United States pork has been considered the major source of *T. gondii* infection in humans (Tenter *et al*., 2000). Nevertheless, the type of meat associated with the infection probably depends on the major food livestock animals, and this varies between countries. For example, in France the risk was associated with beef consumption, in Norway with lamb and in Poland the risk was associated with consumption of pork. In Poland, the prevalence of *T. gondii* in pigs was estimated to be around 36% while in Norway the prevalence in pigs dropped to 3% but in sheep it was reported to reach the 18% level (Tenter *et al*., 2000).

1.5.3.1 Pork as a source of *T. gondii*

Although clinical toxoplasmosis is usually rare in pigs, pork is considered to be a more common source of tissue cysts when than poultry and beef (Dubey, 1991, Hill and Dubey, 2002). This has been suggested to be the result of higher susceptibility of swine when compared to poultry or cattle (Hill and Dubey, 2013). In addition, pigs are omnivores and have cannibalistic behavior, thus they may be exposed to a higher rate of infection than herbivorous species (Smith and Frenkel, 1995). Another important point to take into consideration, is that pork is the most commonly consumed meat in the human population (FAO, 2014). Thereby pigs may be a major source of human infection; in fact, in Europe and the USA, pork has been considered an important reservoir of *T. gondii* infection for humans. Tissues cysts have been found in virtually all commercial cuts of pork (Dubey, 1986, Dubey *et al*., 1986a, Dubey *et al*., 1984). Furthermore, cysts have been demonstrated to remain for up to 865 days in pork meat (Dubey, 1988). Even though the role of pork can be relevant, there is currently no prophylaxis available for this animal species. There is a commercially available vaccine, Toxovax®, which has been shown to experimentally reduce the tissue cyst burden in pork (Burrels *et al*., 2015).

However, the vaccine is currently only in use for the immunization of ewes against abortion.

Monitoring of this disease is still scarce and the USA is currently the only country in which *T. gondii* is included among the diseases to monitor in a national surveillance program (National Animal Health Monitoring Systems (NAHM)) of swine. Furthermore, postmortem monitoring at the slaughterhouse for this parasite is nonexistent in any country. *T. gondii* is not considered to be a disease to control in the international trading of pork, nor are there legal regulations requiring treatment to inactivate the parasite. Nevertheless, some of the mandatory treatments required to kill the pathogen *Trichinella spp* such as curing, freezing or cooking are also effective in killing *T. gondii.* These procedures are implemented in many countries in Europe ((EC) N 2075/2005) ad the USA (CFR 318.10, Title 9). The parasite, however, can survive some curing, cooking, and freezing procedures. Furthermore, cured meats are usually prepared by mixing meats from different animals. This practice, increases the risk of transmission as one or few infected animals could lead to the contamination of an entire batch of the meat product (Bayarri *et al*., 2012a). Several epidemiological studies have found a strong association between consumption of cured, smoked and dried meats and acute toxoplasmosis in pregnant women (Cook, 2000; Jones *et al*., 2009). Contrary to bacterial pork pathogens such as *Salmonella* or *Campylobacter jejuni, T. gondii* does not undergo exponential multiplication during storage at suboptimal temperatures after slaughter. However, it is possible that meat infected with *T. gondii* could contaminate grinding equipment, knifes or other surfaces (Hill and Dubey, 2013, Baer *et al*., 2013). Hence one infected animal could lead to contamination of a larger quantity of meat. The role of pork in *T. gondii* transmission may vary between populations due to a dietary habit or religious restrictions. Furthermore, *T. gondii* prevalence in pigs varies also between countries and between farms within the same country. Prevalences as low as 0% and as high as 95% have been found in this animal species. Nonetheless, even where *T. gondii* infection in pigs is low, the risk of transmission through pork may be very high as it has been estimated that one pig can be consumed by 200 to 400 individuals (Fehlhabe, 2001).

1.6 Toxoplasmosis in pigs (*Sus scrofa*)

1.6.1 Types of pig production systems

Pig farms can be classified according to their management, animal confinement and sanitary measures. Intensive farming is the most common system worldwide; it is found in nearly every country. In intensive farms pigs are kept indoors throughout their entire life, but these are separated in different areas for pregnant sows, empty sows, sows with their litters, boars, weaned, growers and finishers. Animals are kept in pens in groups depending on the weight and age. The pen floor is usually made of concrete or plastic and metal slats to facilitate waste removal. Intensive farms are characterized by rigorous biosecurity and sanitary measures such as use of vaccines, strict hygienic procedures, control of plagues, feeding with commercial sterilized food, record keeping and control of visitors. Depending on the geographical area the infrastructure of the farms varies; for example, in cold or temperate countries buildings are closed and fan ventilated while in tropical countries intensive farms are open in both sides and trees are used to cool the environment (figure 1.5). In addition, the farming regulations also vary between countries and therefore, the sanitary and welfare standards also vary (FAO, 2010).

Figure 1.5 Example of a swine intensive farm in a tropical area (Yucatan, Mexico).

Another type of farming is the extensive model, also called free range, alternative or 'animal friendly'. Extensive farms are characterized by outdoor access, ample space for the pigs, lower capital investment and the possibility of expansion is easier than intensive farming (Honeyman, 2001). Traditionally in extensive farming pigs were kept outdoors however these days, although it is rare, it is possible to find extensive farms which keep pigs indoors using large spaces of bedding in hoop-barns. This is also called 'enriched environment' since the installations are supplemented with materials such straw bedding or toys to improve animal welfare (Honeyman, 2005). In contrast, biosecurity and sanitary measures are less rigorous in extensive farming in comparison to intensive. For example, biosecurity practices such as exclusion of contact with other animals, pig segregation and the use of exclusive sterilized food are not feasible in this production system. There is another type of farming known as organic which has gained popularity during the recent years. This type of farming, in a similar manner to the extensive farming, is more common in Europe and USA and it is characterized by outdoor access, extensive pig living space and straw bedding. But in addition, in an organic farm, pigs must be fed with food cultivated in organic farms in which the use of pesticides and chemical fertilizers is prohibited. Additionally, the use of antibiotics and drugs in pigs is restricted to one treatment per life and the waiting time before slaughter is longer than in a non-organic pig farm (Kijlstra *et al*., 2004). Finally, there is a much simpler system of pig maintenance known as a backyard system or smallholder. This is the most traditional method of keeping pigs which is still being used in numerous countries. The backyard system is in general, a free-range system for familial self-sufficiency. In this system pigs usually roam free around the household or are housed in low input and low cost pens. Pigs are fed with leftovers, garbage and by scavenging in the surroundings. The sanitary conditions are minimal or absent and pigs usually coexist with more animal species such as chickens and cats. The use of a local breed of pig is common in this type of system as they can remain productive despite the poor living conditions. Although this type of farming can be found in every country, it is more commonly found in developing countries or among low-income families and populations in developed countries (FAO, 2010).

1.6.2 Epidemiology of *T. gondii* in pigs

Most epidemiological surveys in swine, as in humans, provide infection rates as seroprevalence data. Epidemiology in pigs is complex as it varies greatly according to geographical area, farm management, rearing and age. Moreover, the source of the pig often differs, making direct comparisons between studies problematic. For example, in several studies in which pigs were sampled at abattoirs, the husbandry system remains unknown. In other number of studies in which mixed husbandry systems were used, the number of samples or prevalence per system was not given. Other studies mentioned that pigs were sampled at farms, however the type of farm was not stated. In addition, the age of the hogs is only provided in around half of the total studies. Furthermore, the diagnostic method used varies greatly between studies. Table 1.1 in Appendix I shows a comprehensive compilation of the *T. gondii* seroprevalence studies in the domestic pig between 1990 until 14th October 2016 by country, farming system and age of the pigs when available. Nevertheless, it has been a challenge to make a comparison between geographical areas and production systems due to the scattered data.

In North America, most of the studies have been carried out in the USA. *T. gondii* seroprevalence in pigs raised in intensive farms in this country is monitored by the National Animal Heath Monitoring System (NAHMS). The NAHMS provides an in-depth study across several states of the country which monitor the disease in livestock on a frequent basis. Values reported in the last NAHMS varied from 2.6% to 3.8% (Hill *et al*., 2010, Hill, 2014). Gebreyes *et al*., (2008) also studied the prevalence in the USA and noted the low prevalence of 1.1%. Poljak *et al*., (2008) investigated the seroprevalence in the intensive farming system in Canada and found the low prevalence of 0.75%. In contrast, in Mexico, *T. gondii* seroprevalence in hogs raised in the same type of farms varied from values as low as 0% to values as high as 100% in farms further South (Alvarado-Esquivel *et al*., 2015, Ortega-Pacheco *et al*., 2011). Pigs raised in free range conditions in Mexico and the USA showed in both countries extremely high values ranging from 9.1% to 75% and from 6.8% to 92.7% respectively (Alvarado-Esquivel *et al*., 2011b, Jiménez-Coello *et al*., 2013, Dubey *et al*., 2002a, Dubey *et al*., 2008, Dubey *et al*., 2012c) (figure 1.6A and 1.6B).

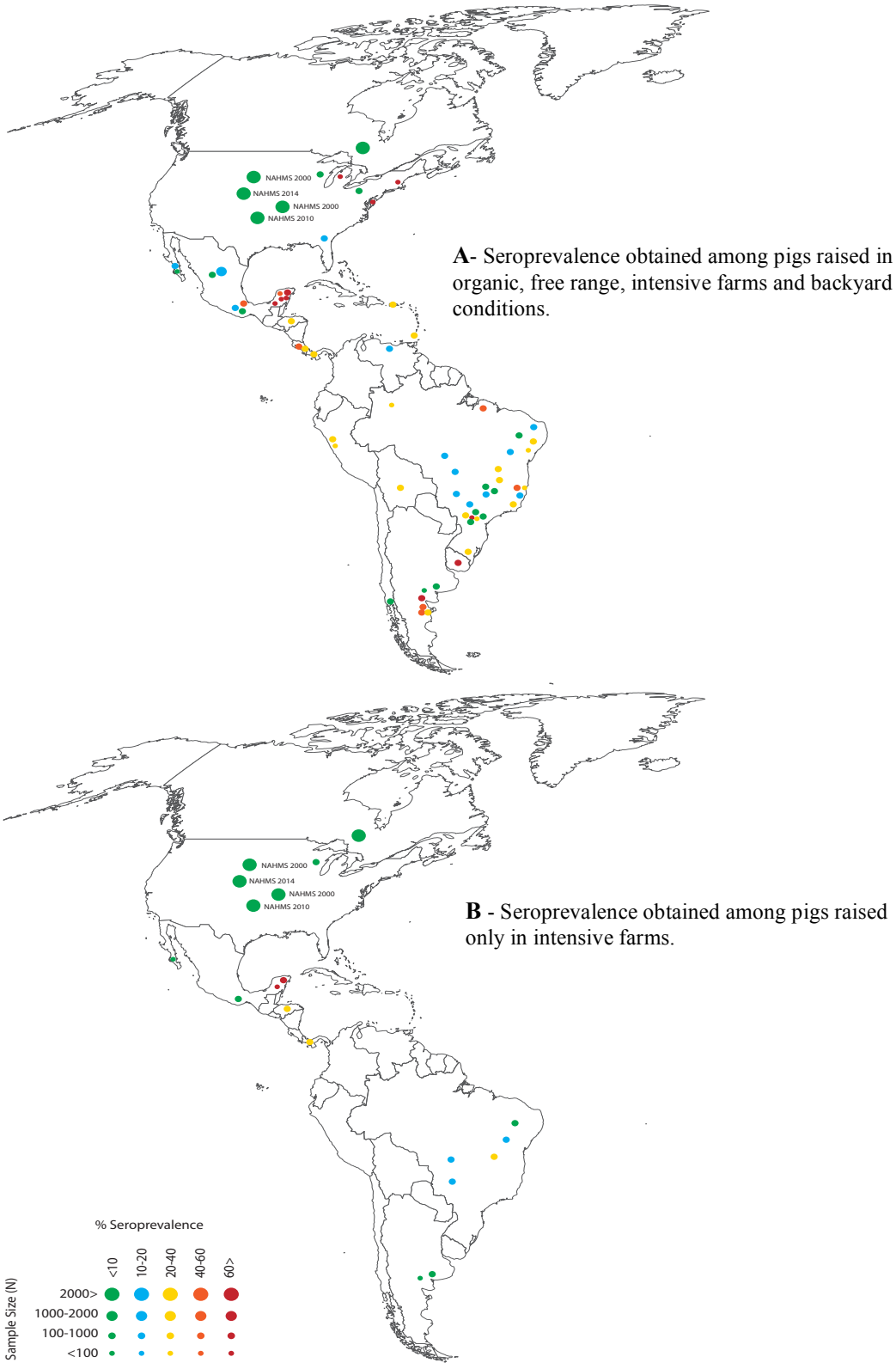

Figure 1.6 Seroprevalence of *T. gondii* in pigs from North, Central and South America 2000-2016. Prevalences data were obtained from the collection of studies in table 1.1. A-The map shows the seroprevalences obtained in pigs raised in backyard, organic, free range and intensive systems. B- The map shows seroprevalences obtained only among pigs raised in intensive farms.

In South America, pigs sampled in intensive farms or at the abattoir showed higher values in comparison to USA and Canada (figure 1.6B). Values of 8.8%, 32%, 32.1%, 27.7%, from 0% to 36.2% and from 4.5% to 37.8% were obtained in Chile, Honduras, Panama, Peru, Brazil and Argentina (Muñoz-Zanzi *et al*., 2012, Pezerico *et al*., 2007, Bezerra *et al*., 2009, Correa *et al*., 2008, Saavedra and Ortega, 2004, Venturini *et al*., 2004). In contrast, free range pigs showed a seroprevalence of 48.5% in Bolivia, from 75.2% to 83.2% (depending on the test used) in Argentina and from 17% to 48.8% in Brazil (Orellana *et al*., 2004, Bonna *et al*., 2006, Piassa *et al*., 2010, Basso *et al*., 2013, Venturini *et al*., 2004) (figure 1.6A, 1.6B).

Regarding Europe, prevalence appears to be higher in central countries and southern countries. For example, the seroprevalence among intensive farms in Switzerland ranged from 14% in finisher pigs (5-6 month old) to 36% in sows or breeding pigs (2-5 years old). In Poland, Germany and Czech Republic surveys in abattoirs also showed prevalences from 14% to 36% in finisher pigs (Berger-Schoch *et al*., 2011, Sroka *et al*., 2008, Sroka *et al*., 2011, Sedlák and Bártová, 2007, Bártová and Sedlák, 2011). In Romania prevalences ranged from 0% to 12.4% on intensive farms while in free range farms the prevalence reached 30.2%. Serbia showed prevalences as high as 49% in sows but in finisher pigs it ranged from 2.2% to 15.2% among samples collected at the abattoir and in intensive farms (Klun *et al*., 2006, Klun *et al*., 2011). Studies in intensive farming in Spain and Italy noted a prevalence of 24.5% in finishers and sows and 16.2% in finishers only respectively (Herrero *et al*., 2016, Veronesi *et al*., 2011). In contrast, the higher prevalences of 73.5% and 95.2% were obtained among pigs raised in free range and organic farms also in Spain and Italy (Hernández *et al*., 2014, Bacci *et al*., 2015). Conversely, pigs in Switzerland and Germany raised in extensive systems obtained lower or similar prevalences (2.8%-13%) than reported in confined pigs (Berger-Schoch *et al*., 2011, De Buhr *et al*., 2008). Countries located more westerly, the Netherlands and France, had the lowest prevalence in pigs which varied from 0% to 2.7% in intensive farms and from 1.2% to 10.9% in organic farms (Djokic *et al*., 2015, Van der Giessen *et al*., 2007). Steinparzer *et al*., (2015) investigated the *T. gondii* seroprevalence in pigs raised in farms from Austria and noted very low prevalence of *T. gondii* in confined pigs which ranged from 1.8% to 4.3% depending on the serological test used. However, a much higher seroprevalence was obtained in Austria, which comprised from 63.5% to 69.2% (depending on the test), for pigs raised in organic farms (Steinparzer *et al*., 2015). The prevalence among slaughtered pigs in North European countries

such as Ireland, UK, Latvia and Sweden ranged from 0.4% to 7.4% among finisher pigs and younger. In contrast, sows in Sweden had a prevalence of 17.3% (Lundén *et al*., 2002, Opsteegh *et al*., 2016, Powell *et al*., 2015, Halová *et al*., 2013, Deksne *et al*., 2012).

In Asia, most of the epidemiological studies in pigs have been concentrated in China in which a seroprevalence ranging from 4.3% to 31.3% was obtained in confined pigs (Chang *et al*., 2013, Xu *et al*., 2014) while in backyard and free range systems the prevalence ranged from 53% to 70% (Yu *et al*., 2011, Li *et al*., 2015). Studies in Taiwan have shown prevalences ranging from 10.1% to 28.8% in slaughtered pigs (Fan *et al*., 2004, Tsai *et al*., 2007). Huong and Dubey, (2007) found a prevalence of 27.7% among hogs raised in intensive farms. The few other studies performed in Asia obtained much lower values of 0% and 5.3% in Japan and Malaysia respectively among pigs sampled in intensive farms and 6.3% in Indonesia among pigs sampled at the slaughterhouse (Inoue *et al*., 2001, Matsuo *et al*., 2014, Chandrawathani *et al*., 2008).

Finally, in Africa, epidemiological studies are also limited for this animal species. Studies have shown that the seroprevalence in pigs is in general high. For example, the higher value was reported in Moghazy *et al.,* (2011) in which a value ranging from 35.5% to 56.6% was found among pigs sampled at the abattoir in Egypt. The rest of the studies carried out in this continent reported prevalences ranging from 19.8% to 32.1% in Zimbawe, Nigeria, Ethiopia and Burkina Faso for pigs raised in intensive farming or studies at the abattoirs (Hove *et al*., 2005, Onyiche and Ademola, 2015, Gebremedhin *et al*., 2015, Moghazy *et al*., 2011, Bamba *et al*., 2016). Arko-Mensah *et al*., (2000) reported 40.6% seroprevalence among pigs raised in a free-range system in Ghana. Pigs raised in the same farming system showed a prevalence of 35.7% in Zimbabwe (Hove *et al*., 2005).

The prevalence data clearly shows that outdoor rearing is associated with a higher prevalence of *T. gondii* in pigs, this occurs probably because of an increased probability of oocyst ingestion and of tissue cysts consumption from intermediate hosts like mice or birds. Several epidemiological studies have found higher prevalences of *T. gondii* antibodies in backyard, organic and extensive farms than in intensive farms (table 1.1, Appendix I). For example, Venturini *et al*., (2004) studied the disease between different swine farm systems in Argentina and obtained the high percentage of prevalence of 40.2% in outdoors farming while

in intensive farms it was 4.5%. In the Netherlands, this pattern is also observed although due to the lower prevalences of this area in comparison to South America, the difference was less marked. For instance, Kijlstra *et al*., (2004) compared the seroprevalence to *T. gondii* between different farming systems and found 4.7%, 1.2% and 0% of seroprevalence among pigs raised in free range, organic and intensive farms respectively. A more recent study in the Netherlands reported similar results of 5.6%, 2.7% and 0.4% of seroprevalence among pigs raised in free range, organic and intensive farms respectively (Van der Giessen *et al*., 2007). The reason why free range farming had higher prevalence than organic farming is not clear as both are extensive systems with outdoor access. Van der Giessen *et al*., (2007) noted that prevalence between free range farms ranged from 0% to 40% suggesting that farm management of each individual farm played an important role independently of the outdoor access. Interestingly, Berger-Schoch *et al*., (2011) found a slightly higher prevalence among finisher pigs raised in intensive farms (14%) when compared with pigs raised in free-range farms (13%). This shows that free roaming is not the only determinant for an increased risk of *T. gondii* infection, but also the level of biosecurity measures in the routine of the farm.

Interestingly, a decline of seroprevalence in pigs has been observed in some industrialized countries. For example, results obtained through the NAHMS in USA, clearly showed a drop in the *T. gondii* prevalence since 1990. For example, the *T. gondii* seroprevalence was 43% in sows in 1991, this dropped to a 20% in 1996, 15% in 1998 and finally, the last study in sows showed a 6% of prevalence in 2000. NAHMS surveys in finisher and younger pigs also showed a decrease from 23% in 1991 to 3.2 % in 1998, 0.9% in 2000, 2.8% in 2010 and the last survey the seroprevalence was 3.8% in finishers in 2014 (table 1.1 in appendix I). This decrease over time has also been observed in some European countries; for example, in Germany the prevalence has declined in finisher pigs raised in intensive farms. This prevalence was 20.5% in the year 2003 and dropped to 5.2% in 2005 and this decreased to 3.8% in 2008 among pigs raised in intensive farming (De Buhr *et al*., 2008, Schulzig *et al*., 2005, Fehlhaber *et al*., 2003). The Netherlands experienced a much more marked decline of *T. gondii* seroprevalence as it dropped from 2.1% in 1991 to 1.8% in 1995 to reach the lowest 0% in 2004 and remain <1% in intensive farming (Berends *et al*., 1991, Van Knapen *et al*., 1995, Kijlstra *et al*., 2004, Van der Giessen *et al*., 2007, Swanenburg *et al*., 2015). This reduction in *T. gondii* prevalence in swine has been attributed to sanitary improvements in intensive farming. Routine practices such

rodent control, hygienic feed handling procedures (closed feeders, automatic drinkers, secure storage of food) or rigorous exclusion of cats and other animal species which are extensively used in industrialized countries reduce the risk of exposure of pigs to *T. gondii* (Jones *et al*., 2007).

In addition, *T. gondii* seroprevalence increases with increasing pig age. Higher seroprevalence has been demonstrated in breeding pigs (sows and boars) than in finishers (fattening pigs at slaughter age) in several studies. For example, Klun *et al*., (2011) noted a seroprevalence of 30% in sows while in finisher pigs it was only 8.3% in intensive farming in Serbia. Likewise, Pastiu *et al*., (2013), also in intensive farming, found a seroprevalence in sows of 12.4% while in finisher pigs was as low as 0% in Romania. Interestingly, Berger-Schoch *et al*., (2011) found 36% prevalence in sows raised in intensive farming while in finisher pigs raised in free range it was 13%. This contrast in prevalence is explained probably because fattening pigs are slaughtered around five to six months while reproductive pigs have a life span of two to five years and it has been demonstrated that prevalence increases with the age in pigs (García-Bocanegra *et al*., 2010a, García-Bocanegra *et al*., 2010b, Villari *et al*., 2009, Dubey, 2009b) like in other animal species.

1.6.3 Sources of *T. gondii* in pigs

Ingestion of oocysts, cannibalism and eating infected mice or other animals have been all reported as sources of *T. gondii* infection for pigs (Lehmann *et al*., 2003, Dubey, 1986, Vidotto *et al*., 1990, Dubey *et al*., 1986b). An extensive number of studies has suggested oocysts to be the primary source of *T. gondii* infection for pigs (Okamoto *et al*., 1989, Penkert, 1973, Weissenböck and Dubey, 1993, Garcia *et al*., 1999, Matos *et al*., 1999; Tsutsui *et al*., 2003, García-Bocanegra *et al*., 2010a). The reason for this was because most outbreaks in pigs have been connected with the ingestion of food or soil contaminated with oocysts. In addition, it is still unknown how many bradyzoites are needed to infect a pig, but it has been demonstrated that pigs can became infected with as little as one oocyst (Dubey *et al*., 1996a, Dubey *et al*., 1996b). Meerburg *et al*., (2006) studied the source of infection in 2791 pigs from 41 organic farms in which the prevalence ranged from 0% to 97% (mean 10%) and found that oocysts and feeding goat whey to pigs were closely related to the transmission of the disease. Mateus-

Pinnilla *et al*., (1999) evaluated the effect on *T. gondii* prevalence in seven pig farms after vaccination of the trapped cats. The vaccine consisted of three oral administrations of bradyzoites of the mutant strain T-263 which was shown to be capable of interrupting the oocyst shedding process (Freyre *et al*., 1993). Mateus-Pinilla *et al*., (1999) observed that the prevalence in the finishing pigs at five farms decreased from 6.7%, 2.2%, 2.2%, 14.4%, 5.6% to 1.1%, 1.1%, 0%, 0%, and 1.1% respectively after three years of the completion of the trial. The remaining two farms showed no effect on the prevalence. Sows were also sampled in six of the farms, for these animals the prevalence decreased in four farms from 23.3%, 25.3%, 22.5% and 6.6% to 6.6%, 3.2%, 20% and 3.3% respectively. On the other hand, two farms suffered an increased prevalence in sows after the trial from 3.3% and 26.2% to 10% and 29.6% respectively. Mateus-Pinilla *et al*., (1999) concluded that the main source of infection on those farms were oocysts due to the general decrease of the incidence of the disease after the trial. However, the vaccine could have been decreasing the seroprevalence also in other small mammals such as rodents which could be prey for pigs. Several studies have found an association between a high *T. gondii* seroprevalence in pigs and the presence of rodents or other small mammals within the farm (Meerburg *et al*., 2006, Dubey and Beattie, 1988, Assadi-Rad *et al*., 1995, Villari *et al*., 2009). Rodents are interesting because these can harbour the infection for several generations without the necessity of the presence of the felid host (Owen and Trees, 1998). Lubroth *et al* (1983) suggested that eating mice was the most likely source of infection in pigs from two farms in Georgia. In contrast, some authors suggested that the role of rodents in pig farms was as a source for cats rather than being the direct source of *T. gondii* for pigs (Garcia *et al*., 1999, Weigel *et al*., 1995b). Kijlstra *et al*., (2008) designed an experiment to try to gain a better understanding of the issue which consisted of a 4-month rodent control campaign and serological follow up of the pigs in three farms from the Netherlands. The three farms had a prevalence of 10.9%, 16.7% and 8.9% before the experiment which dropped to 3.3%, 10% and 0% respectively after the rodent control. Furthermore, it was noted that two months after the campaign had finished, the prevalence again increased in two of the farms from 3.3% to 5.2% and from 0% to 4.7%. Kijlstra *et al*., (2008) suggested that due to the rapid effect upon *T. gondii* prevalence on the farms, the most probable situation was that the rodents were being eaten by those pigs. Dubey *et al.,* (1986b) studied the sources of infections in a *Toxoplasma* enzootic farm (96% of seroprevalence) and found that cannibalism was the main source of

infection. Cannibalism is a common habit among pigs and one body can be sources of infection for several pigs. In addition, tail and ear biting are frequent findings on confined farms and *T. gondii* has been found in tails (Dubey *et al.,* 1988). Dubey *et al*., (1995a) studied the source of *T. gondii* infection in pigs in 47 farms in USA and isolated *T. gondii* from rodents, food, and soil. Dubey *et al*., (1995a) concluded that all of these routes were probably acting as sources of infection of *T. gondii*. Hill *et al*., (2010) also investigated the source of infection in USA by surveying 6238 pigs on 185 farms and by interviewing farmers concluding that ingestion rodents, oocysts and pig carcasses were all possible source of *T. gondii* in the USA.

1.6.4 Clinical manifestations in pigs

1.6.4.1 Acquired toxoplasmosis

Toxoplasmosis in pigs is in general subclinical and piglets have been reported to be more susceptible than adult pigs to the clinical infection (Dubey, 1986). Clinical toxoplasmosis in pigs, has been reported mostly in cases of outbreaks. One of the reasons could be because of the non-specificity of the symptomatology of the disease, resulting in it remaining in most cases unnoticed. The most frequent symptoms are dyspnea, cough, fever, anorexia, weakness and reproductive problems in the pregnant sow. Although respiratory symptoms are the most common clinical manifestation of the infection in this animal species, gastrointestinal and neurological problems such as diarrhoea and ataxia have been also noticed. Clinical toxoplasmosis has been reported mostly in China, Korea, Taiwan, and Japan (Okamoto *et al*., 1989, Roh *et al*., 1997, Liao *et al*., 2006, Haritani *et al*., 1988, Chang *et al*., 1990, Kumagai *et al*., 1988). Liao *et al*., (2006) reported the death of two sows after acute toxoplasmosis in China. Clinical toxoplasmosis has been reported also in Austria, where 13 pigs were ill after ingestion of contaminated food with oocysts. Manifestation of the disease included fever, dyspnea, bloody diarrhoea and convulsions. Seven of these animals died (Weissenböck and Dubey, 1993). In 1994, four outbreaks occurred in four fattening farms in Italy. The clinical signs were observed in all pig groups (weaned, growers and finisher). Depending on the farm, the morbidity and mortality ranged from 50% to 60% and 10% to 42% respectively. The observed clinical signs among the affected pigs were anorexia, fever, depression, prostration, nasal discharge and skin cyanosis predominantly in ears, snout, neck and ventral abdomen (Gelmetti *et al*., 1999). In

addition, in China, in an intensive farm, another outbreak caused 57% of morbidity and 2% of mortality in 960 fattening pigs. The clinical signs observed were anorexia, fever and depression (Li *et al*., 2010).

1.6.4.2 Congenital toxoplasmosis

Infected sows, symptomatic or asymptomatic, can transmit the infection to the foetus. A congenital infection in the foetus can lead to an abortion, reabsorption, stillbirth or birth of a live infected piglet which could die, manifest symptoms or remain asymptomatic. Kumagai *et al*., (1988) reported a sow that gave birth to four stillborn and three live piglets in Japan. The three survivors were ill with neurological symptoms and died two weeks after birth. On necropsy piglets showed toxoplasmic encephalitis, pneumonitis and lymphadenitis. No information was given regarding the dam. Haritani *et al*., (1988) reported also in Japan toxoplasmic encephalitis and pneumonitis in four stillborn and one live piglet. In 1996, neonatal toxoplasmosis was described in Brazil where the parasite was demonstrated in tissues of ten neonatal infected piglets, four stillborn and two aborted foetuses (Giraldi *et al*., 1996). A more recent case of congenital toxoplasmosis was reported in a backyard farm in Thailand (Thiptara *et al*., 2006). A clinically healthy sow gave birth to three dead piglets and six live ones which developed clinical signs three weeks after birth. The clinical manifestations in those piglets were anorexia, fever, dyspnea, diarrhoea and convulsions. One of those pigs died of acute toxoplasmosis (Thiptara *et al*., 2006). In Korea, there was a large outbreak of abortions in a large intensive farm. A total of 84 sows were affected, the symptomatology included fever, anorexia, vomiting, depression, weakness and abortions. The rate of abortion was 44% and the sow mortality was 19% (Kim *et al*., 2009). Venturini *et al*., (1999) studied the level of *T. gondii* seroprevalence in 738 stillborn piglets and only 2% were demonstrated to have developed antibodies against *T. gondii*. This could be an indication that the level of congenital transmission is low in pigs from Argentina. However, it may be that some piglets did not react to the infection or the method used was not sensitive enough. Basso *et al*., (2015) studied the seroprevalence of *T. gondii* in 108 sows with reproductive problems raised in 59 different farms from Switzerland. The reproductive problems were abortions, stillbirths and mummifications. The frequency of anti *T. gondii* antibodies was higher than in Venturini *et al*., (1999) study (3.8%).

1.7 Key questions and general hypotheses

Despite the fact that toxoplasmosis has a high seroprevalence among the human population of Yucatan and pork consumption was suggested to be a risk factor; the studies related to *T. gondii* in pigs were limited and performed only with a non-validated serological test. Nevertheless, Yucatan is a tropical area which has shown higher *T. gondii* seroprevalence in intensive pig farms in comparison to Canada, USA and northern areas of Mexico (figure 1.6B). A much higher prevalence was expected in Yucatan than the USA and northern areas of Mexico as publications reported prevalences as high as 100% (Ortega-Pacheco *et al.,* 2011). This high prevalence was not expected to be an isolated event as a cross-sectional study in which 429 pigs were sampled from 39 different intensive farms of the area showed 95.8% of seroprevalence (Ortega-Pacheco *et al.,* 2013). In addition, Yucatan is the most southern state of Mexico, which borders with the highly prevalent Central and South American countries. Furthermore, Ortega-Pacheco *et al.,* (2013) noted 'relaxed' sanitary standards in the intensive pig farms in this area. Therefore, a hypothesis of high *T gondii* prevalence in pigs from Yucatan was to be tested. Well validated serological tests were used along with PCR screening to estimate the prevalence among hogs across different farms.

The other key question of this thesis was related to the population genetic structure of *T. gondii* in the geographical area of study. A large body of literature has demonstrated a much higher genetic diversity in Central and South American *T. gondii* isolates in comparison to those from North America. Mexico is fascinating as it is located on the border of Guatemala; which is amongst the countries which had the higher *T. gondii* genetic diversity from South America (Rajendran *et al.,* 2012); and also, the border with the USA; which is amongst the countries with a lower *T. gondii* genetic diversity on the American continent (Dubey and Su, 2009). *T. gondii* isolates from Northern areas of Mexico, which also reported much lower *T. gondii* seroprevalence than in Yucatan, showed an intermediate diversity; however, a higher *T. gondii* genetic diversity was expected in Yucatan. Therefore, a hypothesis of greater genetic diversity in *T. gondii* strains of pigs from Yucatan in comparison to Northern areas of Mexico and the rest of the North American continent was to be tested. The hypothesis of higher *T. gondii* genetic diversity on pig farms from Yucatan was based on the higher seroprevalence reported in the pig industry of the area, the rural conditions of those pig farms and the proximity to South America.

CHAPTER 2

Detection of *T. gondii* IgG antibodies in pig serum

2.1 Introduction

The main aim of this chapter was to investigate the seroprevalence of *T. gondii* in pigs from Yucatan by using a set of well validated serological tests in this animal species. Yucatan is a state located in the Southeast of Mexico. Mexico is interesting as it bridges Latin America, a region where in general *Toxoplasma* has high prevalence in humans, and USA, where the prevalence has been reported to be much lower. The last national survey in USA in 2010 showed a seroprevalence of 12.4% in the human population (Jones *et al*., 2014). In contrast, in Latin America prevalence is much higher in the general population in many countries such as Argentina (49%-59%), Brazil (50%-92%), Costa Rica (55%-76%), Guatemala (44%), Colombia (46%-54%), Cuba (44%-71%) and Mexico (44%) (Pappas *et al*., 2009, Dubey, 2010a, Flegr *et al*., 2014, Caballero-Ortega *et al*., 2012).

This distribution of disease prevalence has a similar pattern in pigs, being lower in USA and higher in Latin America (figure 1.6A, 1.6B). In the USA, the last national survey showed a prevalence of 3.8% among 5688 adult pigs (18-30 weeks) by ELISA (Hill *et al*., 2014). In general, higher values in pigs have also been found in Latin American countries. Panama showed a prevalence of 32% by Indirect Hemagglutination Test (IHAT) among 290 pigs raised in intensive farms (Correa *et al*., 2008), Honduras showed a prevalence of 32% by ELISA among 150 pigs raised in intensive systems (Zúniga and Lorca, 2010). Mexico showed the highest prevalence of 95.8% in Yucatan in a study of 429 adult pigs (18-20 weeks) by ELISA (Ortega-Pacheco *et al*., 2013) (figure 1.6B, table 1.1). However, these prevalences are difficult to compare between studies as different serological techniques were used.

The reason why the prevalence is in general higher in Central and South America is not fully understood. However, it is suggested that one factor might be the the climate, as tropical, warmer and humid climates are more favourable for the oocyst survival than hot, dry and artic climates (Gangneux and Dardé, 2012, Tenter *et al*., 2000, Meerburg and Kijlstra.,

2009). As discussed in section 1.6 prevalence of *T. gondii* in pigs is also influenced by management systems (Dubey, 2009b). In poorly managed and non-confinement systems the prevalence of *T. gondii* is general higher. Interestingly, sporadic studies in the USA had shown prevalences comparable to Latin America studies (70.8%-92.7%) in *T gondii* endemic farms with poor management conditions such as open cat access, lack of rodent control, non-confinement measures or where the pigs were fed with carcasses from other animals, including pigs (Dubey *et al*., 2002, Dubey *et al*., 2008, Gamble *et al*., 1999). Non-confinement systems or less 'technified' farms such backyards or smallholdings are more common in low income countries (FAO, 2010). In addition, biosecurity standards in intensive farms may differ between countries. For example, in tropical areas such as Brazil, Panama, Costa Rica or Mexico most intensive farms remain open to facilitate the ventilation (figure 1.5). This allows easier access for other animals such as cats or birds which could be infected with *T. gondii*. These differences could influence the generalized higher prevalence of *Toxoplasma* in pigs in Central and South America (FAO, 2010).

This study is focused particularly in Yucatan because it has been reported as a highly endemic area of toxoplasmosis with prevalences reaching 70% in the human population (Caballero-Ortega *et al.,* 2012). Not only were high rates reported in humans but also in pigs; according to Ortega-Pacheco *et al*., (2013) disease levels reached 95.8% in adult pigs (18-20 weeks) raised in intensive farming systems. Despite the fact that pork is likely to be an important source of *T. gondii* infection, there is currently no testing for *T. gondii* in pigs at the time of slaughter. Due to this high potential risk, adequate surveillance and monitoring of the disease in humans and animals in Yucatan is essential to control the incidence of this parasitic disease. The aim of my research was to investigate the prevalence of the disease in the swine intensive production system. The prevalence was investigated in two different ecological areas. Management and farm characteristics were obtained for all farms by interviewing the farmers. The work also compares serological detection methods and discusses the importance of using a standardized diagnosis test in order to have a correct knowledge of the disease impact.

2.1.1 Serological methods used in pigs

The Sabin-Feldman Dye Test (DT) owes the name to its discoverers Albert B Sabin and Harry A Feldman, (1948). It was the first serological test that could detect low levels of anti-*T. gondii* antibodies in human serum; and after more than 60 years, the DT remains the gold standard for diagnosis of human toxoplasmosis. The DT is considered as the reference test according to WHO for human diagnosis (Reiter-Owona *et al*., 1999). DT detects IgA, IgM and IgG antibodies (Jenum *et al*., 1998, Reiter-Owona *et al*., 1999). In this test, the patient's serum is incubated with live tachyzoites, complement from human serum and methylene blue as a dye. The presence of antibodies against *T. gondii* in the serum induces a complement-mediated cytolysis of the tachyzoite membrane and as a result, tachyzoites do not retain the vital dye methylene blue. The DT is mainly used in human diagnosis but it is restricted to a low number of laboratories, due to the fact that it is an expensive, time consuming and hazardous technique. One main asset of the test, however is that it is not species specific. The DT has therefore been used for many years as a serological diagnosis technique in many animal species. After the DT was discovered, it has been used in pigs for many years (Weinman and Chandler, 1956, Schenk *et al*., 1976, Tamayo *et al*., 1990, Freyre *et al*., 1991, Hejlíček and Literák, 1993, Hejlíček *et al*., 1997, Vostalová *et al*., 2000) and it is considered highly specific and sensitive. Hellesnes *et al*., (1978) found that the success of *T. gondii* isolation was related to DT titre: *T. gondii* was isolated from 7 of 18 pigs which were DT positive at a 1:4 dilution, from 8 of 10 pigs with 1:10 dilution, from 5 of 5 with 1:50 dilution and from none from 30 DT negative pigs (table 2.1).

Table 2.1 Comparison between DT titre and *T. gondii* isolation success. Isolation from diaphragms of 63 naturally infected pigs by bioassay in mice. Data taken from Hellesnes *et al.,* 1978.

DT titre	Number of pigs	Number of isolates	Isolation success
<4	30	0	0%
4	18	7	38.8%
10	10	8	80%
50	5	5	100%
Total	63	20	31.7%

In 1980 Desmonts and Remington described the *Toxoplasma* Agglutination Test (AG) and suggested that it was as sensitive and specific as the DT. Seven years later, the AG was

improved by Dubey and Desmonts (1987) to produce the Modified Agglutination Test (MAT). The MAT test detects IgG antibodies (Dubey, 1997c). In this test dilutions of sera are added to U bottomed 96 well microtiter plates and the serum is incubated with formalin fixed *Toxoplasma* tachyzoites, Evans blue dye and 2-mercapthoetanol. In the presence of positive serum, tachyzoites agglutinate, while in the presence of negative serum the tachyzoites will precipitate at the bottom of the well creating a blue dot. As with the DT, the MAT is not considered species-specific (OIE, World Organization of Animal Health, 2008) hence it has been widely used in many animal species. The sensitivity and specificity have been validated in pigs (Dubey, 1997c, Dubey *et al.,* 1995c). Dubey *et al*., (1995b) found also that the higher the MAT titre the more likely the success of *T. gondii* isolation in pigs. However, some isolates were obtained from pigs with antibody titres below the cut off (table 2.2).

Table 2.2 Comparison between MAT titres and *T. gondii* isolation success. Isolation from hearts of 1000 naturally infected sows by bioassay in mice and cats. Data taken from Dubey *et al.,* (1995b).

MAT titre	Number of Sows	Number of isolates	Isolation success
<20	778	29	3.7%
20	35	13	37.1%
40	21	8	38.1%
80	50	30	60%
200	32	24	75%
400	31	24	77%
≥800	53	42	79.2
Total	1000	170	17%

ELISA techniques (Enzyme-Linked Immunoabsorbent Assay) are usually based on antibody recognition of immobilised antigen. Bound antibodies are selected by the addition of a secondary enzyme-linked antibody, which produces a colorimetric reaction that can be measured. ELISA is a flexible method that can be adapted to specific purposes for example for use in different species or for detection of different sub types of immunoglobulins. IgM, IgA and IgE can be measured, however in livestock detection of IgG is more common. ELISA is a popular and practical method because it can be automated to rapidly screen large numbers of samples. One issue with ELISA however is that most of them are developed for humans. ELISAs used for animal diagnosis are usually in-house (Figueroa *et al*., 2006, Castillo-Morales *et al*., 2012) or are commercial tests available for humans which have been modified by adding

a species specific conjugate (Schaefer *et al*., 2011, Schaefer *et al*., 2012, Nematollahi *et al*., 2014, Jimenez-Coello *et al.,* 2013, Ortega-Pacheco *et al.,* 2013, Hernández-Cortazar *et al.,* 2014, Dzib-Paredes *et al*., 2016). Moreover, only a few of these tests have been validated in animals. In addition, as most are in-house, the antigens, reagents and controls are rarely standardized so it is difficult to compare results between laboratories. Because of the flexibility of the method, ELISA techniques are widely used for sero-diagnosis of *T. gondii* in humans and animals, including pigs. The performance of ELISA also has been compared with *T. gondii* isolation in pigs. Gamble *et al*., (2005) found that of the 70 positive bioassayed pigs 62 were ELISA IgG positive.

In the IFAT (Indirect Fluorescent Antibody Test) patient sera is incubated with whole killed tachyzoites and the presence of antibodies to *Toxoplasma* is revealed by the addition of a fluorescent-labelled anti-species antibody. This is a simple method but a fluorescence microscope is required to read the result. Omata *et al.,* (1994) correlated the results of bioassay in 109 pigs with IFAT titre and obtained higher isolation success in animals with a higher titre with IFAT, as with MAT, some isolates were obtained with animals with low titres (table 2.3).

Table 2.3 Comparison between IFAT titre and *T. gondii* isolation success. Isolation from diaphragms of 109 naturally infected pigs by bioassay in mice. Data taken from Omata *et al*., 1994.

IFAT titre	Number of Sows	Number of isolates	Isolation success
<16	40	2	5%
64-256	57	7	12.3%
≥1024	12	5	41.6%
Total	109	14	12.8%

Finally, there are other serological techniques such as the LAT (Latex Agglutination Test) in which the antigen is coated with latex particles and it agglutinates in the presence of anti *T. gondii* antibodies and the IHAT (Indirect Hemagglutination Test), where antigen is coated with red blood cells and hemaglutination occurs in the presence of positive sera. The use of IHAT and LAT in pigs is much lower than ELISA, IFAT and MAT and few studies have been published (Durfee *et al.,* 1974, Tsubota *et al*., 1977, Dubey *et al*., 1995c, Dubey *et al*., 1997a) and in these the sensitivity is generally lower.

Sensitivity and specificity have been compared between MAT, ELISA, LAT, IHAT and IFAT (table 2.4). Dubey *et al*., (1995c) compared the performance of MAT, LAT, IHAT and ELISA in 1000 naturally infected sows based on the isolation of *T. gondii* and obtained a sensitivity and specificity of 82.9% and 90.29% for MAT, 29.4% and 98.3% for IHAT, 45.9% and 96.9% for LAT, and 72.9% and 85.9% for ELISA respectively. Sroka *et al*., (2008) compared the MAT, LAT, ELISA based on the IFAT results and the specificity and sensitivity was 100% and 77.8% for the MAT test, followed by a 95.7% and 88.9% for the ELISA test and a 91.4% and 47.2% for LAT test. Overall, IHAT and LAT have shown to be less sensitive than ELISA and MAT.

This low performance in terms of sensitivity of IHA and LAT was also noted in experimentally infected pigs, when 2 of 16 pigs remained seronegative after being inoculated with 1000 oocysts. In this study the most sensitive tests were, in order, the MAT, DT and ELISA (100% of sensitivity), however one of the negative inoculated pigs developed high antibody titres in the ELISA test (Dubey *et al.,* 1997a). Similarly, when pigs were infected with a low number of 1-10 oocysts, MAT, DT and ELISA also were more sensitive since these detected antibodies in 39 of 40 inoculated animals when only 26 and 11 were detected via LAT and IHA respectively (Dubey *et al.,* 1996b).

Cross-reactivity against other pathogens has been investigated with MAT, ELISA and DT. No cross-reactivity was found when the specificity of anti-*Toxoplasma* IgG-ELISA was evaluated against several pig pathogens including *Ascaris suum, Trichinella spiralis, Isospora suis, Samonella spp, Yersnia or Actinobacillus spp* (Lind *et al.,* 1997). Furthermore, an anti-*Toxoplasma* IgG avidin-biotin ELISA did not cross-react with *Actinobacillus pleuropneumoniae*, Hog Cholera virus, Porcine Reproductive and Respiratory Syndrome virus, Pseudorabies virus and *Ascaris suum* (Lin and Hung, 1996). However, when Lind *et al.,* (1997) tested the specificity of IgG-ELISA with the closely related coccidian *Sarcocystis miescheriana* they found 3 out of 9 pigs infected with 50 000 sporocysts of this parasite showed cross reactivity with *Toxoplasma*. This cross-reactivity was not found by MAT test after infecting 8 pigs with 500,000, 1 million or 3 million of sporocysts of *Sarcocystis miescheriana* (Dubey, 1997c). Lin and Hung, (1996) did not evaluate the avidin-biotin ELISA with *Sarcocystis miescheriana.* It is suggested that this cross reactivity was related to the source of the antigen;

in ELISA, the antibody could have reacted to intracellular antigens from the parasite lysate while in MAT this phenomenon did not occur as intact tachyzoites were used as antigen (Dubey, 2009b). The specificity of the MAT test has been validated against other swine pathogens such as *Ascaris suum, Trichuris suis, Trichinella spiralis,* and several viruses including Porcine Parvovirus, Porcine Reovirus type 3, Porcine Rotavirus, Pseudorabies, Swine Influenza, Porcine Epidemic Diarrhea virus, Encephalomyocarditis virus, Hemagglutinating Encephalomyelitis virus and no cross reactivity has been observed (Dubey, 1997c). Finally, Freyre (1991) did not find any cross-reactivity in pigs infected with *Sarcocystis spp* by DT.

Table 2.4 Sensitivity and specificity of different serological tests in naturally infected pigs.

Test	Definitive test	Pig number	Sensitivity	Specificity	Reference
MAT	DT	180	95.3%	82.8%	Moghazy *et al*., 2011
ELISA			90.6%	80.1%	
IHAT			80.4%	85.3%	
MAT[a]	IFAT	106	77.8%	100%	Sroka *et al*., 2008
LAT			47.2%	91.4%	
ELISA			88.9%	95.7%	
MAT	Isolation	274	85.7%	94.6%	Gamble *et al.,* 2005
ELISA[b]			88.6%	98%	
MAT	Isolation	1000	82.9%	90.29%	Dubey *et al*., 1995c
ELISA			72.9%	85.9%	
IHAT			29.4%	98.3%	
LAT			45.9%	96.9%	
DT	Isolation	58	100%	65.8%	Hellesnes *et al*., 1978
IFAT	Isolation	109	85.7%	40%	Omata *et al.,*1994
IHAT	Isolation	31	22.2%	100%	Durfee *et al.,* 1974
LAT	Isolation	430	96%	94%	Tsubota *et al*., 1977

[a] Commercially available MAT (Toxo-Screen DA, Biomerieux).
[b] Commercially available ELISA (*Toxoplasma* Microwell Immunoassay Kit, Safe-Path Laboratories, Carlsbad, CA).

2.1.2 Prevalence of *T. gondii* in Mexico in pigs

Pork meat is among the main protein sources in Mexico (Galván-Ramírez *et al*., 2010a). Even though pork has been considered as a major source of *T. gondii* infection (Dubey *et al.,* 1995a, Jones and Dubey, 2012), it has only been studied in a few states. In general, as with humans, lower prevalence is found in northern and central areas with dryer and extreme temperatures than in the southern areas with tropical warm and humid climates.

One epidemiological survey was carried out in an abattoir located in Morelos, in which samples were originated from four states located in the central area of the country: Puebla, Michoacan, Morelos and Mexico, 1203 pigs were sampled and a prevalence of 8.9% to IgG antibodies against *T. gondii* was detected via ELISA (García-Vázquez *et al*., 1993). A more recent study was carried out in the northern states of Sonora and Durango on 1074 backyard pigs. The prevalence of IgG antibodies by MAT was 9.1% in Sonora (n=555) and 16% in Durango (n=519). Risk factors such as geographic area, age and breed were also studied. The prevalence was found to be higher in mountain areas (32.1%) than valley and semi-desert areas (13-14%), higher in mixed-breeds compared to pure-breeds (15.7% and 10.3%) and in 8-month pigs (19.5%) than younger (10.9%) (Alvarado-Esquivel *et al*., 2011a). Alvarado-Esquivel *et al.,* (2015) carried out an epidemiological survey in another state located in northern Mexico, Baja California Sur and found a prevalence of 13.5% in 291 backyard pigs by MAT. An additional 17 pigs raised in an intensive farm were sampled in this study and the prevalence was 0% by the same serological technique. In this study prevalence was higher in older pigs, being 9.4% in 5-8 month old pigs, 13.2% in 9-11month old pigs and 17%in pigs older than 12 months.

Southern states such as Oaxaca, Veracruz and Yucatan showed higher seroprevalence of *T. gondii* than those cited above. In Oaxaca a prevalence of 17.2% was noted in a study of 525 pigs aged from 2 to 36 months by MAT. When serological results were analysed by risk factors, higher prevalence was found in free range backyard pigs (17%) than in pigs raised indoor in an intensive farm (0.5%) and in pigs older than 9 months (40%) than younger pigs (10.6%). Prevalence was also higher in pigs raised in a tropical climate (65%) than warm and warm-humid climates (14.2%), and in pigs raised at 100-660 m above sea level (24.5%) compared to those at 20-60 m of altitude (14.2%) (Alvarado-Esquivel *et al*, 2012c). The overall

prevalence in 403 backyard pigs from Veracruz was 45.3% and when analysing by risk factors, seropositivity was also higher in pigs raised in tropical-humid (52.7%) climates than in other climates (42%), in pigs fed with leftovers (50.3%) than processed food (31.8%) and in free ranging pigs (77.85%) than the others kept in pens or pigsties (45%) (Alvarado-Esquivel *et al*, 2014). Finally, in parallel to the data of humans, levels of antibodies against *T. gondii* in Yucatan in pigs were also significantly higher (75%-92.5%) than those found in other states (Ortega-Pacheco *et al.,* 2013, Jiménez-Coello *et al*., 2013). However, in these studies the serum IgG antibody levels were measured with a commercial indirect ELISA kit (Human-GmbH, Wiesbaden, Germany).

The current study focuses on the intensive commercial farming system, as this is the most extensive in use in Yucatan. In a previous study of intensive farming, a level of 92.5% of IgG antibodies against *T. gondii* was found (Ortega-Pacheco *et al.,* 2013). Backyard pigs are in general associated with higher prevalence of *T. gondii*, so it seems controversial that in these studies it was higher in intensive farming than a backyard system. Backyard pigs are frequently free ranged and this has been associated with higher risk of infection with environmental oocysts than pigs raised in intensive farms which are kept indoors. On the other hand, Jiménez-Coello *et al*., (2013) suggested that in backyard production systems, pigs are fed mostly with leftovers, which are stored for shorter periods in comparison with the commercial food used in intensive farms that is stored for longer time. This time of storage could increase the probability of contamination with oocysts.

Table 2.5 summarises the different prevalences obtained across Mexico in different management systems, states and serological tests. The prevalence of *Toxoplasma* infection in pigs raised in the intensive production system is much lower (0%-0.5%) in comparison to pigs raised in the backyard system, except in studies carried out in Yucatan in which the prevalence in pigs raised in intensive farming system was much higher (95%-100%) (Ortega-Pacheco *et al*., 2011, Ortega-Pacheco *et al*., 2013). However, in addition to the farming differences the studies were also located in different areas within Mexico. In the case of Yucatan and Oaxaca, both are located in the south of Mexico but environmental conditions still differ, for example Yucatan has a tropical rainy climate while in Oaxaca the climate is warm sub-humid (INEGI, 2012). Furthermore, the diagnostic techniques were different; in Yucatan an ELISA test which

has not been validated in pigs was used in contrast with the other two studies in which the MAT test was used (table 2.5). The use of standardised diagnosis methods is very important in order to analyse results from different studies with accuracy.

Table 2.5 Prevalence of anti *T. gondii* IgG antibodies in pigs from Mexico. Details of diagnostic test used, validation of the test and state location within the country are given. Grey cells refer to studies in pigs raised in intensive farming system as the present study.

Test	Validated in pigs	TP[c]	PS[d]	State	Location	Reference
ELISA[a]	NO	8.9%	Unknown	Morelos	Central	García-Vázquez *et al*., 1993
MAT	YES	9.1%	Backyard	Sonora	North	Alvarado-Esquivel *et al*., 2011a
MAT	YES	16%	Backyard	Durango	North	Alvarado-Esquivel *et al*., 2011a
MAT	YES	13.7%	Backyard	BCS[e]	North	Alvarado-Esquivel *et al*., 2015
MAT	YES	0%	Intensive	BCS[e]	North	Alvarado-Esquivel *et al*., 2015
MAT	YES	17.2%	Backyard	Oaxaca	South	Alvarado-Esquivel *et al*., 2012c
MAT	YES	0.5%	Intensive	Oaxaca	South	Alvarado-Esquivel *et al*., 2012c
MAT	YES	45.3%	Backyard	Veracruz	South	Alvarado-Esquivel *et al*., 2014
ELISA[b]	NO	56%	Backyard	Yucatan	South	Dzib-Paredes *et al*., 2016
ELISA[b]	NO	96.6%	Mixed	Yucatan	South	Hernández-Cortazar *et al*., 2016a
ELISA[b]	NO	75%	Backyard	Yucatan	South	Jiménez-Coello *et al*., 2013
ELISA[b]	NO	100%	Intensive	Yucatan	South	Ortega-Pacheco *et al*., 2011
ELISA[b]	NO	95.8%	Intensive	Yucatan	South	Ortega-Pacheco *et al*., 2013

[a] in-house ELISA.
[b] Commercial ELISA kit validated to use in human sera (Human-GmbH, Wiesbaden, Germany).
[c] Total prevalence.
[d] Production system; Mixed: both intensive and backyard.
[e] Baja California Sur.

2.1.3 Characteristics of the study area

The geographical area selected for sampling was located in Yucatan (19° 30' and 21° 35' North latitude and 90° 24' West of the meridian of Greenwich), Yucatan is a state situated on the North portion of the Peninsula of Yucatan, in Mexico (figure 2.1).

Most of the terrain of Yucatan is calcareous; limestones, chalks and gypsums are the best represented. The surface is flat but undulating, the highest elevation is 275 m at the South of the state (Ticul) (Barber *et al*., 2000). The predominant climate is tropical rainy with a short winter and dry season (October-May) (White and Hood, 2004). The annual rainfall ranges from 400 mm to 2000 mm and the mean temperature is 26 ºC with a maximum of 36 ºC and a

minimum of 16 ºC and the relative humidity ranges from 65% to 100%, being the mean value over 78% (INEGI, 2012).

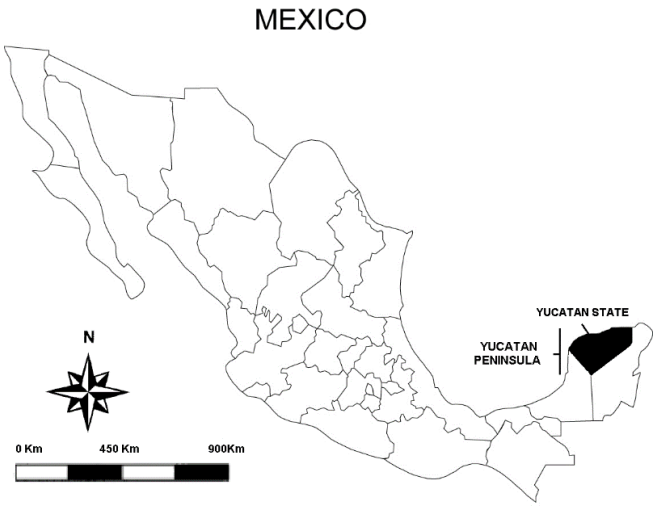

Figure 2.1. Geographical location of Yucatan.

Due to the absence of mountain ranges, there is a pattern of rain distribution, ranging from the driest areas in the North-East to the most humid areas in the South-West. The distribution of the plant communities follows this rainfall pattern, therefore a transitional situation exists between different vegetation types, each of them belonging to different bioclimates (Barber *et al*., 2000).

The Yucatan peninsula can be differentiated into three main vegetation types, tropical deciduous low forest, tropical sub-deciduous medium forest and tropical semi-evergreen medium forest (Barber *et al*., 2000, García and González, 2010, Ibarra-Manríquez *et al.,* 2002) (figure 2.2). Only the first two vegetation types are present in the state of Yucatan, which was our area of sampling. Trees up to 15 m and guacos (climbing vines) characterize tropical deciduous low forest whereas in tropical sub-deciduous medium forest; trees are higher than 15 m. In the dry season, the semi-deciduous foliage falls from between 50-75% of the trees in comparison, in the deciduous forest 100% of the leaves fall. *Piscidia piscipula* and *Lysoma bahamensis* are representative in the tropical deciduous low forest and *Enterolobium cyclocarpum, Cedrela Mexicana* and *Hura poliandra* of the tropical sub-deciduous medium forest in Yucatan (Barber *et al.,* 2000, Miranda and Xolocotzi, 1963). There are no studies that

compare the bioclimate between those two vegetation types, however according to Miranda and Xolocotzi, (1963), in the deciduous forest the annual precipitation mean is between 500-1100 mm and in the semi deciduous forest it is nearly 1200 mm or higher and the dry season is slightly shorter than in the deciduous forest.

The state of Yucatan is an important producer of pork, not only for local consumption, also for export. Yucatan is one of the six most important state producers of pork, generating more than 100,000 tonnes per year (SAGARPA, 2013). Swine production can be divided into intensive and backyard husbandry; the first one is the most common farming system in Yucatan, having 21000 units whilst the backyard production is represented by 3800 units. Farms are located across the state; however, 47% of the production is concentrated near the main city, Merida (INEGI, 2007). This work focuses on intensive farms, the most common husbandry system of the state.

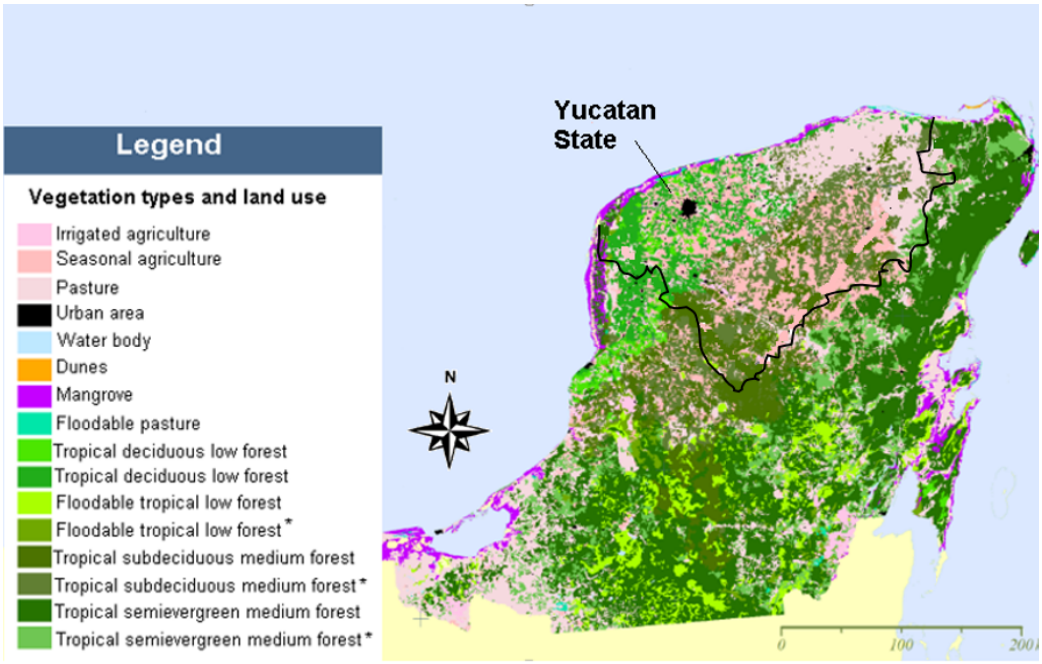

Figure 2.2 Vegetation types and land use of Yucatan Peninsula. Map adapted from García and González, (2010). * The main vegetation type coexists with secondary vegetation in order to recover vegetation that has been lost for hurricanes, fires or farming. Licence number provided by Springer to reuse this figure: 3981381158405.

2.2 Aims and objectives of the chapter

The main aim of this thesis was to gain knowledge on the epidemiology of *T. gondii* in domestic pigs raised in intensive farms in Yucatan. This study was pioneering in using a serological diagnosis method validated in swine in this endemic area of toxoplasmosis. Hence, this work has contributed to the expansion of knowledge of a public health concern. This research then aimed to improve the monitoring of *T. gondii* in pig farms and abattoirs in order to yield a safer meat. Thus, the first objective was to investigate the validity of the ELISA test employed in the published studies. The second objective was to generate new serological data using the MAT. The third objective was to confirm the MAT results with the gold standard DT and with a commercial ELISA kit which has been well validated in pigs. The fourth objective was to estimate the seroprevalence of *T. gondii* in different age groups of pigs to determine the first age profile of anti-*T. gondii* antibodies in pigs from Yucatan. Understanding the transmission dynamics at the farm level is crucial to implement control and prevention strategies. The fifth objective was to evaluate whether the seroprevalence varied between farms and if this is the case, farm management and characteristics would be analysed to understand which factors could have an impact on parasite transmission. Finally, to determine whether the prevalence of infection differs between two environmental zones, the tropical low deciduous forest, located at the north of the state and tropical sub-deciduous medium forest located in the south.

2.3 Material and Methods

In accordance with ethical considerations the project was approved by the ethics panel of ethics of the University of Salford with the reference number CST 13/72 (appendix VI). Animals were treated with good animal practice and blood samples were collected by trained and qualified personnel.

2.3.1 Pilot study

In 2013 a pilot study was completed to gain some knowledge of the prevalence and the characteristics of the farms of the area. To do that, a set of 53 domestic pigs (*Sus scrofa domesticus*) of different ages were sampled for blood extraction from two farms (farm A and farm B) selected by opportunistc sampling based on proximity and accessibility. The presence of IgG *T. gondii* antibodies was measured using the commercial IgG ELISA kit (Human Toxo IgG Human-GmbH, Wiesbaden, Germany) as described by Ortega-Pacheco *et al*., (2011), Jiménez-Coello *et al.,* (2013) and Ortega-Pacheco *et al*., (2013) (see section 2.3.3. for details of the method) in the research centre Dr Hideyo Noguchi in Merida, Yucatan. The OD (Optical Density) was analysed by pig age and gender. The main study design was based on the prevalence and the age distribution of the disease obtained in the pilot study.

2.3.2 Study design

Following the pilot study, a further investigation was carried out to study the relationship between age and infection in 2014. Based on the published literature, an increased seroprevalence with age was expected. Thus, a hypothesis of higher seroprevalence in older pigs was to be tested. Due to the aims of the study, only intensive farms with full cycles were sampled; a full cycle farm is a farm in which animals are kept from birth to the slaughter. A second sampling strategy was carried out to investigate the relationship between environment and *T. gondii* prevalence. To carry this out, adult pigs (20 weeks old) were sampled in farms located within the two vegetation types, tropical deciduous low forest and tropical sub-deciduous medium forest. The tropical subdeciduous medium forest has less drastic environmental conditions as the dry season lasts for shorter and, the remained vegetation

protects the soil from overheating. This conditions are favorable for the oocyst survival and therefore, higher seroprevalence was expected in the tropical subdeciduous medium forest. Thus, a hypothesis of higher seroprevalence in the tropical subdeciduous medium forest was to be tested.

The approximate total sample size for the first study was calculated using the prevalence obtained in the pilot study, with an absolute error of 7% and a 95% of confidence level and a design effect (D) of 3.52, using the software Epi-info 7.1.3 (CDC, Atlanta, USA., 2007). Where D = 1+ rho (n-1), being n the number of animals to sample in each cluster and rho is the intra-cluster correlation coefficient with a value of 0.04 (Ortega-Pacheco *et al.,* 2013). A multistage stratified cluster sampling was carried out in which each cluster or farm was stratified in the four age groups or strata (8 weeks, 12 weeks, 16 weeks and 20 weeks). The number of samples collected per each age group (16) was calculated with a power test using the software Epi-info 7.1.3 (CDC, Atlanta, USA, 2007) with an alpha level of 95% and a power of 0.8. In which the estimated prevalence of the uninfected animals was up to 20% and the prevalence of the infected animals was at least 70%. Therefore, 64 samples per farm were collected (16 x 4 age groups). The total number of farms was calculated dividing the total sampled size obtained with Epi-info by the samples to collect in each farm, the number of farms was rounded to six; consequently, 384 samples were obtained in total for this study.

A second sampling was completed to compare the two habitats in order to study the effect of the environment with the transmission of *T. gondii.* The number of samples per farm was calculated using Lot Quality Assurance Sampling (LQAS) in a LQAS calculator (http://www.brixtonhealth.com/hyperLQAS.html), where the upper threshold was considered 0.7 and the lower threshold 0.2 based on the prevalence obtained in the pilot study and previous studies in this geographical area. Type I and II errors were considered as 0.05. The total number of samples per farm calculated was 10 with a decision rule of 4. This means that if four or less animals of 10 are positive, the true prevalence is lower than 70% and the alternative hypothesis is rejected. On the contrary, if more than four of 10 animals are positive, then the prevalence is greater than 20% and the null hypothesis is rejected. Sixty animals of 20 weeks old were sampled in total. LQAS is a random sampling which was originally developed in 1920 for quality control in industry and is where its name comes from as in industry products are usually

made in lots. LQAS is a low cost methodology as it utilizes a small sample size. For this reasons the use of this sampling method in health surveillance has been growing since 1980 (Lanata and Black, 1991, Robertson and Velazquez, 2006).

To study the risk factors associated with toxoplasmosis transmission, a survey was conducted by interviewing farmers of the 12 farms (questionnaire appendix II.2). Information such as the size of the herd, presence and number of cats, presence of birds, cannibalism and mice, dimensions of the farm, the percentage of abortions, the type of feeders and drinkers, the warehouse and corral characteristics, pig movement thought the farm, pest control routine, the origin of the water and the sanitary program of the farm were collected. In addition, gender and age were recorded from all animals. The questionnaire was designed by reviewing risk assessment studies on toxoplasmosis in pigs (Lehmann *et al*., 2003, Meerburg *et al*., 2006, Lubroth *et al* 1983, Ortega-Pacheco *et al*., 2013, Dubey and Beattie, 1988, Assadi-Rad *et al*., 1995, Villari *et al*., 2009, Garcia *et al*., 1999, Weigel *et al*., 1995b, Dubey *et al.,* 1986b, Dubey *et al.,* 1995a, Hill *et al*., 2010).

Phlebotomy was performed in the external jugular vein using a vacutainer system, where blood was transferred directly in two 5 ml vacutainer tubes per animal, one with EDTA for DNA extraction and another with a serum separator gel for serological screening. The vacutainer system ensured the sterility of the blood and avoided cross contamination. Samples were transported refrigerated in a cooler to the research centre Hideyo Noguchi in Merida for subsequent examination. Serum tubes were centrifuged at 1100 g for 15 minutes to remove the clot and the serum was apportioned into two 1.5 ml clean polypropylene tubes and stored at -20°C until they were analysed.

2.3.3 Detection of IgG antibodies in pig serum

The prevalence data in swine previously published from the region of study has been obtained exclusively with the commercial IgG ELISA kit Human Toxo IgG (Human-GmbH, Wiesbaden, Germany) (Ortega-Pacheco *et al*., 2011, Jiménez-Coello *et al.,* 2013 and Ortega-Pacheco *et al*., 2013). Thus, this diagnosis method was employed during the first screening of the serum during the stay at the Hideyo Noguchi research centre. This kit is designed for the detection of IgG antibodies to *Toxoplasma gondii* only in human serum and according to the

manufacturer information it has a 99.2% specificity and a 96.1% sensitivity in humans. The Human Toxo IgG ELISA kit is based on the classical indirect ELISA technique. The microwells of the plate provided by the manufacturer are coated with *Toxoplasma* antigen prepared from the sonication of the whole tachyzoites grown in HeLa cells. All the incubations and washing steps were performed following the manufacturer instructions with the modification described by Ortega-Pacheco *et al*., (2011), Jiménez-Coello *et al.,* (2013) and Ortega-Pacheco *et al*., (2013) by adding a secondary goat anti- pig IgG antibody labelled with horse radish peroxidase (HRP) (Santa Cruz Inc. CA, USA) at a dilution of 1:5000 in the conjugate of the kit and by diluting the serum samples and pig controls at 1:100 with the dilution buffer provided with the kit. The swine controls included a commercial negative control (Santa Cruz Inc. CA, USA), one field positive control and two field negative controls kindly provided by the research centre Dr Hideyo Noguchi. The optical density (OD) was measured in a spectrophotometer at 450 nm (Multiskan Multisoft Primary EIA). The cut off was calculated as the mean of OD of the negative serum controls plus three standard deviations (SD) in each plate (Sroka *et al.,* 2008). All samples were screened with this test.

The repeatability of the assay was calculated with the coefficient of interplate coefficient of variance (CV):

$$CV = SD_{replicates}/\text{mean } OD_{replicates} \text{ X } 100$$

Where $SD_{replicates}$ is the standard deviation of the OD of positive and negative controls (commercial) in all the plates (n= 20) and mean $OD_{replicates}$ is the mean of OD of those controls in all plates.

Following the first screening, all the serum samples were transferred to the UK and tested using the standard MAT method at Salford University. The MAT test used was the commercial kit available as Toxo-Screen DA, bioMerieux. (Dubey, 2010a) which has been used widely in the literature in several animal species, including pigs (Sroka *et al*., 2008, Gebremedhin *et al.,* 2015). The MAT test was used according to the manufacturer instructions except that pig serum was tested at 1:10, 1:25 and 1:500. Positive samples were end-titrated by doubling dilutions when the serum was positive in any of the dilutions. Samples that showed an inconclusive agglutination at 1:10 were tested at 1:4. Serum was tested at the higher dilution of

1:500 in the first screen to avoid false negatives due to the prozone phenomenon. A prozone phenomenon is an inhibition of the agglutination caused by an excess of antibodies or antigens (Desmonts and Remington, 1980). Controls of the kit: positive, negative and antigen control (antigen, dye and PBS) were included in all tests. The diluted serum was added to the wells with 2-mercaptoethanol (0.2 mol/L) and the antigen diluted with a dye, the plate was sealed and incubated for 17-18 hours at room temperature. A red dot or a ring at the centre of the bottom of the well was a negative reaction, as a result of the sedimentation of the tachyzoites. A well with an even distributed dye was a positive reaction, indicating the agglutination of the tachyzoites in a mat covering the bottom of the well. Non-specific agglutination was suppressed by 2-mercaptoethanol which denatures the IgM or IgM-like substances. A positive result was considered when the reciprocal titre was ≥25.

In addition, a set of 50 samples was sent to the *Toxoplasma* Reference Unit in Swansea with the collaboration of Dr Edward Guy to screen with the DT as a gold standard. Additionally, another set of samples were screened with the commercial indirect IgG ELISA test ID Screen® (IDVet, Montpellier, France). ID Screen® is a multispecies ELISA to detect anti *Toxoplasma* antibodies in dogs, cats, swine and ruminants. This test has been validated in pigs internally (by IDvet) using IFAT as a gold standard (n=277). The sensitivity and specificity according to the manufacturer are 100%. This test was performed according to the manufacturer instructions. The cut off recommended by the manufacturer was as follows:

$$S/P\ \% = (OD_{sample} - OD_{NC} / OD_{PC} - OD_{NC}) \times 100$$

Where OD_{sample} is the average of the OD of the two replicates for a given sample and OD_{NC} and OD_{PC} are the average of the OD of the two replicates of the negative and positive controls. Samples with a S/P % ≥ 50 are classified as positives, samples with ≤ 40 are classified as negative and samples with in between values as a doubtful. This cut off was examined by a ROC (Receiver Operating Characteristic) curve. The serum was diluted 1:10.

2.3.4 Statistical analysis

Statistical analysis was performed with the package data Epi-info (v. 7.1.3) and SPSS (v. 19). Prevalence data was expressed as a percentage by dividing the number of serological positive samples between the total number of samples tested and the 95% of Coefficient Interval (CI) was calculated based on a binomial distribution. To study the relationship of prevalence between the variables gender, sex, farm, and environment, variables were analysed in a univariate analysis using the Fisher Exact test, Chi-square test or univariate binary logistic regression. The Fisher Exact test was used instead of the Chi-square test when the number of observations was lower than five in any of the cells. Agreement between serological tests was calculated with the kappa coefficient with a 95% of CI and the diagnostic accuracy and the cut off of the ELISA test was calculated with a Receiver Operating Characteristics (ROC). Statistical significance was set at a p value of <0.05. Odds ratio (O.R.) with a CI of 95% was used to estimate the magnitude of association with the outcome. Odds ratio (O.R.) is a measure of association between an exposure and an outcome (Szumilas, 2010), the higher the ratio, the stronger the association.

2.4 Results

2.4.1 Results of the pilot study

In order to produce an accurate design of the main study, seroprevalence was investigated beforehand. During the pilot study carried out during the first year of my PhD, blood samples were collected from 53 pigs of different ages from two farms; farm A and farm B (figure 2.3).

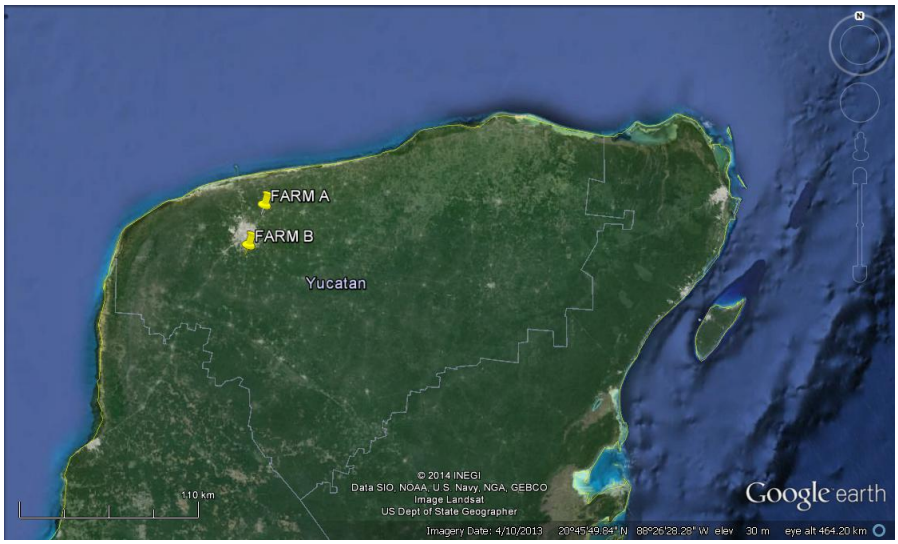

Figure 2.3 Location of farm A and B. Snapshot taken with Google Earth.

The overall prevalence of toxoplasmosis was found to be 85% (95% CI: 72%-93%). The prevalence in farm A was 73% (95% CI: 52%-88%) and in farm B was 96% (95% CI: 81%-99%) (table 2.6). When analyzed by sex and age results showed an increase of the OD with increasing the age (table 2.7) and this association showed a significant p-value with the Pearson's chi-square test ($p = \leq 10^{-3}$) with a $\chi^2 = 20.6$. However, Fisher Exact test did not show any statistical association between seropositivity and gender as p-value was not significant ($p = 1$) (table 2.8).

Table 2.6 *T. gondii* seropositive animals by farm: pilot study.

Pilot farm	Samples tested	Positives	Negatives
A	26	**19 (73%)**	7
B	27	**26 (96%)**	1
Total	53	**45 (85%)**	8

Table 2.7 Distribution of anti *T. gondii* antibodies by age.

Age in weeks	Samples tested	Positives
8	5	1 (20%)
10	5	4 (80%)
12	5	4 (80%)
14	5	5 (100%)
16	10	9 (90%)
17	1	0 (0%)
20	5	5 (100%)
24	3	3 (100%)
25	10	10 (100%)
26	4	4 (100%)

Table 2.8 Distribution of seropositivity by gender.

	Positives	Negatives	Total
Female	28 (84.8%)	5	33
Male	17(85%)	3	20
Total	45 (85%)	8	53

2.4.2 Main study: sampling, farm location and characteristics

The state of Yucatan can be divided in two ecological zones (figure 2.4). To study the relationship between age and infection, only the tropical deciduous low forest was sampled (area 1 in the map- figure 2.4) and to compare the prevalence between environment or ecological areas additional sampling was carried out in the tropical sub-deciduous medium forest (area 2 in the map- figure 2.4).

To carry out the age profile study (area 1), 6 farms were selected (farm 1-farm 6) and 384 animals were sampled in total (64 for each farm). Farms were selected using opportunistic sampling. In addition, farm 1 was the same farm as the pilot B. For the second study (area 2), 6 additional farms were selected (farm 7-farm 12) also by opportunistic sampling. 60 samples were collected in total for this purpose (10 for each farm). 20 week old pigs were selected for

the second study. However, 18 week old pigs were sampled in five instances because older pigs were not available. For the statistical study comparing both environments, these were considered 20 week old pigs. However, in the binary logistic regression these were considered as 18 weeks as the purpose was to analyse an age pattern. All sampled animals were domestic pigs (*Sus scrofa domesticus*).

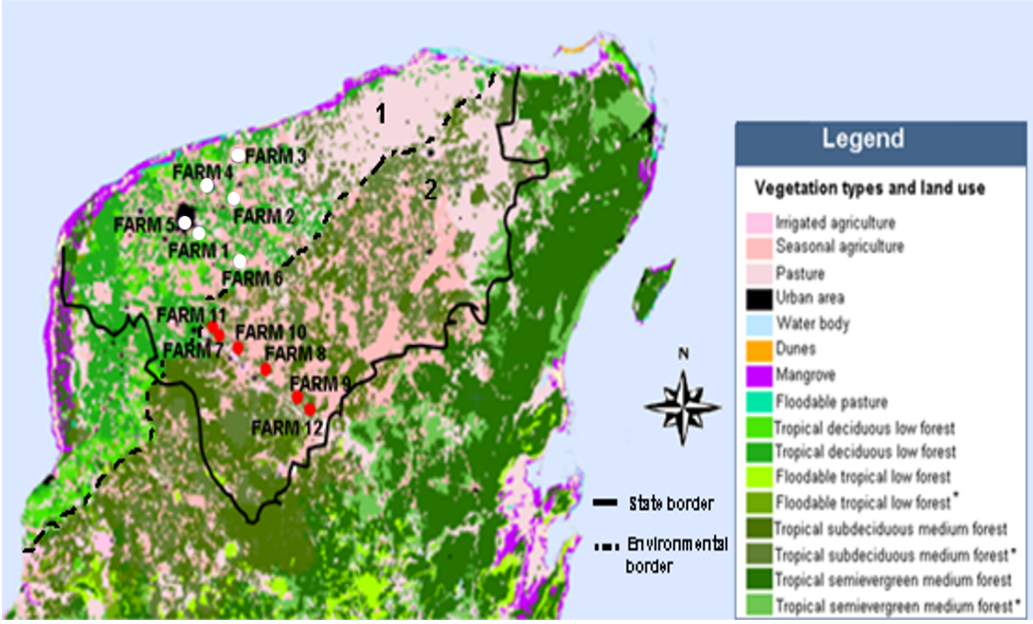

Figure 2.4 Geographical location of the farms 1-12. White dots correspond to the farms sampled in first study and red dots in the second study. Farms were placed with Google earth using the coordinates and the Snapshot was merged with the map from García and González, (2010) in order to obtain the ecological zones of the sampling areas.1 and 2 numbers correspond to tropical deciduous low forest and tropical sub-deciduous medium forest respectively. Licence number provided by Springer to reuse this figure: 3981381158405.

All farms were intensive and full cycle and they raised pigs destined for human consumption. The average herd size was 765 (SD ± 826). The minimum herd size was 33 pigs (farm 7) and the maximum was 3272 pigs (farm 3). Although two mating strategies were used, mating with boars was more frequent (75% of the farms) than insemination using purchased semen (25%) (http://www.picgenus.com/). Different swine breeds were noted; 33% of the farms used patented genetic lines such as PIC 337 or PIC 410, 25% used crosses between pure breeds such as Landrace with Pietrain and/or Duroc, 8% used a mixture of patented genetic lines with crosses with pure breeds and 33% were of unknown breed. The breed was independent to the

mating strategy used, however the 33% with unknown pig breed were all mating using boars. The average farm size was 11.3 ha (SD ± 28). The largest farm was 100 ha (farm 3) and the smallest farm was 1 ha (farm 9). All farms were open to the environment allowing access to wildlife. Warehouses, where the pig food was stored, were open to the environment in 85% of the farms. In addition, in 85% of the farms the feeders were also open to the environment. Rodent control was lacking in 23% of the farms. 30% of the 77% of farms which reported use of rodent control as a routine, used cats as a control strategy. Independently of using rodent control, 70% of the farms were reported to observe mice and/or rats. Furthermore, two of the farms which were reported to have observed rodents, also reported pigs being observed eating them. 85% of the farms reported cat ownership and also, 85% of the farms observed stray cats. The average number of owned cats per farm was 9.5 (SD ± 9.3). The maximum number of owned cats per farm was 30 (farm pilot B) and the minimum was 2 (farm 3, 11, 12). The water for all the farms was obtained from wells and only 23% of the farms performed sanitary treatment of the water. A detailed description of the management and characteristics by farm is available in Appendix III.1. Some farm pictures are available in AppendixIII.2.

2.4.3 Seroprevalence of IgG antibodies

When results obtained with the MAT test and the Human Toxo IgG ELISA were compared these were unexpectedly contradictory (Table 2.9).

Table 2.9 Comparison between ELISA and MAT seroprevalences. Prevalence data is given with a 95% CI.

	ELISA	MAT
Farm 1	48.4% (36%-61%)	0% (0%-0.5%)
Farm 2	75% (63%-85%)	0% (0%-0.5%)
Farm 3	53.1% (40%-66%)	0% (0%-0.5%)
Farm 4	78.1% (66%-87%)	1.5% (0%-8.4%)
Farm 5	66% (53%-77%)	0% (0%-0.5%)
Farm 6	79.6% (68%-89%)	1.5% (0%-8.4%)
Farm 7-12	96.6% (88%-99%)	3.33% (0.4%-11.5%)
TOTAL	67% (95% CI: 62%-72%)	1.5% (95% CI: 0.56%-3.3%)

The overall prevalence obtained in the age profile study with the Human Toxo IgG ELISA was 67% (95% CI: 62%-72%). The prevalence was 9.4% (95% CI: 4%-17%) in the 8

week animals but increased with the age reaching the 95.8% (95% CI: 89.7%-98.8%) in the oldest pigs (table 2.10).

Table 2.10 Prevalence analysed by farm and age group using the Human Toxo IgG ELISA. Prevalence data is given with a 95% CI.

	Week 8	Week 12	Week 16	Week 20
Farm 1	0/16	5/16	10/16	16/16
Farm 2	0/16	16/16	16/16	16/16
Farm 3	0/16	8/16	14/16	12/16
Farm 4	2/16	16/16	16/16	16/16
Farm 5	3/16	10/16	13/16	16/16
Farm 6	4/16	15/16	16/16	16/16
TOTAL	9.4% (4%-17%)	72.9% (63%-81%)	88.5% (80%-94)	95.8% (89%-98%)

In addition, the prevalence obtained with the Human Toxo IgG ELISA in the tropical sub-deciduous medium forest (area 2), was slightly higher (96.6%, 95%CI: 88%-99%) when compared with the 20 week old pigs from the tropical deciduous low forest (95.8%, 95%CI: 90%-99%) (table 2.11). However, this difference was not statistically significant (Fisher Exact p = 1).

Table 2.11 Prevalence rates by environment obtained using the Human Toxo IgG ELISA. Prevalence data is given with a 95% CI. Prevalences obtained in 20 week old pigs sampled in the tropical deciduous low forest.

Environment	Farm	Positives	Negatives	Total	Prevalence
Tropical deciduous low forest	Farm 1	16	0	16	95.8% (89%-98%)
	Farm 2	16	0	16	
	Farm 3	12	4	16	
	Farm 4	16	0	16	
	Farm 5	16	0	16	
	Farm 6	16	0	16	
	TOTAL	92	4	96	
Tropical subdeciduous medium forest	Farm 7	10	0	10	96.6% (88%-99%)
	Farm 8	9	1	10	
	Farm 9	9	1	10	
	Farm 10	10	0	10	
	Farm 11	10	0	10	
	Farm 12	10	0	10	
	TOTAL	58	2	60	

Results from this ELISA test were highly inconsistent with the MAT test. Of the 53 animals sampled in 2013 in the pilot study only two animals had antibody titres higher than the

MAT cut off (≥25), one from each farm. In addition, six animals showed agglutination at 1:10 and two at 1:4 dilutions. From the main epidemiological study carried out in 2014 four animals had antibody titres higher than the cut off and four and eight animals showed agglutination at 1:10 and 1:4 dilutions respectively (table 2.12). Samples with titres ≥25 were classified as positives, those that showed agglutination below the cut off were classified as doubtful and the ones showing no agglutination at all were classified as negative samples. Doubtful samples were categorized as negative when the statistical analysis was performed.

Table 2.12 MAT results by farm and age.

Farm	Positives	Titre	Age in weeks	Doubtful	Titre	Age in weeks
Pilot A	1	1000	12	6 2	10 4	20 (1) 25(4) 25
Pilot B	1	25	24	0	0	-
Farm 1	0	-	-	2	4	20
Farm 2	0	-	-	0	-	-
Farm 3	0	-	-	1	4	20
Farm 4	1	25	12	2 1	10 4	20 12
Farm 5	0	-	-	1	10	20
Farm 6	1	50	8	1 4	10 4	20
Farm 7-10	0	-	-	0	-	-
Farm 11	2	25	20	0	-	-
Farm 12	0	-	-	0	-	-

The ELISA OD was plotted against MAT titre and no correlation was found (figure 2.5). Samples that showed agglutination in MAT did not have any pattern of OD distribution with values that ranged from 0.4 to 1.7. MAT negative samples also had random OD values in ELISA, mainly above the cut off and values ranged from 0.17 to 1.56. Additional samples which were used for the isolation study (Chapter 4) were also plotted on the graph (grey dots). Controls used in the ELISA were tested by MAT and as expected, negative controls used in ELISA were negative by MAT in all dilution and positive control was positive by MAT with a titre of 16000.

The cut off in ELISA was calculated for each plate and the value varied from 0.328 to 0.638. The inter plate variation was investigated, the CV was 25.9% for the negative and 16.2% for the positive control. The OD was corrected for each sample according to Lind *et al*., (1997) however the results did not improve (data not shown).

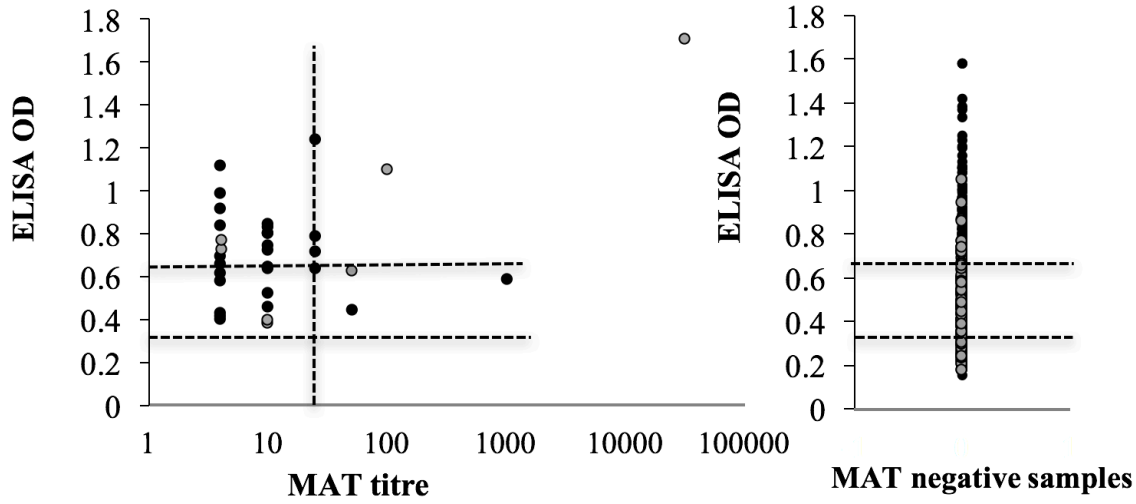

Figure 2.5 ELISA OD plotted against MAT titre. Left: distribution of OD on animals that showed MAT agglutination. Right: Distribution of OD in MAT negative samples. Black dots correspond to the animals studied in this chapter and grey dots are pigs that that correspond to a further chapter. The dashed vertical line represents the cut off for the MAT test and the double dashed horizontal lines correspond with the cut off range in ELISA.

A contingency table was performed to calculate the sensitivity and specificity of the ELISA test based on the MAT as a gold standard. The specificity of the test was as low as 37.4% (95%CI: 33.5%-41.3%) due to the high level of false positives samples and the sensitivity was 100% (95%CI: 66.4%-100%) probably by chance due to the few positive animals by MAT and the high number of false positives (table 2.13). The kappa coefficient was 0.017 (95%CI: 0.007-0.029, p-value= 0.006) showing very low agreement with the MAT test. Kappa values <0-0.2, 0.21-0.39, 0.40 - 0.59, 0.60 - 0.79 and ≥ 0.80 were interpreted as low, fair, moderate, substantial and almost perfect agreement, respectively (Landis *et al*., 1977). Results of this test were discarded as they were considered unreliable.

Table 2.13 Contingency table of ELISA test and MAT results.

		MAT			
		Positive	Negative	Questionable	Total
ELISA	Positive	9	367	23	399
	Negative	0	230	3	233
	Total	9	597	26	632

Following these findings, first a set of 36 samples were examined with the ELISA test in Salford University using exactly the same protocol as in Mexico to exclude any possible risk

of degradation during the transfer to the UK; the results mirrored the previous ELISA data (table 2.14). Of 27 samples which tested positive with the ELISA performed in the Hideyo Noguchi, 25 tested positive in the duplicated performed in Salford University. The ODs obtained in Salford University were in general higher than in comparison with the previous data obtained in Mexico.Thus the possibility of sample degradation was discarded and the protocol of the ELISA test was reassessed. A nonspecific reaction caused by addition of the secondary anti-pig antibody to the secondary anti-human antibody (the kit conjugate) was suspected as they were mixed together.

Table 2.14 ELISA OD and MAT results I. ELISA 1 refers to the test performed in the Hideyo Noguchi Research Centre and ELISA 2 refers to the ELISA repeated in Salford University.

ELISA2 (OD)	Cut-off	Test	ELISA1 (OD)	Cut-off	Test	MAT titre
2.821	0.934	+	1.7	0.573	+	32000
2.052	0.934	+	1.088	0.573	+	100
1.595	0.934	+	1.233	0.573	+	25
1.292	0.934	+	0.799	0.466	+	10
1.383	0.934	+	0.911	0.573	+	4
1.282	0.934	+	1.108	0.573	+	4
2.35	0.934	+	1.419	0.323	+	-
2.03	0.934	+	1.163	0.412	+	-
1.452	0.934	+	0.858	0.394	+	-
1.502	0.934	+	0.975	0.573	+	-
1.545	0.934	+	1.107	0.573	+	-
1.464	0.934	+	0.966	0.519	+	-
1.564	0.934	+	1.135	0.514	+	-
1.61	0.934	+	0.965	0.519	+	-
1.213	0.934	+	1.336	0.528	+	-
1.292	0.936	+	0.799	0.466	+	-
1.868	0.934	+	1.586	0.528	+	-
1.941	0.934	+	1.585	0.546	+	-
1.323	0.934	+	1.383	0.546	+	-
1.557	0.934	+	1.375	0.573	+	-
1.62	0.934	+	1.194	0.546	+	-
1.513	0.934	+	1.009	0.573	+	-
1.19	0.934	+	0.715	0.466	+	-
0.991	0.934	+	0.971	0.573	+	-
1.014	0.934	+	0.454	0.514	+	-
0.773	0.934	-	0.442	0.514	-	-
0.782	0.934	-	0.516	0.514	+	-
0.702	0.934	-	0.412	0.514	-	-
0.66	0.934	-	0.566	0.514	+	-
0.627	0.934	-	0.414	0.514	-	-
0.548	0.934	-	0.369	0.514	-	-
0.234	0.934	-	0.291	0.391	-	-
0.129	0.934	-	0.183	0.514	-	-
0.181	0.934	-	0.24	0.514	-	-
0.557	0.934	-	0.371	0.514	-	-
0.653	0.934	-	0.392	0.514	-	-

Subsequently, a set of samples was tested only with the secondary anti-pig antibody by diluting it in the dilution buffer (PBS, 1% BSA) instead of adding it into the conjugate of the kit. The conjugate of the kit contains a rabbit anti-human IgG labeled with HRP, 0.1 g/l thimerosal and 100 µg/ml of gentamicine. The OD values were lower than when the conjugate of the kit was used (table 2.15) but still the results did not show a good agreement with the MAT (kappa=0.288, 95%CI -0.049-0.625, p = 0.06).

Table 2.15 ELISA OD and MAT results II. Cut off values of 0.151 and 0.236 showed a sensitivity and specificity of 60% and 47% and 30% and 95% respectively.

Anti-human + pig Ab (OD)	Test	Anti-pig Ab (OD)	Test cut off 0.151	Test cut off 0.236	MAT titre
1.7 **	+	0.388**	+	+	32000
1.66	+	0.271	+	+	16000
0.562	+	0.114	-	-	1000
1.088	+	0.237	+	+	100
0.442	+	0.143	-	-	50
0.622	+	0.138	-	-	50
0.632	+	0.139	-	-	25
0.708	+	0.159	-	-	25
0.789	+	0.161	+	-	25
1.233	+	0.153	+	-	25
0.724	+	0.17	+	-	10
0.849	+	0.169	+	-	10
0.647	+	0.168	+	-	10
0.735	+	0. 148	-	-	10
1.108	+	0.141	-	-	4
0.911	+	0.136	-	-	4
0.581	+	0.15	-	-	4
1.336	+	0.161	+	-	-
1.103	+	0.175	+	-	-
1.586	+	0.238	+	+	-
1.034	+	0.168	+	-	-
1.586*	+	0.225	+	-	-
1.104	+	0.223	+	-	-
1.383	+	0.138	-	-	-
0.911	+	0.161	+	-	-
0.41	-	0.093	-	-	-
0.31	-	0.075	-	-	-
0.29	-	0.076	-	-	-
0.402	-	0.098	-	-	-

A ROC curve was performed to analyse the diagnostic accuracy of the test and calculate the cut off value with the best sensitivity and specificity (figure 2.6). The area under the curve or AUC was 0.561 (95%CI: 0.333-0.788, p-value=0.59). A perfect test has an AUC of 1, the minimum AUC has been considered 0.5 as a chance level while AUC=0 means that a

test has incorrectly classified all subjects with disease as negative and all subjects with no disease as positive (Hajian-Tilaki, 2013). The accuracy of this test was considered moderate. A test with an AUC below 0.5 represents a pointless test. The ROC curve could not compute a cut off with a better balance of sensitivity and specificity than 50% (95%CI: 18.7%-81.3%) and 47.4% (95%CI: 24.45%-71.14%) or 30% (95%CI: 6.7%-65.3%) and 94.7% (95%CI: 74%-99.8%) respectively for 0.151 and 0.236. The cut off 0.236 was the best option as it had better agreement (k=0.288) with the MAT results than 0.151 (k=-0.024, 95%CI -0.367-0.319, p = 0.8). Results from this ELISA protocol were also discarded as the test was shown to be inaccurate.

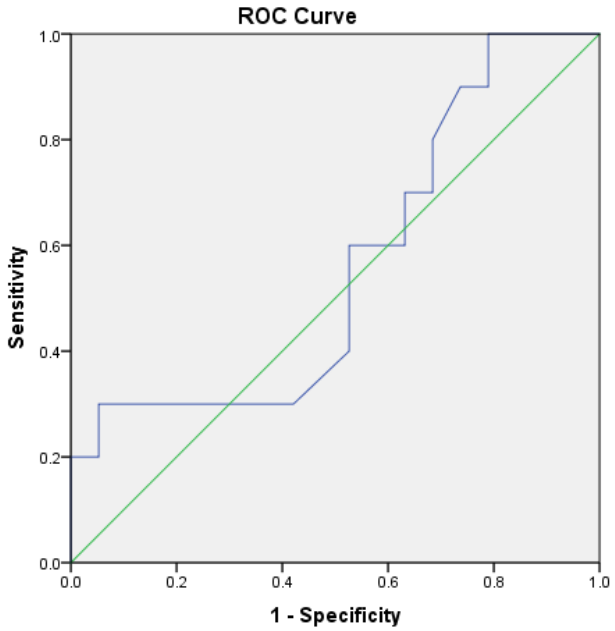

Figure 2.6 ROC curve obtained for the ELISA OD values. The diagonal green line represents the chance level 0.5. The blue line represents the curve with the OD value for each sensitivity and specificity value.

A chessboard titration was performed to find out whether a different dilution of secondary antibody and serum samples could improve the performance of the test (Crowther, 2000). A true negative sample (MAT, DT and ID screen® negative) which tested positive with ELISA (* in table 2.15) was selected to perform the titration and a true positive sample (MAT titres 32000, 500IU DT, ID screen® positive (** in table 2.15). Serum was diluted at 1:25, 1:50, 1:100 and 1:200. Secondary antibody was tested at 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000. High ODs were obtained for the negative serum for all serum dilution and

specially when it was combined with higher dilution of the secondary antibody such 1:250, 1:500 and 1:1000 (figure 2.7.). These high ODs are a result of nonspecific binding of the negative serum to the coated wells. In addition, the secondary antibody was tested without samples for all dilution and no nonspecific binding was found (OD 0.08 to 0.04) at any of the dilution. Positive sera showed a good titration for 1:250, 1:500, 1:1000 dilution of the secondary antibody (figure 2.7).

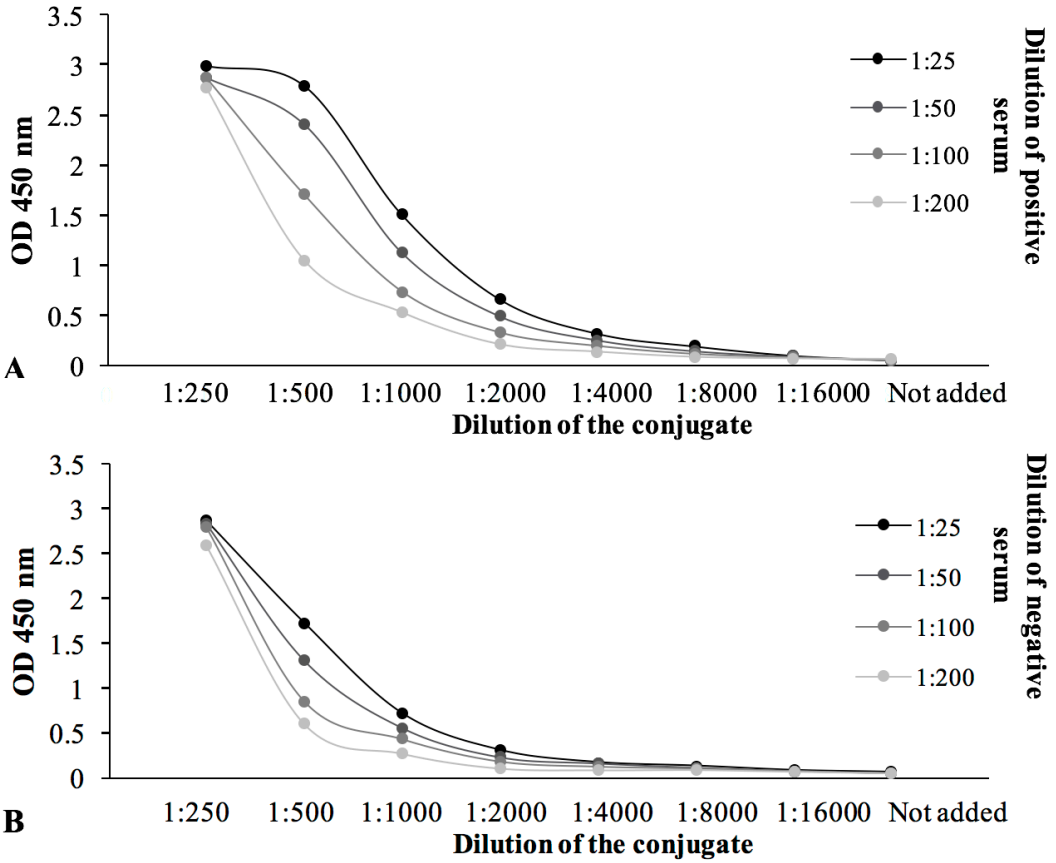

Figure 2.7 Optimization of the ELISA by chessboard titration. Figure A shows a titration of the secondary antibody anti-pig against the pig positive serum. Figure B shows a titration of the secondary antibody anti-pig against the pig negative serum.

Binding ratios (BR) were calculated to find the optimum dilution of the reagents (Crowther, 2000). A BR is the OD value of the positive serum divided by the OD value of the negative serum at a given dilution. Extremely low binding ratios were observed for all dilutions. A binding ratio of one indicates that the OD of the positive and negative serum have the same

value. All values were very close to 1 (table 2.16). Low binding ratios at higher dilutions of the secondary antibody and serums are due to nonspecific binding of the negative serum. Low binding ratios at lower dilutions of the secondary antibody and serum are due to failure to detect antibodies in positive serum. The best combination of dilution of the reagents was a dilution of 1:2000 for the secondary antibody and a dilution of 1:25 of the serum. However, this combination still showed a low BR (2.18) being the OD of the positive and negative serums 0.665 and 0.305 respectively.

Table 2.16 Binding ratios for pig serum. The light grey cells show the dilution of the conjugate that gives the highest values for BR.

Dilution Conjugate	BR at dilutions: 1:25	1:50	1:100	1:200
1:250	1.045	1.016	1.025	1.069
1:500	1.620	1.844	1.996	1.737
1:1000	2.108	2.034	1.720	1.948
1:2000	2.180	2.175	1.844	1.972
1:4000	1.917	1.610	1.598	1.540
1:8000	1.523	1.313	1.127	0.911
1:16000	1.288	1.257	1.067	0.944
Not added	0.966	1.120	1.021	1.130

As a result of the above tests, MAT was chosen as a diagnostic test and the overall prevalence (n=632) obtained with the MAT test was 1.4% (95%CI: 0.6%-2.7%). In the pilot study it was 3.77% (95%CI: 0.46%-12.98%), being the prevalence in farm pilot A and pilot B (farm 1) of 3.8% (95% CI:0%-19.6%) and 3.7% (95% CI:0%-18.9%) respectively. The total prevalence in the main epidemiological study was 0.9% (95%CI: 0.25%-2.3%). The prevalence among all animals in the tropical deciduous low forest was 0.5% (95%CI: 0%-1.8%). The prevalence in the tropical sub-deciduous medium forest was 3.33% (95%CI: 0.4%-11.5%); this prevalence was higher than the prevalence obtained for the 20 week old pigs from the tropical deciduous low forest was 0% (95%CI: 0%-0.37%). However, this difference did not show any statistical significance, as the fisher exact test p-value was 0.14.

MAT positive animals were found in only 3 farms from the main study, one animal per farm in the tropical deciduous low forest, thus the prevalence was 1.5% (95%CI: 0%-8.4%) in each of those farms and two belonging to the farm number 11 located in the tropical subdeciduous medium forest where the prevalence reached the 20% (95%CI: 2.5%-55%) in this

farm, however the total number of animals analysed on this farm was only 10. Prevalence between farms was analysed statistically to find possible risk factors associated with farm management using the Fisher Exact test but these differences in the prevalence were not statistically significant for the farms that were sampled 10 animals (p-value = 0.153) and for the farms that were sampled 64 animals (p=1). Because the prevalence between farms did not differ statistically, farm characteristics collected by a questionnaire could not be used to build a model which explained the risk factors associated with the disease. The questionnaire and farm characteristics are in appendix II and III.

To carry out an age profile, animals used in the isolation study (Chapter 4) were included in the logistic regression to increase the sample size. Therefore, overall (sample size = 632) one animal of 8 weeks was infected, two of 12 weeks, none of 16 weeks, two of 20 and one of 24 weeks. A binary logistic regression was preformed to reveal whether age has an effect in the infection state. The p-value was close to significance, but the results showed no age effect in the prevalence (table 2.17).

Table 2.17 Results of the binary logistic regression for MAT results.

Variable	N (pigs)	O.R. (95% CI)	χ^2	D.F.	P-value
Age	632	1.15 (1-1.3)	4.13	1	0.051

From the 6 positive animals, 2 were females and 4 were males. To perform the statistical analyses, animals from the isolation study were included (except 9 in which information of the gender was not provided by the abattoir). The difference in *T. gondii* positives between genders was not statistically significant by the fisher exact text (p = 0.2) (table 2.18).

Table 2.18 Distribution of seropositives by gender.

	MAT +	MAT -	Total
Female	2	298	300
Males	7	316	323
Total	9	614	623

To corroborate the MAT results, 50 samples were sent to the Reference Centre of Toxoplasmosis in Swansea to test with the DT. DT analysis showed a good agreement with the MAT test. Results were expressed as IU/ml. The cut off for the DT was considered at > 4 IU/ml

and 2 and 4 IU/ml were considered as questionable. Some of the animals that were tested correspond with the isolation study, which will be addressed in Chapter 4. Three animals which were positive with the MAT test were also positive with the DT. From 3 MAT questionable animals in the MAT, 2 were questionable and one was negative with the DT. Thus from 45 animals that were negative with the DT 44 were negative with the MAT and one doubtful (table 2.19).

Table 2.19 Contingency table of DT and MAT results.

		DT			
		Positive	Negative	Questionable	Total
MAT	Positive	3	0	0	3
	Negative	0	44	0	44
	Questionable	0	1	2	3
	Total	3	45	2	50

The DT is considered to be the gold standard for *T. gondii* serological diagnosis in humans. Although it has not been validated in pigs as exhaustively as the MAT test, the DT has been used also as gold standard in this species (Moghazy *et al*., 2011) as it is considered not to be host species specific. Results of MAT and DT were compared in a contingency table and specificity and sensitivity were calculated for the MAT test based on the DT results as a gold standard. Specificity and sensitivity had a value of 97.8% (95% CI: 88.2%-99.4%) and 100% (95% CI: 29.2%-100%) respectively (table 2.19). The agreement of both tests was calculated with the kappa coefficient, having a value of 0.901(95% CI: 0.536-1, $p \leq 10^{-3}$). MAT was considered to have an excellent agreement with the DT. Furthermore, the MAT titres were comparable with the DT (table 2.20). Animals, which had low IU/ml such 2 or 4, had the low antibody titre in MAT of 1:4 and the higher the IU/ml the higher was the MAT titre in the corresponding sample.

Table 2.20 Results of DT and MAT titres.

Disease status	DT (IU/ml)	MAT titre	Animals
Negatives animals	-	1 -1:4 44 - negative	45
Positive animals	500 IU/ml 32 IU/ml 8 IU/ml	1:32000 1: 100 1:25	3
Questionable	2 IU/ml 4 IU/ml	1:4 1:4	2

To reinforce the MAT and DT results a set of 90 samples was selected to test with the commercially available ELISA kit ID Screen® Toxoplasmosis Indirect Multi-Species. The selected samples were the 50 tested also with the DT and 40 additional samples which were selected in accordance with MAT titres. Thus a total of 9 MAT positive sample with titres ≥ 25, 26 MAT samples with titres < 25 and 55 MAT negative samples were tested with this ELISA multi-species.

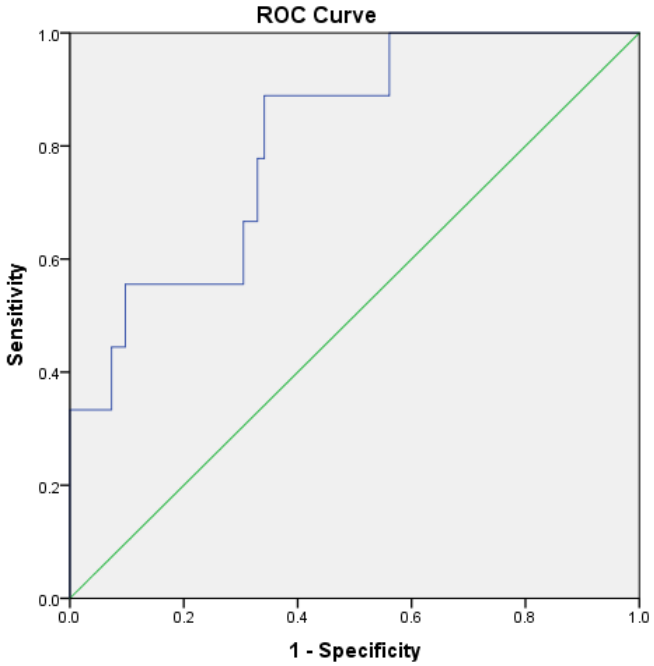

Figure 2.8 ROC curve obtained for the ID Screen® OD values. The diagonal green line represents the chance level 0.5. The blue line represents the curve with the OD value for each sensitivity and specificity value.

A ROC was also performed for this ELISA multispecies to analyse the accuracy of the test and to evaluate the cut off provided by the manufacturer instructions (figure 2.8). The area under the curve or AUC for this ELISA was 0.810 (95%CI: 0.676-0.945, p-value=0.002). The accuracy of this test is considered good (Hajian-Tilaki, 2013). A cut off value of 0.277 showed the best balance of specificity and sensitivity. The sensitivity and the specificity at this cut off was 56% (95%CI: 21.2%-86.3%) and 91.4% (95%CI: 83%-93.5%) respectively. This cut off obtained with the ROC curve had a much higher sensitivity than the 33.3% (95%CI: 7.5%-70.1%) obtained with the cut off provided by the manufacturer. On the other hand, the specificity according with the cut off provided by the manufacturer was slightly higher 97.5%

(95%CI: 91.4%-99.7%). The cut off obtained with the ROC curve was preferred as the sensitivity is higher with no considerable loss of specificity. Furthermore, the kappa agreement with the MAT was higher using the cut off computed with the ROC curve. When the OD was plotted against MAT titres, a positive correlation was found with the MAT titres and ELISA kit ID Screen® ODs. OD values increased with MAT titres (figure 2.9).

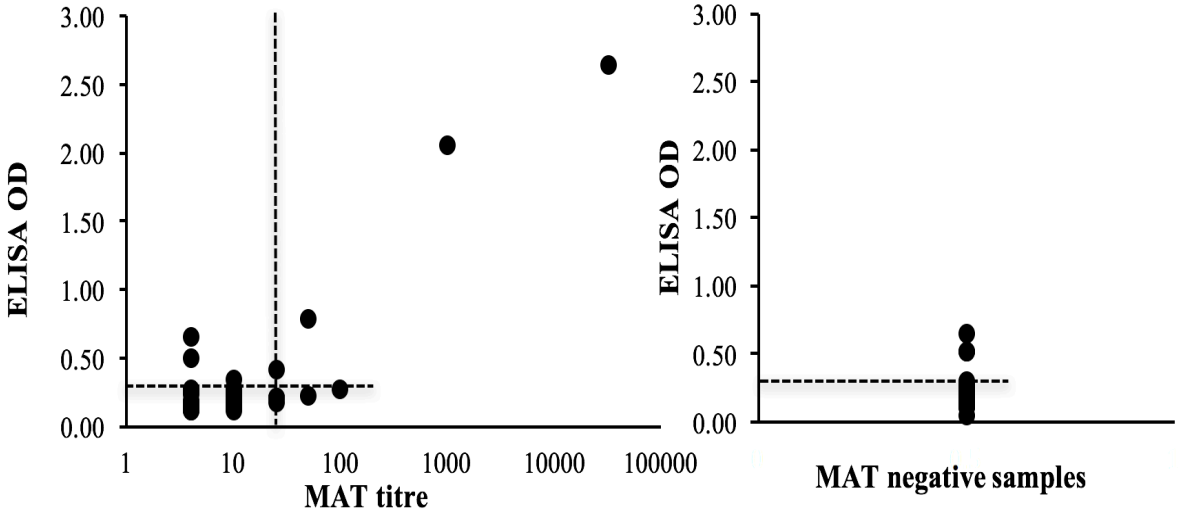

Figure 2.9 ELISA ID Screen® OD plotted against MAT titre. Left: distribution of OD on animals that showed MAT agglutination. Right: Distribution of OD in MAT negative samples. Black dots correspond to the animals studied in this chapter and grey dots are pigs that that correspond to a further chapter. The dashed vertical line represents the cut off for the MAT test and the double dashed horizontal lines correspond with the cut off range in ELISA.

Of 9 MAT positives samples, 5 tested positive with this ID Screen® ELISA. 7 additional animals tested positive with this ELISA, of which 3 were MAT negative and 4 were animals with MAT titres < 25 (table 2.21). The 5 concordant positive samples had MAT titres of 32000, 1000, 1000, 50, 25. This ELISA was less sensitive in comparison to the MAT. All samples with MAT titres >100 were positive with the ELISA but only one of the samples with 50 and 25 titres were positive. Thus 100%, 50%, 25% and 15% of the animals with MAT titres > 50, >25, = 25 and < 25 respectively tested positive with ID Screen® ELISA. The number of false positives was 3 of 55 (5%) negative samples. The 4 samples with a MAT titre < 25 which tested positive with this ELISA were two 20 week old pigs raised in farm 6 and two 25 week old pigs raised in farm 1 (isolation study) and pilot B. The 3 MAT negative samples which

tested positive with this ELISA were one 25-week old pig raised in farm 1 (isolation study) and two 12 and 16 week old pigs raised in farm 2. The level of agreement between the MAT and ID Screen® was moderate with a kappa value of 0.41 (95% CI: 0.124-0.69, $p \leq 10^{-3}$). The agreement between these two tests using the cut off provided by the manufacturer instructions was lower (k= 0.385, 95% CI: 0. 099-0.722, p = 0.000).

Table 2.21 Results of ID Screen® and MAT titres.

		MAT			
		Positive	Negative	Questionable	Total
ID Screen®	Positive	5	3	4	12
	Negative	4	52	22	78
	Total	9	55	26	90

Thus, taking into account the ID Screen® ELISA positives that were doubtful and negative with MAT, the prevalence of farm 2 increased from 0% (95%, CI: 0%-0.5%) to 3.1% (95%, CI: 0%-10%), the prevalence of farm 6 increased from 1.5% (95%, CI: 0%-8.4%) to 4.7% (95%, CI: 0.9%-13.1%), the prevalence in farm pilot B (farm 1) increased from 3.7% (95%, CI: 0%-18.9%) to 7.4% (95%, CI: 0%-24.3%). The prevalence overall (n= 632) increased from 1.4% (95%, CI: 0.6%-2.7%) to 2.5% (95%, CI: 1.4%-4%), the prevalence in the pilot study increased from 3.77% (95%, CI: 0%-12.9%) to 5.5% (95%, CI: 1.2%-15.6%). The prevalence in the main epidemiological study increased from 0.9% (95%, CI: 0.2%-2.2%) to 1.8% (95%, CI: 0.7%-3.5%). Finally, the prevalence in the tropical deciduous low forest increased from 0.5% (95%, CI: 0%-1.5%) to 1.5% (95%, CI: 0%-3.3%).

A binary logistic regression was performed including those ID Screen® ELISA positives animals which tested negative or doubtful with MAT to re-evaluate the association between age and *T. gondii* infection. Results in this case showed statistical significance (table 2.22). Thus age was explanatory variable for disease, in other words older pigs were more likely to be seropositive.

Table 2.22 Results of the binary logistic regression including ID Screen® results.

Variable	N (pigs)	O.R. 95% CI	χ^2	D.F.	P-value
Age	632	1.15 (1.04-1.3)	10.2	1	0.003

MAT, ID Screen® positive animals and MAT, ID Screen® positive animals and animals with MAT titres <25 were plotted against age and a clear positive correlation can be observed (figure 2.10).

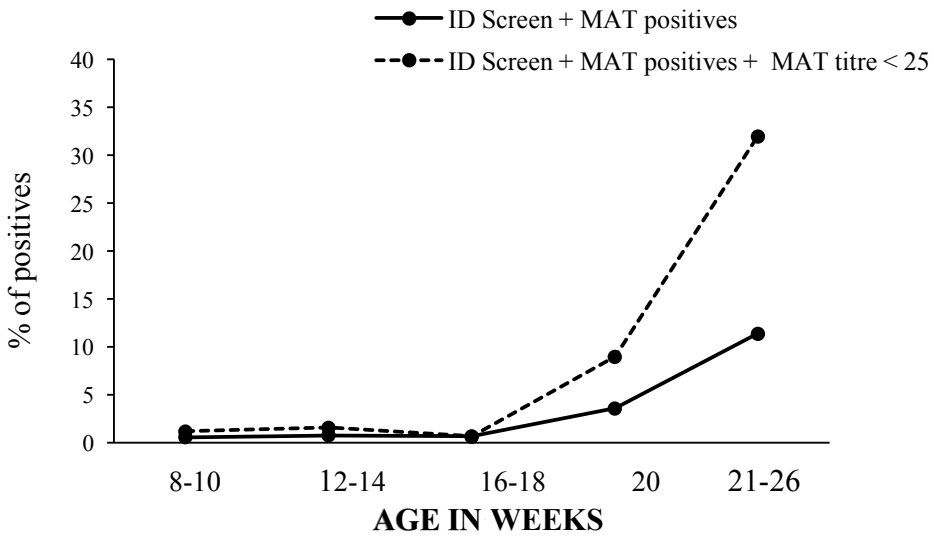

Figure 2.10 Distribution of seropositive animals by age. Black line refers to MAT and ID Screen® positive animals. Dashed line refers to MAT, ID Screen® positive animals and animals with MAT titre <25 (n=632).

The risk of seroconversion was measured with O.R. for a given age. Pigs older than 20 weeks had a risk of being seropositive (MAT positive and ID Screen® positive) 20.13 times higher (O.R.= 20.13, 95%, CI: 2.3-177; p = 0.0001) than 8-10 week pigs, 5.2 times higher (O.R.= 5.2, 95%, CI: 1.2-22.4; p = 0.008) than 12-14 week old pigs, 18.2 times higher (O.R.= 18.2, 95%, CI: 2-160.4; p = 0.0002) than 16-18 week old pigs and 3.4 times higher (O.R.= 3.4, 95%, CI: 0.9-11.8; p = 0.02) than 20 week old pigs. 20 week old pigs had a risk of being seropositive 5.8 times higher (O.R.= 5.8, 95%, CI: 0.7-49.5; p = 0.03) than 8-10 week pigs, 1.5 times higher (O.R.= 1.5, 95%, CI: 0.4-6.1; p = 0.2) than 12-14 week old pigs and 5.3 times higher (O.R.= 5.3, 95%, CI: 0.6-44.8; p = 0.0002) than 16-18 week old pigs.

Other risk factors were also re-analysed statistically including those additional 7 positive animals with the ID Screen® ELISA to study whether there was a relationship between gender and disease, between environment and disease and between farms. However, the Fisher

Exact test did not show a statistical association including those animals either for gender and infection (p= 0.3), for environment and infection (p= 0.15) and between farms (p=0.24 among 64-animal farms and p=0.153 among the farms which had 10 animals each).

Finally, a contingency table was performed with the results of the ID Screen® and the DT. Of 3 DT positives samples, 2 tested also positive with the ELISA. The number of false positive according to the DT was 2 (table 2.23). The 2 samples with IU/ml<8 were negative with the ELISA. The two concordant positive samples had the higher IU of 500 and 32 with the DT. The samples which tested positive with the ELISA and were negative with the DT were two 16 and 12 week old pigs raised in farm 2. The sensitivity and specificity of this ELISA based on the DT was 66.7% (95%CI: 9.4%-99.2%) and 95.7% (95%CI: 85.5%-99.5%) respectively and the agreement was moderate (kappa= 0.54, 95%CI: 0.05-0.99, $p \leq 10^{-3}$). The kappa agreement, the sensitivity and specificity of the ELISA also improved when the cut off generated with the ROC curve was used. When the cut off o provided by the manufacturer instructions was used, kappa value was 0.370 (95%CI: -0.188-0.92, p = 0.007) with a sensitivity and specificity of 33.3% (95%CI: 9.4%-99.2%) and 97.8% (95%CI: 0.8%-90.6%) respectively.

Table 2.23 Results of ID Screen® and DT titre.

		DT			
		Positive	Negative	Questionable	Total
ID Screen®	Positive	2	2	0	4
	Negative	1	43	2	46
	Total	3	45	2	50

Below follows a table which summarizes the results generated by the different assays for each pig found to be positive (=18) by one or more of the validated assays.

Table 2.24 Seropositive animals using MAT, DT and IDScreen®

Pig [1]	MAT [2]	DT [3]	IDScreen	Age [4]	Farm	Environment [5]
1	1:25	np	+	25	Pilot B-1	TDLF
2	1:1000	np	+	12	Pilot A	TDLF
3	1:10	np	+	20	Pilot B-1	TDLF
4	–	–	+	12	2	TDLF
5	–	–	+	16	2	TDLF
6	1:4	4IU	–	16	3	TDLF
7	1:32000	500 IU	+	24	1	TDLF
8	1:100	32 IU	+	25	1	TDLF
9	1:4	2 IU	–	12	4	TDLF
10	1:25	8 IU	–	12	4	TDLF
11	1:25	np	–	20	11	TSMF
12	1:25	np	–	20	11	TSMF
13	1:50	np	–	8	6	TDLF
14	1:4	np	+	20	6	TDLF
15	1:4	np	+	20	6	TDLF
16	–	np	+	25	1	TDLF
17	1:10	np	+	23	1	TDLF
18	1:50	np	+	20	1	TDLF

[1]: hypothetical number look appendix V for individual identification (red rows).
[2]: Animals with MAT titres < 25 were only included in the table when positive by another additional test.
[3]: DT: Dye test, np: not performed.
[4]: Age in weeks.
[5]: TDLF: Tropical Deciduous Low Forest, TSMF: Tropical Subdeciduous Medium Forest.

2.5 Discussion

This study is pioneering in using a serological test for *T. gondii* which is validated to look at infection in pigs in Yucatan. The 1.4% (95%CI: 0.6%-2.7%) of seroprevalence obtained with the MAT is at odds with the previous epidemiological report of toxoplasmosis in pigs from Yucatan which reported 95.8% - 100% seroprevalence in the intensive farming system (Ortega-Pacheco *et al.,* 2013, Ortega-Pacheco *et al.,* 2011). However, the in-house ELISA test used in Ortega-Pacheco *et al*., (2011), Ortega-Pacheco *et al*., (2013), was shown here to have no agreement with other established tests including MAT (kappa coefficient = 0.017), DT and ID screen®. Previously reported seroprevalences obtained in intensive farming systems (0%-0.5%) in another Mexican states such Oaxaca and Baja California Sur (Alvarado-Esquivel *et al*., 2012c, Alvarado-Esquivel *et al*., 2015) also showed low prevalence and support the findings of this study (0.5%, 95%CI: 0%-1.8%).

When the in-house ELISA was duplicated at Salford University, the results were equivalent to those obtained in Merida (table 2.14). Thus, the likelihood of degradation of immunoglobulins during the import transfer was discarded. The reason why the ELISA test showed such a high number of false positives (390/623) when both antibodies were used together is not totally understood. OD increased with the age of the animals, being at the highest in older pigs independently of their *Toxoplasma* status (figure 2.5, table 2.10, table 2.11, table 2.12). Results of the ELISA test improved when only one secondary antibody (anti-pig) was used (table 2.14 -15). Therefore, these results led to the conclusion that the original assay which added both anti-human and anti-pig secondary antibodies could have led to a nonspecific interaction creating a high colorimetric background. As the OD increased with the age of the pigs, this was probably a factor present in the pig serum. In addition, when the ELISA was duplicated in Salford two samples that were negative in the first ELISA, were diagnosed as positive in Salford due to an increase of the OD of the cut off (table 2.14). This could be due to the poor repeatability of the assay. In fact, the interplate variance was considered high (CV=25.9%) and acceptable (CV=16.2%) for the negative and positive reference sera (Crowther, 2000). The OD of the controls varied between plates generating a variance from 0.328 to 0.638 in the cut off ODs. The OD was corrected for each sample according to Lind *et al*., (1997) but this did not improve the results of the test. Hence, I suggest this ELISA protocol

(Ortega-Pacheco *et al.,* 2013, Ortega-Pacheco *et al.,* 2011, Jiménez-Coello *et al*., 2013, Dzib-Paredes *et al*., 2016, Hernández-Cortazar *et al*., 2016a) is not suitable for the detection of IgG *T. gondii* antibodies in pig serum.

Although the results improved when the conjugate was prepared only with the secondary anti-pig antibody, these still differed from the MAT results. Sensitivity was as low as 30%. Pigs with MAT titres 1:1000, 1:50 and 1:25 were not diagnosed as positive. Although the specificity increased substantially (94.7%), false positives were still present as one negative sample (MAT, DT and ID Screen® negative) still was positive with this in-house ELISA and two negative samples (MAT, DT and ID Screen® negative) had a relatively high OD. In addition, the accuracy calculated with the ROC as moderate (AUC=0.561, 95%CI: 0.333-0.788, p-value=0.59) was not statistically significant. An attempt to optimize the test was performed by cross titration of the conjugate with a true positive and negative pig serum (positive and negative concordant with MAT, DT and ID Screen®). For serum and conjugate dilutions which gave the best ODs for the positive serum, the true negative showed also high ODs. A good titration of positive serum is characterized by a plateau phase in which the OD is at its maximum followed by a proportional decrease of the OD and ending with a low OD which characterizes the end point (figure 2.7). As a result of this nonspecific binding, extremely low BRs were produced in the chessboard titration. Optimal binding ratios have values > 15 (Crowther, 2000) and the highest BR obtained for this ELISA was 2.18 (table 2.16). The dilutions of the conjugate were chosen below and above the recommended by the manufacturer (1:250-16000 dilution) to cover the recommended range (500-10000 dilution). The dilutions of the positive and negative pig serum were chosen based on the dilutions used in other studies for this animal species (Kijlstra *et al*., 2004, Hill *et al.,* 2006, Sroka *et al.,* 2008, Gamble *et al.,* 2005). A development of an in-house ELISA for pigs is recommended rather than a modification of this human based ELISA kit since the cost of this is more elevated than an in-house ELISA; this human based ELISA did not perform better than in-house ELISAs developed in other studies and was in fact, shown to be not reliable. In-house ELISAs have the possibility of further optimization, for example, the optimization of the antigen which in this case was not possible. For situations in which an in-house ELISA is not feasible (for example, when there is lack of facilities to produce antigen, or difficulties of acquiring antigen or other reagents due to local regulations or lack of stocks) a more exhaustive optimization of this protocol is needed.

Pig serum was diluted 1:100 in the present ELISA, Lind *et al*., (1997) obtained cross-reactivity against *Sarcocystis miescheriana* at higher dilutions (1:400). It was suggested before that *T. gondii* lysate may react with antibodies to other pig pathogens (Dubey, 2009b). In the current case, the antigen was obtained from lysed parasites grown in HeLa cells. Better specificity was obtained with the ELISA test ID Screen® in which the wells were coated with the P30 antigen (native antigen) of *T. gondii*. Perhaps the low specificity was due to a cross-reactivity against *T. gondii* organelles or residuals of HeLa cells present in the antigen but further investigations are necessary to support this hypothesis.

The cut off titre in MAT of 1:25 was selected as in studies described by others (Dubey, 1997c, Gamble *et al*., 2005, Hill *et al.,* 2006, Venturini *et al*., 2004, Piassa *et al*., 2010, Gauss *et al*., 2005, Alvarado-Esquivel *et al*., 2011a, Alvarado-Esquivel *et al*., 2012c, Alvarado-Esquivel *et al*., 2014). A previous report initially suggested a cut off of 1:10 instead of 1:25 as the sensitivity increased from 85.7% to 95.7%, however a decrease of the specificity was observed from 94.6% to 89.2% (Gamble *et al*., 2005). The close cut off of 1:20 titre has also been used (Dubey *et al*., 1995a, Dubey *et al*., 1995b) with reliable results, nevertheless the specificity against other pathogens in pigs was validated at 1:25 titre by Dubey (1997c). Some studies used a higher cut off at 1:40 titres but a considerable loss of sensitivity (77.8%) was observed (Sroka *et al*., 2008) when compared with the sensitivity (85.7%) obtained at 1:25 (Gamble *et al.,* 2005). Low antibody titres were considered as 'doubtful' as *T. gondii* has been isolated from pigs with MAT titres 1:10 and <10 but not considered as positives as the specificity has not been validated at such low dilutions (Dubey, 1997c).

MAT and DT had a strong agreement (kappa = 0.901) in the present work. Sensitivity and specificity for MAT were 100% and 97.8% respectively based on the DT as a gold standard (Moghazy *et al*., 2011) Other studies have shown also a good agreement between these two tests (table 2.4). In addition, titres in MAT were parallel to those obtained with DT (table 2.20). The cut off of > 4IU in the DT which was used here followed a recent study by the WHO in which a cut off > 4IU was the consensus for most of the reference laboratories of *Toxoplasma* participants from France, UK, Israel, Norway and Germany (Reiter-Owona *et al*, 1999). Animals with lower IU than 4IU were considered as 'doubtful' as some laboratories consider those low titres also positives (Reiter-Owona *et al*, 1999). Studies in pigs have expressed the

cut off as the dilution of the serum instead of IU. A Cut-off of 1:4 to 1:10 has been used in pigs with high specificity and sensitivity (Dubey *et al*., 2002, Dubey *et al*., 1996b, Hellesnes *et al*., 1978). However, the dilution of the serum was not provided by the the *Toxoplasma* Reference Unit in Swansea. Depending on the laboratory 4IU could correspond to a titre between 1:4-1:16 in serum (Reiter-Owona *et al*, 1999).

The multispecies ID Screen® ELISA was chosen as it has been validated in pigs and it has also been widely used in this animal species by several authors (Hotea *et al*., 2010, Bokken *et al*., 2012, Gómez-Laguna *et al.,* 2011, Calero-Bernal *et al.,* 2013, Hernández *et al*., 2014, Steinparzer *et al.,* 2015, Papatsiros *et al.,* 2016). Furthermore, ID Screen® showed slightly better agreement with the MAT test (k= 0.67) when compared with two other commercially available ELISAs validated in pigs (k= 0.62-64) (Steinparzer *et al.,* 2015). In addition, in Bokken *et al*., (2012) the accuracy of the test was higher (AUC= 0.999) than one in-house ELISA (AUC= 0.906) and another commercially available ELISA validated in pigs (AUC= 0.930) when compared in experimentally infected pigs. The cut off produced with the ROC curve showed better balance of sensitivity and specificity (56% and 91.4%) than with the cut of suggested by the manufacturer instructions (33.3% and 97.5%) using the MAT as a gold standard. The balance of sensitivity and specificity also improved (66.7% and 95.7% instead of 33.3% and 97.8%) with the cut off calculated with the ROC curve when the test was compared with the DT. The agreement with the MAT(k=41) and DT (k=0.54) also increased when the cut off generated with the ROC was used instead of the cut off suggested by the manufacturer (k =0.385 and k= 0.370). Hence an evaluation of the cut off is suggested for a more accurate diagnosis when using this test. Better accuracy was also obtained in Bokken *et al*., (2012) with the cut off calculated with the ROC curve, rather than with the cut off provided with the test. Although the agreement of ELISA ID Screen® with MAT and DT was moderate, overall, the accuracy of the test was good (AUC=0.81) (Hajian-Tilaki, 2013). The test showed a good specificity when compared with the MAT test and DT (91% and 95.7%). However, the sensitivity of the assay was lower than expected (56% and 66.7% with the MAT and DT respectively). Lower performance of ELISA has been reported before in comparison with the MAT and DT (Dubey *et al*., 1995c, Moghazy *et al*., 2011). Steinparzer *et al.,* (2015) is the only study in which IDvet Screen® has been compared with the MAT test in pigs and its agreement was higher (k=0.67) than in the present study (k=41). Nevertheless, the cut off used in

Steinparzer *et al.,* (2015) for the MAT test was 1:40 instead of 1:25 and a loss of sensitivity has been reported in MAT when a cut off of 1:40 (77.8%) was used instead of 1:25 (85.7%) (Sroka *et al*., 2008, Gamble *et al.,* 2005). Even though ID Screen® showed a substantial agreement (k=0.67) in Steinparzer *et al.,* (2015), the sensitivity of the assay still was low (57.5%) when compared with the MAT test. Furthermore, pigs with MAT titres < 180 did not test positive with the IDvet Screen® in Steinparzer *et al.,* (2015). Studies in which ID Screen® was compared with the MAT test in other animal species such goats and sheep also reported a difficulty when using the test to detect positive animals with MAT titre < 100 (Gebremedhin *et al.,* 2013). The reason why this test has low sensitivity in comparison with other serological tests is unclear. The test was validated in pigs with IFAT, which has been shown to be less sensitive than the DT and less specific than MAT and DT (table 2.4). Gamble *et al.,* (2005) compared the performance of an ELISA based on the P30 antigen and the whole tachyzoite in naturally infected pigs. The sensitivity was much lower (57.1%) when only the P30 antigen was used than when the whole tachyzoite was used (88.6%), however the study did not mention whether there was a difference in specificity.

Whether the animals which tested positive with ID Screen® and tested negative or doubtful with the DT and MAT are true or false positives is unclear. DT and MAT has shown to be more sensitive than this ELISA. On the other hand, sensitivity and specificity of MAT and DT are rarely 100% and it is a challenge to find a diagnostic test with such level of accuracy. Further testing of those animals is necessary to reach a conclusion. Bioassay in mice and cats has shown to be the gold standard for demonstration of the parasite in livestock (Dubey *et al*., 2009a Liu *et al*., 2015, Gangneux and Dardé, 2012). Bioassay is a good definitive test to use when results are unclear. Nevertheless, those unclear animals seemed to follow the same pattern as MAT positive animals. For example, ID Screen® positive animals increased with age. Of the 12 positive animals, nine were 20 week old or older, two were 12 week old and one was 16 week old. Two of the three farms with the additional ELISA positive animals were farms that already had MAT positive animals (farm 6, farm pilot B).

The 1.4% of *Toxoplasma* prevalence estimated here, suggests that the risk of human infection derived from the consumption of pork from pigs raised in intensive farms is much lower than previously believed (Ortega-Pacheco *et al.,* 2013). It is noteworthy to mention that

there are studies which have isolated the parasite from seronegative pigs. Omata *et al*., (1994) found that 2 of 14 isolates were from seronegative pigs by IFAT (titres <16). Dubey *et al*., (1995b) obtained 29 of 170 isolates from seronegative pigs by MAT (titre 1:10). Dubey *et al*., (2002a) obtained 2 of 51 isolates that were from seronegative pigs by MAT (titre <10) and DT (titre <10). Moreover, in one experiment one pig fed with 10 oocysts did not develop antibody titres detectable by MAT, DT, IHAT, LAT and ELISA (Dubey *et al*., 1996b), however infection was confirmed as *T. gondii* was isolated from its tissue. Gamble *et al*., (2005) obtained 14% of the *T. gondii* isolates from pigs with MAT titres <25). Djocik *et al*., (2016) also obtained one *T. gondii* isolate from a MAT negative pig. These findings suggested that a low percentage of positive pigs may remain undetectable with serological tests. Another reason for this may be because the antibody titres had not reached detectable levels as IgG antibodies in pigs appear between 10 and 17 days post infection (Lind *et al*., 1997; Jungersen *et al*., 1999). Another possibility is that a low percentage of pigs did not react immunogenically to the infection or perhaps, reacted very little that the serological tests were not sensitive enough to detect those levels. Those animals with low levels of IgG in MAT (< 1:25 titre) could be animals in which IgG levels have not reached yet detectable levels at higher dilutions. Even though, a small number of animals could be false negatives, MAT test has been shown to be a very reliable and accurate test. This technique is the preferred *T. gondii* serological diagnostic test in pigs for many researchers as it has been shown to be highly sensitive and specific (table 2.4).

The use of additional techniques such as PCR or IgM detection could be useful to detect the parasite at an earlier stage of infection. The advantage of PCR is that the animal can be diagnosed soon after infection while IgM can not be detected before 8-10 days post infection (Lind *et al*., 1997; Jungersen *et al*., 1999). Thus, although IgG antibody detection is useful to monitor the disease at the farm level, it could not be a true indicator of the number of pigs carrying the parasite. Additional techniques such PCR and/or IgM detection in combination with MAT could be useful to assess the level of toxoplasmosis *in vivo* at the farm level.

As several studies have found a differences in *T. gondii* prevalence between different environmental conditions in the same area (Alvarado-Esquivel *et al*, 2012c, Alvarado-Esquivel *et al*, 2014), two different environments were sampled. In this study the 3.33% of prevalence obtained in the tropical sub-deciduous medium forest was higher than the 0% obtained in pigs

with the same age in the tropical deciduous low forest. Tropical sub-deciduous medium forest is characterized by having a shorter dry season, slightly elevated annual rainfall and less fall of foliage coverage in the dry season than the tropical deciduous low forest (Barber *et al.,* 2000, Miranda and Xolocotzi, 1963). These conditions may help parasite survival as it has been suggested that oocysts survive for longer periods in humid conditions and that moisture increases the oocyst survival in periods of heat (Afonso *et al.,* 2006, Lélu *et al.,* 2012). However, this difference was not supported statistically neither by taking only MAT results ($p = 0.14$) nor by including ID Screen® positives ($p=0.15$). Possibly a larger sample size could improve the significance. A Power test using the actual seroprevalence data indicated an unfeasibly high sample size of 2900 pigs would be needed in each environment to assess any difference due to environmental conditions. As such it was not possible in the confines of this thesis work to determine whether the conditions of the Tropical Subdeciduous Medium Forest caused any *T. gondii* epidemiological differences compared to the ropical Deciduous Low Forest.

Previous studies have demonstrated an increase of *T. gondii* prevalence with pig age (García-Bocanegra *et al*., 2010a, Dubey *et al*., 1991, Weigel *et al*., 1995a, Villari *et al*., 2009). To increase the sample size for the age analysis, animals from the isolation study were included. The number of positive animals increased slightly with the age (one animal of 8 weeks was positive, 2 of 12 weeks, 3 of 20 weeks and 3 of >20 weeks). In addition, all animals with antibody titres <25 were 20 weeks or older except one that was 12 weeks old (table 2.11). Moreover, positive ELISA ID Screen® results with pigs which were MAT negative and MAT titre <25 were 12-16 weeks and 20-25 weeks old respectively. This increase of anti *T. gondii* antibodies with age supports the notion that horizontal transmission is more relevant than vertical transmission in this species in agreement with other studies (García-Bocanegra *et al*., 2010b, García-Bocanegra *et al*., 2010c, Dubey, 2009b). Moreover, the possibility of passive seroconversion in the 8-12 week old pigs was rejected as maternal antibodies transferring from the mother to the sucking piglet in colostrum cannot be detected after 6 weeks of life (García-Bocanegra *et al*., 2010c). In addition, the placenta in sows is epitheliochorial, which means that the transfer of antibodies from the mother to the offspring does not occur during pregnancy (Dubey and Urban, 1990, Pastoret *et al*., 1998). In the binary logistic regression, although it was close ($p = 0.051$), age was a non-significant factor in disease when only positive MAT animals were used. However, when positive ID Screen® animals were included, age was a

significant factor (p = 0.003). Since the prevalence is low in this study, a larger sample size could provide a much stronger association between age and disease. It has been suggested that horizontal transmission is the most important route of infection in humans (Tenter *et al.,* 2000) and cats (Afonso *et al.,* 2006) while in rodents (Marshall *et al*., 2004) and more controversially in sheep (Williams *et al*., 2005, Innes *et al.,* 2009) vertical transmission is believed to be more important.

The prevalence of *Toxoplasma* infection among farms differed in both environments. Some farms had 0% of MAT positive animals (farm 2-3, farm 5, farm7-10, farm 12), two farms had 1.5% of MAT positive animals (farm 4 and farm 6), two farms had 3.8% of MAT positive animals (farm pilot A and pilot B) and one farm had 20% of MAT positive animals (farm 11). When the additional positives with the ID Screen® were included the prevalence increased to 4.7% in farm 6, to 3.1% in farm 2 and to 7.4% in farm pilot B. Several studies suggested that individual farm regimens and farm characteristics can have an effect on *T. gondii* prevalence. Factors such as source of water (Villari *et al.,* 2009), rodent control (Kijlstra, *et al*., 2008, Weigel *et al*., 1995b, Assadi-Rad *et al*., 1995, Lehman *et al*., 2003), cat access (Weigel *et al*., 1995b, Assadi-Rad *et al*., 1995, Lehman *et al*., 2003), size of the farm (Kijlstra, *et al*., 2004), and pig breed (Alvarado-Esquivel *et al*., 2011a, Damriyasa *et al*., 2004) were risk factors to *T. gondii* infection in pig farms. The theory that particular farm management or characteristics could have influenced the difference of prevalence between farms located in the same environment, is supported by the fact that seroprevalence differed between farms. All farms were intensive and full cycle thus differences in prevalence did not differ because of different production systems. Thus, management and farm characteristics could have an influence in *T. gondii* transmission. However, no statistical significance in prevalence was found between farms in either the tropical deciduous low forest (p = 1) nor the tropical sub-deciduous medium forest (p = 0.153) including when the ID screen® positive animals were included in the fisher test (p=0.24 and p=0.153). Future studies in which prevalence is compared at the farm level should consider higher sample size and only older pigs (> 20 weeks old) as the prevalence was higher at this age. Interestingly, all farms with MAT and ELISA ID Screen® positive animals had rodents, stray cats, lack of water treatment, open feeders to the environment and open warehouses (appendix III.1). Pigs remained in the farrowing area for one month, until piglets were weaned, afterwards, they remained in the nursery area for 4-6 weeks before they were

finally moved to the fattening area (around 10 -12 weeks). In addition, the floor in the nursery area was noted; there were two types of floors in this farm area: a raised perforated floor and low concrete floor. A raised perforated floor avoids the accumulation of water, excrement or any other waste and therefore decrease the possibility of contact with oocysts within cat faeces. It has also been mentioned that cats do not like being on a perforated floor (Skjerve *et al*., 1998). Therefore, the presence of a low concrete floor could be a risk factor for *T. gondii* transmission in pig farms. A curious finding is that farm 6, which had the higher prevalence in the tropical deciduous low forest and the youngest positive pig (8 weeks), was the only one in which the floor of the nursery area was a low concrete floor (appendix III). For future studies of risk factors at farm level this could be an interesting risk factor to include in the analysis. There are no studies that have compared the transmission between different types of floors on pig farms, but it has been demonstrated that using raised perforated floors in sheep farms reduces the risk of *T. gondii* transmission (Skjerve *et al*., 1998, Hernández-Cortazar *et al*., 2014).

The results showed no statistically significant differences in prevalences between gender ($p = 0.2$), even when the ID screen® positive animals were included in the Fisher Exact test ($p = 0.3$). There are no studies in pigs which demonstrate differences in prevalence between genders. Some studies reported higher prevalences in sows (Alvarado-Esquivel *et al*, 2012c, Klun *et al*., 2011, Lunden *et al*., 2002), however, this difference is attributed to the usual higher age at which sows are slaughtered. Sows are reproductive pigs which have a life time of nearly 30 months in contrast fattening pigs have an average span of life of 6 months.

The pilot study showed higher prevalence (3.77%-5.5%) than the main study (0.9%-1.8%), However older pigs were sampled in the pilot study and the proportion of ≥ 20-week old pigs was larger than any other age group (table 2.7). Thus, these differences in prevalence could be due to the higher proportion of old pigs in comparison with the main study. It is very unlikely that this discrepancy is due to particular differences in farm management and characteristics as farm pilot B is the same farm as farm 1. In fact, 2 of 7 pigs raised in farm 1 in 2014 (main study) were positive when sampling at slaughter age (average 25 weeks). All the samples of the present study were collected in the wet season (June-October) during the three years of the study. Thus influence of the seasonality was discarded. Seasonal difference in *T. gondii* prevalence was suggested in one study in which higher prevalence was found in winter than in summer in pigs

at slaughter age from the Netherlands (Swanenburg *et al*., 2015). However, the reason was not clear and details such as number of farms, pigs and production system were not given in the study.

It is important to mention that although the backyard production system in Mexico has little sanitary management, it is a common source of food for families in rural areas in Yucatan. Currently, the published studies on this group of animals are based on the ELISA evaluated in this thesis and which results have shown to be unreliable (Jiménez-Coello *et al*., 2013). The prevalence obtained in backyard pigs in southern states in Mexico with the MAT test was much higher (17%-45.3%) than the prevalence obtained in intensive farms (0.5%) using the same well validated test (Alvarado-Esquivel *et al*., 2012c, Alvarado-Esquivel *et al*., 2014). Future studies could focus on this production system as the published work suggests that the prevalence in the backyard system could be higher and there is a lack of epidemiological studies in such production systems in Yucatan.

CHAPTER 3

Detection of *T. gondii* DNA in pig blood

3.1 Introduction

The main aim of this chapter was to assess the *T. gondii* prevalence in pigs from Yucatan using PCR on blood. Blood extracts from pigs of different age groups were analyzed to investigate the age profile of the active infection at the farm level. This study is pioneering in analyzing the prevalence of *T. gondii* DNA in blood in pigs of different ages. Historically, the majority of epidemiological studies in *T. gondii* in animals and humans have been based on serological methods. However, in the last two decades DNA based diagnosis of *T. gondii* by PCR has become more common and has turned into an indispensable laboratory test (Ivović *et al*., 2012). One advantage of PCR is its versatility, as it can be used on any biological sample. Detection of *T. gondii* may be performed in peripheral blood when there is suspicion of active infection but can also be performed in tissue in the case of chronic infection. In cases of suspected congenital toxoplasmosis PCR has been performed on umbilical cord, amniotic fluid (AF), or fetal tissues of animals and humans (Wiengcharoen *et al*., 2004, Bezerra *et al*., 2014, Remington *et al*., 2004, Goldstein *et al.,* 2008). PCR revolutionized the diagnosis of human congenital toxoplasmosis and it has become the first choice diagnostic method *in utero* toxoplasmosis infection. The reason for this is that PCR has been shown to have high sensitivity and specificity in several studies in AF (Hohlfeld *et al*., 1994, Gratzl *et al*., 1998, Foulon *et al*., 1999) showing better performance than bioassay in mice or culture isolation (Hohlfeld *et al*., 1994, Foulon *et al*., 1999). In patients with suspected ocular and cerebral toxoplasmosis, PCR can be performed in the vitreous or aqueous fluid of the eyes, in the retina or in cerebrospinal fluid (CSF) (Mesquita *et al*., 2010, Montoya, 1999). In addition, PCR can be used in meat and meat derivatives (Aspinall *et al*., 2002, Warnekulasuriya *et al*., 1998), milk (Mancianti *et al*., 2013, Dubey *et al*., 2014, Mancianti *et al*., 2014, Tavassoli *et al*., 2013a), fruits and vegetables (Lass *et al*., 2012) and water (Wells *et al*., 2015) for monitoring of *T. gondii* in products for human consumption.

3.1.1 PCR techniques used in *T. gondii* diagnosis

Polymerase Chain Reaction is a technique that amplifies exponentially a specific DNA sequence (the target), in this case a sequence from the *T. gondii* genome that may be present in a clinical sample, to produce a DNA product. The amplified DNA product is resolved through a gel by electrophoresis to separate the expected size of the target DNA fragment and this is visualized with a dye, which binds to DNA and fluoresces under UV light. Comparisons between different PCR methods have shown that the performance of the test depends on variables such as the tissue used, the type of PCR and the selection of the target to be amplified (Jones *et al*., 2000, Chabbert *et al*., 2004, Lau *et al*., 2010). Detection of *T. gondii* in clinical samples can be challenging due to the high density of host DNA present such as in blood or even more challenging in tissue or meat extracts. Sensitivity of *T. gondii* detection in tissues or meats is in general lower than in blood or other body fluids where the contaminating host-derived DNA level is low (Jauregui *et al*., 2001). In addition, it has been suggested that preservatives found in meats such as salt can lead to a PCR inhibition (Warnekulasuriya *et al*., 1998). For these reasons the use of highly sensitive PCR methods such N-PCR (Nested-PCR), RT-PCR (Quantitative or Real Time PCR) or LAMP (Loop mediated isothermal Amplification) are recommended to avoid false negatives. N-PCR, is a modification of C-PCR (Conventional PCR) in which the product of the first PCR is used as a template with another set of primers (nested primers). This type of PCR allows the detection of low copy numbers of the target DNA with high specificity and sensitivity (Hierl *et al*., 2004, Vitale *et al*., 2013, Lau *et al*., 2010, Wiengcharoen *et al*., 2004). The sensitivity of N-PCR has been estimated to be around 1-10 parasites per reaction in recent studies (Su *et al*., 2010, Khan *et al*., 2005a, Vitale *et al*., 2013) while the detection limit with C-PCR has been estimated to be 10-100 times less sensitive (Nagy *et al*., 2006, Su *et al*., 2010, Piergili, 2004). RT-PCR is a widely used technique in which the amplified DNA is monitored in real time. RT-PCR uses a fluorescent dye such as SYBR Green dyes or fluorophore labelled probes which bind to the product, thus the fluorescence intensity is proportional to the amount of DNA amplified. In addition, RT-PCR allows the quantification of the target in the samples. RT-PCR has been suggested to be a more sensitive method than N-PCR, being capable of detecting as few as 0.1 parasites per reaction when it is using along with multi-copy genes (Reischl *et al*., 2003, Lin *et al*., 2012). However, some studies have obtained comparable (Calderaro *et al*., 2006, Vitale *et al*., 2013) or even superior accuracy in N-PCR

(Hierl *et al*., 2004) when compared with RT-PCR. The downside of RT-PCR is that this high sensitivity may lead to false positives and the necessity of expensive reagents and special equipment (Wang *et al*., 2013). LAMP is a relatively new modification of the C-PCR in which the DNA is amplified under isothermal conditions, therefore without the necessity of a thermal cycler. The visualization of the product can be done by observation of the turbidity or color change of the reaction tube by naked eye (Fallahi *et al*., 2015, Wang *et al*., 2013). This is a technique for *T. gondii* detection that has been extended for use in the field or in low-income countries since it is cheap and it does not require special equipment. Its sensitivity has also been reported to be higher than N-PCR (Fallahi *et al*., 2015, Lin *et al*., 2012). However, caution should be exercised with this technique as although it has high sensitivity, there is a risk of carry over contamination due to the robustness of the LAMP product (Dhama *et al*., 2014) which could cause some unreliability in the results. For example, Francois *et al*., (2011) found a variation of the minimal amplification time from 60 to120 minutes in positive samples and one of the negative controls showed amplification after 180 minutes.

Different DNA targets have been used for PCR detection of *T. gondii*. The target used in the current study was the single copy P30 gene. This gene, is one of the most widely used targets for *T. gondii* diagnosis. Savva *et al*., (1990) was the first in developing a PCR protocol for detection of *T. gondii* targeting this gene. The P30 gene, also called SAG1 gene, encodes the *T. gondii* major surface antigen (Burg *et al*., 1988). The P30 gene has been widely used in C-PCR, N-PCR, RT-PCR and LAMP and in different species including pigs (Yu *et al*., 2013, Warnekulasuriya *et al*., 1998, Wang *et al*., 2013). Other single copy genes used for *T. gondii* diagnosis include SAG2, SAG3, GRA1 and GRA6 (Liu *et al*., 2015, Lau *et al*., 2010, Mason *et al*., 2010). Repetitive sequences such as the B1 gene, ribosomal genes and the 529bp repetitive element are more popular than single copy genes due to an increase of the sensitivity in comparison with one single copy genes. Nevertheless, this sensitivity can be compensated using N-PCR (Jones *et al*., 2000, Warnekulasuriya *et al*., 1998) or RT-PCR (Buchbinder *et al*., 2003, Yu *et al*., 2013). It has been estimated that the number of copies per parasite for the B1 and 529bp repetitive element are 35 and 200-300 respectively. Conversely, recent studies have shown that their validity in terms of quantification is questionable. Wahab *et al*., (2010) suggested that the 529bp repetitive fragment was absent in some *T. gondii* strains and his study found a 4.8% rate of false negatives using this target. Secondly, Costa and Bretagne, (2012)

quantified that the number of copies of the B1 gene and the 529bp repetitive element were between 5-12 and 4-8 times lower than expected. These findings question the reliability on these multi copy genes as a diagnostic method. Other multi-copy genes used as a target in *T. gondii* amplification are the ribosomal genes such 18s rDNA and ITS-1. Although these gene targets have been estimated to have 110 copies per parasite, they were shown to have the same sensitivity as B1 (Hurtado *et al*., 2001; Calderaro *et al*., 2006) or lower (Okay *et al*., 2009).

3.1.2 PCR techniques used in pigs

Most molecular detection methods in pigs are developed in tissues such as organs or pork derivatives such as sausages, minced pork or cured meats (Da Silva *et al*., 2005, Belfort *et al.,* 2007, Tsutsui *et al.,* 2007, Jauregui *et al.,* 2001, Halová *el al*., 2013) for monitoring of *T. gondii* in food products for human consumption. Only four studies have been developed in peripheral swine blood (Lin *et al*., 2012, Yu *et al*., 2013, Ortega-Pacheco *et al*., 2013, Wang *et al*., 2013). Additionally, *T. gondii* DNA has been detected in the retinas (García *et al*., 2008) and semen (Moura *et al*., 2007a) from experimentally infected pigs.

Some studies in pigs have assessed the sensitivity of different PCR methods. However, it is difficult to compare the performance of those methods due to the heterogeneity of the tissue and target used (table 3.1). In general, N-PCR, the technique used in this study, shows higher sensitivity than C-PCR. For example, Yu *et al*., (2013) found the higher limit of 1000 parasites per reaction targeting the single copy gene SAG1 by C-PCR in blood samples. When the same target was used in N-PCR, Warnekulasuriya *et al*., (1998) found a lower detection limit of 250 parasites by N-PCR using the same target but in meat samples. Parallel results were obtained when multi copy genes were used instead of SAG1; C-PCR targeting the 529 bp repeat element had a detection limit of 10-50 parasites per reaction in blood (Lin *et al*., 2012) and tissue (Tsutsui *et al.,* 2007) respectively, but when N-PCR was used using the ITS-1 in meat, the detection limit was 2 parasites per reaction (Vitale *et al*., 2013).

The most common targets used in pigs include SAG1, SAG2, ITS-1, B1 and the 529 repetitive element. Other single copy genes such SAG2, SAG3 and GRA6 have used with genotyping purposes and rarely to assess the contamination of *T. gondii* in pig tissues. Even

though SAG1 is a single copy gene, it is still one of the preferred targets for *T. gondii* DNA detection in pigs. This gene has also shown comparable results when compared with multi copy genes in pigs using RT-PCR. For example, Yu *et al*., (2013) obtained a detection threshold of 1 parasite per reaction targeting the SAG1 gene and the detection threshold obtained in other studies with the same technique using multi copy genes range from 0.1 to 10 parasites per reaction (table 3.1).

The present study is the first to use the N-PCR technique targeting the SAG1 gene in blood from pigs. Furthermore, the region amplified in this study comprises six SNPs (Single Nucleotide Polymorphism), which are able to discriminate between types I or II and III strains (Su *et al*., 2010).

Table 3.1 Comparison of sensitivity of different PCR methods used in pigs. Information is organized from the lowest to the highest detection limit.

Method	Target	Tissue	Detection limit [a]	Reference
RT-PCR	529bp	Blood	0.1 parasite	Lin *et al*., 2012
RT-PCR	SAG1	Blood	1 parasite	Yu *et al*., 2013
RT-PCR	ITS-1	Muscle	1 parasite	Jauregui *et al*., 2001
LAMP	529bp	Blood	1 parasite	Lin *et al*., 2012
RT-PCR	B1	Minced pork	1 parasites	Sutthikornchai *et al*., 2013
N-PCR	ITS-1	Fresh sausages	2 parasites	Vitale *et al*., 2013
C-PCR	529bp	Blood	10 parasites	Lin *et al*., 2012
C-PCR	529bp	Muscle	50 parasites	Tsutsui *et al*., 2007
N-PCR	SAG1	Cured meats	250 parasites	Warnekulasuriya *et al*., 1998
C-PCR	SAG1	Blood	10^3 parasites	Yu *et al*., 2013

[a] The detection limit is the number of *T. gondii*/reaction of PCR.

3.1.3 PCR diagnosis of *T. gondii* in blood from pigs

Most reports of molecular diagnosis in peripheral blood have been developed for humans. In livestock, including in pigs, *T. gondii* DNA detection focuses on tissue rather than blood. This preference could be because tachyzoites are circulating in blood only during the active infection while tissues cysts can persist presumably beyond 865 days in pigs (Dubey, 1988).

The reason why there are only a few studies in which the prevalence of *T. gondii* is investigated by PCR in blood in pigs rather by serology could be because the active infection

lasts for 1-3 weeks after the pig has become infected with the parasite, while IgG antibodies can remain detectable 2 years after infection in this species (Dubey *et al*., 1997a). However, in contrast, as PCR is independent from the immune status of the animal, there is no delay between the infection of the pig and the positive detection. It has been estimated that pigs develop IgM and IgG detectable antibodies 8-10 days and 10-17 days post infection respectively (Lind *et al*., 1997, Jungersen *et al*., 1999) whilst it has been reported that *T. gondii* tachyzoites circulate in blood as early as one hour after infection in murine models (Chinchilla *et al*., 1993, Guerrero *et al*., 2015). The confirmation of this rapid invasion within one hour of the circulation system has not been verified in swine, however *T. gondii* DNA was detected within two days after infection by PCR in pigs (table 3.2). Wang *et al*., (2013) infected 15 pigs intraperitoneally with tachyzoites of the virulent strain GJS and those were bled from day 2 to 35 days post inoculation. Parasites were detected in blood by LAMP and RT-PCR targeting the SAG1 gene in 5 pigs on day 2 after challenging. The day 4-5 post challenge *T. gondii* was detected in 100% of the pigs with both methods. The detection was possible only in a few animals after the 21th day post inoculation (table 3.2).

Table 3.2 Detection of *T. gondii* from 15 challenged pigs by SAG1-PCR. Adapted from Wang *et al*., (2013). Free to reuse and adapt: https://creativecommons.org/licenses/by-nc/3.0/legalcode.

	2PI[a]	3PI	4PI	5PI	6PI	7PI	10PI	14PI	21PI	28PI	35PI	CNT[b]
LAMP	5/15	14/15	15/15	15/15	13/15	10/15	8/15	6/15	3/15	1/15	0/15	0/15
RT-PCR	5/15	14/15	15/15	15/15	13/15	10/15	8/15	6/15	3/15	1/15	0/15	0/15

[a] Days Post Inoculation (PI).
[b] Negative controls were the 15 pigs before challenging.

Published studies have shown that DNA detection in pig blood samples is a rapid, specific and sensitive method to process high number of samples in short time (Yu *et al*., 2013, Lin *et al*., 2012, Wang *et al*., 2013, Ortega-Pacheco *et al*., 2013). Yu *et al*., (2013) found 4.9% and 6% of PCR positives targeting the SAG1 gene in 181 slaughtered pigs in China by C-PCR and RT-PCR respectively (table 3.3). Wang *et al.,* (2013) found the slightly higher prevalence of 7.8% and 6.9% by RT-PCR and LAMP respectively, also targeting the SAG1 gene in 216 pigs at slaughter age in China. Lin *et al*., (2012) also studied the prevalence in pigs at slaughtered age in China but using the 529 bp repetitive element by C-PCR, LAMP and RT-PCR and obtained a prevalence of 0.4%-4.2% in 284 pigs. Although Lin *et al*., (2012) used a multi copy

gene, they obtained lower prevalence in comparison with the previous studies which used the SAG1 gene (table 3.3). When Ortega-Pacheco *et al*., (2013) studied the prevalence in 18-20 week old pigs in Yucatan by C-PCR targeting the B1 gene, they obtained the much higher prevalence of 50.8% (table 3.3). All the studies are based in old pigs at slaughter age (20-25 weeks) or close to that age (18-20 weeks).

Table 3.3 *T. gondii* DNA frequency in naturally infected pigs by PCR in blood.

Method	Target	N	Age pigs	Prevalence	Location	Reference
RT-PCR	SAG-1	216	Slaughter	7.8%	China	Wang *et al*., 2013
LAMP	SAG-1	216	Slaughter	6.9%	China	Wang *et al*., 2013
RT-PCR	SAG-1	181	Slaughter	6.07%	China	Yu *et al*., 2013
C-PCR	SAG-1	181	Slaughter	4.97%	China	Yu *et al*., 2013
C-PCR	529bp	284	Slaughter	0.4%	China	Lin *et al*., 2012
LAMP	529bp	284	Slaughter	3.2%	China	Lin *et al*., 2012
RT-PCR	529bp	284	Slaughter	4.2%	China	Lin *et al*., 2012
C-PCR	B1	429	18-20 weeks	50.8%	Yucatan	Ortega-Pacheco *et al*., 2013

3.2 Aims and objectives of the chapter

The main aim of this chapter was to determine the frequency of *T. gondii* DNA circulating in blood in pigs from Yucatan. More specifically, the objectives were: firstly, to optimize three published N-PCR protocols which are SAG1 N-PCR, SAG2 N-PCR and B1 N-PCR (Su *et al*., 2010, Mason *et al*., 2010); secondly, to evaluate the sensitivity of these N-PCR protocols with a high density of host DNA; thirdly, to analyze the prevalence of *T. gondii* DNA in swine blood in different age groups; fourthly, to discuss the disease status of the pigs of this study using the serological and PCR results; lastly, to propose the PCR as a diagnostic tool for *T. gondii* detection in swine blood.

3.3 Materials and methods

3.3.1 Cell and parasite culture

T. gondii DNA was purified from the RH strain maintained for many years in our laboratory (Prof Judith Smith's collection). Parasites were thawed from liquid nitrogen and cultured in Madin-Darby Bovine Kidney (MDBK) cells (ECACC, UK) expected to be *T. gondii* DNA free. Cells were maintained in 10 ml of Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich) with 1% of non-essential amino acids (NEAA, Sigma-Aldrich) in 10% of fetal calf serum (FCS, Thermo Fisher Scientific) in 25cm^2 cell culture filter cap flasks (Thermo scientific, Nunclon Delta) at 37°C and 5% CO_2 atmosphere. The medium was changed every three days and cells were passaged once per week and seeded at 2x10^5-1x10^6 cells per flask. Passages were carried out by washing in 10 ml of PBS pH 7.2 (Sigma-Aldrich) and trypsinizing in 4 ml of 0.25% trypsin and 0.02% EDTA (Sigma-Aldrich). To help the detachment, cells were gently agitated by tapping. The trypsin was neutralized with 6 ml of media and cells were counted using a haemocytometer and aliquoted as appropriate.

Parasites were seeded at 10^6 doses when the confluence of the monolayer was around 80-90%. Tachyzoites that had failed to infect the monolayer and debris of cells were removed at 24 hours post-infection by changing the media. Parasites were allowed to grow until the monolayer was disrupted. The remaining cells in the monolayer were removed by trypsinizing and parasites released by passing the cells through a 27G needle. Free parasites either from media or released from cells, were centrifuged at 300 g for 10 minutes, resuspended in 1ml of EMEM, counted and inoculated to new flasks.

3.3.2 DNA extraction from cells and RH strain

DNA from *T. gondii* was purified from the free tachyzoites contained in the media. This media was harvested, centrifuged as above and parasites were counted. The pellet was washed twice in 1ml of PBS. The pellet was resuspended in 1ml of PBS and stored at -20°C until DNA extraction. 5x10^6 MDBK cells were prepared for DNA extraction as parasites. MDBK DNA was extracted to prepare the spiked samples with *T. gondii* as PCR standards. DNA from both

was extracted using the Qiagen DNeasy Blood and Tissue kit following the manufacturer instructions. DNA concentration and purity were measured with a spectrophotometer (Nanodrop ND-1000).

3.3.3 PCR standards preparation

To prepare the standards, 10-fold serial dilution of *T. gondii* DNA was spiked into MDBK cells at 100ng/µl. The concentration of RH DNA ranged from 10ng/µl to 100fg/µl. Additional dilutions were made with 10, 50, 300, 400 and 500 fg to achieve more sensitivity. The *Toxoplasma* genome is haploid and it is considered to have a size of ~65 Mb (Khan *et al*., 2005b). The number of femtograms (fg) of one *Toxoplasma* parasite genome was calculated based on the assumption that the average molecular weight of one bp is 650 Dalton (Da), thus following the equation: *T. gondii* genome (fg) = Genome size (bp) x Molecular weight (650 Da/bp) x (1.66 x 10^{-9} Da/fg) (Dolezel *et al.,* 2003). A unique *T. gondii* parasite genome mass is therefore ~ 70 fg. Hence the *T. gondii* concentrations of the standards ranged from 1.4x10^{5}/µl to 0.14 parasites/µl approximately. Standards were tested in triplicate with each marker. The detection of limit was the lower parasite concentration in which the PCR was positive in all the replicates.

3.3.4 DNA extraction of swine blood

Phlebotomy and transport of the blood to the research centre were performed as described in section 2.3.2. To avoid cross contamination, filter tips were used in all the steps, gloves were changed between samples if necessary and extraction reagents were aliquoted. *T. gondii* tachyzoites travel in the blood flow mainly inside leucocytes (Unno *et al*., 2008, Courret *et al.,* 2006) and for this reason, to increase the sensitivity of *T. gondii* detection in blood, DNA was extracted from the leucocyte layer (Brenier-Pinchart *et al.,* 2015). To isolate the leucocyte fraction, uncoagulated blood was centrifuged at 1300 g for 30 minutes. The fine white layer, corresponding to the leucocytes, was removed carefully with filter tips to avoid contamination with RBC and placed in sterile 2ml microcentrifuge tubes. The RBC remaining in the leucocyte fraction were lysed according to the Gallardo and Pelayo (2013) protocol. Three volumes of erythrocyte lysis solution (0.83% Ammonium Chloride, Sigma-Aldrich) were added to the

leucocyte fraction. This mixture was vortexed briefly and incubated on ice for 15 minutes followed by a centrifugation at 1050 g for 15 minutes. The supernatant was carefully removed using filter tips and the washing, incubation and centrifugation steps were repeated once more. Following this, the pellet of leucocytes was washed twice with phosphate buffered saline (PBS) at pH 7.4 and resuspended in 200 µl of PBS for DNA extraction. DNA was extracted with the Qiagen DNeasy Blood and Tissue Kit following the manufacturer instructions. DNA was eluted in 200 µl using the elution buffer AE of the kit and the volume was halved into two 1.5 ml microcentrifuge tubes and stored at -20C. Half of the DNA was transferred to Salford University, where it was tested. DNA concentration and purity were measured with the spectrophotometer Nanodrop 1000.

3.3.5 PCR protocols

SAG1 N-PCR and SAG2 N-PCR protocols were performed according to Su *et al*., (2010) with modifications. Reaction conditions were optimized by testing different concentrations of $MgCl_2$, Taq polymerase, primers, the addition of Q solution and an increase in the number of cycles, to increase sensitivity. The optimized amplification was performed in a volume of 25 µl with 1.25 units of Hot Start Plus Polymerase (Qiagen), 2 µl of DNA (1 µl of standard DNA), 2.5 µl of 10x clear buffer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 0.35 µM of each external forward 5'GTTCTAACCACGCACCCTGAG3' and 5'GGAACGCGAACAATGAGTTT3'and reverse 5'AAGAGTGGGAGGCTCTGTGA3' and 5'GCACTGTTGTCCAGGGTTTT3' SAG1 and SAG2 primers respectively. The nested reaction was carried out in a volume in 25 µl with 1.25 units of Hot Start Plus Polymerase (Qiagen), 2 µl of the PCR product obtained in the first round, 2.5 µl of 10x CoralLoad buffer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each internal forward 5'CAATGTGCACCTGTAGGAAGC3', 5'ACCCATCTGCGAAGAAAACG3' and reverse 5'GTGGTTCTCCGTCGGTGTGAG3', 5'ATTTCGACCAGCGGGAGCAC3' SAG1 and SAG2 primers respectively. The PCR conditions were carried out at 95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes. For the nested or internal reaction, the mixture was treated at 95°C for 5 min, followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1.5 minute. Reactions were performed in a Stratagene Robocycler Gradient 96 Gradient Thermal Cycler.

B1 N-PCR protocol was performed according to Mason *et al*., (2010) with adaptations. The external and nested amplifications were performed in 20 µl of reaction volume with 1 unit of Hot Start Plus Polymerase (Qiagen), 2 µl of 10x buffer, 2 mM of $MgCl_2$, 2 µl of DNA for the external reaction and 2 µl of the PCR product of the first round for the nested reaction, 100 µM of each dNTP were used in the external reaction and 200 µM in the internal, 0.2 µM of each external forward 5'GGAACTGCATCCGTTCATGAG3' and reverse 5'TCTTTAAAGCGTTCGTGGTC3' primers and internal forward 5'TGCATAGGTTGCAGTCACTG3' and reverse 5'GGCGACCAATCTGCGAATACACC3' primers. The PCR conditions for both rounds were carried out at 95°C for 5 minutes, followed by 35 cycles of 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 45 seconds and a final extension of 5 minutes at 72°C. Reactions were performed in a Stratagene Robocycler Gradient 96 Gradient Thermal Cycler.

PCR-water (Qiagen) was used as a negative control in both rounds of the N-PCR and *T. gondii* RH strain DNA was used as a positive control. To avoid cross contamination with PCR products, these were manipulated always in a separate room from the PCR set up room. PCR reagents and DNA were stored in small aliquots to avoid multiple pipetting within the same tube. PCR products were visualized with GelRed™ (biotium) staining on a 1% TBE (Tris-borate-EDTA) with 1.5% of agarose for SAG1 and SAG2 genes and 2.5% for B1 gene. The expected size of PCR products were 94bp, 390 bp and 546 bp for B1 N-PCR, SAG1 N-PCR and SAG2 N-PCR respectively.

Standards were tested in the first instance with SAG1 and SAG2 markers and swine samples were tested only with the SAG1 gene. After a first screen with SAG1, a set of samples was repeated with this marker to analyze repeatability. In addition, a set of samples was tested with B1 and a titration was then performed with this marker. A set of SAG1 N-PCR DNA products were sequenced to confirm that the expected size band was *T. gondii* SAG 1 gene and to investigate any possible cross contamination between samples. DNA products were purified with the commercial kit Wizard® Gel and PCR clean-up system (Promega) following the manufacturer instructions. Cleaned products were sent to the commercial company Source Biosciences where they were processed by Sanger sequencing. DNA sequences were aligned by ClustalW using default parametres in MEGA 6.06 software and DNA sequences were

compared against the NCBI GenBank database using Basic Local Alignment Tool (BLAST).

3.3.6 Statistical analysis

PCR prevalence was expressed as the sero-prevalence as described in Chapter 2, with 95% of CI based on a binomial distribution. To study the relationship of PCR prevalence between gender and different farms and environments, data was analysed by the Fisher Exact or Chi-square tests. The relationship between age and PCR positives was analysed by binary logistic regression.

3.4 Results

3.4.1 SAG1, SAG2 and B1 N-PCR in standards

A titration of *T. gondii* DNA in the presence of a high density of host cells was performed to evaluate the sensitivity of the genetic markers SAG1 and SAG2. Expected band sizes of 390 bp for SAG1 and 546 bp for SAG2 were obtained (figure 3.1). Both markers showed the same sensitivity when the published protocol by Su *et al*., (2010) was applied. However, SAG1 N-PCR was more sensitive than SAG2 N-PCR after optimization. The detection limit of SAG2 N-PCR was 14.3 parasites per reaction. The attempts at increasing the sensitivity were unsuccessful with this marker. SAG1 N-PCR allowed the detection of 7.1 and 5.7 parasites per reaction after optimization of the reaction (figure 3.1).

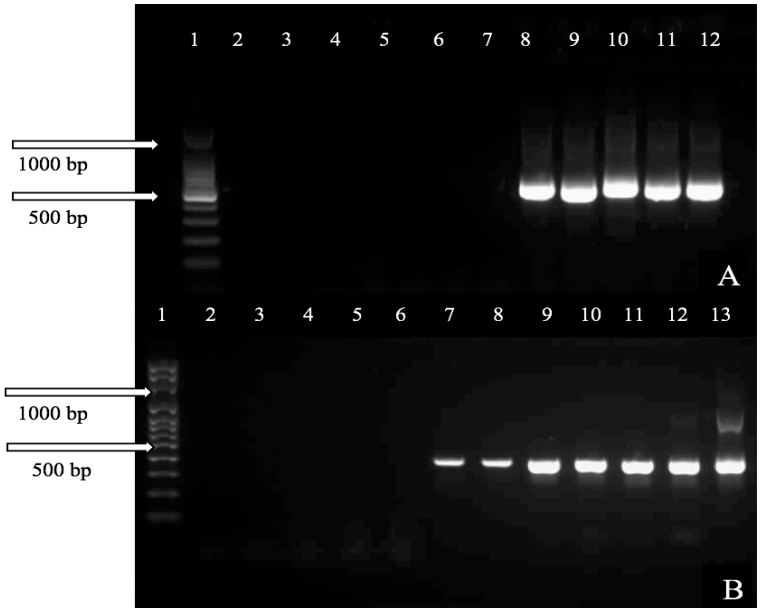

Figure 3.1 10-fold serial dilutions of *T. gondii* DNA spiked in MDBK cells using SAG1 and SAG2 N-PCRs. A: SAG2 N-PCR. Lanes 1-12 as follows 100bp ladder (bioline), external negative control (H_20), internal negative control H_20, MDBK cells, 100 fg, 400 fg, 500fg, 1pg, 10pg, 100pg, 1ng, 10ng. B: SAG1 N-PCR. Lanes 1-13 as follows 100bp plus ladder (bioline), external negative control (H_20), internal negative control (H_20), MDBK cells, 100 fg, 300fg, 400 fg, 500fg, 1pg, 10pg, 100pg, 1ng, 10ng.

Thus, SAG1 was the genetic marker of choice for diagnosis of *T. gondii* DNA in pig blood. In addition, because DNA samples of farm 2 were selected to test with the genetic marker B1, the sensitivity of the B1N-PCR protocol was also investigated. The product obtained had the expected size of 94 pb (figure 3.2). B1 N-PCR resulted in a more sensitive detection than SAG1 N-PCR as the highest sensitivity achieved was 50 fg per reaction (figure 3.2). This is equivalent to 0.7 tachyzoites. Hence, B1 N-PCR was used to confirm the SAG1 N-PCR positives.

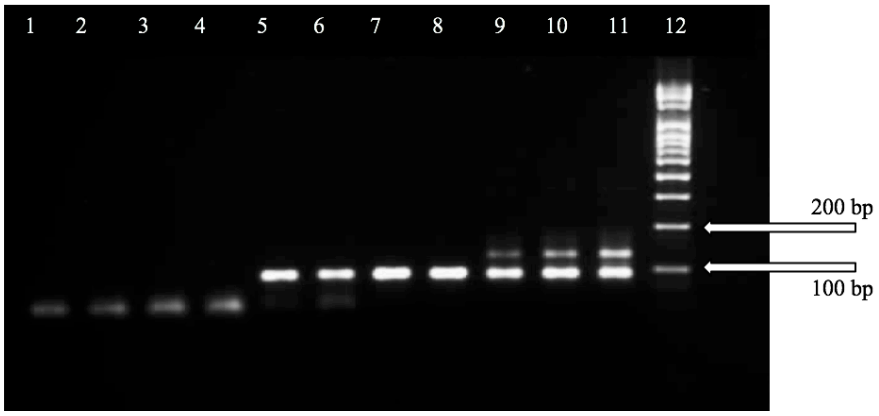

Figure 3.2 10-fold serial dilutions of *T. gondii* DNA spiked in MDBK cells tested with B1 N-PCR. Lanes 1-12 as follows external negative control (H_20), internal negative control H_20, MDBK cells, 10fg, 50fg, 100 fg, 1pg, 10pg, 100pg, 1ng, 10ng.

3.4.2 PCR screening of pig blood

Blood extracts from all animals sampled in the present work (n=632) were screened with the SAG1 N-PCR. Nevertheless, this chapter focuses on the PCR blood screening of those from the main epidemiological study (n=444). PCR results of animals sampled for the isolation study will be discussed in section 4.4. Expected band sizes of 390 bp for SAG1 N-PCR were obtained during the blood screening for *T. gondii* in these samples (figure 3.3).

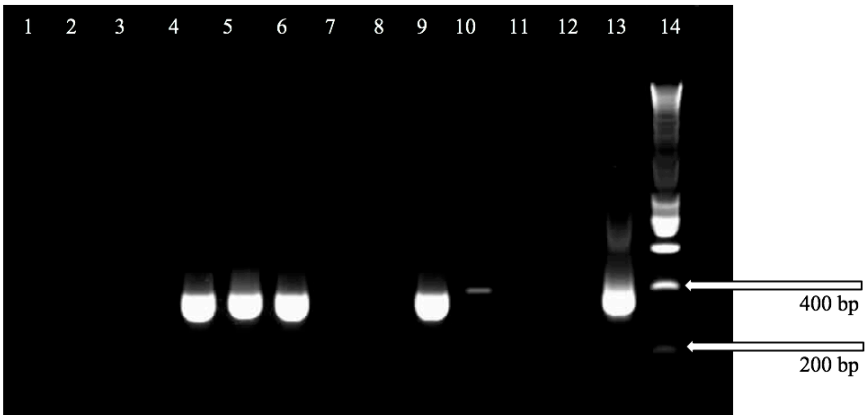

Figure 3.3 Example of SAG1 N-PCR in swine blood extracts. Lanes 1 and 2: external and internal negative controls, lane 13: RH positive control, lane 14: 1kb ladder (bioline), lanes 3,7,8,11 and 12: examples of negative blood samples, lanes 4,5,6, 9 and 10: examples of positive blood samples.

To assess the reproducibility of the assay, farms 1 and 2 were tested in triplicate. Only two farms were chosen for this purpose due to time and financial constraints. On farm 1, 16 animals tested positive in the first screen, 13 on the second and 17 on the third. Positive animals rose from 16 to 18 to 20 after the three repeats. Farm 2 showed a similar pattern. 14 animals were positive in the first screen, 16 in the second and 14 on the third. Positive animals raised from 14 to 16 to 19 after all the repeats. The PCR prevalence was accumulative with the number of repeats in both farms. Thus repeatability was moderate. PCR prevalence data are given with the accumulative prevalence after all the repeats for those samples stated above. For the statistical analysis, however, only the data generated in the first screen was used as in this way a positive test was equally probable for all the samples.

The total number of SAG1 N-PCR positive animals in the 632 animals analyzed was 134 (21.2% CI: 18%-24.6%). *T. gondii* DNA was detected in many more animals than detected in the MAT test ($\chi^2 = 113.7$, df=1, $p = \leq 10^{-3}$). Even including ID Screen® positive animals, the difference was substantial ($\chi^2 = 106.5$, df=1, $p = \leq 10^{-3}$). The number of SAG1 N-PCR positive animals in the pilot study was 4 (7.5%, 95% CI: 2%-18.2%), 2 from each farm. The number of positive pigs in the main epidemiological study (n=444) was 105 (23.6%, 95% CI: 19.9%-28.1%).

The number of SAG1 N-PCR positive animals was different between farms (table 3.4 and 3.5). All farms had PCR positive animals except farm number 10. The PCR prevalence for

farm 1 to farm 6 in tropical deciduous low forest was 31.2% (95% CI: 20%-44%), 29.6% (95% CI: 18.9%-42%), 14% (95% CI: 6.6%-25%), 26.5% (95% CI: 16.3%-39%), 25% (95% CI: 15.2%-37%) and 12.5% (95% CI: 5.5%-23%) respectively (table 3.4).

Table 3.4 PCR prevalence analysed by farm and age group. Prevalence data is given with a 95% CI.

	Week 8	Week 12	Week 16	Week 20	TOTAL
Farm 1*	7/16	3/16	4/16	6/16	31.2% (20%-44%)
Farm 2*	4/16	4/16	7/16	4/16	29.6% (18.9%-42%)
Farm 3	2/16	4/16	2/16	1/16	14% (6.6%-25%)
Farm 4	3/16	6/16	5/16	3/16	26.5% (16.3%-39%)
Farm 5	3/16	7/16	4/16	2/16	25% (15.2%-37%)
Farm 6	1/16	3/16	2/16	2/16	12.5% (5.5%-23%)
TOTAL	20.8% (13%-30%)	28% (19%-38%)	24% (15%-34%)	19% (12%-28%)	23.2% (20%-28%)

* Cumulative PCR prevalence after the repeats.

The PCR prevalence for farm 7 to farm 12 in the tropical sub-deciduous medium forest, was 60% (95% CI: 12%-73%) for farm 7, 30% (95% CI: 2.5%-55%) for farm 8 and 11, 20% (95% CI: 0.2%-44%) for farm 9 and 12 and 0% (95% CI: 0%-30%) for farm 10 (table 3.5).

Table 3.5 PCR prevalence by environment. Prevalence data is given with a 95% CI.

		Positives	Negative	Total	Prevalence
Tropical deciduous low forest	Farm 1*	6	9	16	19% (12%-28%)
	Farm 2*	4	12	16	
	Farm 3	1	15	16	
	Farm 4	3	13	16	
	Farm 5	2	13	16	
	Farm 6	2	14	16	
	TOTAL	18	78	96	
Tropical sub-deciduous medium forest	Farm 7	6	4	10	27% (18%-41%)
	Farm 8	3	7	10	
	Farm 9	2	7	10	
	Farm 10	0	10	10	
	Farm 11	3	7	10	
	Farm 12	2	8	10	
	TOTAL	16	44	60	

* Cumulative PCR prevalence after the repeats.

However, these differences were not statistically significant as p = 0.21 (χ^2 = 7.1, df=5) for the farms located at the tropical deciduous low forest. For the farms located in the tropical sub-deciduous medium forest with a sample size of 10 animals, the p value was 0.1 with the Fisher Exact test.

SAG1 N-PCR prevalence was compared between the two environments. The PCR prevalence obtained in the tropical sub-deciduous medium forest 27% (95%CI: 18%-41%). This prevalence was higher when compared with the 19% (95%CI: 12%-28%) for the 20 week old pigs from the tropical deciduous low forest. However, this difference did not show any statistical significance, as p value was 0.23 (χ^2 = 1.4, df=1).

In addition, PCR prevalence was compared between ages (Figure 3.4). To do this, all animals were included (n=632). 12-14 week old pigs showed the higher percentage of positivity of 26.8% (95%CI: 19.2%-35.5%), followed by a 20.5% (95%CI: 14.5%-27.5%) at 20 week old pigs, 20.2% (95%CI: 14.3%-27%) at 8-10 weeks of age, 18.9% (95%CI: 12.3%-25.4%) at 16-18 weeks and pigs older than 20 weeks showed the lowest percentage of PCR positivity of 16% (95%CI: 6.6%-30%). Age was not considered an explanatory variable for PCR positivity according to the binary logistic regression (table 3.6).

Table 3.6 Results of the binary logistic regression.

Variable	N (pigs)	O.R. (95%CI)	χ^2	D.F.	P-value
Age	632	0.98 (0.95-1)	6.28	1	0.45

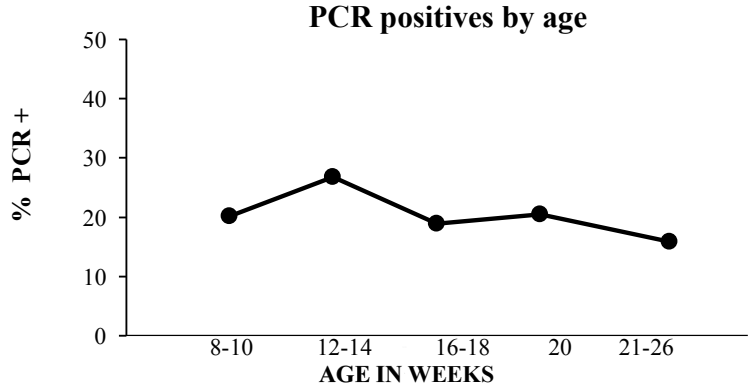

Figure 3.4 PCR positive animals by age. The percentages were calculated taking into account all animals (n=632).

PCR prevalence was also compared between genders. To do that, animals from the isolation study also were considered (excluding 9 animals in which the gender was unknown). From the 133 PCR positive animals (n=623) 75 were males and 58 were females. This difference was not statistically significant ($\chi^2 = 0.59$, df=1, p = 0.4).

The correlation between the MAT and PCR data was poor. Of the 9 MAT positive animals, only 3 pigs were positive by PCR, two with 1:25 titres and one with 1:32000 titres. Of the 26 animals with antibody titres below the cut off, one animal was positive with MAT titres at 10 dilutions and 3 animals with MAT titres at 1:4 dilution. Of the 7 additional animals which tested positive with ID Screen® only one animal was PCR positive. 127 animals which did not show anti *T. gondii* antibodies were positive by SAG1N-PCR.

To support SAG1 N-PCR prevalence, animals from farm 2 were chosen to screen also with B1 N-PCR. Those 64 samples were tested in triplicate with this genetic marker. All PCR products showed the expected band size (94bp) (figure 3.5).

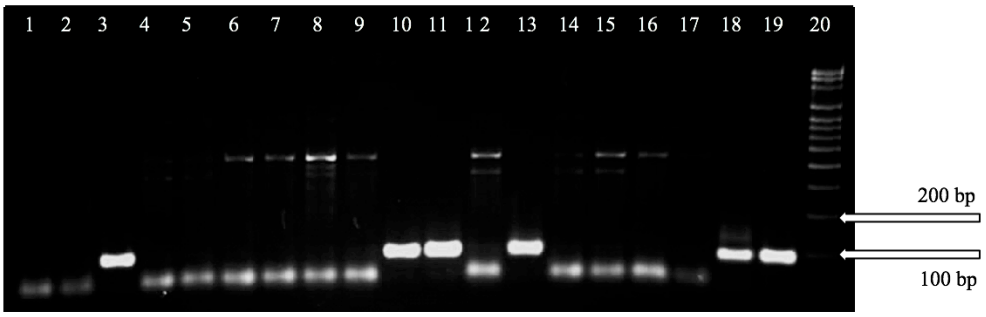

Figure 3.5 Example of B1 N-PCR in swine blood extracts. Lanes 1 and 2: external and internal negative controls, lane 19: RH positive control, lane 20: 100bp plus ladder (bioline), lanes 3,10, 11, 13 and 18: examples of *T. gondii* positive blood samples.

In the same way as SAG1 N-PCR, positivity was also accumulative with the B1 N-PCR protocol. 14 samples tested positive in the first screen, 16 in the second and 15 in the third. The number of positives rose from 14 to 20 and to 23 after the third screen. From the 19 samples from farm 2 that tested positive with SAG1N-PCR, 17 were confirmed with B1 at least in one of the replicates.

Although the number of positives increased with the replicate number, accumulative cross contamination of the DNA was not suspected with either the SAG1 N-PCR or with B1 N-PCR. Several samples that were positive eventually were negative in subsequent testing. This poor repeatability could have occurred due to a limiting number of targets as a result of low parasitemia levels in the blood flow.

SAG1 N-PCR products of positive animals from farm 2 were sequenced to confirm the identity of *T. gondii* and to analyse the homology of the DNA sequence among samples. All SAG1 DNA products had 99%-100% identity to the *T. gondii* SAG1 gene (table 3.7). All sequences belonged to the type I SAG1 allele but 9 of 19 had SNPs which were different from each other. Thus, cross contamination between samples was not suspected. Both samples in which positivity was not confirmed with the B1 gene belonged to the most common haplotype detected which was clonal type I.

Table 3.7 Description of the SAG1 sequences from farm 2. Consensus sequence is the one shared by at least two of the three archetypal types I, II and III alleles. "." indicates identity to the consensus sequence. Grey cells indicate nucleotide polymorphisms not shared by alleles from Type I, II and III archetypal types. Pale grey rows are the samples not confirmed by B1 N-PCR.

Position	29	33	51	56	70	86	103	112	128	147	182	196	211	212	239	250	260	284	289	300
Consensus	T	G	T	A	T	C	G	T	C	T	C	G	A	A	G	G	T	C	G	C
GT1 (Allele I)	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
ME49 (Allele II)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
VEG (Allele III)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TgPigMe1	.	A	C	.	.	T	C	.	.	.	.	A	.	G	.	.	.	T	.	.
TgPigMe2	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe3	.	A	C	.	.	T	C	.	.	C	.	A	.	.	.	.	.	T	.	.
TgPigMe4	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe5	.	A	C	G	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe6	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe7	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe8	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	A	.
TgPigMe9	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe10	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe11	.	A	C	.	.	T	C	.	.	.	T	A	.	.	.	.	.	T	.	.
TgPigMe12	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe13	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe14	.	A	C	.	.	T	C	.	.	.	.	A	T	.	.	.	.	T	.	.
TgPigMe15	Y	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe16	.	A	C	.	.	T	C	Y	.	.	.	A	.	.	.	.	.	T	.	Y
TgPigMe17	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe18	.	A	C	.	.	T	C	.	.	.	.	A	.	.	.	.	.	T	.	.
TgPigMe19	.	A	C	.	A	T	C	.		.	.	A	.	.	.	R	W	T	.	.

* Y=C or T, R=A or G, W=A or T, S=G or C

PCR prevalence was also analysed by sampling date to investigate any trend of accumulative contamination due to contamination of instruments, reagents or human errors (figure 3.6). PCR prevalence was lower in the pilot study during 2013 in both farms. In 2014 PCR prevalence fluctuated randomly by sampling date, showing no trend to indicate contamination. Samples taken in 2015 for the isolation study were included (Chapter 4). All these animals sampled in 2015 were from the farm 1. PCR prevalence did not increase with the date of sampling in these animals either.

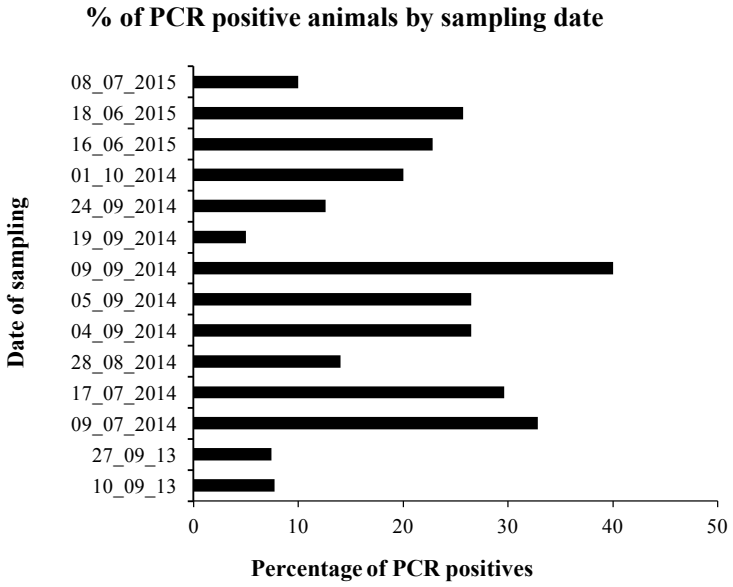

Figure 3.6 Percentage of PCR positive animals by sampling date. Animals sampled on the same day were merged to calculate the current percentages, independently of the farm which they were samples. 2013: animals sampled in the pilot study, 2014: main epidemiological study, 2015: animals sampled for the isolation study.

3.5 Discussion

SAG1 and SAG2 were chosen firstly because they are among the most popular PCR target genes used for *T. gondii*; secondly, they have been used in pigs (Yu *et al*., 2013, Wang *et al*., 2013, Aspinall *et al*., 2002); thirdly, they have allowed us to distinguish between *T. gondii* strains (Su *et al*., 2010) and genotyping is one of the aims of this study. Lastly, SAG1 and SAG2 were among the primers which had higher rates of amplification success in a previous genotyping study from our team in which 12 genetic markers were used, (Alruhaili M, data not published). Therefore, the sensitivity of SAG1 N-PCR and SAG2 N-PCR were compared.

Sensitivity of single copy genes by N-PCR was first suggested to be around 10 parasites spiked in human CSF per reaction (Khan *et al*., 2005a). Further studies achieved greater sensitivity. For example, Nowakouska *et al*., 2016 found a detection limit of 2.5 and 5 parasites in human AF and CSF respectively by N-PCR targeting the SAG2 gene. Nowakouska *et al*., (2016) showed higher sensitivity than the 5.9 or 14.2 parasites per reaction obtained in the present study with SAG1 and SAG2 respectively, but a lower concentration of host DNA was present. More challenging studies using higher densities of host DNA have found closer sensitivity than in the present study. For example, Mason *et al*., (2010) found 6 and 22 parasites per reaction by N-PCR targeting the SAG2 and SAG1 gene respectively in sheep host DNA. Warnekulasuriya *et al*., (1998) found a detection limit of 250 trophozoites per reaction in cured meat using SAG1 N-PCR. In this study, the sensitivity of the SAG1 N-PCR was higher than in those studies mentioned above, and the sensitivity of SAG2 N-PCR was lower than that achieved by Mason *et al*., (2010). However here, different regions of the SAG1 and SAG2 genes were amplified. These finding could suggest that these different primers published for the same genes could have differences in amplification efficiency. Another aspect could be the quality of the DNA used, since Warnekulasuriya *et al*., (1998) suggested that the low sensitivity obtained in his study could be due to PCR inhibitors present in the meats because of its high concentration of salt. Due to DNA quality used in this study for SAG1 and SAG2 protocols being identical and that both targets are single copy genes, it is more likely that the differences in the sensitivity reflect differences in efficiency based on the PCR primers. In this study, the SAG2 sequence was 546 bp while SAG1 was 390 bp, and it is believed that shorter DNA fragments amplified more efficiently than longer ones in PCR (Shagin *et al.,* 1999). The SAG1 sequence amplified

in Mason *et al*., (2010) and Warnekulasuriya *et al*., (1998) was larger (522bp) than in this study (390bp) and the SAG2 sequence amplified in Mason *et al*., (2010) was smaller (241bp) than in this study (546 bp). These findings could suggest that the length of the sequence amplified could also have influenced in the efficiency of the PCR of this study.

N-PCR targeting the B1 gene showed higher sensitivity than the single copy genes SAG1 and SAG2. This protocol was chosen because B1 is a multi-copy gene; multi-copy genes are believed to be more sensitive than single-copy genes (Ivović *et al*., 2012 Lin *et al*., 2012, Sutthikornchai *et al*., 2013, Vitale *et al*., 2013). Studies showed high level of sensitivity of this marker in high density of host tissue (Jones *et al*., 2000, Mason *et al*., 2010). Mason *et al*., 2010 obtained a sensitivity of 0.9 parasites per reaction in 100% of the replicates and a sensitivity of 0.02 parasites per reaction in 50% of the replicates. The sensitivity achieved in this study with this marker was 0.7 parasites per reaction in all the replicates. However, the number of replicates in the present study was 3 whereas Mason *et al*., (2010) did 6 replicates. The sensitivity of B1 in this study and in the studies mentioned above was higher than the sensitivity of the ITS-1N-PCR suggested in Vitale *et al*., 2013 (table 3.1) in pork sausages.

Although the sensitivity of B1 was higher than SAG1, the confirmation of *T. gondii* DNA in two samples was unsuccessful. Sensitivity of PCR targeting the B1 gene may vary between samples as the number of copies across *T. gondii* genome may vary between *T. gondii* strains. Costa and Bretagne (2012) observed that the type I RH strain had a greater copy number (~7) than other type I strains (~5.7-6.4) and than type II (~2.5-3.7) and type III strains (~4-5.8). In the present study, standards were made with the RH strains. There are no published studies in Yucatan which reveal which *T. gondii* strains are found in the area, but studies in several animal species from the north of Mexico have found predominantly type III strains and recombinant strains (Dubey *et al*., 2009, Dubey *et al*., 2004b, Alvarado-Esquivel *et al*., 2011b). The SAG1 sequenced products revealed a type I allele in this gene. However, genotyping based on one single gene is not discriminative enough to distinguish between the three clonal types, recombinant and atypical strains. Thus the B1 gene may have showed higher sensitivity in some samples than others depending on the *T. gondii* strain. However, more studies are necessary in which the copy number of the B1 gene is investigated in further *T. gondii* strains, as Costa and

Bretagne (2012) only tested twelve strains. In addition, there is a lack of genotyping data on *T. gondii* in the locality of Yucatan

The specificity of SAG1 N-PCR was investigated by sequencing. The specificity was 100% as all the sequenced samples had 100% identity with *T. gondii* SAG1 gene. B1 products were not sequenced. However, the specificity of these primers has been tested against other pathogens such *N. caninum, Cp. abortus, S. gigantea, P. carinii, C. albicans, and A. niger* (Owen *et al*., 1998, Wastling *et al*., 1993).

SAG1 N-PCR was used to detect *T. gondii* DNA in swine blood. A protocol was developed for this PCR technique using published primer sequences (Su *et al*., 2010) with a threshold detection limit of 5.9 parasites in high density of host DNA. The overall prevalence by PCR in the main epidemiological survey was 23.6%, 95% CI: 19.9%-28.1%). Here, the PCR prevalence is much lower than the 50.8% (95% CI: 45.9%-55.6%) obtained by Ortega-Pacheco *et al*., (2013) in the same area of study. The reason for this could be a more sensitive PCR protocol as the multi copy gene B1 was used. In comparison to this study, Yu *et al*., (2013), Lin *et al*., (2012) and Wang *et al*., (2013) obtained lower prevalences in abattoirs from China, when using RT-PCR (6-7.8%), a multi-copy gene (0.4%-3.2%) or a combination of both (4.2%). The reason for this could be a lower incidence of the disease in the Chinese pig industry.

To date, the level of agreement between PCR and serological data is unknown. The number of PCR positive animals obtained was much higher (21.2% CI: 18%-24.6%) (n= 632) than the number of MAT positive animals 1.4% (95%, CI: 0.6%-2.7%) (n= 632) even when the animals which had low antibody titers (<25) were included (5%, 95% CI: 3.1%-7.6%) and when ID Screen® positive animals were included (6%, 95% CI: 4.3%-8.2%). From the above published studies in which swine blood is tested with PCR, only Ortega-Pacheco *et al*., (2013) used serological methods along with PCR. However, in this case much less prevalence was obtained by PCR (50.8%, 95% CI: 45.9%-55.6%) than by IgG-ELISA (95.8%, 95% CI: 93.7%-97.8%), although the serology method used has been shown to be unreliable when compared with MAT and DT (section 2.4-5). It was expected that a proportion of seronegative animals would be positive by PCR as detectable IgG antibodies only develop between 10-17 days after infection (Lind *et al*., 1997, Jungersen *et al*., 1999) and *T. gondii* DNA in blood has been detected in pigs within two days after infection and remained detectable until 28 days post-

infection (Wang *et al*., 2013). Maybe those PCR positive and seronegative pigs are animals in which the IgG levels had not risen to detectable levels. Another possibility is that some infected animals may not react immunogenically as suggested before (Dubey *et al*., 1996b, Dubey *et al*., 2002a, Dubey *et al*., 1995b, Omata *et al*., 1994). The reason why a low percentage of pigs may not develop antibody response to *T. gondii* is unknown. In other species an immune tolerance to *T. gondii* has been suggested to explain this event. When immune tolerance is developed, the immunological response is delayed or suppressed. Immune tolerance to *T. gondii* has been suggested in rodents (Rejmanek *et al*., 2010, Araujo and Remington, 1974, Suzuki and Kobayashi, 1990, Owen and Trees, 1998), rabbits (Araujo and Remington, 1975) and humans (McLeod *et al*., 1985, Hara *et al.,* 1996, Yamamoto *et al.,* 2000) which had been passively immunized by congenital transmission. For example, Suzuki and Kobayashi (1990) found that 12 of 13 mice born from infected dams did not developed antibodies detectable by LAT after infection. Rejmanek *et al.,* (2010) demonstrated that only 5 of 30 congenitally infected mice (*P. manuculatus*) had detectable antibodies levels by MAT and IFAT by 16 weeks after birth. Conversely, 14 of those 30 mice were positive by PCR (including the 5 seropositive from above). Owen and Trees, (1998) also found that MAT was less efficient than PCR to detect *T. gondii* infection in congenitally infected mice (*M. musculus*). Hafid *et al.,* (2001) found PCR more sensitive than ELISA-capture and immunoblotting in experimentally infected mice (not congenitally) by intraperitoneal inoculation with RH strain. Studies have found PCR as sensitive as mice inoculation and more sensitive than some serological tests in naturally infected sheep (Owen, Clarkson and Trees, 1998, Williams *et al*., 2005). Immune tolerance has not been demonstrated in pigs. Sows were not included in the sampling and passively transmitted antibodies cannot be detectable in the pigs sampled in this survey because of the age (García-Bocanegra *et al*., 2010c). Therefore, we have no information of the *Toxoplasma* status of the sows from the sampled farms. Nevertheless, results from this study have shown that old pigs have a greater chance of having antibodies against *T. gondii* (O.R.=20.13, p = 0.0001). In future studies, sows could be included in the sampling to allow a more detailed study of *T. gondii* transmission at farm level.

Cross-contamination between samples was not suspected because firstly a high percentage (9/19) of SAG1 N-PCR sequences differ between samples. From 19 SAG1 N-PCR products, 9 were unique (table 3.7). Secondly, although the positivity was accumulative, some

samples that were positive tested negative in further PCRs. Thirdly 17 of 19 SAG1 N-PCR (90%) from farm 2 were confirmed by amplification of the B1 gene. Fourthly, PCR prevalence was investigated by date of sampling and no pattern of build-up contamination was observed (figure 3.5). Lastly, measures to prevent cross contamination were strictly applied during all the processes.

A total of 12 SNPS were described in SAG1 sequences from farm 2. It may seem a low level of variation among samples, however it is important to mention that SAG1 is one of the less polymorphic loci in *T. gondii*. Actually, SAG1 is dimorphic when other SAG genes and other loci are trimorphic. In other words, SAG1 is not capable of providing a distinction between the three archetypal types I, II and III alleles. It has been estimated that a level of variation in SAG genes of 1-5% over 0.5-2kb occurs when 'rare recombinant' strains are compared with the classic clonal types I, II and III (Lekutis *et al*., 2001). It is suggested that the low variation is because SAG1 encodes the sequence for the major surface antigen which plays an important role in modulating host cell invasion, the host immune system and virulence (Lekutis *et al.,* 2001).

The concordance between MAT results and PCR was poor. Farms that had higher seroprevalence such as Pilot A, pilot B and farm 6 did not showed higher PCR prevalence. (section 2.4). In addition, only three MAT positive animals, one ID Screen® positive and four from the 20 questionable pigs were positive by PCR. This poor agreement could be explained because IgG antibody titres are related to old or chronic infections. Animals with IgG antibodies titres may either have cleared the infection; parasites have abandoned the blood flow to colonize preferred organs such muscle and brain to subsequently transform into tissue cysts or the parasite burden in blood is lower than the detection limit of the current PCR technique. In contrast, those animals that have IgG antibody titres and are positive to PCR may be animals in which a latent infection has been reactivated or animals which have been re-infected.

The PCR prevalence was different between farms (table 3.4 and 3.5). As suggested before, these variance could be due to differences in farm management and farm characteristics. The presence of tachyzoites in blood in all ages sampled suggests that *T. gondii* was circulating across the different areas of the farm (nursery and fattening area). Although the difference was not statistically significant ($p= 0.21$, $\chi^2 = 7.1$, $df=5$ and $p=0.1$ in Fisher exact test), the higher

percentage of PCR positive animals was at 12-14 weeks old. Pigs were moved from the nursery area to the fattening area at 10-12 weeks. This could suggest that the majority pigs became infected in the fattening area. The fact that higher seroprevalence was found in oldest ages (O.R. =20.13, p = 0.0001) supports this theory.

The 27% of PCR prevalence obtained in the tropical sub-deciduous medium forest was also higher when compared with the 19% obtained in the tropical deciduous low forest, although the difference was not statistically significant (p =0.23, $\chi^2 = 1.4$, df=1). These results supported the MAT results obtained in those two environments and reinforced the hypothesis that environmental conditions in the sub-deciduous medium forest could help parasite survival as discussed in section 2.5. The statistical analyses did not show significant difference neither in the PCR and MAT results between these two environments but as suggested before, a larger sampled size may help to find a significant association in future studies.

PCR prevalence was higher in males than females, however as in the serological results, no association was found between gender and *T. gondii* infection ($\chi^2 = 0.59$, df=1, p = 0.4). There are no studies which have reported an association of *T. gondii* infection with gender.

PCR results showed that the 1.4% of positive animals obtained by MAT may underestimate the number of infected pigs as the SAG1N-PCR showed a percentage of 21.2% of pigs with the parasite circulating in blood. These results suggest that monitoring of *T. gondii* based solely on serological methods could underestimate the real prevalence of the parasite in pig farms. The combination of both techniques is recommended in order to estimate the true levels of the parasite. Since it is unknown whether those tachyzoites detected in blood were viable or whether those reached the target organ to form tissue cysts; an assessment of the contamination of *T. gondii* in meat is necessary to evaluate the real risk delivered from pork consumption for the population of Yucatan.

CHAPTER 4

Isolation and genotyping of *T. gondii* in pigs

4.1 Introduction

The main aims of this chapter were to isolate *T. gondii* from pigs destined for human consumption and produce genotyping data for the first time in the locality of Yucatan. In addition, the infection of the parasite was investigated in market age pigs destined for human consumption indirectly, using the serological assays MAT, DT and IDScreen® described in Chapter 2 and also, using the SAG1 N-PCR protocol proposed in Chapter 3.

T. gondii was first isolated in 1937 by A Sabin and P Olitsky from guinea pigs during a study involving a neurotrophic virus. The parasite was then isolated by inoculation of guinea pig brain tissue into laboratory mice and then rabbits. Sabin and Olitsky, (1937) also demonstrated 1- the pathogenicity of this parasite in guinea pigs, mice, rabbits, chickens and rhesus monkeys, 2- the multiplication of this organism was only possible in living cells and 3- the experimental infection could be performed by several routes including oral, nasal, subcutaneous, intracranial and intravenous. In 1939, a 6-year-old boy was hit in the head with a baseball bat. Following the incident, the boy experienced intensive headache, weakness, and ataxia. The health of the young boy got worse and he died few days after being admitted in the hospital with convulsions. Under suspicion of a Polio infection, neural tissues were inoculated into mice, as a surprise, these developed a deadly acute toxoplasmosis (Sabin, 1941). The isolate was named after the initials of the child and became one of the most well-known *T. gondii* strains which is the highly pathogenic RH strain. Isolation studies of the RH strain allowed a better understanding of the virulence of this pathogen. In 1958 Cook and Jacobs demonstrated the ability of the *T. gondii* to grow in a large variety of host cells. Since that time, we have come to know that this parasite is capable of infecting nearly all species of warm blooded vertebrates and that it could be found in virtually any tissue. Cook and Jacobs, (1958) also observed that the RH strain could destroy monolayers within a few days of inoculation of the parasite in the culture. After these events, isolation of *T. gondii* was used as a technique to validate infection

diagnostically in cases of human and animal disease by animal inoculation and *in vitro* culture. In the late 1980s *T. gondii* isolation using laboratory animals was the first choice for detection of the parasite (Gangneux and Dardé, 2012). This technique, also called bioassay, has been considered the gold standard for the demonstration of *T. gondii* infection (Liu *et al*., 2015). One of the reasons for such reliability of this technique is the high sensitivity as *T. gondii* can be isolated by bioassay in mice and cats by inoculation with clinical samples with the lowest parasite burden of one single microorganism. This high sensitivity has the advantage of being able to demonstrate the parasite presence especially in large animals where the burden in tissues is low and they are unevenly distributed (Dubey and Beattie, 1988). Another advantage of this technique is that *T. gondii* can be isolated from virtually any body fluid or tissue. In human diagnosis*, T. gondii* has been successfully isolated from body fluids such as blood (Fernandez *et al*., 1991, Hofflin and Remington, 1985), vitreous humor (Miller *et al*., 2000), amniotic fluid (Teutsch *et al*., 1980) and tissues such as placenta (Pardini *et al*., 2014) and neural tissue (Wolf *et al*., 1939).

However, isolation techniques are tedious, time consuming, expensive and hazardous (Redondo *et al*., 1999). The recent development of highly sensitive PCR techniques have achieved a detection threshold down to less than one single microorganism (Lin *et al*., 2012, Jones *et al*., 2000). Nowadays demonstration of the parasite tissue relies mostly on PCR rather than isolation of the parasite by bioassay or *in vitro* culture. PCR techniques have been demonstrated to be fast, safe and sensitive. However, isolation studies are still used as a supplement in some reference centres or in specific cases when PCR results are inconclusive. Currently isolation techniques are usually reserved for genotyping, epidemiological and biological purposes (Gangneux and Dardé, 2012) rather than diagnosis.

In *T. gondii* epidemiology, the detection of the parasite in meats is an important area of study as an evaluation of the potential risk to humans. Over recent years a number of epidemiological studies have determined *T. gondii* viability in different meats such as pork (Bayarri *et al*., 2012b), chicken (Boughattas and Bouratbine, 2015), lamb (Da Silva and Langoni, 2001), goat (Dubey *et al.,* 2011b), horse (Shaapan and Ghazy, 2007), rabbit (Zhou *et al.*, 2013), ostrich (Da Silva and Langori 2016) and turkey (Lindsay *et al*., 1994). However, due to the fact that DNA detection is straightforward, most studies have assessed the prevalence of

T. gondii in meats by PCR (Warnekulasuriya *et al*., 1998; Aspinall *et al*., 2002, Yildiz *et al*., 2015). Nevertheless, results from these studies should be interpreted with caution as DNA detection does not give information about the viability of the parasite. Bioassay in mice and cats is considered the gold standard of isolating *T. gondii* from cysts in animal tissue (Dubey, 2010a, Homan *et al*., 2000, Gangneux and Dardé, 2012). Dubey *et al*., (2005) investigated *T. gondii* viability of 6282 samples (2094 each of beef, chicken and pork) from meat stores across the USA by cat and mice bioassay. Eight pork samples were positive and none from chicken and beef. Two of the eight samples were purchased from the same store, whether those pieces were from the same pig is unknown but it is a possibility. Dubey *et al*., (2005) suggested that meat was a risk factor for consumers. This study was an important result in epidemiology as it corroborated previous studies which suggested that pork was the most important meat source of *T. gondii* infection for humans in the USA (Mead *et al*., 1999). Nevertheless, the fact that viable *T. gondii* was found in 0.1% of the total meat or in 0.3% of pork does not explain how 23% of the USA human population acquire *T. gondii.* Bioassay studies have been also very important in the epidemiology of *T. gondii* transmission as they can identify which treatments undertaken in meats or additives can kill the parasite. Hill *et al*., (2004) found that meat cysts treated with 1% of sodium chloride (SC), 0.25% of sodium tripolyphosphate or 0.1% of sodium diacetate either in combination or alone for 7 days did not affect the parasite viability. On the other hand, the study demonstrated that a 7-day treatment with 2% of SC or 4% of potassium or sodium lactate reduced the infectivity to cats. It is noteworthy however that Sommer *et al.,*(1965) found that encysted *T. gondii* could survive for 4 days in 8% of SC. Djurkovic-Djakovic and Milenkovic, (2000) demonstrated by bioassay in mice that untreated cysts in tissues could survive for 7 weeks at 4ºC and up to 48 hours at -20ºC without loss of infectivity.

Zintl *et al.,* (2009) proposed the use of *in vitro* culture to assess *T. gondii* viability in tissue as an alternative to bioassay. However, this work was challenging. They relied on PCR detection at the end of the culture period as live parasites could not be identified in 9 of 15 *T. gondii* cultures from experimentally infected sheep. Is important to mention that Warnekulasuriya *et al.,* (1998) succeeded in isolating *T. gondii* from one sample of 67 cured meats by *in vitro* culture. This supported a study by Sommer *et al.,* (1965) which found that *T. gondii* could survive the process of curing. Nevertheless, several difficulties were faced in the studies by Warnekulasuriya *et al.,* (1998) and Zintl *et al*., (2009) where cultures were destroyed

after 48h and a loss of cells was observed in some flasks. Warnekulasuriya *et al*., (1998) attributed the problems to a cytopathic effect of the high content of salt in the meats and Zintl *et al.,* (2009) reported a 22% of loss of cultures due to a bacterial and fungal infection. Both studies reported difficulty in identifying *T. gondii* in cultures by microscopic observation.

Studies clearly show that it is difficult to isolate *T. gondii* as a standard diagnostic method. Therefore, it is possible that bioassays are biased in that only a subset of isolates can be retrieved. However, despite these issues collection of *T. gondii* isolates has been very important in allowing the study of genetic diversity of the parasite in detail across the world and among different host species. Isolation of the parasite has allowed intense genotyping via techniques such as high throughput sequencing. Genotyping has shown the existence of diverse *T. gondii* strains. The knowledge of the *T. gondii* strains which circulate in a particular area is important as a tool to track infection. Lehman *et al.,* (2003) predicted the source of infection in a pig farm due to the *T. gondii* genotypes circulating among the different hosts within the farm and its surroundings. Lehman *et al.,* (2003) isolated *T. gondii* from pigs, cats, chickens, wild birds, wild mammals and applied high resolution genotyping using RFLP and microsatellites. Lehman *et al.,* (2003) hypothesized that 1- those pigs were infected as a result of three different events as three different genotypes were identified in pigs, 2-oocysts were the main cause of infection in that farm as all chickens and the vast majority of pigs harboured the two genotypes found in cats, 3- the farm was a focus of infection for the near environment due to the higher prevalence and shared *T. gondii* genotypes in the wild life trapped near the farm in the nearby surroundings. Hence, knowing the *T. gondii* strains of a particular environment may help better understanding of the epizootology of the disease. Lehman *et al.,* (2003) concluded that the dynamics of transmission in that particular farm was characteristic of an enzootic situation rather than epizootic (outbreak).

In addition, *T. gondii* isolation is very useful to allow us to assess the phenotype of the strains. Virulence, growing rates and migration rates vary between *T. gondii* strains. For example, in mice type I strains are well known to cause fatal outcome in 100% of the cases while type II and III strains are considered to be less pathogenic. In addition, studies noted that type I strains grow more rapidly and produce higher parasite loads than the slow growing type II and III strains. *T. gondii* genotype is also correlated with the human disease. Type I and

atypical strains have been associated with more severe outcomes in ocular and congenital diseases (Khan *et al.,*2006b, Switaj *et al*., 2006, Ajzenberg *et al*., 2002).

In the current study we note that Mexico is an interesting area because it borders North and South America which have very different prevalence patterns (section 2.1). Another reason which makes Mexico a fascinating country is the variation of *T. gondii* genetic population structure between North America and South or Central America. The lack of genotyping studies in Yucatan aroused interest in achieving the first *T. gondii* isolation from the area.

4.1.1 Genetic population structure of *T. gondii*

4.1.1.1 Clonal population structure

Because *T. gondii* has a worldwide distribution, can infect almost all mammals and birds and has a sexual cycle, it is expected that it will show a high diversity among its isolates. However, early studies in the 1980s which used MLEE (Multilocus enzyme electrophoresis) for *T. gondii* typing had already discovered that *T. gondii* genetic diversity was limited (Dardé *et al*., 1988, Dardé *et al*., 1992, Dardé, 1996). MLEE is a typing technique capable of classifying strains into groups (called zymodemes) depending on the migration speed of intracellular enzymes in electrophoresis (Tibayrenc, 2009). In two initial studies 35 isolates were typed with European and USA origins. Only 5 zymodemes were identified in these studies (Dardé *et al.,* 1988, Dardé *et al.,* 1992). A subsequent study of MLEE classified 86 isolates from USA, Europe and South America into 12 zymodemes (Dardé, 1996). These studies revealed that the majority of the isolates fell into 3 zymodemes (Z1, Z2, Z3).

Further genotyping studies which used techniques with higher resolution such as microsatellites (MS) or PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) also identified that the majority of isolates (>90%) fell into three clonal predominant lineages which were named as types I, II and III; those I, II and III genotypes were frequently corresponding to Z1, Z2 and Z3 respectively. (Howe *et al*., 1997, Sibley and Boothroyd, 1992, Howe and Sibley, 1995, Costa *et al*., 1997). MS are short segments of DNA (1-6bp) which are repeated in tandem across the genome. MS are highly variable, the number of repeats vary within a population and they can be detected by PCR (Costa *et al*., 1997). Ajzenberg *et al*., (2002) genotyped 86 *T.*

gondii isolates from patients with congenital toxoplasmosis in Europe using 8 MS and found that 85% belonged to type II, 8% to type I and 2% to type III. The remaining 5% were called atypical or recombinant genotypes and as they had different MS allelic combinations than type I, II or III genotypes or unusual zymodemes (different from Z1, Z2, Z3) (Ajzenberg *et al*., 2002).

PCR-RFLP is based on the digestion of a PCR product with one or more restriction enzymes. These enzymes recognize SNPs among a DNA sequence, thereby the product is fragmented creating different patterns that can be revealed by gel electrophoresis (Su *et al*., 2010). Howe and Sibley, (1995) conducted a comprehensive study in which 106 *T. gondii* isolates from animals and humans with European or North American origin were genotyped by PCR-RFLP using 6 loci. Howe and Sibley, (1995) also found using this technique that the majority of isolates were grouped in type I (18%), II (54%), or III (25%) lineages. Howe and Sibley, (1995) also noticed that isolates had frequently only 2 or 3 alleles per each locus.

Shortly after the finding of Howe and Sibley, (1995) studies based on sequence analysis which allowed more detailed analysis of polymorphic sites, revealed that the allelic variation between the three clonal types was in reality limited to two allelic types (Meisel *et al*., 1996, Luton *et al*., 1995, Khan *et al*., 2006a). Grigg *et al.,* (2001a) analyzed 15 polymorphic loci across the *T. gondii* genome of 18 strains from a variety of seven hosts and 3 continents (Europe, America and Africa). The level of polymorphisms between the three types was estimated and found to be limited to two allelic types. Due to this biallelic structure, Grigg *et al.,* (2001a) suggested that the three major lineages had emerged as a result of meiotic recombination from two ancestral allelic types, called A (Adam) and E (Eve) (figure 4.1). Su *et al*., (2003) found that a frequency of 1 SNP every 100bp differed between the A and E lineages and that the within-lineage allelic diversity was virtually absent.

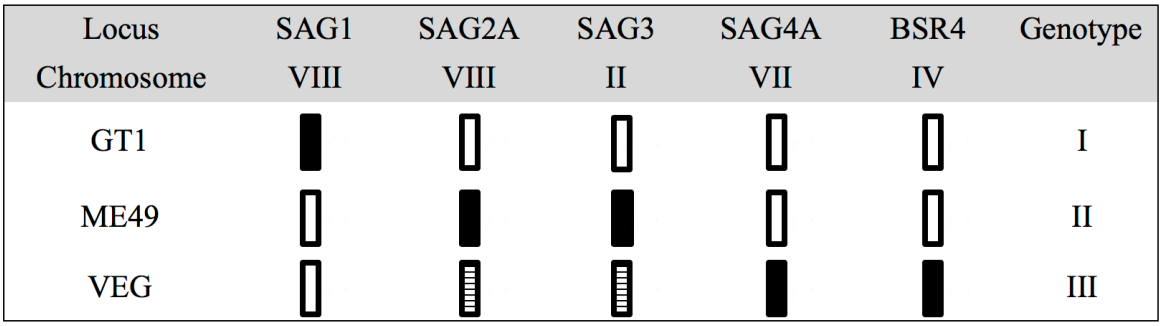

Figure 4.1 Example of the allelic dimorphism in the three main lineages by DNA sequencing analysis in 5 loci. Adapted from Grigg and Suzuki, (2003). The black rectangle refers to the E allele, the white to the A allele and the dashed one refers to type III allele (A allele with one (SAG2A) or three (SAG3) nucleotide polymorphism). GT1, ME49 and VEG are reference strains of the clonal type I, II and III lineages respectively License number provided by Elsevier to adapt and reuse this figure: 3981511179922.

4.1.1.2 Recombinants and atypical genotypes

The classical clonal structure of *T. gondii* was based on a limited sample collection mainly from humans and domestic animals from Europe and North America. Genotyping studies from a larger variety of hosts and remote areas showed that the *T. gondii* genetic population structure was of greater diversity in tropical areas such South and Central America. This diversity was described by a mixture of recombinant and atypical strains with a lower frequency of the classical clonal types.

Recombinant strains are characterized by having genotypes with mixed I, II and III alleles (Grigg *et al* 2001b, Howe and Sibley, 1995). These strains still have the same A and E alleles but their segregation is different from the clonal type I, II and III strains. Recombinant strains have been isolated mostly from tropical areas such the Caribbean, South America, Reunion Island and Africa (Ferreira *et al*., 2006, Pena *et al*., 2008, Ajzenberg *et al*., 2004). The isolation of strains with recombinant genotypes is less common in North America, but also present. For example, in the USA, recombinant isolates have been obtained from wild life (Ajzenberg *et al*., 2004), pigs (Mondragon *et al*., 1998), patients with AIDS and from human congenital toxoplasmosis cases (Howe and Sibley, 1995). Recombinant isolates are rarer in Europe but also they have been described in isolates from patients with congenital

toxoplasmosis in France (Ajzenberg *et al*., 2004). Examples of strains with recombinant genotypes are P62, P80, P89 and P105 from pigs from USA (Mondragon *et al*., 1998).

Atypical strains (or also called exotic strains) are characterized by having genotypes with alleles which are different from those in type I, II and III lineages. These alleles are frequently referred to as 'unique' (u). Although these strains were more divergent, they still had low levels of polymorphism and at some loci retained the dimorphic allele structure of the three clonal types (Grigg *et al*., 2001a, Su *et al.,* 2003). Atypical isolates have been obtained mostly from tropical areas such in Africa, South and Central America (Carme *et al*., 2002, Dardé *et al*., 1998, Grigg *et al*., 2001a). But also, isolates with atypical genotypes have been obtained from patients with congenital toxoplasmosis in France (Ajzenberg *et al*., 2004); and from wild life in Canada and the USA (Lehman *et al*., 2000, Miller *et al*., 2004). Examples of atypical strains are VAND obtained from a patient with disseminated toxoplasmosis in French Guiana and X and A obtained from sea otters in USA.

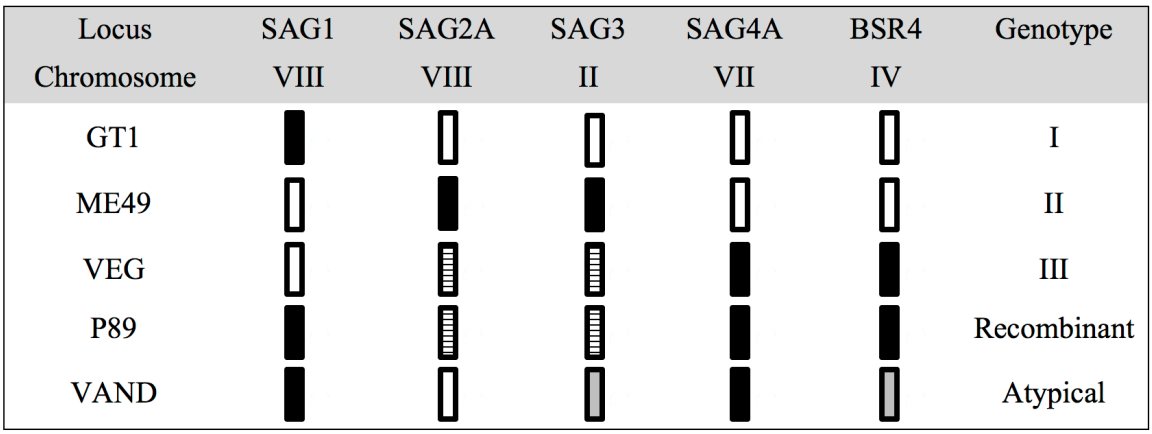

Figure 4.2 Example of the allelic combination in atypical and recombinant genotypes by DNA sequencing analysis in 5 loci. Adapted from Grigg and Suzuki, (2003**).** The black rectangle refers to the E allele, the white to the A allele, the dashed to type III allele (A allele with one (SAG2A) or three (SAG3) nucleotide polymorphism) and the grey rectangle to a unique allele (> 0.4% of polymorphism from either E or A). GT1, ME49 and VEG are reference strains of the clonal type I, II and III lineages respectively. P89 and VAND are examples of strains with recombinant and atypical genotypes respectively. License number provided by Elsevier to adapt and reuse this figure: 3981511179922.

4.1.1.3 *T. gondii* major haplogroups

As *T. gondii* genotyping studies progressed by using techniques with high resolution such as DNA sequencing and by doing more intensive sampling of isolates across different geographical areas and different hosts, the *T. gondii* population genetic population structure was becoming more complex and harder to classify. For example, the categorization of the 'atypical' genotype or 'unique' alleles simply because they show variation in one particular attribute is not totally adequate in terms of genetic diversity. There is no precise definition of how different one allele or genotype needs to be in order to classify them as 'unique' or 'atypical'. In order to allocate isolates in an appropriate classification, high definition genotyping techniques together with genetic population structure and phylogenetic analyses have classified *T. gondii* genotypes into haplogroups (Sibley *et al*., 2009).

The first classification of haplogroups was performed by Khan *et al.,* (2007). Khan *et al.,* (2007) analyzed 46 *T. gondii* isolates from a wide range of host species (humans, wild animals and domestic animals) from Europe, Africa, North and South America by analysis of eight intron sequences from 4 genes and the apicoplast. Introns are non-coding regions of a gene which are frequently used as genetic markers as they are believed to be more variable than coding regions (Igea *et al*., 2010). This analysis allowed the classification of *T. gondii* into 11 haplogroups. Not long after this finding, Khan *et al.,* (2011b) conducted a study in which *T. gondii* genotypes were clustered into 3 more additional haplogroups; 14 haplogroups in total. Khan *et al.,* (2011b) analyzed eight intron sequences from five genes in 74 isolates from humans, wild and domestic animals from Europe, Africa, North and South America and Asia.

The most recent published study in which the *T. gondii* genetic population structure was classified into haplogroups was Su *et al.,* (2012). Su *et al.,* (2012) classified *T. gondii* genotypes into 15 haplogroups. In the first instance, 956 isolates were collected from all around the world. These were compared by PCR-RFLP using 12 markers (SAG1, 3'SAG2, 5'SAG2, Alt-SAG2, SAG3, GRA6, BTUB, PK1, L358, C22-8, C29-2 and Apico) distributed across 8 of 14 chromosomes and the apicoplast (Su *et al.,* 2010). 138 different genotypes were found by this technique and these were then analyzed with 15 microsatellite markers distributed across 10 chromosomes and by sequencing 4 introns from 3 genes. The 138 genotypes clustered into

15 haplogroups which in addition were joined together to from 6 major clades (A-F) (figure 4.3). To date, Su *et al.,* (2012) is the most exhaustive study which classifies *T. gondii* into haplogroups.

The classification of the *T. gondii* genetic population structure into 15 haplogroups and 6 clades showed a strong geographic separation, with widespread clonal genotypes in the Northern hemisphere and more diverse genotypes in South America (Su *et al*., 2012). This classification agreed which previous reports which suggested that the genetic population structure of *T. gondii* in some geographical areas such Africa, Central and South America was divergent from those in North America and Europe (Lehman *et al*., 2006, Ferreira *et al*., 2006, Ajzenberg *et al*., 2004).

Clade B is composed of haplogroups 4 and 8 which have been reported exclusively in South America. Clade E is composed of haplotype 9 which is widely distributed in South America and rare in North America. Clade F is composed of haplogroups 5, 10 and 15 which have been isolated exclusively from the Amazonia. Clade C is composed of haplotype 3 (type III genotype) which has worldwide distribution but is more frequently found in North America and Africa. Clade A is composed of haplogroups 1 (type I genotype), 14 and 6 which has been isolated worldwide (Africa (6, 14), South America (6) and North America (1)). Clade D is composed of haplogroup 2 (type II genotypes) which is widely distributed in North America and Europe and haplogroup 12 (X and A genotype) widely distributed in North America.

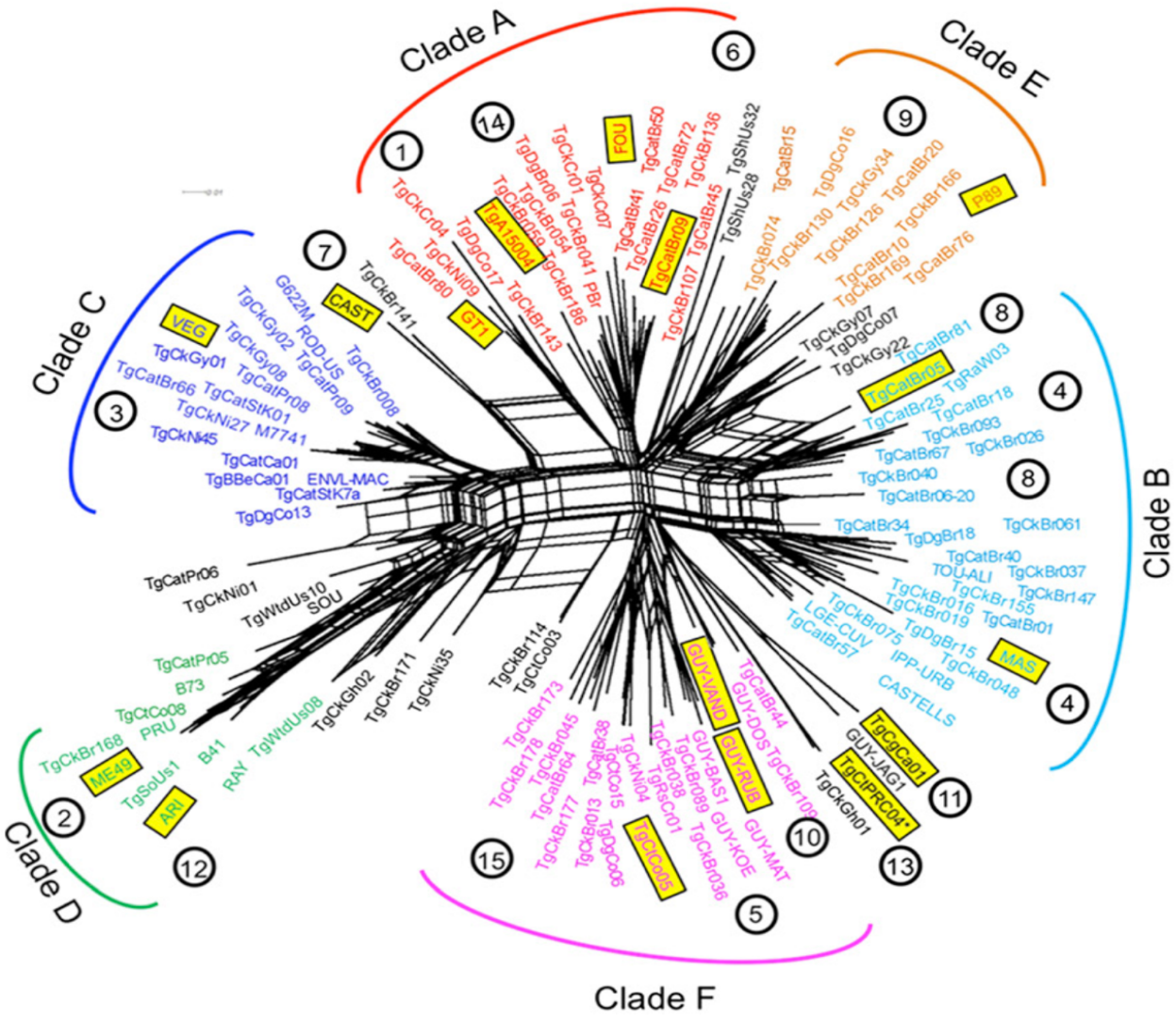

Figure 4.3 Classification of *T. gondii* genetic population structure in 15 haplogroups and 6 major clades (A-F). Taken from Su *et al*., 2012 (with permission from PNAS, no license number is needed)**.** Representative strains for each haplogroup are indicated by yellow boxes and haplogroups are shown in circled numbers.

Lorenzi *et al.,* (2016) performed a comparative genome analysis of 62 *T. gondii* isolates dispersed among all the 15 haplogroups. Even though Lorenzi *et al.,* (2016) used higher resolution genotyping, the isolates were segregated into 6 clades as in the Su *et al.*, (2012) study. No additional haplogroups were identified by using more resolved genotyping techniques. This suggested that the current *T. gondii* classification into haplogroups and clades is very robust and that the discovery of a larger number of clades is not expected (Lorenzi *et al.,* 2016). Nevertheless, it is noteworthy to mention that this conjecture could be a bias as intensive

sampling of further isolates from unexplored geographical areas is still to be done. Lorenzi *et al.,* (2016) revealed a clade-specific inheritance of conserved chromosomal haploblocks. The shared haploblocks were also evident on isolates from different clades in some cases eroding the boundaries between clades. This inheritance of large conserved haploblocks was a major factor driving the shape of the global population structure of the parasite. Lorenzi *et al.,* (2016) suggested that this structure could be caused by a recent genomic admixture that has not been modified due to infrequent recombination events.

4.1.2 Geographical distribution of *T. gondii* strains

Many studies have been conducted across the globe to study the *T. gondii* strains circulating in different countries. The most common technique used is PCR-RFLP and less frequently microsatellites and sequencing. In Europe, strains mainly belong to the main type II clonal lineages in humans and livestock (Ajzenberg *et al.,* 2002, De Sousa *et al.,* 2006). Genotype III is found mostly in southern Europe (De Sousa *et al*., 2006, Fuentes *et al*., 2001) and type I is less common but there are also some reports (Ajzenberg *et al.,* 2002). Atypical or recombinant strains are rare in Europe and sometimes associated with infections acquired abroad or with ingestion of imported food (Ajzenberg *et al*., 2009, Pomares *et al*., 2011). In Africa, 50-90% of the genotypes are clonal type II and III (Al-Kappany *et al*., 2010, Mercier *et al*., 2010) and atypical strains are more common than in Europe. One atypical genotype referred to as Africa 1 (within haplogroup 6) and one recombinant genotype referred to as Africa 3 (within haplogroup 14) are largely distributed in Africa (Mercier *et al*., 2010, Dardé *et al*., (2014). In Asia, the *T. gondii* genetic diversity is overall low and 50-70% of the isolates fall into an atypical genotype referred to as Chinese 1 (within haplogroup 13). The rest of the isolates are related type I genotypes and although type II and III are rare, they are also found (Dardé *et al*., 2014). North American studies have been mainly conducted in the USA. In North America, type II and III genotypes are predominant while type I is rare. In addition, atypical and recombinant strains are also found, more frequently in wildlife. An extended atypical genotype is the type 12, this genotype includes the X and A genotypes isolated from sea otters (Miller *et al*., 2004). This genotype fallsl into the haplogroup 12 and it is so extended in USA that it was named ‘the fourth clonal lineage’ (Dubey *et al*., 2011a). Finally, South American

isolates have shown the greater diversity with the highest diversity in Amazonia where atypical strains are commonly found and clonal types usually represent 0-5% (Ajzenber *et al*., 2004, Mercier *et al*., 2011). Type I and III isolates are also found and type II isolates are rare (Dubey and Su 2009).

4.1.2.1 North American and South American *T. gondii* strains

Many studies have noted that isolates from South and Central America are different from those in North America (Rajendran *et al*., 2012, Dubey and Su, 2009, Lehmann *et al*., 2006, Pena *et al*., 2008). Dubey and Su, (2009) compared the genotype of 253 *T. gondii* isolates from the USA (182 from pigs, 56 from lambs and 15 from deer) with 149 isolates from Brazil from free range chickens by PCR-RFLP using the 12 markers described by Su *et al*., 2010 (SAG1, 3' SAG2, 5'SAG2, Alt-SAG2, SAG3, GRA6, BTUB, PK1, L358, C22-8, C29-2 and Apico). Dubey and Su, (2009) observed that the *T. gondii* genetic population structure in Brazil was characterized by an absence of type II genotypes and by a high diversity in other genotypes. Among the 149 Brazilian isolates only one isolate was of clonal type I and five isolates were of clonal type III. The remaining isolates had recombinant or atypical genotypes, mainly with type I and III alleles. From 149 isolates, 58 different genotypes were identified and 29 genotypes had a single isolate each. In contrast, among the 253 isolates from USA, 52% were clonal type II, 23% were clonal type III and the remaining 25% were mixed genotypes or atypical more frequently with type II and III alleles. 18 genotypes were identified and only 8 of them had a single isolate each. Pena *et al.,* (2008) noted that strains from Brazil not only were genetically different, they also differed in virulence. Of 125 isolates, 33% were 100% lethal to mice and fell into a genotype referred to as BrI (within haplogroup 6), 42% had intermediate virulence (30-100% lethal to mice) and fell into two genotypes referred to as BrII and BrIV (within haplogroup 4) and 19% were avirulent to mice and fell into a genotype referred to as BrIII (within haplogroup 9). Dubey and Su, (2009) did not give information on the virulence to mice of the isolates analyzed; however, in Velmurugan *et al.,* (2009), the virulence of the 182 isolates from pigs in USA from Dubey and Su (2009) was considered and none of them was virulent to mice. Dubey *et al*., (2012b) reviewed all the genotypes published in Brazil and found that of 363 reported isolates, one was clonal type II, one was clonal type I, 12 were clonal type III, 40 were BrI, 23 were BrIII, 20 were BrII, 11 were BrIV and the rest of 248 isolates was represented

by 98 different genotypes which were recombinant or atypical (more frequently a mixture of type I, III and unique alleles and less commonly II alleles). Pena *et al.,* (2008) and Dubey *et al*., (2012b) suggested that *T. gondii* diversity in Brazil is high and that those BrI-IV genotypes could represent a few successful clonal lineages.

Interestingly, when Dubey *et al.,* (2011a) analyzed *T. gondii* genetic diversity of 169 isolates from wildlife (opossums, coyotes, bottle-nose dolphins, raccoons, woodrats, gray wolves, brown bears, sea otters, red foxes, red tailed hawk and arctic foxes) in the USA and Canada by PCR-RFLP using the 12 markers described by Su *et al*., (2010), this was slighter more diverse than the studies reported in farm animals or humans (Dubey and Su, 2009). 47% of the 169 isolates fell into the type 12 genotypes (mixed I, II and unique alleles), 28% of the isolates were clonal type II, the 10% were clonal type III, 15% of the isolates had recombinant or atypical genotypes. 22 genotypes were identified in total, 11 had a single isolate each and 8 had 2 isolates each.

However, studies in South America were focused in Brazil until Rajendran *et al.,* (2012) investigated *T. gondii* genetic diversity in more countries in South and Central America. A total of 164 isolates from chickens in Chile (n=22), Costa Rica (n=32), Nicaragua (n=44), Grenada (n=9), Guatemala (n=3), Argentina (n=10), Venezuela (n=7), Peru (n=5) and from cats (n=16) and chickens (n=16) in Colombia were analyzed by PCR-RFLP using the 12 markers described by Su *et al*., (2010). Rajendran *et al.,* (2012) found that the genetic diversity differed within and between populations. Countries with higher diversity were Guatemala, Brazil and Colombia and with less diversity were Costa Rica, Grenada and Chile. Chile was the least diverse and the genetic population structure was different from the rest of South and Central America. The genetic population structure in Chile was clonal with a high frequency of type II genotypes. The genetic diversity of isolates also differed within countries. For example, higher genetic diversity was observed in isolates from cats in Colombia than in isolates from chickens in Colombia. The genetic diversity also was higher in the North of Brazil than in the South. Nevertheless, the diversity among all the isolates of the study was high. A total of 42 genotypes were identified; 15 had a single isolate each and 10 had two isolates each. 20 isolates had a clonal type III genotype, 13 were clonal II except in one locus that was type I (Chile), 4 had the

clonal type II genotype (Chile), 2 had the clonal type I genotype and the rest of 38 genotypes were recombinant or atypical high higher frequency of type I and III alleles.

It is obvious that genetic diversity from South and Central America is higher than in North America. In the study by Su *et al*., (2012) isolates from Central, South America, French Guiana and Caribbean islands frequently fell into haplogroups 4, 5, 6, 8, 9, 10, 14 and 15. In contrast, North American isolates fell more frequently into haplotypes 2, 3 and 12.

In addition, the comparison of *T. gondii* isolates from domestic animals and wildlife in USA showed a higher genetic diversity in wildlife. This may suggest the existence of an independent domestic and wild cycle in *T. gondii.* It has been suggested before that in environments where a large variety of hosts participate in the *T. gondii* life cycle, for example in the rainforests such the Amazonia or in French Guiana where many species of felids, mammals and birds are resident, *T. gondii* genotypes are highly diverse to facilitate its survival in the different host species. In contrast, in domestic environments, where the hosts that participate in *T. gondii* life cycle are defined by a few domestic species and humans, the clonal types I, II and III seemed to be the most successfully adapted among the domestic hosts (Lehman *et al*., 2003).

It is interesting that genetic diversity also varied with South American populations such as in Chile with a population more similar to North America or Europe. The farming system of the chickens did not influence this discrepancy as all chickens in the study were free range. Less genetic diversity may be expected in chickens raised in cages due to a more limited environment contact. Dubey *et al.,* (2010) also reported a high frequency of type II genotypes in Fernando de Noronha. Fernando de Noronha is an island located 350 km offshore of the Brazilian coast. Rajendran *et al*., (2012) suggested that type II strains were likely spread from Europe as Fernando de Noronha was occupied by Europeans. Rajendran *et al*., (2012) also conjectured that a similar event could have happened in Chile as the trading with Europe, North America and Asia is frequently via the western coast of this country. In addition, the Andes Mountains divides the country and therefore limits the migration of animals from and to other countries and could have influenced this particular *T. gondii* genetic population structure.

4.1.2.2 Genetic diversity of *T. gondii* in Mexico

To my knowledge there are no genetic characterization studies of *T. gondii* in Yucatan and to date, only seven genotyping studies of *T. gondii* have been reported in Mexico. From these seven, no one has ever isolated or genotyped *T. gondii* in pigs. These studies have found a predominance of type I and III genotypes, recombinant and atypical strains. Two of the atypical strains were unique two were reported before in the USA and one showed a widespread distribution (table 4.1).

A study located near the state of Mexico, in the center of the country carried out by Dubey *et al.,* (2004b) using PCR-RFLP at the SAG 2 locus, identified that of 6 free-range chickens, 5 were type III and one was type I. These isolates were avirulent to mice. Further PCR-RFLP genotyping using the 12 loci described in Su *et al*., (2010) (B1, SAGI, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) from 4 of the isolates with type III at SAG2 locus revealed that those were clonal type III (Dubey *et al*., 2009). On the other hand, in patients with congenital toxoplasmosis in Mexico City, clinical samples from four mother-child pairs were genotyped by PCR-RFLP using the four loci SAG2, SAG3, GRA6, BTUB. Only type I genotypes with unique alleles were found (Rico-Torres *et al*., 2012).

Three studies conducted in Durango also using PCR-RFLP in the same 12 loci discovered a high percentage of strains with recombinant and atypical genotypes among wild and domestic animals. Dubey *et al*., (2009) obtained 9 isolates from five cats and three dogs. Two isolates were obtained from the same cat, indicating a multiple infection with two *T. gondii* strains. Five different genotypes were identified among the nine isolates. Genotypes had mixed type I and III alleles and the other two had mixed I, II and III alleles. They were not virulent to mice. One genotype was unique, two had been reported previously in sheep from USA and the other was found previously in cats from China, in dogs from Vietnam, Sri Lanka and Columbia and in chickens from Brazil (Dubey *et al*., 2009). Studies in wildlife also in Durango revealed two atypical strains obtained from one puma (*Felis concolor*) and one pigeon (*Columba livia*) (Dubey *et al*., 2013, Alvarado-Esquivel *et al*., 2011b). The puma isolate, had a mixed genotype with type I and II alleles and the pigeon isolate was identical to one of the isolates obtained in cats in Dubey *et al*., (2009). This isolate was found also in China, USA, Vietnam, Sri Lanka

and Columbia indicating a pandemic distribution of this genotype (Alvarado-Esquivel *et al*., 2011b). The isolate obtained from the puma was reported for the first time and it was virulent to mice (Dubey *et al*., 2013). In a more recent study carried out by Rico-Torres *et al*., (2015), *T. gondii* was isolated from 1 cat in the state of Colima and was an atypical strain also with mixed I, II and III alleles. This genotype was reported before in humans from USA, chickens from Brazil and cats and chickens from Colombia.

Table 4.1 Description of the genotypes of the *T. gondii* isolates in Mexico

Isolate	SAG1*	SAG2	SAG3	BTUB	GRA6	c22.8	c29.2	L358	PK1	Apico*	Genotype	State	Reported**	References
Reference GT1	I	I	I	I	I	I	I	I	I	I	**I**	-	WW	ToxoDB http://toxodb.org/toxo/
Reference ME49	II-III	II	II	II	II	II	II	II	II	II	**II**	-	WW	
Reference VEG	II-III	III	III	III	III	III	III	III	III	III	**III**	-	WW	
TgDgMx1,2TgCatMx1	II-III	III	III	II	II	II	III	II	II	I	Recombinant	Durango	USA	Dubey *et al.*, 2009 Alvarado-Esquivel *et al.*, 2011b
TgCatMx1b	I	III	I	I	III	I	III	III	I	ND	Recombinant		Unique	
TgCatMx2-5, TgPigeonMx1	u-1	II	III	III	II	II	III	II	II	I	Atypical		Colombia Brazil USA China Vietnam Sri Lanka	
TgDgMx2	II-III	III	I	I	I	III	III	III	I	I	Recombinant		USA	
TgCkMx1-4	II-III	III	III	III	III	III	III	III	III	III	**III**	Mexico	WW	Dubey *et al.*, 2009 Dubey *et al.*, 2004b
TgPumaMx1	I	II	II	II	II	II	u-1	I	II	I	Atypical	Durango	Unique	Dubey *et al.*, 2013
No named	I	I	I	I	I	II	I	III	I	III	Recombinant	Colima	USA Brazil Colombia	Rico-Torres *et al.*, 2015

* ND not done; I, II, III refer to the alleles of types I, II, and III lineages; u1 refer to alleles which differ from the type I, II, or III lineages.

** WW reported worldwide, unique first time reported.

4.1.3 Demonstration of *T. gondii* in pig tissue

T. gondii tissue cysts can occur in many organs such as heart, kidney, liver, brain, skeletal muscle, eye, intestine, salivary gland or diaphragm. Tissue cyst distribution seems controlled by the host and strain of *T. gondii* (Dubey *et al*., 1998a). Experiments have also demonstrated that in pigs the distribution of the tissue cysts seems dependent on the initial dose of the infection. This phenomenon has not been observed in cats and rodents (Dubey, 1997b). For example, in Dubey *et al*., (1996a) 42 pigs were fed approximately with 10, 1 and less than 1 oocyst of the VEG strain. *T. gondii* was then isolated from 93% of the tongues, 72% of the brains and 45% of the hearts of the 29 pigs bioassayed in mice. That experiment showed that the tongue was the organ of preference rather than heart and brain. Moreover, the association between dose of inoculum and number of mice infected was positive for the tongue but not for the other organs. This preference for tongue was observed as well in naturally infected pigs; in Dubey *et al*., (1986a), 4 pigs with acquired toxoplasmosis were sacrificed and the parasite was isolated from tongue, arm picnic and pork shoulder of three pigs, from heart, diaphragm ham, tenderloin and spareribs of two pigs and from the brain and bacon of 1 pig. However, when pigs were fed with high doses ($100\text{-}10^5$) of oocysts, cysts were more randomly distributed (Dubey, 1988, Dubey *et al*., 1986a, Dubey *et al* 1984, Lindsay *et al*., 1993, Pinckney *et al*., 1994). These studies could suggest that tongue is the preferred organ in naturally infected pigs; this is an advantage for studies as tongue can be easily biopsied. They also suggested that naturally infected pigs are infected with low doses of oocysts, as the distribution was the same for naturally infected pigs and pigs infected with low doses.

There are many studies in pigs in which *T. gondii* was demonstrated in tissue. PCR and bioassay are preferred to histopathology probably because the latter was the least sensitive (García *et al*., 2006). García *et al*., (2006) compared the sensitivity of PCR, isolation and histopathology in ten *T. gondii* experimentally infected pigs and found that, 16.6% of 150 tissue samples were positive by PCR, 55.1% of 98 muscle samples were positive by mouse bioassay and none was positive by histopathology. The low performance of histopathology has been noted in other animals such as sheep (Silva and Langoni, 2001) and infected mice (Yai *et al.,* 2003, Bezerra *et al*., 2012) when compared with isolation and/or PCR. It has been estimated that the parasite burden in pork is no more than 1 cyst in 25-100 g of tissue (Dubey *et al.,* 2005,

Dubey, 2009b) and the distribution is not homogeneous (Opsteegh *et al*., 2010, Gangneux and Dardé, 2012); the low sensitivity of histopathology was attributed to the small amount of tissue of approximately 10mg used in the preparations (García *et al*., 2006, Dubey, 2009b).

4.1.3.1 Demonstration of *T. gondii* DNA in pig tissue by PCR

Several studies showed that PCR was a rapid and a sensitive method to detect the presence of *T. gondii* in pork meat (Wang *et al*., 2012, Aspinall *et al*., 2002). Studies in Brazil showed a high percentage of pork contaminated with *T. gondii* DNA (table 4.2). For example; Oliveira *et al*., (2003) and Da Silva *et al*., (2005) investigated the presence of *T. gondii* in 70 pork sausages from commercial stores. Oliveira *et al.,* (2003) found the presence of *T. gondii* DNA in 47% of the sausages by C-PCR by targeting the multicopy 529bp repeat element; in contrast, Da Silva *et al*., (2005) found the presence of *T. gondii* DNA in a lower number (27.2%) of samples by N-PCR targeting the single copy gene SAG2. Belfort-Neto *et al*., (2007) detected *T. gondii* DNA in 66% of 50 tongues and 34% of 50 diaphragms by SAG 2 N-PCR collected from abattoirs also in Brazil. One study in Mexico evaluated the presence of *T. gondii* in 48 cuts of pork purchased in local stores by B1-PCR. *T. gondii* DNA could not be detected in any of cuts of pork (Galván-Ramírez *et al*., 2010a).

Other countries such China and Thailand investigated contamination with *T. gondii* in meats purchased from stores by RT-PCR targeting multi copy genes and obtained lower prevalences than in the Brazilian studies (table 4.2); Wang *et al*., (2012) demonstrated 18% of meat samples contaminated with *T. gondii* in China and Sutthikornchai *et al*., (2013) found only a 1.4% of meat samples contaminated with *T. gondii* in Thailand.

Warnekalasuriya *et al*., (1993) found only 1.5% of pork sausages contaminated with *T. gondii* DNA by SAG1 N-PCR in UK. However, a more recent study in UK noted the higher percentage of 33.3% of commercial cuts of pork contaminated with *T. gondii* by SAG2 N-PCR (Aspinall *et al*., 2002).

In general, a higher frequency of *T. gondii* DNA was present in pork samples in Brazil in comparison with the frequency obtained in China, Thailand, UK and Mexico. This presence

of *T. gondii* in pork agreed with the published epidemiological studies in pigs (Chang *et al*., 2013, Halová *et al*., 2013, Samico-Fernandes *et al*., 2012, Galván-Ramírez *et al*., 2010a).

Table 4.2 Frequency of *T. gondii* DNA in pork meat from naturally infected pigs.

Tissue	Samples	PCR +	Country	Method	Reference
Sausages	70	27.2%	Brazil	SAG2 N-PCR	Da Silva *et al*., 2005
Diaphragms	50	34%	Brazil	SAG2 N-PCR	Belfort-Neto *et al*., 2007
Sausages	70	47%	Brazil	529bp C-PCR	Oliveira *et al*., 2004
Tongues	50	66%	Brazil	SAG2 N-PCR	Belfort-Neto *et al*., 2007
Lymph node	434	7.8%	China	B1 N-PCR	Zhou *et al*., 2010
Muscle	416	18%	China	529bp RT-PCR	Wang *et al.,* 2012
Lymph node	101	56.4%	Japan	SAG2 N-PCR	Zakimi *et al*., 2006
Loin, Leg	48	0%	Mexico	B1 N-PCR	Galván-Ramírez *et al*., 2010a
Minced	145	1.4%	Thailand	B1 RT-PCR	Sutthikornchai *et al*., 2013
Cured meats	67	1.5%	UK	SAG1 N-PCR	Warnekalasuriya *et al*., 1998
Muscle	57	33.3%	UK	SAG2 N-PCR	Aspinall *et al*., 2002

Some studies however, categorized PCR as an insensitive method of detecting tissue cysts in pig tissue when compared to bioassay (Tsutsui *et al*., 2007, Hill *et al*., 2006, García *et al*., 2006). Bayarri *et al.,* (2012a) suggested that the low sensitivity of PCR observed in some studies in comparison with bioassay is due to the low starting material for the DNA extraction which in general was less than 15 g, while isolation studies used 50-100g for mice isolation and 500g for cat isolation. Currently however there are protocols in which the amount of starting material for DNA extraction can be increased to 100g such by digesting the samples (Oliveira *et al*., 2004, Wang *et al*., 2012, Sutthikornchai *et al*., 2013) and by magnetic capture and PCR (MC-PCR) (Opsteegh *et al*., 2010). From these studies, Oliveira *et al*., (2004) detected 47.7% of *T. gondii* DNA among 70 pork sausages contaminated with *T. gondii* DNAwhile no isolation was obtained by mouse bioassay. Opsteegh *et al*., (2010) compared MC-PCR with mouse bioassay in 4 experimentally infected pigs with $5x10^5$ oocysts of the DX strain and by either method only 3 of 4 pigs tested positive. Tsutsui *et al*., (2007) infected 10 pigs with 4 $x10^4$ oocysts of the VEG strain and 27 positives samples were obtained from 40 samples (4 cuts of each pig) by mouse bioassay while only 9 of those 40 samples were detected positives by RT-PCR targeting the 529bp repeat element. In contrast, Oliveira *et al.,* (2004) detected *T. gondii* DNA in 47% of the 70 sausages by C-PCR targeting the 529bp repeat element but the parasite could not be isolated by mouse bioassay. Yai *et al*., (2005) also noted higher sensitivity using

B1 N-PCR than bioassay in mice. In this experiment 8 pigs were challenged with $5x10^4$ oocysts of the AS-28 strain, 5 of 8 were positive by B1 N-PCR, and 4 of 8 by mice bioassay. It seems unclear which method is more sensitive to detect the presence of *T. gondii.* There are only a few studies which compare bioassay and PCR in pig tissue and the studies differed in PCR protocols, *T. gondii* strains, sample size and tissue or organ analyzed. In addition, methods are not totally comparable as bioassay gives information about the parasite viability. Hence, studies in which higher sensitivity was noted in PCR may reflect nonviability of the parasite instead of better performance of the PCR method. In any case, bioassay remains as a gold standard technique to demonstrate *T. gondii* in tissue as is the only method in which the parasite viability can be evaluated.

4.1.3.2 Isolation of *T. gondii* in pigs

Nearly all isolation studies in pigs are performed on meat samples rather than in peripheral blood. One of the reasons is probably because tissue cysts can persist longer than tachyzoites. *T. gondii* was successfully isolated in tissue cysts of pigs as long as 865 days post inoculation (d.p.i.) with 1000 oocysts of the GT1 strain (Dubey, 1988). Whereas with tachyzoites, as was mentioned in Chapter 2, they persisted in blood from 2 to 28 d.p.i. There are no studies in pigs that demonstrate the earliest time needed to form tissue cysts. In murine models, tissue cysts could be demonstrated as early as 5 d.p.i. with tachyzoites, 7-9 d.p.i. with cysts and 9-10 d.p.i. with oocysts (Dubey and Frenkel, 1976). Nevertheless, there is one study in which *T. gondii* was successfully isolated from peripheral blood of naturally infected pigs. Klun *et al*., (2011) isolated *T. gondii* from the blood of 16 of 22 seropositive pigs by mouse bioassay. Blood was collected at the time of slaughter (Klun *et al*., 2011). The other reason why *T. gondii* isolation is performed in tissues is because it has a special interest from the public health point of view as a foodborne disease. Some studies have isolated *T. gondii* by bioassay from tissue samples collected directly from abattoirs and some from cuts of meat or sausages purchased in stores (table 4.3). The success of isolation was not comparable as some studies performed isolation only in seropositive animals (table 4.3).

Table 4.3 Summary of the *T. gondii* isolation studies in tissue from naturally infected pigs.

Tissue	Origin	Samples	Isolation +	Country	Reference
Diaphragm	Abattoir	109	12.8%[m]	Argentina	Omata *et al*., 1994
Brain, heart, diaphragm	Abattoir[i]	253	0.4%[m]	Austria	Edelhofer, 1994
Heart, brain, tongue	Abattoir[pm]	28[+]	25%[m]	Brazil	Dos Santos *et al.,* 2005
Brain	Abattoir[pm]	12	50%[m]		Teixeira *et al*., 2006
Sausage	Shop	149	8.7%[m/s]		Dias *et al*., 2005
Sausage	Shop	70	0%[m]		Oliveira *et al*., 2004
Heart, brains	Shop	35	14.3%[m]		Teixeira *et al*., 2011b
Brain and tongue	Shop	20	55%[m]		Bezerra *et al*., 2012
Brain and heart	Abattoir[pm]	36[+]	47.2%[m]		Cademartori *et al*., 2014
Heart, brain, liver, lung, diaphragm	Abattoir	50[+]	2%[m]		Samico-Fernandes *et al*., 2015
heart, diaphragm	Abattoir	2800	0%[m]	Canada	Gajadhar *et al*., 1998
Muscle	Shop	14[++]	7%[m]	China	Wang *et al.,* 2012
Brain and heart	Abattoir	70	4.2%[m]		Li *et al*., 2015
hearts	Abattoir	233[+]	9.8%[m]		Wang *et al.,* 2016
Brain, diaphragm	Abattoir[i]	2447	1.6%[m]	Czech Rep	Hejlíček and Literák, 1993
Brain, diaphragm	Abattoir	57	10.2%[m]		
Heart	Abattoir[f]	160[*]	25%[m]	France	Djokic *et al*., 2016a
Loin, leg	Shop	48	2.1%[m/s]	Mexico	Galván-Ramírez *et al*., 2010a
Brain, heart,	Abbatoir[si]	37[+]	40.5%[m]	Portugal	De Sousa *et al*., 2006
Tongue, loin, rib	Shop	25	8%[m/s]	Spain	Bayarri *et al*., 2012b
Cured ham	Shop	25	0%[m]		
Cured meats	Shop	67	1.5%[v]	UK	Warnekulasuriya *et al*., 1998
Diaphragms	Abattoir	NG	Yes[m,c]	Uruguay	Freyre *et al*., 1991
Heart	Abattoir[pm]	38[+]	36.8%[m]	USA	Dubey *et al*., 2008
Heart	Abattoir[I]	1000	17%[m,c]		Dubey *et al*., 1995a
Heart	Abattoir[pm]	300	9.6%[c]		Dubey *et al*., 2002a
Heart, tongue	Abattoir[pm]	55[**]	92.7%[c]		Lehmann *et al*., 2003
Loin	Shop	2094	0.3%[c]		Dubey *et al*., 2005
Heart	Abattoir[o]	33	51%		Dubey *et al*., 2012c
Diaphragms	Abattoir	NG	Yes[m]	Yugoslavia	Wikerhauser *et al*., 1988

Samples: [*] Hearts from 69 seropositive and 91 seronegative pigs, [**] Hearts from 30 seropositive pings and pool of hearts and brains of 25 not tested pigs, [+] From seropositive pigs only, [++] From positive pigs in ELISA meat juice and PCR, NG = data not given.
Origin: [i] Intensive farming, [si] Semi-intensive farming, [mx] Mixed farms with intensive and extensive farming, [o] organic farms, [pm] Type 'backyard' farms with poor management.
Isolation + (positive samples in bioassay) [m] Mice bioassay, [c] Cat bioassay, [s] serology only, [v] *in vitro* culture.

Dubey (2009b), reviewed *T. gondii* isolation from pigs and observed that the efficiency of the technique increased when pigs were seropositive than when pigs were bioassayed without knowledge of the serological status. The techniques used in the studies summarized in table 4.3

also differed; some studies used cat bioassay and some mice bioassay. Cat bioassay is very efficient because cats shed usually millions of oocysts after ingesting the small dose of one single bradyzoite. In contrast, mice needed a dose >10 of bradyzoites to become infected (Dubey, 2001). Furthermore, in the cat bioassay a larger quantity of meat can be used (500g or more) than in the mouse bioassay (50-100g). In addition, the number of mice/cats employed in the summarized studies in table 4.3, also differed between studies.

It is important to mention that isolation studies that assess meats from stores showed, in general, a lower success rate of *T. gondii* isolation than studies which used meat from abattoirs, where maybe the meat was fresher. Furthermore, the isolation success was even lower when meats were processed such as in ham or sausages. This supported previous studies which suggested that some storage and treatments with salts or other meat enhancers can affect the viability of tissue cysts (Hill *et al*., 2004, Djurkovic-Djakovic and Milenkovic, 2000). Nevertheless, because the parasite can survive in some storage and treatment conditions such as cooking, curing or drying, pork can play an important role in *T. gondii* transmission.

In South America, isolation studies have been developed in Argentina, Brazil and Uruguay. In Argentina, Omata *et al*., (1994) isolated *T. gondii* from 12.8% of 109 diaphragms from naturally infected sows sampled from abattoirs by mouse bioassay. In Brazil, three isolation studies have been carried out from abattoirs. Dos Santos *et al*., (2005) and Cademartori *et al.,* (2014) isolated *T. gondii* from seropositive pigs by pooling heart and brain of each pig and obtained isolates from 47.2% of seropositive pigs. Teixeira *et al.,* (2006) isolated *T. gondii* from 50% of 12 brains from pigs with unknown antibody status to *T. gondii*. Dos Santos *et al*., (2005), Cademartori *et al.,* (2014) and Teixeira *et al.,* (2006) used the bioassay in mice. Several studies, also in Brazil, attempted isolation from cuts of pork from commercial stores and isolation frequencies were higher when organs such as brain and tongue were used instead of sausages. Dias *et al*., (2005) bioassayed 149 sausages in mice and 13 (8.7%) samples produced seroconversion in mice, however the parasite was only isolated from one sample (0.6%). Oliveira *et al*., (2004) bioassayed 70 sausages and no mice seroconverted and no *T. gondii* isolates were obtained. On the other hand, when Teixeira *et al.,* (2011b) and Bezerra *et al*., (2012) bioassayed brains, hearts and tongues purchased from shops in Brazil the frequency of *T. gondii* isolation was much higher. Teixeira *et al.,* (2011b) isolated *T. gondii* in 3 of 19 brains

and in 2 of 15 hearts (14.3% in total) from 5 different local shops. Bezerra *et al*., (2012) purchased 20 pig heads in a local market and bioassayed in mice the brain and tongue of each head. Bezerra *et al*., (2012) isolated *T. gondii* from 11 heads (55%). Freyre *et al.,* (1991) isolated *T. gondii* from pools of diaphragms from DT positive pigs in Uruguay by cat and mouse bioassay. Freyre *et al* (1991) did not mention how many individuals per pool were bioassayed.

Regarding North America; Dubey *et al.,* (2005) carried out a big isolation study in which 2094 cuts of loin were purchased in different meat stores across USA and viable *T. gondii* was isolated from only 0.3% using cat bioassay. Galván-Ramírez *et al*., (2010a) studied the presence of *T. gondii* in 48 cuts of loin and leg also purchased in meat stores in Mexico. One mouse (2.1%) seroconverted but *T. gondii* could not be isolated from the mouse tissue. When isolation studies were performed in abattoirs in USA, the frequency of isolation increased with ranges from 9.6% to 92.7% (Lehmann *et al.,* 2003, Dubey *et al*., 1995a, Dubey *et al*., 2002a, Dubey *et al*., 2008). Lehmann *et al.,* (2003) obtained 92.7% of isolates by feeding cats a pool of tongue and heart each of 25 pigs and hearts of 30 MAT positive pigs. Dubey *et al*., (2008) obtained viable *T. gondii* from 36.8% of 38 hearts of MAT positive pigs by feeding the heart to cats. Dubey *et al*., (1995a) obtained a frequency of 17% of isolates from 1000 naturally infected sows by cat and mouse bioassay. Dubey *et al*., (2002a) isolated *T. gondii* from 9.6% of 300 pigs by also feeding hearts to cats. In Canada, Gajadhar *et al.,* (1998) failed to isolate viable *T. gondii* from the hearts and diaphragms of 2800 pigs from different slaughterhouses across the country. There are no studies in Mexico which studied the viability of *T. gondii* in fresh meat from the abattoir.

In Europe, studies have shown a lower presence of viability of *T. gondii* in meats in countries from central areas such Austria and Czech Republic than in south western areas such as Portugal and France. Edelhofer, (1994) isolated viable *T. gondii* from 0.4% of 253 pigs from an abattoir in Austria by mouse bioassay of pools of heart, brains and diaphragm of each pig. Hejlíček and Literák, (1993) investigated the presence of viable *T. gondii* in heart and diaphragms collected at the abattoir from pigs raised in different husbandry systems. When pigs were raised in intensive farming, *T. gondii* was isolated from only 1.6 % of pools of heart and diaphragm of each of 2449 pigs by mouse bioassay. However, when Hejlíček and Literák, (1993) investigated the frequency of *T. gondii* isolated in hearts and diaphragms from 57 pigs

in small holders, the percentage raised to 10.2% following the same methodology. De Sousa *et al*., (2006) isolated *T. gondii* from 15 (40.5%) pools of brain and heart of each of 37 seropositive pigs from a slaughterhouse in Portugal by mouse bioassay. Djokic *et al*., (2016a) isolated *T. gondii* from 40 (58%) hearts of 69 seropositive pigs and from one (1.1%) of 91 seronegative pigs from 26 different abattoirs in France using mouse bioassay. In Spain, there are no studies which attempted the isolation of *T. gondii* in meat collected from abattoirs, but Bayarri *et al.,* (2012b) investigated the viability of the parasite in 25 cuts of pork which comprised loins, ribs and tongues in Spain. Two (8%) ribs of the total samples, seroconverted mice but viable *T. gondii* could not be isolated from any of them. Bayarri *et al.,* (2012b) also investigated the presence of *T. gondii* in 25 pieces of 'serrano' ham, a popular dry-cured product in the Mediterranean diet but in this case, no mice seroconverted and no *T. gondii* isolates were obtained. In Yugoslavia, Wikerhauser *et al.,* (1988) isolated viable *T. gondii* from three of 10 pools containing 10-14 diaphragms each. However, in this case a representative number of *T. gondii* viability in the samples cannot be provided as pools were used instead of individual samples.

In China, two isolation studies have been carried out (Wang *et al*., 2012, Li *et al.,* 2015) by mice bioassay. Wang *et al*., (2012) isolated *T. gondii* from one sample (8%) of 14 cuts of pork which tested positive by PCR and MAT in meat serum. Li *et al.,* (2015) isolated *T. gondii* in 4.2% of 70 pigs by using the brain and heart collected at the slaughterhouse.

4.1.3.3 Genetic diversity of *T. gondii* isolates from pigs

Table 4.4 shows a detailed compilation of the genotypes found in *T. gondii* isolates from pigs across the world. The majority of genotyping studies carried out in *T. gondii* isolates from pigs used PCR-RFLP using the 12 loci described by Su *et al.,* (2010) (SAG1, 3' SAG2, 5'SAG2, Alt-SAG2, SAG3, GRA6, BTUB, PK1, L358, C22-8, C29-2 and Apico). However, there are some studies in which PCR-RFLP is based only in one locus (Aspinall *et al*., 2002, Dos Santos *et al*., 2005, Da Silva *et al*., 2005). As the resolution provided only by one locus is too poor to distinguish among genotypes, these studies are not shown in table 4.4. In addition, Teixeira *et al.,* (2011b) used MLST and also PCR-RFLP using the 12 loci described by Su *et*

al., (2010). Both studies are shown in the table to compare the discriminative power between both techniques.

In the USA, genotyping data have been generated from 199 *T. gondii* isolates from pigs isolated from a variety of sources described in the previous sections (tables 4.2 and 4.3) Dubey *et al*., (2008) genotyped 14 isolates (TgPigUSA1-14) by using PCR-RFLP using the 12 markers described by Su *et al.,* (2010) and obtained 4 genotypes. The clonal type II genotype had the higher frequency with 10 isolates (TgPigUSA1-4, 6-9, 12-13), a variant of genotype III had three isolates (TgPigUSA5, 10,14) and a genotype with mixed alleles had one isolate (TgPigUSA11). In the most extensive study, Velmurugan *et al.,* (2009) genotyped the isolates previously obtained in Dubey *et al*., (2002a), Dubey *et al.,* (2008), Lehman *et al.,* (2003), Dubey *et al.,* (2005), Dubey *et al.,* (1995a) using the same method as above and named them as TgPigUSA15-182. From 168 isolates analyzed, 56 were clonal type II and 36 were identical to clonal type II except at locus Apico that were type I. This type II genotype was present in 55% of the isolates. The next more common genotype was clonal type III which was present in 49 isolates. All together these type II and type III clonal lineages were present in the 83% of the isolates (table 4.4). Eighteen isolates (10%) had the type 12 genotype highly extended in the USA, 5 isolates (5%) had the BrIII genotype highly extended in Brazil and 3 isolates had a genotype with mixed alleles I/III previously reported in Brazil. The 2 remaining isolates had a single genotype each either with mixed alleles or unique, one of them reported previously in Brazil and the other for the first time reported referred as to 'unique'. In addition, Dubey *et al*., (2012c) studied the genotypes circulating in *T. gondii* isolates (TgPigUSA183-199) from pigs raised in organic pig farms and obtained parallel results to those mentioned above. From 17 isolates, 11 were clonal type II, 4 were clonal type III and one had mixed alleles.

Table 4.4 Description of the genotypes of the *T. gondii* isolates from pigs.

Genotype	Isolate	SAG1*	SAG2	SAG3	BTUB	GRA6	c22.8	c29.2	L358	PK1*	Apico	Method	Country	References
II	TgpigUS, TgpigCn (N=67)[1]	II-III	II	II	II	II	II	II	II	II	II	RFLP	France, USA, China	Djokic *et al.*, 2015, Wang *et al.*, 2016, Vermurugan *et al.*, 2009, Dubey *et al.*, 2008
II-variant	TgpigUS, TgpigC(N=37)[2]	II-III	II	II	II	II	II	II	II	II	I			
III	TgpigUS (N=49)[3]	II-III	III	III	III	III	III	III	III	III	III		USA	Dubey *et al.*, 2012c Vermurugan *et al.*, 2009, Dubey *et al.*, 2008,
Recombinant	TgpigUS (N=17)[4]	II-III	II	II	II	II	II	II	I	II	I			
Atypical	TgpigUS 89	u-1	II	II	II	II	II	II	I	II	I			
Recombinant	TgpigUS (N=8)[5]	I	III	III	III	III	II	III	III	III	III			
Atypical	TgpigUS 182	I	III	III	II	I	I	I	I	u-1	ND			
Recombinant	TgpigUS ,10,14	I	III	III	III	III	III	III	III	III	I			
Recombinant	T TgpigUS 11	I	II	III	II	II	II	ND	III	II	ND			
Recombinant	TgpigUS 192	II-III	I	ND	ND	III	II	I	ND	III	ND			
Recombinant	TgpigBr (N=6)[6]	I	I	III	III	ND	I	ND	ND	I	III	RFLP	Brazil	Bezerra *et al.*, 2012
Atypical	TgpigBr 7	I	I	III	III	ND	u-1	ND	ND	ND	III			
Recombinant	TgpigBr 9	I	I	III	II	ND	I	ND	ND	I	III			
Atypical	TgpigBr 10	u-1	I	III	III	ND	III	ND	ND	I	III			
Atypical	TgpigBr 13	I	I	III	III	ND	I	ND	ND	u-1	III			
Recombinant	TgpigBr 16	I	I	III	I	ND	I	ND	ND	I	III			
Recombinant	TgPigPE01	I	II/I	III	III	III	I	I	I	III	I			Samico-Fernandes *et al.*, 2015
Atypical	TgpigBr1,2	I	u-1	III	I	u-1	u-1	I	III	u-1	I	MLST		Teixera *et al.*, 2011b
Atypical	TgpigBr3	u-1	u-2	III	I	u-2	u-2	u-1	u-1	u-1	I			
Atypical	TgpigBr4	II-III	III	III	III	u-3	III	III	III	u-2	III			
Atypical	TgpigBr5	u-1	u-2	III	u-1	II	u-1	u-1	u-2	u-3	III			
Recombinant	TgpigBr1,2	I	I	III	I	II	I	I	I	I	I			
Atypical	TgpigBr3	u-1	u-1	III	I	II	II	I	I	I	I	RFLP		
III	TgpigBr4	II-III	III	III	III	III	III	III	III	III	III			
Atypical	TgpigBr5	u-1	u-1	III	III	II	II	I	III	III	III			
Atypical	TgpigGZ1-3, TgpigCn (N=13)[7] Tgpigfx171	u-1	II	III	III	II	II	III	II	II	I	RFLP	China	Wang *et al.*, 2012, Wang *et al.*, 2016 Li *et al.*, 2015
I-variant	Tgpighf2, 3	ND	I	I	I	I	II	I	I	I	I			Wang *et al.*, 2012

[1] TgpigUs 1-4, 6-9, 12, 13, 112, 113, 129, 137, 144, 160, 161, 163, 165, 169, 170, 172, 178, 17, 19, 21- 24, 28-31, 33-35, 38, 43, 50-54, 56, 62- 64, 66-68, 72-74, 77, 81, 83, 85, 86, 88, 91, 94, 95, 98, 99, 180, 181, 183-191, 193, 194, TgpigCn14-56. The name of the isolates from France was not given. [2] TgpigUs 27, 32, 36, 37, 39, 44, 48, 49, 59, 69, 93, 97, 100-108, 114-128, TgpigCn5-6, 14-56. [3] TgpigUs 109-111, 130-136, 138-159, 162, 164, 166-168, 171, 173-177, 179, 18, 41, 61, 70, 71, 87, 195,196,198,199. [4] TgpigUs 16, 20, 25, 26, 40, 42, 47, 60, 65, 75, 78-80, 82, 84, 90, 96. [5] TgpigUs 15, 45, 46, 55, 57, 58, 76, 92. [6] TgpigBr6, 8, 11, 12, 14, 15. [7] TgpigCn 3-12, 5-5, 8-16, 8-18, 10-34, 19-24, 27, 8, 29-5, 33-6 , 40-10, 58-14, 61-11, 80-11* ND not done; I, II, III refer to the alleles of types I, II, and III lineages; u1-3 refer to alleles which differ from the type I, II, or III lineages.

Regarding *T. gondii* genotypes circulating in pigs in South America, Brazil is the country in which most studies have been completed. Genetic diversity of *T. gondii* isolates from pigs, as in other animal species, was higher in South America. Bezerra *et al.,* (2012) genotyped 11 isolates (TgPigBr6-16) also by PCR RFLP but using seven loci (SAG1, SAG2, SAG3, BTUB, C22-8, PK1, Apico). None of the isolates was clonal type I, II or III. All of them had genotypes with mixed alleles, predominantly I and III and some of them had unique alleles (table 4.4). Six of 11 isolates had the same genotype, characterized by type I genotype at SAG1, SAG2, C22-8 and PK1 loci and III at BTUB, SAG3 and Apico loci. In Teixeira *et al*., (2011b) only 5 isolates were genotyped (TgPigBr1-5). However, in Teixeira *et al.,* (2011b) MLST and RFLP were used, thus the resolution obtained in this study was higher than in the previous studies mentioned before. When RFLP was performed only 2 atypical alleles were obtained but when MLST was used, 13 additional alleles were obtained. One isolate was clonal type III by PCR-RFLP but when the loci were sequenced, two had atypical alleles. As in Bezerra *et al.,* (2012), isolates had more frequent type I and III alleles (table 4.4). Studies that have used only the SAG2 locus to investigate *T. gondii* genotype, obtained type I and III alleles (Dos Santos *et al*., 2005, Da Silva *et al*., 2005). Dos Santos *et al*., (2005), on the contrary only used one locus for PCR-RFLP and found that from 7 isolates from pigs in Brazil, two had type I SAG2 allele and five have type III allele by PCR RFLP.

In contrast with the genotypes reported in North and South America in isolates from pigs, European pigs had high frequency of type II *T. gondii* genotypes. Djokic *et al*., (2016a) found that the 41 isolates obtained from pigs from abattoirs in France were type II by PCR RFLP using 12 loci according to Su *et al*., (2012). De Sousa *et al* (2006), conducted genotyping only in the SAG2 locus and found that from 15 isolates from pigs in Portugal 11 had the type II allele and 4 had the type III allele in Portugal. Conversely, when Aspinall *et al.,* (2002) genotyped the 19 *T. gondii* positive pork samples from the UK, she found that 16 were type I at the SAG2 locus and 3 samples showed contamination with multiple strains (I+II). Aspinall *et al.,* (2002) used monolocus RFLP and sequencing analyses. The presence of multiple strains in the samples of meat could not be directly attributed to a coinfection in the original animals as samples of meats were probably a mixture of more than one pig (pork pate, pork pie and reformed ham). This study showed an unusually high frequency of Type I alleles to be in Europe compared to other studies.

T. gondii isolates obtained in China have shown a high frequency of the Chinese 1 genotype. The three *T. gondii* isolates obtained in Li *et al.,* (2015) and the single isolate obtained in Wang *et al.,* (2012) had the Chinese 1 genotype. In addition, Wang *et al*., (2012) obtained one more genotype by direct genotyping in the pork meat samples and this was a mixed genotype with I and II alleles. Wang *et al*., (2016) genotyped 15 isolates and found that 13 had the Chinese 1 genotype, and the other two had the type II genotype

4.2 Aims and objectives of the chapter

The aims of this chapter were to isolate *T. gondii* from pigs, produce genotyping data and assess the infection with *T. gondii* in pigs destined for human consumption. More specifically, the objectives were to evaluate the viability of *T. gondii* in tissues of slaughtered pigs sampled at the abattoir by mouse bioassay and to evaluate the frequency of the parasite in pork by SAG1 N-PCR. These techniques in combination with serology were used to provide an accurate monitoring of the disease levels of this pathogen in pigs at market age. In addition, another objective was to isolate *T. gondii* from acutely infected animals using *in vitro* and *in vivo* isolation; thus, blood was inoculated into cell culture and into mice. Another objective was to carry out a sympatric study to investigate the possible source of *T. gondii* on pig farms. Lastly, to achieve the first isolate of *T. gondii* from Yucatan and to generate for the first time genotyping data of the strains circulating in this geographical area. *T. gondii* genotyping data were obtained by using MLST with the panel of most frequently used loci (Su *et al*., 2010, Su *et al*., 2012) to discover the local genotypes. The achievement of isolation of the parasite using mouse bioassay enabled the production of high resolution genotyping data by intensive sequencing. Finally, the genotypes obtained in this work were compared with a set of 49 North and South America prototypic strains to reveal the degree of similarity among these two diverse populations and therefore, to expand the knowledge of the parasite's genetic population structure in the country of study.

4.3 Materials and methods

In accordance with ethical considerations, animals were manipulated and sacrificed according to the Mexican Official Standards for animal care (NOM-033-ZOO-1995 and NOM-062-ZOO-1999) by trained and qualified personal. In addition, the project was approved by the ethics panel of the University of Salford with the reference number CST 13/72 (Appendix VI).

4.3.1 Serological and *T. gondii* DNA screening

Pig serum was screened with the commercial IgG ELISA kit Human Toxo (Human-GmbH, Wiesbaden, Germany) and MAT as described section 2.2.3. DNA was extracted from blood, tissues, digestions and cultures with the commercial kit DNeasy blood and tissue kit (Qiagen) following the manufacturer instructions and screened with SAG1 N-PCR and B1 N-PCR as described in section 3.3.

4.3.2 *T. gondii in vitro* isolation from tissue

4.3.2.1 Sampling

During the first visit in June 2013, six animals which presented poor growth and respiratory problems from the farm pilot A were euthanized with pentobarbital (Pisabental®) by the Veterinary surgeon on the farm. Samples of tongue, brain and muscle were collected and dissected in sterile conditions and placed in sterile containers to keep contamination to a minimum. In addition, three tongues of pigs intended for human consumption were collected from the abattoir located at the Autonomous University of Yucatan. The dissection instruments were changed between samples to avoid cross-contamination. One tube with EDTA for blood PCR and one tube for serum for the MAT screen were collected for all animals. Samples were transported refrigerated in a cooler to the research centre Hideyo Noguchi for further examination.

4.3.2.2 Methodology

A piece of 30-50 mg of each organ was stored as above for DNA extraction. *In vitro* culture was attempted only with the tongues. Three tongues from the farm pilot A and the three tongues from the abattoir were used. The tongues were processed based on Miller *et al*., (2001) and the Dubey, (1998d) protocols with adaptations. The surface of the organ was disinfected to avoid external contamination. To do that, the organ was washed 3 times with sterile gauze with sodium hypochlorite at 20% followed by rinsing in 70% ethanol and dried with sterile gauze. The dissection of the tongue was carried out in sterile conditions (type 2 flow cabinet and sterile material). 25 grams were dissected from the interior of the tongue, avoiding the surface, fat and connective tissue. The muscle was minced with disposable blades and sterile forceps in a petri dish with PBS (7.2) with 100 IU/ml of penicillin, 10 µg/ml of streptomycin, and 0.25 µg/ml of Amphotericin B (Life Technologies). The homogenate was incubated with 100 ml of sterile saline solution at 37°C for 30 minutes in a sterile jar, following that, a second incubation in a shaking oven for 60 minutes at 37°C in 125 ml of pre warmed sterile pepsin solution (porcine stomach pepsin, 1.8 g (Sigma Aldrich); NaCl, 2.5g; HCl, 3.5 ml and distilled water to make 250 ml) was carried out. The digestion was filtered through two layers of sterile gauze and split into 50 ml sterile centrifuge tubes. The homogenate was centrifuged at 1200 g for 10 minutes. The supernatant was poured off and the pellet resuspended in saline solution pooling the suspensions into four tubes. The previous step was repeated but including washing with PBS (pH 7.2) to prevent the fat sticking to the tubes. Following another centrifugation, the supernatant was discarded and the sediment resuspended in EMEM with phenol red (Sigma Aldrich) and pooled in one tube. The EMEM changed from pink to yellow because of the acidic pH of the pepsin. The pepsin was neutralized with sodium bicarbonate 1.2% (Sigma Aldrich) pH 8.3 until the EMEM returned to pink. 1ml of the digestion was stored at -20°C for DNA extraction and 200µl of the digestion were placed in duplicate into two wells of a 24 well cell culture plate previously seeded. MDBK cells were maintained as in section 3.3.1 and were plated in a 24 well plates at a concentration of 10^5 cells per well. This concentration of cells was chosen because in a previous study of our team good results were obtained for *T. gondii* maintenance (Appleford and Smith, 1997). When the wells were inoculated with the digestion mixture, 100 IU/ml of penicillin, 10 µg/ml of streptomycin, and 0.25 µg/ml of Amphotericin B (Life Technologies) were added to the wells to prevent bacterial and fungal proliferation. Plates were maintained at

37 °C and 5% CO_2 atmosphere. 24 hours post-infection the media was changed and monolayers were washed twice with PBS to remove the debris of the inocula. The cells were observed daily to study changes in the monolayer. If parasites were observed, those were passaged by transferring the free parasites in the media into new wells previously seeded with cells. On the contrary, 'blind' passages were performed when the monolayer was overgrown. To passage the monolayer, cells were trypsinized and lysed by several passages through a 27G needle and this homogenate was transferred to another well previously seeded with cells. Media was changed once per week and the following day after passages. Due to the shortage of this first trip cultures were only maintained for 17 or 15 days. After this period of time the surviving cultures were harvested for DNA extraction.

4.3.3 *T. gondii* bioassay from pig tissue

4.3.3.1 Sampling

Pigs for mouse bioassay were sampled at the slaughterhouse located at the Veterinary University of Yucatan in Merida. Due to proximity, most of the pigs that were raised on farm 1, also located at the university, were slaughtered in this abattoir. In addition, this abattoir slaughtered pigs raised on additional farms on request. Pigs slaughtered on request were pigs raised in backyard production systems. The weekly average of pigs slaughtered was around 1.3. The system sampling was therefore subject to availability. Samples were collected from 2013 to 2015 during the periods of my stay in Yucatan (from 3 to 15 weeks, depending on the year, during summer season). The organ sampled for isolation was tongue (Dubey *et al*., (1986a). Sex and age of pigs were recorded. Information on the origin of the pigs was provided by the abattoir as farm 1 or backyard origin but no further details were given regarding the backyards. Two blood tubes were also collected from these animals and samples were transported as described in section 4.3.2.

4.3.3.2 Methodology

Tongues were processed following Dubey, (1998d) protocol. The connective tissue, fat and skin were trimmed and discarded. The muscle was cut into small pieces with scissors,

disposable scalpels and forceps. A small section of 30-50 mg of muscle were placed in an Eppendorf tube and stored at -20ºC for DNA extraction. The remaining muscle was weighed and 50g was ground with a kitchen blender. When the muscle was ground, 125 ml of saline solution (0.85% NaCl) was added for further liquefaction. The homogenate was poured into a 1000 ml beaker; the blender was rinsed with 125 ml of saline solution and this was added to the homogenate. After this, the homogenate was pre-warmed at 37ºC. Separate dissection utensils, gloves, cutting mats, blender pots and beakers were used for the specimens to avoid cross contamination. The homogenate was mixed with 250 ml of pepsin solution which was prepared beforehand by the addition of 2.6g of porcine stomach pepsin, (Sigma Aldrich), 5g of NaCl and 7 ml of HCl into 493 ml of distillated water. The pepsin solution was also pre-warmed at 37ºC. Both solutions were mixed and incubated at 37ºC in a shaking oven for 60 minutes. The homogenate was filtered through 2 layers of gauze and divided into 50 ml centrifuge tubes. Centrifugation, washing and neutralization steps were carried out as described in section 4.3.2. After the last centrifugation, 1ml of the digestion was transferred to a tube and stored at -20 ºC until DNA extraction. The rest of the sediment was mixed with 5 ml of saline that contained 1000 IU of penicillin and 100µg/ml of streptomycin and 500 µl was inoculated intraperitoneally to 2-4 BALB/c mice using a 27G needle. Mice were individually marked by ear cutting and monitored during my stay.

For mouse screening, approximately 100 µl of blood was extracted by tail snip and placed in an Eppendorf tube. Blood, once coagulated, was centrifuged at 1500 g for 15 minutes for serum separation. The serum was screened with the commercial IgG ELISA kit Human Toxo IgG (Human-GmbH, Wiesbaden, Germany) as described in Chapter 2 but this time using a rabbit anti-mouse secondary IgG antibody labelled with horse radish peroxidase (HRP) (Santa Cruz Inc. CA, USA). Due to Salford regulations, mice serum was not imported. Therefore, serum was exclusively tested with the IgG ELISA kit Human Toxo IgG. The test was optimized by doing a chessboard titration of a positive mouse serum (P1), a negative mouse (P2) serum and the anti-mouse secondary antibody (by itself not mixed with the human) according to Crowther, (2000). The optimization was performed in duplicate. The mice controls included two positive sera obtained from positive mice experimentally inoculated with a field isolate obtained in this study, a commercial negative control (Santa Cruz Inc. CA, USA), and four non-inoculated mice from the animal house believed to be free of pathogens. The coefficient of

interplate variance (CV) was calculated and corrected according to Lind *et al*., (1997) with the formula:

$$OD_{corrected} = (OD_{sample} - OD_{P2}) \times OD_{mean\ P1} / OD_{P1} + OD_{mean\ P2}$$

P1 was a reference positive serum and P2 was a reference negative serum included in every plate $OD_{mean\ P1}$ and $OD_{mean\ P2}$ are the mean of the OD for P1 and P2 calculated in all plates performed and OD_{P1} and OD_{P2} are the OD for P1 and P2 for the present plate (Lind *et al*., 1997). The optical density (OD) was measured in a spectrophotometer at 450 nm (Multiskan Multisoft Primary EIA) and the cut off was calculated as the mean of OD of the negative serum controls plus three standard deviations (SD).

Inoculated mice were screened in the same visit when the time post inoculation was greater than 2 months (Dubey *et al*., 1995a), otherwise mice were left to be screened during the next research visit (10-12 months p.i.). For isolate maintenance, seropositive mice were euthanized by cervical dislocation and the brain was dissected. A section of 30-50 mg of the brain was placed in an Eppendorf tube and stored for DNA extraction. The remaining brain was homogenized in 1000 µl of saline solution contained 1000 IU of penicillin and 100/µg of streptomycin by continuous passages through a 23G needle. When the suspension was liquefied this was passaged through a 27G needle several times until the liquid passed through without blocking the needle. The suspension then was passaged into 2-4 mice. This protocol was also applied when mice died during the observation period (2 months) as a 'blind' passage (Dubey *et al*., 1995a). Negative mice were euthanized and a section of the brain was stored for DNA extraction. Bodies of the mice which died during my absence were not recovered.

4.3.4 *T. gondii* Isolation from blood

4.3.4.1 Sampling

70 pigs were sampled from farm 1 in June of 2015 in order to attempt *T. gondii* isolation from peripheral blood. This time 2 blood tubes with EDTA and one blood tube for serum were collected from all animals. The 70 pigs were sampled in two lots of 35 pigs. *In vitro*

culture was attempted from the 30 in the first sampling and in 24 of the second. On the other hand, only positive blood samples were inoculated into mice to attempt bioassay isolation.

4.3.4.2 Methodology

For blood isolation the Hitt and Filice, (1992) protocol was applied. 8 ml of blood from each pig were mixed gently with 8 ml of PBS. The diluted blood was split in two aliquots of 8 ml and each was layered onto 6.6 ml of Histopaque® 1.119 (Sigma Aldrich) placed in 15ml tubes. Two tubes were prepared per pig. The tubes were centrifuged at 800 g for 25 minutes. Leucocytes from the interface layer from each pig were carefully removed and placed in one 15 ml tube. The leucocytes were washed twice with PBS by centrifugation at 1000g for 10 minutes to remove any residual Histopaque® 1.119. The leucocytes from each pig were split in three aliquots, for PCR, *in vitro* culture and mice inoculation. The aliquot for *in vitro* culture was inoculated in one well of a 24 well culture plate within the same day of sampling. All the samples were screened for *T. gondii* by SAG1 N-PCR the following day after sampling, the positive ones were then inoculated into mice (within 48 hours after sampling). The cultures and mice were monitored and screened as in section 4.3.3.1 and 4.3.3.2 with the addition that since the length of this visit was longer, the cultures were maintained for longer, from 24 to 22 days. At day 7 and 14 p.i. the media from the cultures were centrifuged and the pellets were observed under the microscope after resuspending in 200µl of PBS. If parasites were observed, they were passaged by transferring the free parasites in the media into new wells previously seeded with cells. On the contrary, 'blind' passages were also performed when the monolayer was overgrown as described in section 4.3.3.1. DNA was extracted from the pellet from the media from days 7 p.i. and/or 14 p.i. and also from the harvested monolayer of the surviving cell cultures at the end at the experiment.

4.3.5 Sympatric study

In June of 2015 in order to study the transmission dynamics on farm 1, *T. gondii* status was studied in the population of rodents which cohabit with the pigs. Farm 1 was chosen for the sympatric study due to its proximity, accessibility and because the farmers previously reported a plague of rats. Forty Sherman traps 7.5 × 23 × 9 cm, (HB Sherman Traps Inc., Tallahassee,

Florida, USA) were placed on the farm by distributing them across the different areas where pigs were raised (maternity, farrowing, weaning, fattening sections), in the warehouse where pig food was stored and in the kitchen area where the workers eat. The bait used was a mixture of oats and vanilla (Panti-May *et al*., 2012). Traps were placed for 8 successive days and these were inspected every morning for trapped animals. In addition to the traps for rodents, due to a reported presence of stray cats surrounding the farm, a trap for cats (Havahart®) was placed also inside the farm during the same 8 days of the sampling. Captured animals were transported to the zoology laboratory of the Faculty of Veterinary at the Autonomous University of Yucatan where they were euthanized with pentobarbital (Pisabental®). Heart, brain and a piece of muscle were collected for each specimen. For each trapped specimen sex, weight, length and age were recorded. Samples were transported then to the Hideyo Noguchi Research Center. Approximately 30-50 mg of each organ was dissected for DNA extraction and PCR screening. Brains which tested positive by SAG1 N PCR were inoculated into 2-4 BALB/c mice and those were monitored and screened as in 4.3.3.2.

In addition to the rodent study, in 2013 due to an overpopulation of cats on pilot farm A, a cat was given to the veterinarian of the clinic of small animals located at the Autonomous University of Yucatan for a humanitarian euthanasia. Brain and heart were collected for bioassay and DNA extraction. Samples were transported to the Hideyo Noguchi Research centre. The organs were digested following section 4.3.3.2 and the pellet of heart and spleen were inoculated into mice. The sampling and euthanasia of the cat were performed by the veterinarian of the above cited veterinary clinic and the bioassay was carried out by the research team of the Hideyo Noguchi research centre. The following work such as mouse screening, genotyping and maintenance of strains was performed by myself.

4.3.6 Genotyping of *T. gondii*

T. gondii genotyping was performed by MLST using the 12 loci (SAG1, 3' SAG2, 5'SAG2, Alt-SAG2, SAG3, GRA6, BTUB, PK1, L358, C22-8, C29-2 and Apico) as described in Su *et al.,* (2010). These 12 loci are distributed across eight chromosomes and the Apicoplast of *T. gondii*. DNA was amplified by N-PCR. In addition, the database was expanded by using four introns distributed across three chromosomes used in Su *et al.,* (2012). A summary of the

primers used for each locus is given in table 4.5. The amplifications and reaction conditions were performed as the SAG1 N-PCR protocol described in Chapter 3. The conditions were the same for all loci except for Apico in which the annealing temperature was 58°C instead of 60°C in the nested reaction (Su *et al*., 2010). PCR-water was used as negative control in both rounds of the N-PCR and *T. gondii* RH strain DNA was used as a positive control. PCR products were visualized with GelRed^{TM} (Biotium) staining on a 1% TBE (Tris-borate-EDTA) gel with 1% to 2% of agarose (Bioline) depending on the fragment size to resolve.

The PCR product of the successful amplification was purified with the commercial kit Wizard® Gel and PCR clean-up system (Promega) following the manufacturer instructions. DNA concentration and purity of the cleaned products were measured with the spectrophotometer 1000 Nanodrop before sequencing. Sequencing was performed by the company Source Bioscience. Both strands were sequenced for each sample (forward and reverse). Sequences were aligned by Clustal Wallis using the default parameters with the software MEGA 6.06. Phylogenetic trees were contrasted using the Neighbor-Joining and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) methods using also the default parameters in MEGA 6.06 (Pairwise deletion, including trasitions and transversions, uniform rates and Maximum Composite Likehood Method to calculate evolutionary distance). *T. gondii* reference sequences were compared and downloaded from the websites ToxoDB (http://toxodb.org/toxo/) and NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using BLAST.

Table 4.5 Primers used in the PCR reactions. (Su *et al*., 2010, Su *et al*., 2012).

Locus	Chromosome	External primer	Nested primer	Product
SAG1	VIII	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390 bp
3'SAG2	VIII	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222 bp
5'SAG2	VIII	The DNA sequence is covered by the external product of Alt-SAG2	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	242 bp
Alt-SAG2	VIII	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546 bp
SAG3	XII	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	225 bp
GRA6	XII	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	344 bp
BTUB	IX	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411 bp
PK1	VI	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	903 bp
L358	V	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418 bp
C22-8	I	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R: AGGTGCTTGGATATTCGC	521 bp
C29-2	III	F: ACCCACTGAGCGAAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R: TGTCTAGGAAAGAGGCGC	446 bp
Apico	Apicoplast	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640 bp
UPRT1	XI	F: CCCGATATTCGACAAACGAC R: GAGCCGTCTGCTTCATGAGC	F: TCAACCGAAGTTTGCTTTCC	397bp
UPRT7	XI	F: TGGTCGTCGTCACTTGTTA R: GCAGCCTCACAACTAAAACT	F: TCTTGTTTGCTTTCCTCGGC	459bp
EF1	X	F: AAATGCACCCTTTTCTTAAA R: CACATGAAGGTACACCAAAA	F: AAATTGTCCCGCCATCAG	447bp
HP2	IX	F: GACAGAAACACGCAGAGAAT R: TAATCTTTGTTCCCATGCTT	F: ATAATACAGTCAGTTCCCTCGAT	470bp

4.4 Results

4.4.1 *T. gondii in vitro* isolation from pig tissue

T. gondii viability in pork was investigated in the tongues of three pigs raised in farm pilot A and in the tongues from three pigs raised in farm 1 (tongues collected from the abattoir) by *in vitro* culture. In addition, the presence of *T. gondii* DNA in tissues was investigated by SAG1 N-PCR in brain, muscle and tongue of the pigs raised in pilot A and in the tongues of the pigs raised in farm 1. Pigs from farm pilot A were 16-17 weeks old, 5 were males and one female. Pigs sampled at the abattoir were 24 weeks old and all were males. These animals were also tested by MAT and only one pig from the abattoir showed MAT titers (1:25). *T. gondii* DNA was amplified by SAG1 N-PCR in two tongues and one brain from the pilot farm A. The brain was from the same animal as one of the tongues. From the samples collected at the abattoir one tongue and one blood sample were positive. The blood sample was from the seropositive animal and the tongue was from one of the other two pigs. None of the digested tongues (digestions) resulted in a PCR positive (table 4.6).

Table 4.6 PCR and *in vitro* cultures of pig tissues of farm pilot A.

		PCR						
Number [a]	**MAT**	**Blood**	**Brain**	**Muscle**	**Tongue**	**Digestion**	**Culture**	**Parasites in culture**
1a	-	-	-	-	-	-	D***	Not done
2a	-	-	-	-	-	-	D	Not observed
3a	-	-	+	-	+	-	D	Not observed
4a	-	-	-	-	-	NP**	NP	NP
5a	-	-	-	-	+	NP	NP	NP
6a	-	-	-	-	-	NP	NP	NP
7a	-	-	NC*	NC	-	-	-	Not observed
8a	-	-	NC	NC	+	-	-	Not observed
9a	1:25	+	NC	NC	-	-	-	Not observed

[a] Hypothetical number, see appendix V for individual identification with the code (n) a.
* Not collected, only tongues were sampled from the abattoir.
** Not performed, only three tongues of the farm were processed.
*** Cultures were discarded due to a fungal or bacterial contamination.
- Negative, + positive.

Cultures were observed daily to identify tachyzoites or bradyzoites but these were not clearly observed in any of the samples during the period of culture. It was difficult to maintain

the sterility. The culture from the first tongue was discarded due to a high overgrowth of fungus 24 d.p.i. In the remaining cultures the viability of the cells was compromised. A cytopathic like effect with cell detachment and lysis was observed. Passages of the cultures to new seeded wells were performed every day and every 2 days due to a continuous loss and detachment of the cells. Bacterial contamination was suspected in two other cultures due to the turbidity and acidity of the media, thus these were also discarded before the end of the experiment. The media was clearer in the other three cultures and pH seemed normal but structures compatible with *Toxoplasma* were not identified in any of them. Viable cultures were harvested at the end of the experiment for DNA extraction and PCR screening but *T. gondii* DNA was not amplified in any of the cultures. Hence, the viability of *T. gondii* in pig tissue was not demonstrated by *in vitro* culture.

4.4.2 *T. gondii* bioassay from pig tissue

T. gondii viability was also investigated in tongues collected from the abattoir by mouse bioassay. Thirty-four pigs in total were sampled from the abattoir located at the university. From 34 pigs, 26 were raised in farm 1 and 8 were raised in the backyard system. In general, pigs were slaughtered between 23-26 weeks old, except 4 pigs raised in backyards which were much younger (16 weeks) and two pigs raised on farm 1 which were slightly younger (20 weeks) than the usual slaughter age (table 4.7). Of 34 pigs, 5 were females, 20 were males and in 9 the gender was not provided by the abattoir.

All animals were screened with MAT. Three of 34 pigs from the abattoir had MAT titers ≥ 25 and 5 pigs had MAT titers < 25. All the positive pigs (MAT titers ≥ 25) were raised in farm 1. From the five pigs with low antibody titers (MAT titers < 25) two were raised in backyard production (23 and 25 weeks) and three in farm 1 (table 4.7). Thus the MAT seroprevalence was 8.8% (95% CI: 1.8%-23.5%) among all pigs slaughtered, 10.7% (95% CI: 2.2%-28.3%) among the older pigs (> 20 weeks), 11.5% (95% CI: 2.5%-30%) among the pigs raised in farm 1, 12.5% (95% CI: 3.7%-32.4%) among the older pigs (> 20 weeks) raised in farm 1 and 0% (95% CI: 0%-3.7%) among pigs raised in the backyard system.

In addition, the MAT positive animals (3), MAT 'doubtful' animals (5), a set of MAT negative animals (7) were tested with the serology kit ID Screen® (table 4.7). Thus taking into

account those ELISA positive animals, the seroprevalence increased to 14.7% (95% CI: 5%-31%) among all pigs slaughtered, to 17.8% (95% CI: 6%-37%) among older pigs, to 19.2% (95% CI: 6%-37%) among pigs raised in farm 1 and to 20.8% (95% CI: 7%-42%) among older pigs raised on farm 1 (table 4.7). Fisher test did not show a statistical association between the variables gender (p=0.57), farm of origin (p=0.23) and age (p=0.35) with the presence of *T. gondii* antibodies in slaughtered pigs.

Table 4.7 Description of the pigs used for tissue bioassay in mice.

					PCR [c]								PCR [c]		
Num [*]	**MAT**	**ID** [d]	**Age** [a]	**Farm**	**D**	**T**	**B**	**Num** [*]	**MAT**	**ID** [d]	**Age** [a]	**Farm**	**D**	**T**	**B**
1b	1:32000	+	24	1	+	+	+	18b	-	ND	25	1	-	-	+
2b	1:100	+	25	1	+	-	-	19b	-	ND	25	1	-	-	-
3b [**]	1:25	+	25	1	-	-	+	20b	-	ND	16	B	-	-	-
4b	1:10	+	23	1	+	+	-	21b	-	ND	16	B	-	-	-
5b	1:10	-	23	B [b]	+	-	-	22b	-	ND	16	B	-	-	-
6b	1:10	-	25	B	-	-	-	23b	-	-	20	1	+	-	-
7b	1:4	-	20	1	-	-	-	24b	-	ND	16	B	-	-	-
8b	1:4	-	25	1	-	-	-	25b	-	ND	25	1	+	-	-
9b [**]	-	ND	24	1	-	-	-	26b	-	ND	25	1	-	-	-
10b [**]	-	ND	24	1	-	+	-	27b	-	ND	25	1	-	-	-
11b	-	ND	26	1	+	-	-	28b	-	ND	25	1	-	-	-
12b	-	ND	26	1	-	-	-	29b	-	ND	25	1	-	-	-
13b	-	-	25	1	-	-	-	30b	-	ND	25	1	-	-	-
14b	-	-	25	1	-	-	-	31b	-	ND	25	1	-	+	+
15b	-	-	25	1	+	-	-	32b	-	ND	25	1	-	-	-
16b	-	-	25	B	-	-	-	33b	-	+	25	1	+	+	-
17b	-	ND	25	B	+	-	-	34b	-	ND	25	1	+	+	-

[a] Pig age in weeks.
[b] Backyard production.
[c] PCR screen: D: digested tongue, T: tongue, B: blood.
[d] ID screen® ND, not done.
[*] Hypothetical number, see appendix V for individual identification as (n) b.
[**] Tongues (n=3) also used for *in vitro* bioassay (section 4.4.1).

In addition, the presence of *T. gondii* DNA was evaluated among the pigs bioassayed by screening with SAG1 N-PCR extracts from tongue, digested tongue and blood of all slaughtered animals. A total of 13 animals tested positive by PCR in tissue. Eleven of the 13 samples tested positive by using the digested tongue and only six of 13 by using the non-digested tongue (table 4.7 and figure 4.4). Four animals were concordant, being *T. gondii* DNA positives in both tongue and digested tongue. All tissue samples were screened in duplicate; a sample was considered positive when at least one of the duplicates tested positive. In addition,

four animals were positive when blood was tested. Hence, the presence of *T. gondii* DNA in pig tissue was 38.2% (95% CI: 22%-56.4%) using both digested and non-digested tongue, 32.3% (95% CI: 17.3%-50.5%) using only digested tongue and 17.6% (95% CI: 6.7%-34.5%) using only non-digested tongue. The Fisher Exact test did not show a statistical significant association between methods (p=0.208). The prevalence of *T. gondii* DNA in blood among the pigs slaughtered was 11.7% (95% CI: 0%-27.4%). Fisher test did not show a statistical association between the variables gender (p=35), farm of origin (p=0.32) and age (p=0.15) with the presence of *T. gondii* DNA in pig tissues.

The agreement between PCR using both methods (digested and non-digested) and serology was fair (kappa = 0.29, 95%CI: 0.0-0.58, p-value= 0.037); the agreement between PCR using only digested tissue and serology was also fair (kappa = 0.373, 95%CI: 0.05-0.7, p-value= 0.014); and the agreement between PCR using only non-digested tissue and serology was moderate (kappa=0.459, 95%CI: 0.07-0.85, p-value= 0.007), probably due to a lower number of samples that amplified.

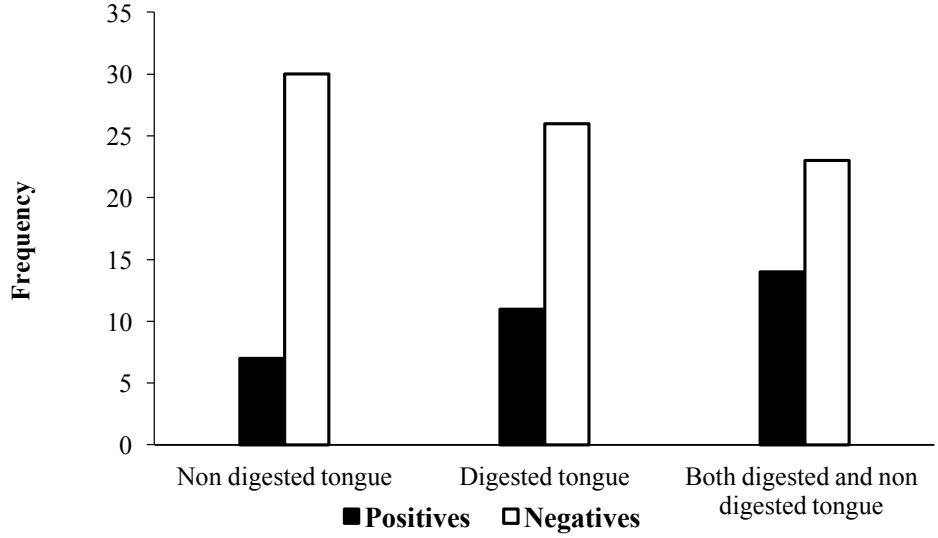

Figure 4.4 PCR positives by method used: digested tongue, non-digested tongue and both. Tongue samples from section 4.4.1 non bioassayed (n=3) have been included in the graph (n=37).

A total of 88 mice were infected with the tongue digested homogenates. Of 88 mice, 33 were dead before the screening was performed, thus 38.6% of the bioassayed mice were lost

before the start of the experiment. The surviving mice were screened with the commercial IgG ELISA kit Human Toxo (Human-GmbH, Wiesbaden, Germany). The ELISA protocol was optimized by cross-titrating the secondary antibody against the positive (P1) and the negative (P2) reference controls (Crowther, 2000). P1 was serum obtained from a positive mouse infected with an isolate obtained in this study (TgCatMx6) and P2 was a commercial mouse negative control (Santa Cruz Inc. CA, USA). Titration of P1 and P2 was done in four two-fold dilutions starting at 1:25 and the mouse secondary antibody was tested in six two-fold dilutions starting at 1:250 (figure 4.5). The starting dilution of the serum was chosen according to other ELISA protocols performed using mouse sera (Al-adhami and Gajadhar, 2014). The range of dilution of the secondary antibody was chosen according to the manufacturer instructions (Santa Cruz Inc. CA, USA).

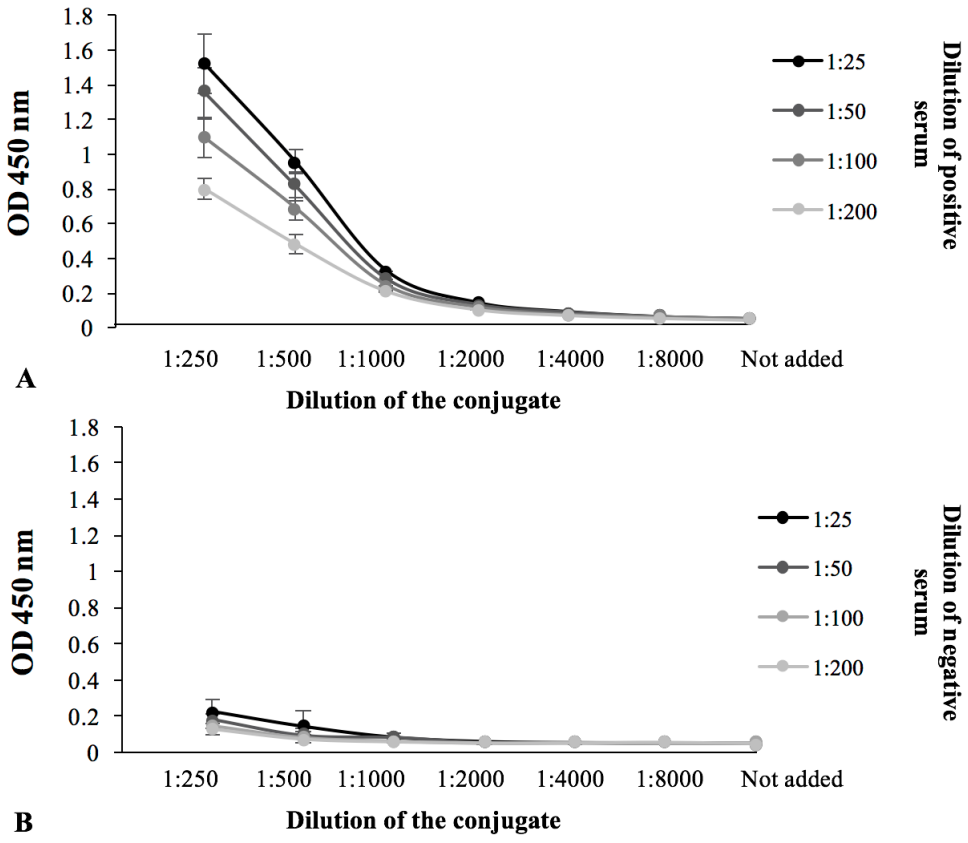

Figure 4.5 Optimization of the ELISA by chessboard titration. Figure A shows a titration of the secondary antibody anti-mouse against the mouse positive serum. Figure B shows a titration of the secondary antibody anti-mouse against the mouse negative serum.

The optimum dilution of the reagents was obtained by calculating the binding ratios (BR). A BR is the OD value of the positive serum divided by the OD value of the negative serum at a given dilution. The preferred dilutions of the reagents are the ones that give the higher values of BR. A combination of a dilution of the mouse secondary antibody at 1:500 with a dilution of sera at 1:50 produced the best results. The binding ratios for all the dilutions are shown in table 4.8.

Table 4.8 Binding ratios. The light grey cells show the dilution of the conjugate that gives the higher values for BR. The dark grey cell indicates the dilution of the serum that gives the highest BR. Values given with SE (±).

Dilution Conjugate	BR at dilutions : 1:25	1:50	1:100	1:200
1:250	7.5 ± 3.47	7.7 ± 2.57	7.4 ± 0.85	6.3 ± 0.67
1:500	9.6 ± 4.87	9.8 ± 2.6	8.9 ± 1.73	6.8 ± 0.61
1:1000	4.4 ± 1.46	3.6 ± 1.04	4.0 ± 0.76	3.7 ± 0.22
1:2000	2.4 ± 0.28	2.4 ± 0.32	2.3 ± 0.21	2.1 ± 0.24
1:4000	1.7 ± 0.23	1.7 ± 0.2	1.4 ± 0.06	1.5 ± 0.15
1:8000	1.2 ± 0.14	1.3 ± 0.03	1.2 ± 0.1	1.1± 0.12
Not added	1 ± 0.02	0.9 ± 0.05	0.9 ± 0.07	1 ± 0

The CV for P1 and P2 was calculated to evaluate the inter-plate variability. A CV of 45% and 38% were obtained for P1 and P2 respectively. The repeatability of the assay was considered poor as CV > 20% is considered an excessive inter-plate variation (Crowther, 2000). Hence the OD was corrected for all the samples according to Lind *et al*., (1997). The distribution of the uncorrected and corrected OD was evaluated by histograms. Corrected OD produced a clearer bimodal distribution capable of distinguishing between seropositive and seronegative mice (figure 4.6).

Seven screened mice seroconverted (figure 4.6, table 4.9). One seropositive mouse was inoculated with tissue from a seropositive pig (pig 3, table 4.7, 4.9), one mouse was inoculated with tissue from a pig with MAT titer 1:10 (pig 6, table 4.7, 4.9) and five mice were inoculated with tissues from four seronegative pigs (pig 10, 11, 13, 15, tables 4.7 and 4.9). The seropositive pig (pig 3) was not SAG1 N-PCR positive in tissue; nevertheless, it was positive for SAG1 N-PCR in blood. The pig with MAT titers 1:10 was also PCR positive in tissue (pig 6). Three (pigs 10, 11, 15) of four seronegative pigs tested PCR positive in tissue and the remaining (pig 13) was PCR negative (table 4.7).

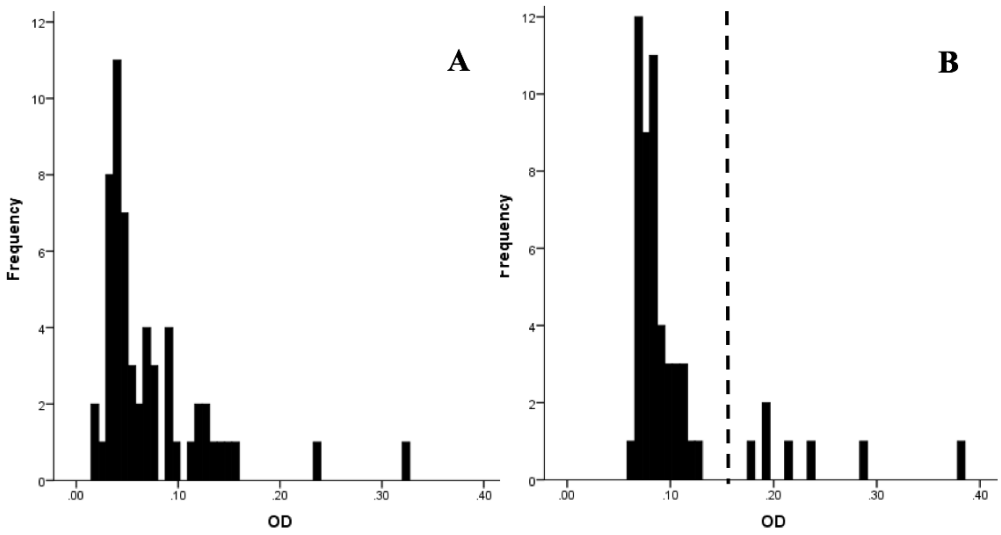

Figure 4.6 Distribution of the uncorrected and corrected OD. A: uncorrected OD, B: corrected OD, n= 55. Dashed line represents the cut off (0.15).

Thus six pigs of 34 (17.6%, 95% CI: 6.7%-34.5%) seroconverted mice by bioassay and 7.9% (95% CI: 3.2%-15.7%) of the inoculated mice presented IgG anti *T. gondii* antibodies. Even though ELISA is considered a sensitive and specific technique, categorizing a positive isolation solely by mice seroconversion could led to a false positive. Mouse seroconversion denotes exposure to the parasite, to confirm the parasite viability, the demonstration of the parasite in mice tissue is recommended. In the present study, the demonstration of *T. gondii* in mouse tissue was performed by PCR screening. Therefore, a positive isolation involved both seroconversion and demonstration of the parasite in mouse tissue.

Of the 55 screened mice, 12 died before the necropsy (21% of losses). The body of three (pigs 13, 15 table 4.7, 4.9) of seven seropositive mice could not be recovered due to mortality, PCR of the brain could not be performed and therefore isolation was not confirmed. The brain of three seropositive mice inoculated with tissues of pigs 3, 10 and 11 tested PCR positive by SAG1 N-PCR. However, the brain inoculated with tissues from the pig with 1:10 MAT titres was SAG1 N-PCR negative. Hence 17.6% (n=6) of the pigs produced mouse seroconversion with their tissues but the parasite was isolated from only 8.8% (n=3) of the pigs (8.8%, 95% CI: 1.8%-23%). Of the seronegative mice, brain was recovered from 36. All the 36

seronegative mice tested SAG1 N-PCR negative. Positive and negative SAG1 N-PCR results were confirmed with B1N-PCR with a 100% agreement.

Although the kappa statistic was not statistically significant, the agreement between pig seroconversion and isolation success was low (kappa=0.16, 95% CI=0.0-0.57, p=0.34); the agreement between pig seroconversion and mouse seroconversion was moderate (kappa=0.25, 95% CI=0.0-0.58, p=0.34); the agreement between mice seroconversion and PCR in pig tissue (both methods) was low kappa=0.098, 95% CI=0.0-0.4, p=0.5) and the agreement between isolation success and PCR in pig tissue (both methods) was also low (kappa= 0.12, 95% CI=0.0-0.5, p=0.5).

Table 4.9 Results of the bioassay in mice. Light grey cells highlight seropositive mice; dark grey cells highlight seropositive and PCR positive mice.

	MICE [a]			MICE [a]	
Number*	**ELISA [c]**	**PCR [b]**	**Number***	**ELISA [c]**	**PCR [b]**
1	3ND/1-	4ND	18	2-	2-
2	2ND/2-	3ND/1-	19	3ND/1-	4ND
3	1+/1ND	1+/1ND	20	4ND	4ND
4	2-	2ND	21	2-	2-
5	2ND/2-	2ND/2-	22	1ND/1-	1ND/1-
6	1ND/1+/2-	1ND/3-	23	4-	1ND /3 -
7	4-	4-	24	4-	4-
8	3ND/1-	3ND/1-	25	1ND/1-	1ND/1-
9	2-	2-	26	1ND/1-	1ND/1-
10	1+/1-	1+/1-	27	1ND/1-	1ND/1-
11	1+/1-	1+/1-	28	1ND/1-	1ND/1-
12	1ND/1-	1ND/1-	29	2-	2-
13	2+	2ND	30	2-	2-
14	2-	2-	31	2ND	2ND
15	1+/1ND	2ND	32	2ND	2ND
16	2-	2ND	33	2-	2-
17	1ND/1-	1ND/1-	34	2ND	2ND

* Refers to the pig number on table 4.7.
[a] Numbers refer to the number of mice, ND: not done (mice died before screen).
[b] PCR in brain.
[c] ELISA in mice serum.

4.4.3 *T. gondii in vitro* isolation from blood

Seventy pigs were sampled from farm 1 in order to attempt *T. gondii* isolation from blood. Pigs were between 8-12 weeks of age. None of the pigs was MAT positive. The blood of 54 pigs was inoculated into pre-seeded 24 well culture plates. Cultures were observed daily. 30 of 54 (55.5%) of the cultures were discarded before the end of the experiment due to bacterial and fungal contamination. It was difficult to identify the parasite. Most of the cultures presented a cytopathic like effect and inclusions. Nevertheless, organisms compatible with *T. gondii* tachyzoites were suspected in 10 cultures inoculated with blood, five of which were from pig blood that was SAG1-PCR positive (table 4.10). The rest of the cultures presented inclusions but the distribution and shape were not typical of *T. gondii*. Inclusions were smaller than tachyzoites and were randomly distributed instead of forming rosettes. DNA was extracted from the media on days 7p.i. and 14p.i. On the day 7 p.i. two samples from media were positive (16 and 17 table 4.10). Monolayers from these cultures were passaged into two new wells. On the days 14 p.i. 5 samples from media were PCR positive (including the positives on the day 7 p.i.). Three of these positive samples were from blood PCR positive pigs and 3 of these samples had organisms compatible with *T. gondii* (table 4.10).

Table 4.10 Results of the *in vitro* cultures of blood. Light grey cells highlight visually positive cultures; dark grey cells highlight SAG1 N-PCR positives cultures.

		CULTURE [b]						CULTURE [b]			
Num*	**PCR[a]**	**Visual**	**PCR[1]**	**PCR[2]**	**Mice**	**Num***	**PCR[a]**	**Visual**	**PCR[1]**	**PCR[2]**	**Mice**
1c	+	+	-	-	2ND	13c	-	-	-	-	NB
2c	-	+	-	+	2ND	14c	-	+	-	-	NB
3c	-	-	-	+	NB	15c	-	+	-	-	NB
4c	+	-	-	+	NB	16c	+	+	+	+	2-
5c	-	+	-	+	2-	17c	+	+	+	+	1ND/1-
6c	+	-	-	+	NB	18c	-	-	-	-	NB
7c	+	-	-	-	NB	19c	-	-	-	-	NB
8c	-	-	+	+	2-	20c	-	-	-	-	NB
9c	+	+	-	+	1ND/1-	21c	-	-	-	-	NB
10c	+	-	-	-	NB	22c	-	+	-	-	1ND/1-
11c	+	+	+	+	2-	23c	-	-	-	-	NB
12c	-	-	+	+	1ND/1-	24c	-	-	-	-	NB

[*] Hypothetical number, see appendix V for individual identification as (n) c.
[a] PCR in pig blood.
[b] Visual: microscope observation of the cultures, PCR[1] in media of cultured blood using. PCR[1] SAG1 N-PCR protocol, PCR[2] idem using B1 N-PCR protocol after importation. ND: not done (mice died before screen) NB: not bioassay.

Cultures were kept until day 22 p.i. (end of the research visit). At the end of the experiment, monolayers from PCR positive medias and monolayers with structures compatible with *T. gondii* which were also PCR positive either in media or in the blood used from culture were inoculated into 2 mice as a procedure to preserve the isolates. Twenty mice were inoculated in total with the cultures, 8 of 20 (40%) mice died before screening and none of the surviving mice had anti-*T. gondii* antibodies (table 4.10). When the DNA was imported to Salford University, further testing was carried out on the 24 DNA media extracts with the B1 N-PCR protocol. The 5 samples which tested positive with the SAG1 protocol were confirmed; in addition, 6 further samples tested positive by B1 N-PCR of which 3 were from cultures in which *T. gondii* had been classified visually as suspicious (table 4.10). Therefore, isolation of *T. gondii* by *in vitro* culture was confirmed in 20% (n=11) of the 54 cultures.

4.4.4 *T. gondii* bioassay from blood

From the 70 pigs, 17 were SAG1 N-PCR positives (24.3%, 95% CI: 14.8%-36%). Thus these 17 leucocyte pellets were inoculated into 2 mice each. Thirteen of the 34 inoculated mice died before screening. Thus the loss of bioassayed mice in this experiment was 38.2%. Two mice of 34 showed IgG anti-*T. gondii* antibodies by ELISA (table 4.11).

Table 4.11 Results of the bioassayed blood. Light grey cells highlight seropositive mice.

			MICE[a]					MICE[a]	
Num*	PCR[c]	MAT	ELISA	PCR[b]	Num*	PCR[c]	MAT	ELISA	PCR[b]
1d	+	-	1ND/1-	1 ND /1-	10d	+	-	2-	2-
2d	+	-	1+/1-	1+/1-	11d	+	-	1 ND /1-	1 ND /1-
3d	+	-	2ND	2 ND	12d	+	-	1 ND /1-	1 ND /1-
4d	+	-	1ND/1-	1 ND /1-	13d	+	-	1 ND /1-	1 ND /1-
5d	+	-	1ND/1-	1 ND /1-	14d	+	-	2-	2-
6d	+	-	2ND	2 ND	15d	+	-	1 ND /1-	1 ND /1-
7d	+	-	1ND/1-	1 ND /1-	16d	+	-	2-	2-
8d	+	-	2-	2-	17d	+	-	1+/1-	2-
9d	+	-	2-	2-					

[*] Hypothetical number, see appendix V for individual identification.
[a] Numbers refer to the number of mice, ND: not done (mice died before screen).
[b] PCR in mouse brain.
[c] PCR in pig blood.

Mouse brains were screened with SAG1 N-PCR and B1 N-PCR. All brain DNA extracts were negative by SAG1 N-PCR, however, one of the brains from a seropositive mouse was positive with B1 N-PCR. Thus isolation of *T. gondii* was demonstrated from 1 of 17 (6%) of the sampled pigs.

4.4.5 Sympatric study

Traps for cats and rodents were placed in farm 1 to study *T. gondii* infection in hosts surrounding the farm and to investigate the possible source of infection for pigs. Attempts to capture cats were unsuccessful. Forty Sherman traps were located to trap rodents. A high population of rats was observed in the farm (appendix III, A3). During the first two days the attempts to capture rats were unsuccessful. Traps were placed across all areas of the farm. After studying the area, it was noticed that the rats were located mainly in the fattening area of the farm and they were consuming pig food. Pig food was used for the remaining days as a bait instead of oats and vanilla. Three rats were captured with this bait. Only traps with pig fattening food and no other food such as farrowing or weaning food had trapped rats and hence fattening food was used throghout. All the traps with the trapped animals were located in the fattening area. The fourth day no rats were captured. On the fifth day 30 of the 40 traps were placed in the fattening area and only 10 in other areas. 10 rats were captured the following day. The sixth and seventh day of sampling no rats were captured and the last day of sampling one rat was captured in the kitchen area. All the rodents captured were rats (*Rattus rattus*). Five rats were females and nine were males. Age of rats was classified as adults, sub-adults and juvenile based on weight (Sridhara and Krishnamurthy, 1992). Eight rats were adults; five rats were sub-adults and one was juvenile.

Rat brains were screened for *T. gondii* DNA by SAG1 N-PCR. Three rat brains (rat 1, rat 9 and rat 14) tested positive and these brains were bioassayed in mice (table 4.12). A total of eight mice were bioassayed with the rat brains. Six of eight mice (75%) died before screening. None of the surviving mice seroconverted (table 4.12). Three samples of muscle were positive by SAG1N-PCR after further screening at Salford University. The muscle samples were from different rats than the brains (table 4.12). Therefore, the prevalence of *T. gondii* DNA in rats

was 43% (96%CI = 17%-71%). No statistical difference was found between gender and infection (p=0.9) and between age and infection (p=0.5) by Fisher Exact test.

Table 4.12 Results of sampled rats. Light grey cells highlight bioassayed rats.

				PCR [c]			Bioassayed brains [d]	
Rat	**Trap location**	**Age** [a]	**Sex** [b]	**B**	**H**	**M**	**Number mice**	**Mice**
1	Fattening area	Sb	M	+	-	-	2	1ND/1-
2	Fattening area	A	M	-	-	-	NP	NP
3	Fattening area	A	M	-	-	-	NP	NP
4	Fattening area	A	M	-	-	-	NP	NP
5	Fattening area	A	F	-	-	-	NP	NP
6	Fattening area	A	M	-	-	+	NP	NP
7	Fattening area	Sb	F	-	-	-	NP	NP
8	Fattening area	A	F	-	-	-	NP	NP
9	Fattening area	A	M	+	-	-	2	1 ND /1-
10	Fattening area	J	F	-	-	+	NP	NP
11	Fattening area	A	M	-	-	-	NP	NP
12	Fattening area	Sb	M	-	-	+	NP	NP
13	Fattening area	Sb	M	-	-	-	NP	NP
14	Kitchen	Sb	F	+	-	-	4	4 ND

[a] Sb: sub-adult, A: adult, J: juvenile.
[b] F: female, M: male.
[c] B: brain, H: heart, M: muscle.
[d] NP: no performed, ND: not done.

Additionally, to the sympatric study carried out in farm 1, bioassay was performed with the cat collected in farm pilot A. Brain and heart tested SAG1 N-PCR positive. Six mice were bioassayed with the cat tissues. Three mice were inoculated with brain and three with heart. The three mice inoculated with the brain died and the brain of one of them was passaged into two other mice. In addition, one of the three mice inoculated with heart died and also its brain was passaged into other two mice. One of the surviving mice inoculated with heart was seropositive and its brain tested SAG1 N-PCR (table 4.13). Thus *T. gondii* was successfully isolated from cat tissues. The isolate was named as TgCatMx6 because TgCatMx1-5 has been already published (Dubey *et al.,* 2009). The brain of the positive mouse was passaged into 2 other mice. The isolate was maintained during three years of my PhD by successive passages (five in total) but the body of the infected mice were no recovered in the last trip.

Table 4.13 Bioassay of a cat sampled from farm pilot A. The original passages were performed by the collaborators of the Hideyo Noguchi. The screening of the mice was performed by me (in bold). The light grey cells highlight the positive mouse.

Bioassayed mice (original passages)				**Mice screen**	
Cat tissue	Mouse	Passage	Number mice	**ELISA**	**Brain PCR**
Brain	1	ND	ND	ND	ND
	2	ND	ND	ND	ND
	3	YES	2	2 -	2-
Heart	4	YES	2	1 ND/ 1-	1 ND/1-
	5	ND	ND	ND	ND
	6	Surviving	Surviving	1+	1+

ND: not done.

4.4.6 Genotyping of *T. gondii*

The SAG1 locus was sequenced from the SAG1 N-PCR positive blood samples from pigs raised in farm 1 (20), farm 2 (19), farm 7 (6) and farm 8 (3). In addition, SAG1 N-PCR positive tissue samples of pigs (13), rats (6), and mice (5) and cultures (5) were sequenced. Sequences were aligned and compared to the references type I, II and III genotypes.

Of the 77 SAG1 DNA products, 74 were successfully sequenced of which 68 were from pigs (TgPigMx1-68), 5 from rats (TgRatMx1-5) and one from a cat (TgCatMx6). Genotyping data obtained from pig samples included the 20 blood samples collected from farm 1 (TgPigMx20-39), the 19 blood samples collected from farm 2 (TgPigMx1-19), the 9 blood samples collected in the second environment (TgPigMx42-50), 13 pig tissue samples (TgPigMx40, 41, TgPigMx51-55, 58-60, 62-63, 67), four blood cultures (TgPigMx56, 57, 66, 68) and the three bioassayed pigs (TgPigMx61, 64, 65). This DNA sequence spans six polymorphic sites which allows an attempt to distinguish between type I or II/III SAG1 alleles (table 4.14). All PCR products showed 100% identity with the *T. gondii* SAG1 gene (table 4.14). All DNA sequences shared the type I polymorphic SNPs except two, TgPigMx27 and TgPigMx48. TgPigMx27 had type I in 5 polymorphic places and type II/III in one. TgPigMx48 had two different alleles (type I and type II/III). Of the 74 DNA sequences 49 SAG1 DNA sequences were identical to clonal type I. The 26 remaining sequences were considered atypical and were named as u1-26 (table 4.14). A total of 30 novel SNPs were identified. Three of the SNPs were parsimony-informative, which means that it was shared by at least two sequences.

One of these parsimony-informative SNPs was shared by two sequences with the identical SAG1 allele which was named as u-20; one of them was from a pig raised in pilot farm A and the other was from a pig raised in farm 1. The other two SNPs were shared by two samples which had different alleles (u-25 and u-26) as one of them had another SNP. These pigs were from farm 1.

In addition, five DNA sequences from blood samples presented double peaks at one to seven nucleotides. One DNA sequence from pig tissue had a double peak at one nucleotide. Nucleotide sites were named according to the IUPAC code (Johnson, 2010). Due to the high quality of the DNA sequences and that the double peaks were observed only in a few nucleotide sites, this could be an indicative of co-infection with two different strains. One of the DNA sequences from blood had double peaks at the polymorphic sites which differ between type I and II/III alleles, the remaining were novel SNPs (figure 4.7, table 4.14).

The 74 samples successfully sequenced with the SAG1 locus were tested up to three times (only negative samples were retested) with the genetic marker SAG2 (Alt-SAG2). Of the 74 samples, 39 (53%) were also positive with the SAG2 genetic marker after the three repeats. The reason why fewer samples amplified with this marker could be explained by the lower sensitivity achieved when compared to SAG1 (figure 3.1). Samples which tested positive with SAG2 were 10 pig tissue samples of which 2 were from pigs raised in backyard (TgPigMx55, 63); one in the pilot A farm (TgPigMx40) and 7 in farm 1 (TgPigMx52-54, 58-60, 62); one muscle from a rat (TgRatMx4); one brain sample from a rat (TgRatMx5), 9 blood samples from farm 1 (TgPigMx20, 25, 28-30, 35, 37-39); 8 blood samples from farm 2 (TgPigMx2, 4, 5, 7, 10, 14, 15, 19), 4 blood samples from the second environment (TgPigMx44, 46, 47, 50); one mouse brain sample from the bioassayed cat (TgCatMx6); three mouse brain samples from bioassayed pigs raised in farm 1 (TgPigMx61, 64, 65) and two samples from cultured blood (TgPigMx57, TgPigMx66). Of the 10 swine tissue samples, 8 were extracts from digested tongue and two from non-digested tongue. The 39 amplifications were successfully sequenced showing 100% of identity with the SAG2 gene (table 4.15).

Table 4.14 DNA sequences for the SAG1 locus. Consensus sequence is the one shared by at least two of the three archetypal types I, II and III alleles. "." indicates identity to the consensus sequence. Pink cells indicate SNPs shared with the clonal type I reference strain. Black cells indicate SNPs not shared by alleles from Type I, II and III archetypal types. Grey cells indicate sites shared by two nucleotides. Y=C or T, R=A or G, W=A or T, S=G or C.

Position	29	33	37	46	49	51	56	70	84	86	98	103	112	114	128	133	147	150	154	182	186	191	196	211	212	239	250	260	271	284	289	300	329	340	342	352	Allele
Consensus	T	G	T	A	G	T	A	T	C	C	C	G	T	T	T	G	T	T	T	C	T	T	G	A	A	G	G	T	T	C	G	C	G	A	A	G	
GT1	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	I
ME49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	II
VEG	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	III
TgPigMx(n)** TgRatMx1-4 TgCatMx6	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	I
TgRatMx5	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-1
TgPigMx1	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	T	.	.	.	.	.	.	u-2
TgPigMx3	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-3
TgPigMx5	.	A	.	.	.	C	G	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-4
TgPigMx8	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	A	.	.	.	.	.	u-5
TgPigMx11	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-6
TgPigMx14	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	.	.	T	.	.	.	.	.	.	u-7
TgPigMx15a/b	Y	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-8
TgPigMx16a/b	.	A	.	.	.	C	.	.	.	T	.	C	Y	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	Y	.	.	.	.	u-9
TgPigMx19a/b	.	A	.	.	.	C	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	R	W	.	T	.	.	.	.	.	.	u-10/11
TgPigMx22	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	C	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-12
TgPigMx23	.	A	.	.	.	C	.	.	.	T	T	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-13
TgPigMx24	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-14
TgPigMx27	.	.	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-15
TgPigMx30	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	T	.	.	u-16
TgPigMx33	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	T	.	.	.	.	.	.	u-17
TgPigMx37	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	A	.	.	A	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-18
TgPigMx38a/b	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	R	.	u-19
TgPigMx41	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-20
TgPigMx42	.	A	.	.	.	C	.	T	T	T	.	C	.	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-21
TgPigMx44	.	A	A	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-22
TgPigMx48a/b	.	R	.	.	.	Y	.	.	.	Y	.	S	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	Y	.	.	.	.	.	.	I/II-III
TgPigMx51	.	A	.	.	.	C	.	.	.	T	.	C	.	.	.	.	.	.	.	.	.	C	A	.	.	.	.	.	.	T	.	.	.	.	.	.	u-20
TgPigMx52a/b	.	A	.	.	.	C	.	.	.	T	.	C	.	Y	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	T	.	.	.	.	.	S	u-24
TgPigMx57	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	u-25
TgPigMx61	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	u-26

** TgPigMx2,4, 6-7, 9-10, 12-13, 17-18, 20-21, 25-26, 28-29, 31-32, 34-36, 39-40, 43,45-47, 49-50, 53-56, 58-60, 62-68.

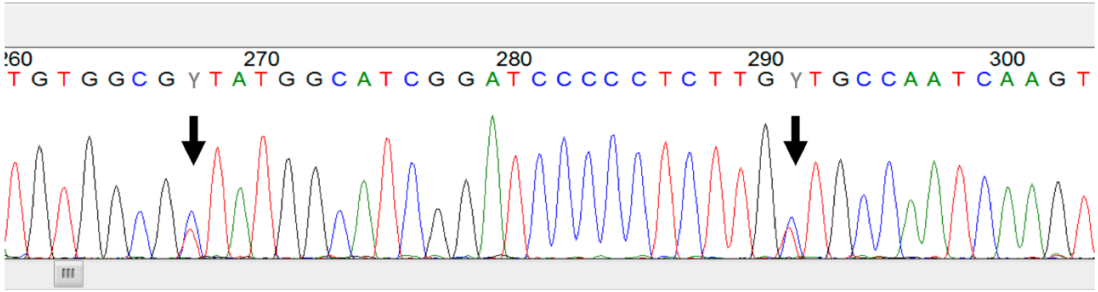

Figure 4.7 Example of double peaks in the chromatogram of the sample TgPigMx48. SAG1 *T. gondii* DNA sequence extracted from pig blood. T or C at the 267 and 291 nucleotide sites belong to the type I or type II and III respectively.

Of the 39 SAG2 DNA sequences 5 were identical to the clonal type I allele and one was identical to the type III allele. No clonal type II allele was obtained with this locus. Of the 33 remaining SAG2 sequences, 7 shared an atypical allele named as u-1 in this study. This atypical allele is characterized by a combination of type I and II SNPs. In addition, 11 sequences shared an atypical SAG2 allele named as u-4. This u-4 allele was identical to u-1 but with a novel SNPs. Three SAG2 sequences shared an allele named as u-13 which has a type I allele with a novel SNP. The remaining SAG2 sequences were different between each other characterized by type I, u-1 and u-4 alleles with novel SNPs. These genotypes were named as u-2, u-3, u-5-18. A total of 21 novel SNPs were identified, four of which were parsimony informative (table 4.15).

In addition, four SAG2 sequences showed double peaks. None of the double peaks were in the polymorphic sites which distinguish between type I, II and III genotypes. However, one of the double peaks was shared by three sequences (table 4.15, figure 4.8). This could indicate a double infection with two parasite strains. Those three samples were obtained from blood from pigs raised in farm 1. In addition, one of them presented double peaks also in the SAG1locus (table 4.14, TgPigMx38).

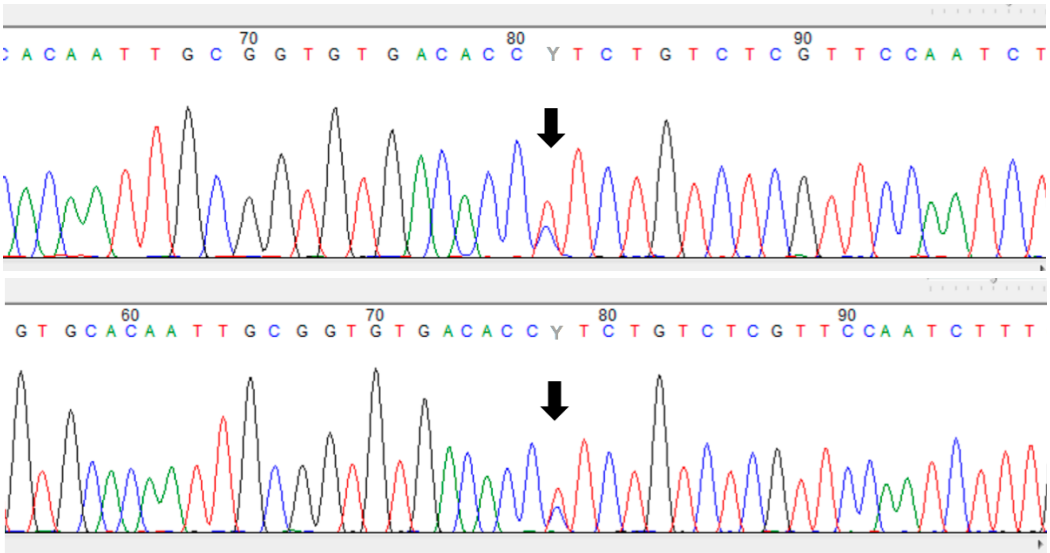

Figure 4.8 Example of the shared double peaks in the SAG2 DNA sequence of TgPigMx38 and TgPigMx20 strains.

Table 4.15 DNA sequences for the SAG2 locus. Consensus sequence is the one shared by at least two of the three archetypal types I, II and III alleles. "." indicates identity to the consensus sequence. Blue cells indicate SNPs shared by the clonal type II reference strain. Green cells indicate SNPs shared by the clonal type III reference strain. Black cells indicate nucleotide polymorphisms not shared by alleles from Type I, II and III archetypal types. Grey cells indicate sites shared by two nucleotides Y=C or T, R=A or G, W=A or T, S=G or C.

Position	15	30	41	42	62	86	109	126	151	175	220	225	227	229	258	281	317	365	387	394	395	398	409	418	437	459	465	466	Allele
Consensus	T	A	T	T	T	T	C	T	T	G	C	T	A	T	T	T	G	A	T	T	T	C	C	G	A	T	G	A	
GT1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
ME49	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	G	C	.	.	.	.	II
VEG	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	III
TgCatMx6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgRatMx4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
TgRatMx5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx2	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-2
TgPigMx4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
TgPigMx5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	G	C	.	.	.	.	u-3
TgPigMx7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
TgPigMx10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx15	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx19	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-5
TgPigMx20a/b	.	.	.	.	Y	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4/6
TgPigMx25a/b	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4/7
TgPigMx28	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	u-8
TgPigMx30	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	u-9
TgPigMx35a/b	.	.	.	.	.	.	.	.	A	.	.	C	R	.	.	.	.	.	.	.	.	.	G	C	.	.	W	.	u-10/11
TgPigMx37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	u-12
TgPigMx38a/b	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4/6
TgPigMx39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx40	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx44	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx46	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx47	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	u-13
TgPigMx50	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	C	G	.	.	.	u-14
TgPigMx52	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx53	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	III
TgPigMx54	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	u-15
TgPigMx55	.	.	.	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	u-16
TgPigMx57	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-1
TgPigMx58	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
TgPigMx59	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	.	u-17
TgPigMx60	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	u-13
TgPigMx61	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx62	.	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4
TgPigMx63	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
TgPigMx64	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	C	.	C	.	G	C	.	.	.	.	u-18
TgPigMx65	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	u-13
TgPigMx66	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	C	.	.	.	G	u-4

In order to expand the genotyping data, the 74 samples sequenced with the SAG1 locus were additionally tested with the genetic marker GRA6. Even though, samples were tested with this genetic marker also up to three times, the number of successful amplifications was much lower when compared to SAG2 and SAG1. However, seven samples that did not amplified with SAG2, resulted in positive amplification with GRA6. After the three repeats, a total of 22 (34%) samples amplified for this gene. Following sequencing, all PCR products showed 100% of identity with the *T. gondii* GRA6 gene (table 4.16). Samples that amplified with this marker were muscle extracts from two rats (TgRatMx4, TgRatMx1), 4 swine blood samples of pigs from the second environment (TgPigMx46, 48-50), two mouse brains from the bioassayed cat and one pig (TgCatMx6 and TgPigMx65), 9 swine blood samples from farm 1, 4 swine tissue samples of which one was raised in backyard (TgPigMx55) and three in farm 1 (TgPigMx53,58, 62). The four swine tissue samples which amplified with this genetic marker were extracts from digested tongue. Of the 23 GRA6 DNA sequences, 7 shared the clonal type I allele, 4 shared the clonal type II allele and 11 sequences had atypical alleles characterised by type I or II background with novel SNPs (table 4.16). The type III allele was not found at this locus. One of the sequences had a combination of SNPs of type II and I lineages which was named as u-3. A total of 9 SNPs were noted among the GRA6 sequences, two of which were parsimony informative as they were shared by two samples each (genotypes u-4 and u-8). The remaining 6 sequences had unique genotypes (u-1, 2, 6-9).

Table 4.16 DNA sequences for the GRA6 locus. Consensus sequence is the one shared by at least two of the three archetypal types I, II and III alleles. "**.**" indicates identity to the consensus sequence. Pink cells indicate SNPs shared with the clonal type I reference strain. Blue cells indicate SNPs shared by the clonal type II reference strain. Green cells indicate SNPs shared by the clonal type III reference strain. Black cells indicate nucleotide polymorphisms not shared by alleles from Type I, II and III archetypal types. Grey cells indicate sites shared by two nucleotides.

Position	43	93	113	116	120	143	152	178	179	222	223	234	243	278	Allele
Consensus	C	A	C	C	G	T	T	C	T	C	A	G	A	C	
GT1	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
ME49	.	.	T	.	.	.	.	.	.	.	.	.	G	.	II
VEG	.	.	.	.	.	.	.	T	.	.	.	A	.	.	III
TgCatMx6	.	.	T	.	.	.	.	.	.	.	.	.	G	.	II
TgRatMx1	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgRatMx4	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx21	T	.	T	.	.	.	.	.	.	.	.	.	G	T	u-1
TgPigMx22	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx23	.	.	.	.	.	G	.	.	.	.	G	.	.	.	u-2
TgPigMx25	.	.	.	.	.	.	.	.	.	.	.	.	G	.	u-3
TgPigMx27	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx28	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx30	.	.	T	.	.	.	.	.	A	.	.	.	G	.	u-4
TgPigMx35	.	G	.	.	.	G	.	.	.	.	.	.	.	.	u-5
TgPigMx37	.	.	.	T	.	G	C	.	.	.	.	.	.	.	u-6
TgPigMx38	.	.	.	.	.	G	.	.	.	T	.	.	.	.	u-7
TgPigMx46	.	.	T	.	A	.	.	.	.	.	.	.	G	.	u-8
TgPigMx48	.	.	T	.	.	.	.	.	.	.	.	.	G	.	II
TgPigMx49	.	.	T	.	.	.	.	.	.	.	.	.	G	.	II
TgPigMx50	.	.	T	.	A	.	.	.	.	.	.	.	G	.	u-8
TgPigMx53	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx55	.	.	.	.	.	G	.	.	.	.	.	.	.	.	I
TgPigMx58	.	.	T	.	.	.	.	.	A	.	.	.	G	.	u-4
TgPigMx62	.	.	.	.	.	.	.	.	.	.	.	.	.	.	u-9
TgPigMx65		.	T	.	.	.	.	.	.	.	.	.	G	.	II

To increase the genotyping resolution, samples which had amplified with more than one genetic marker, were also tested with the SAG3 locus. Thus 46 samples were tested with this locus of which 23 (50%) were positive after a maximum of three repeats. The samples which amplified were 3 mouse brains, one of which was from the bioassayed cat (TgCatMx6) and the remaining two were from bioassayed pigs (TgPigMx61, 64); two muscles samplesd from 2 rats (TgRatMx1 and TgRatMx4), 5 swine blood sample from pigs raised in farm 1 (TgPigMx20, 21, 27, 29, 30, 39), one swine blood sample from farm 2 (TgPigMx7); 8 swine tissue samples of which two were from pigs raised in pilot farm A (TgPigMx40, 41), one was from a pig raised in the backyard system(TgPigMx55) and 5 from pigs raised in farm 1 (TgPigMx53,55, 58, 60 and 62). Of the 23 SAG3 sequences, 10 were clonal type III, 10 were clonal type I and 4 had atypical alleles characterised by type III backgrounds with a novel SNP

(u-1 and u-3) and by a type I background with a novel SNP (u-2 and u-4). Type II alleles were not found at this locus. In addition, one sample with this genetic marker showed double peaks. The sample was from a swine blood extract (TgPigMx7). Therefore, TgPigMx7 had type I and an atypical allele with combined type I and II alleles. Four novel SNPs were identified, however, none was parsimony informative (table 4.17).

Table 4.17 DNA sequences for SAG3 locus. Consensus sequence is the one shared by at least two of the three archetypal types I, II and III alleles. "**.**" indicates identity to the consensus sequence. Pink cells indicate SNPs shared with the clonal type I reference strain. Blue cells indicate SNPs shared by the clonal type II reference strain. Green cells indicate SNPs shared by the clonal type III reference strain. Black cells indicate nucleotide polymorphisms not shared by alleles from Type I, II and III archetypal types. Grey cells indicate sites shared by two nucleotides. R=A or G.

Position	44	56	64	68	100	107	109	116	119	124	139	141	185	Allele
Consensus	C	A	G	A	T	G	G	C	G	C	A	A	G	
GT1	.	.	.	.	.	.	.	A	.	G	.	.	.	I
ME49	T	.	A	G	.	A	.	.	.	.	G	.	.	II
VEG	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgCatMx6	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgRatMx1	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgRatMx4	.	.	.	.	C	.	A	.	.	.	.	.	C	u-1
TgPigMx7a/b	.	.	.	.	.	.	.	A	.	G	R	.	.	I/u-2
TgPigMx20	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx27	.	G	.	.	C	.	A	.	.	.	.	.	.	u-3
TgPigMx29	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx30	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx37	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx39					C	.	A	.	.	.	.	.	.	III
TgPigMx40	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx41	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx53	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx54	.	.	.	.	.	.	.	A	.	G	.		.	I
TgPigMx55	.	.	.	.	.	.	.	A	.	G	.		.	I
TgPigMx56	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx58	.	.	.	.	.	.	.	A	.	G	.	G	.	u-4
TgPigMx59	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx60	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx61	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx62	.	.	.	.	.	.	.	A	.	G	.	.	.	I
TgPigMx63	.	.	.	.	C	.	A	.	.	.	.	.	.	III
TgPigMx64	.	.	.	.	C	.	A	.	.	.	.	.	.	III

At this stage, full classification of genotypes from genotyped strains is difficult to achieve as only information from a few loci could be obtained. In addition, the loci sequenced from samples varied as not all loci from each sample could be successfully amplified. Table 4.18 shows a summary of the strains in which two or more loci were sequenced. It can be said that the *T. gondii* genotypes were diverse as they rarely shared genotypes among the strains. Genotypes presented mixed type I, II, III and u- alleles. The classical clonal types seemed rare

among the *T. gondii* strains sampled in this area of Mexico; although, this is still an early conjecture as for many of the samples only information from one or two loci was obtained.

Table 4.18 Allele combinations of *T. gondii* from pigs, rats and a cat from Yucatan. Grey cells refer to *T. gondii* strains genotyped with four loci.

Isolate/Strain	Genetic Marker				Isolate/Strain	Genetic Marker			
	SAG1	SAG2	SAG3	GRA6		SAG1	SAG2	SAG3	GRA6
GT1	I	I	I	I	TgPigMx35a	I	u-10	-	u-5
ME49	II	II	II	II	TgPigMx35b	I	u-11	-	u-5
VEG	III	III	III	III	TgPigMx37	u-18	u-12	I	u-6
TgCatMx6	I	u-1	III	II	TgPigMx38a	I	u-4	-	u-7
TgRatMx1	I	-	III	I	TgPigMx38b	u-19	u-6	-	u-7
TgRatMx4	I	I	u-1	I	TgPigMx39	I	u-1	III	-
TgRatMx5	u-1	u-1	-	-	TgPigMx40	I	u-4	I	-
TgPigMx2	I	u-2	-	-	TgPigMx41	u-20	-	I	-
TgPigMx4	I	I	-	-	TgPigMx44	u-22	u-4	-	-
TgPigMx5	u-4	u-3	-	-	TgPigMx46	I	u-4	-	u-8
TgPigMx7a	I	I	I	-	TgPigMx47	I	u-13	-	-
TgPigMx7b	I	I	u-2		TgPigMx48a	I	-	-	II
TgPigMx10	I	u-1	-	-	TgPigMx48b	II/III	-	-	II
TgPigMx14	u-7	u-1	-	-	TgPigMx49	I	-	-	II
TgPigMx15a	I	u-4	-	-	TgPigMx50	I	u-14	-	u-8
TgPigMx15b	u-8	u-4	-	-	TgPigMx52a	I	u-1	-	-
TgPigMx19a	u-10	u-5	-	-	TgPigMx52b	u-23	u-1	-	-
TgPigMx19b	u-11	u-5	-	-	TgPigMx53	I	III	III	I
TgPigMx20a	I	u-4	III	-	TgPigMx54	I	u-15	I	-
TgPigMx20b	I	u-6	III	-	TgPigMx55	I	u-16	I	I
TgPigMx21	I	-	-	u-1	TgPigMx56	I	-	III	-
TgPigMx22	u-12	-	-	I	TgPigMx57	u-24	u-1	-	-
TgPigMx23	u-13	-	-	u-2	TgPigMx58	I	I	u-4	u-4
TgPigMx24	u-14	-	-	-	TgPigMx59	I	u-17	III	-
TgPigMx25a	I	u-4	-	u-3	TgPigMx60	I	u-13	I	-
TgPigMx25b	I	u-7	-	u-3	TgPigMx61	u-25	u-4	I	-
TgPigMx27	u-15	-	u-3	I	TgPigMx62	I	u-4	I	u-9
TgPigMx28	I	u-4	-	I	TgPigMx63	I	I	III	-
TgPigMx29	I	u-8	III	-	TgPigMx64	I	u-18	III	-
TgPigMx30	u-16	u-9	I	u-4	TgPigMx65	I	u-13	-	II
					TgPigMx66	I	u-4	-	-

The eight samples in which the 4 loci were successfully sequenced had a different genotype each (table 4.18, figure 4.9). Six of these 8 strains were obtained from pigs and one rat sampled in farm 1 (TgPigMx37, TgPigMx37, TgPigMx53, TgPigMx58, TgPigMx62 and TgRatMx4). This suggests that those animals were infected as a result of different events. Regarding the samples in which sequencing data were obtained for three loci; the strain TgPigMx40 presented the same combination of SAG1, SAG2 and SAG3 alleles as TgPigMx62 (I, u-4, I respectively) and TgPigMx60 presented the same combination of SAG1, SAG2 and

SAG3 alleles as TgPigMx55 (I, u-13, I respectively). TgPigMx40 and TgPigMx60 were obtained from pigs raised in different farms from TgPigMx62 and TgPigMx55. The same combination of alleles was more frequently shared for strains in which only two loci were sequenced, however the power of discrimination obtained is rather low. The combination of allele I in SAG1 and allele II in GRA6 was shared by TgCatMx6, TgPigMx48a, TgPigMx49 and TgPigMx65. Other frequent allele combinations were allele I in SAG1 and u-4 in SAG2 which was shared by six strains (table 4.18).

Among all loci, the predominant allele type was atypical (46%), followed by the type I allele (43%), the type III allele (8%) and finally, the type II allele (3%). Atypical alleles in SAG1 revealed the type I background. Atypical alleles in SAG2 and GRA6 presented as a mixed type I and II background. In contrast atypical alleles in SAG3 presented as type III and type I backgrounds. Therefore, these *T. gondii* strains presented the type I background with combinations of II or III or both II and III alleles (table, 4.18, figure 4.9).

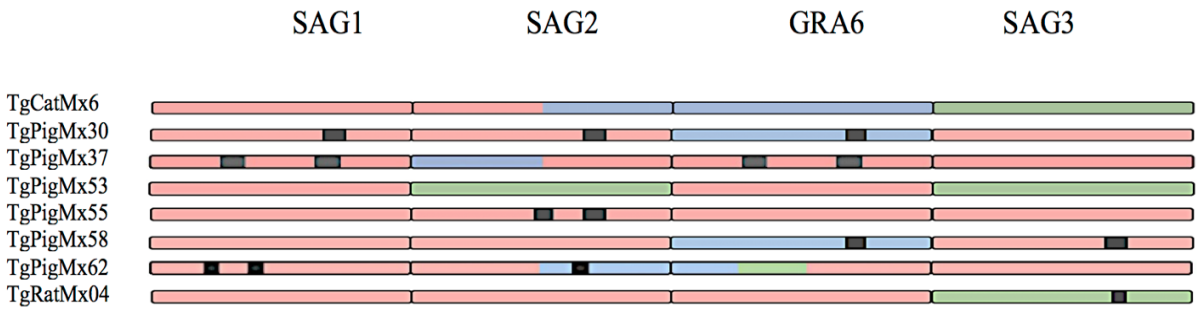

Figure 4.9 Illustration of SAG1, SAG2, SAG3 and GRA6 alleles for the 8 strains/isolate with the four sequenced loci. Pink, blue and green refers to type I, II and III backgrounds. Black areas denote novel SNPs.

For a better understanding of the genetic population structure of the *T. gondii* strains from Yucatan, genotypes were analyzed in a phylogenetic tree with the reference type I, II and III strains. In order to obtain a solid phylogram, this was constructed by concatenating the loci from those strain in which sequencing data were obtained for three or more genes. In the phylogram obtained, *T. gondii* genotypes were segregated in five clades (A, B, C, D and E) (figure 4.10A). In clade A were clustered genotypes closely related to the type I lineage. Clade C comprised genotypes with mixed type I and II alleles. Both clades B and D included mixed

type I and III alleles. Clade B clustered genotypes with type III alleles at the SAG3 locus and type I alleles at SAG2 and GRA6 loci. In clade D were clustered genotypes representing the type III alleles at the SAG3 locus, atypical alleles at SAG2 and type I at GRA6. Finally, in clade E, the strains were clustered with the atypical u-4 allele at the SAG2 locus and type I alleles at the remaining loci. However, the segregation into clusters was not supported by the bootstrap values. A statistically significant bootstrap value has been suggested to be above 50% (Felsenstein J, 1985, Wilcox *et al*., 2002) and the bootstrapping of the external nodes had values as low as 0. However, some internal clusters were the result of a more robust bootstrapping. For example, the genotype TgPigMx55 was clustered with the GT1 type I strain with the bootstrap value of 78. Interestingly, TgPigMx61 and TgPigMx62 were clustered together with a high bootstrap value of 98. The branches of these two genotypes were long indicating them to be divergent from the rest of the *T. gondii* population of Yucatan. Clusters A, B, D and E comprised strains obtained from pigs raised in farm 1, except two strains which were obtained from pigs raised in backyard systems classified in clades A and B (TgPigMx55 and TgPigMx63). The three strains classified in clade C were obtained from animals raised in the pilot farm A (TgCatMx6) and in the second environment (TgPigMx46, TgPigMx50). The tree constructed using the UPGMA method showed slightly different topology. Clades A and B were identical with the exception that the strains TgPigMx21 and TgPigMx29 interchanged positions with the strains TgPigMx37 and TgRatMx1 respectively. Clades C and E were constructed only with the strains ME49 and VEG, and TgPigMx61 and TgPigMx62 respectively. The remaining strains were merged in the remaining clade.

In contrast, better bootstrapping values were obtained in the Neighbor-Joining phylogram when only the genotypes based on four loci were used (figure 4.10B). Interestingly, in this phylogenetic tree, the genotype TgPigMx55 was clustered also with the GT1 strain. TgPigMx62 was clustered with the genotype TgPigMx37 and in this phylogram, it was also found on a long branch. TgPigMx30 was clustered with TgPigMx58. It is noteworthy that the genotype obtained from a rat TgRatMx4 was clustered with the genotype TgPigMx53 obtained from a pig raised in the same farm. Both genotypes appeared to be almost identical in the phylogram. In contrast, the isolate TgCatMx6 obtained from the cat remained unclustered. In addition, TgCatMx6 was connected to the node which contained ME49 and VEG strains. This suggests that this isolate was more closely related to type II or III lineages than the other strains.

All the *T. gondii* strains were obtained from animals raised in farm 1, except TgPigMx55 which was raised in a backyard system. In this case, the UPGMA showed identical clustering except for the strains TgCatMx6 and TgPigMx62 which interchanged their positions. Both A and B trees showed general higher bootstraping values when using the Neighbor-Joining method than UPGMA. The strains which showed differences in segregation when both trees were compared had the lowest bootstrap values, showing therefore more unstability due to weaker statistics.

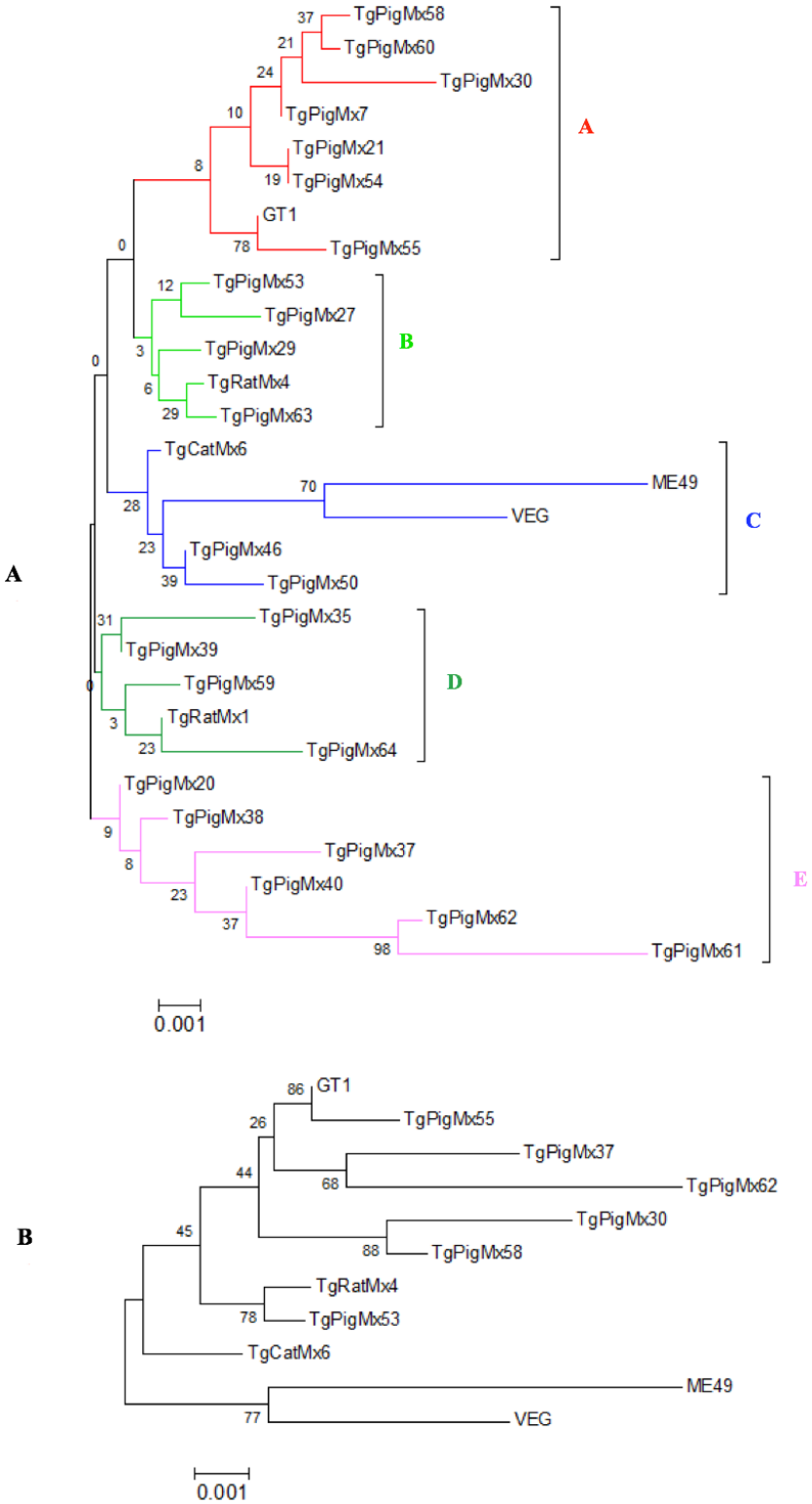

Figure 4.10 Phylogram of *T. gondii* strains obtained from pigs, rats and a cat from Yucatan. The phylogenetic tree was constructed in MEGA6 using the Neighbor-Joining method. The number on the branches indicate the bootstrap value (1000 replicates). The length of the branches was drawn to scale of evolutionary distance (number of base substitution per site), which was calculated using the Maximum Composite Likelihood Method. A: sequencing data of 3 and 4 loci were concatenated to drawn the tree. B: only sequencing data of the strains with four sequenced loci was used.

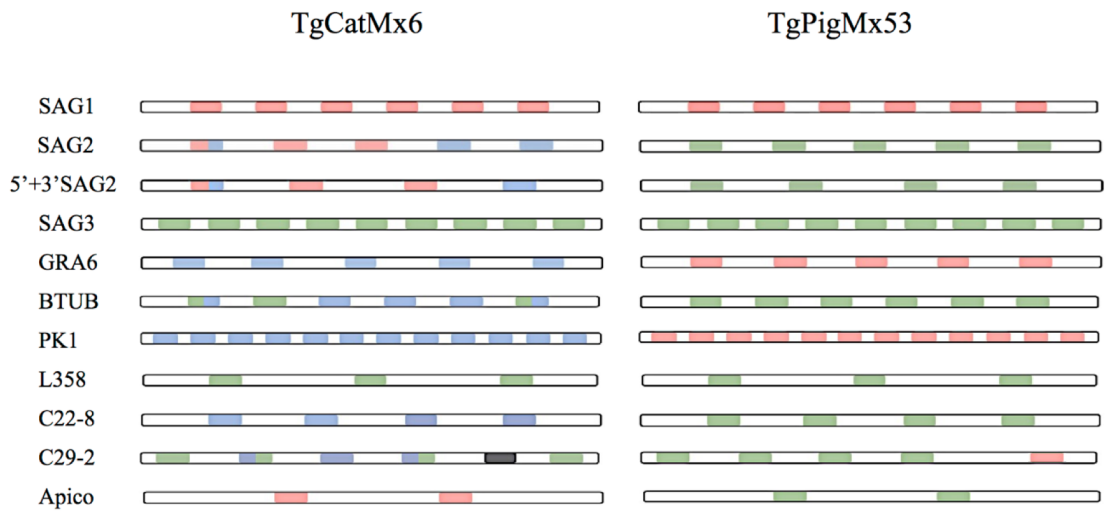

Figure 4.11 Illustration of the SNPs in the 12 loci analyzed in TgCatMx6 and TgPigMx53. Pink, blue and green refers to SNPs shared with type I, II and III respectively. Black areas denote novel SNPs.

In order to improve the genotyping resolution, amplification with the remaining 8 loci to complete the set of 12 (Su *et al*., 2010) was attempted with the samples in which sequencing data for the four loci was achieved (figure 4.9). The remaining loci (5' SAG2, 3'SAG2, BTUB, PK1, L358, C22-8, C29-2 and Apico) were fully completed with the isolate TgCatMx6 and the strain TgPigMx53. TgCatMx6 was an atypical genotype with mixed type I, II, III and atypical alleles. TgPigMx53 was also atypical but with recombined type I and III alleles and one atypical allele (figure 4.11 and table 4.19). TgCatMx6 had type I alleles at SAG1 and Apico loci; type II alleles at Gra6, PK1 and C22-8 loci; type III alleles at SAG3 and L358; mixed I and II alleles at SAG2 (Alt +3'+5'SAG2) and mixed II and III alleles in BTUB and C29-2. C29-2 had also a novel SNP. In contrast TgCatMx53 had type I alleles at SAG1, GRA6 and PK1; no type II alleles; type III alleles at SAG2 (Alt +3'+5'SAG2), SAG3, BTUB, L358, C22-8 and Apico and finally mixed type I and III alleles at the C29-2 locus (figure 4.11 and table 4.19).

Table 4.19 Allele combinations and genotypes of TgCatMx6 and TgPigMx53 by MLST.

Chromosome	VIII			VII	X	IX	VI	V	III	Ib	Apicoplast	Genotype
Locus	SAG1	AltSAG2	3'+5'SAG2	SAG3	GRA6	BTUB	PK1	L358	C29-2	C22-8	Apico	
GT1	I	I	I	I	I	I	I	I	I	I	I	I
ME49	II	II	II	II	II	II	II	II	II	II	II	II
VEG	III	III	III	III	III	III	III	III	III	III	III	III
TgCatMx6	I	u1	u1	III	II	u1	II	III	u1	II	I	Atypical
TgPigMx53	I	III	III	III	I	III	I	III	u2	III	III	Atypical

In order to investigate the similarity of the genotypes of the present study with other genotypes or South/North America isolates, MLST genotypes of TgCatMx6 and TgPig53 were 'transformed' to RFLP genotypes. This approach was implemented because the majority of genotyping studies are based on the RFLP of these 12 loci rather than by sequencing. The number of RFLP genotypes of *T. gondii* isolates generated over the recent years has been substantial, reaching the number of 262 (Work *et al.,* 2016). The RFLP patterns were predicted by identification of the recognition sites of the restriction enzymes (Su *et al*., 2010). Once the RFLP genotypes were completed (table 4.20), these were compared with the genotypes published in ToxoDB (http://toxodb.org). ToxoDB is a genome database resource of *T. gondii* and other protozoa such as *Neospora, Eimeria* and *Sarcocystis*. In ToxoDB there is the most complete collection of genotypes of *T. gondii*. These genotypes are designated as '#' followed by a number. TgCatMx6 had the RFLP genotype number #154, this genotype was obtained from the isolate TgGoatUS20 from a goat in the USA. In contrast the RFLP genotype of TgPigMx53 was not found. DNA sequencing had a greater resolution than RFLP (table 4.19 and table 4.20). By using MLST, TgCatMx6 the three SAG2 loci, Btub, C29-2 had atypical alleles while by RFLP, restriction enzymes only could resolve the atypical allele at the C29-2 locus. MLST also had a greater resolution for TgPigMx53 which using MLST showed this strain to have an atypical genotype and by RFLP it had a recombinant type I/III genotype.

Table 4.20 Allele combinations and genotypes of TgCatMx6 and TgPigMx53 by RFLP.

Chromosome	VIII			VII	X	IX	VI	V	III	Ib	Apicoplast	Genotype
Locus	SAG1	AltSAG2	3'+5' SAG2	SAG3	GRA6	BTUB	PK1	L358	C29-2	C22-8	Apico	
GT1	I	I	I	I	I	I	I	I	I	I	I	I
ME49	II	II	II	II	II	II	II	II	II	II	II	II
VEG	III	III	III	III	III	III	III	III	III	III	III	III
TgCatMx6	I	II	II	III	II	II	II	III	u1	II	I	Atypical
TgPigMx53	I	III	III	III	I	III	I	III	III	III	III	Recombinant I/III

The TgGoatUS20 isolate has been sequenced at the loci UPRT1, UPRT7, EF, HP2 and GRA 6 (Dubey *et al*., 2011b). Thus the loci UPRT1, UPRT7, EF, HP2 were downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/nucleotide) and were used to compare the SNPs between TgGoatUS20 and TgCatMx6 isolates (table 4.21). Genotyping of the four introns UPRT1, UPRT7, EF1, and HP2 were also attempted with the strain TgPigMx53 but only

UPRT7 amplified by PCR. GRA6, UPRT1, UPRT7, and HP2 were identical for both TgGoatUS20 and TgCatMx6 (table 4.21). UPRT1 and UPRT7 were atypical alleles, GRA6 was type II and HP2 type I or II (this locus could not distinguish between type I and II alleles). For the intron EF1 TgCatMx6 had allele type III, however TgGoatUS20 had an atypical allele characterized by mixed type I/III SNPs (table 4.21). TgPigMx53 presented type II or III alleles (type II and III alleles are indistinguishable with this locus). Even though, RFLP patterns of SAG1, SAG2, SAG3, BTUB, PK1, Apico, L358, C29-2 and C22-8 were the same for the isolate TgGoatUS20 and TgCatMx6, it cannot be assumed that those loci were also identical since sequencing data of TgGoatUS20 were not available. Nevertheless, it is obvious that those isolates are closely related as they shared RFLP patterns and four of the five sequenced loci were identical except for one SNP. On the other hand, TgPigMx53 presented type I and III alleles with one u-1 allele. This combination of alleles is more commonly found in South America. However, it has also been reported in North America (Dubey *et al.,* 2011a, Velmurugan *et al.,* 2009, Dubey *et al.,* 2011b).

Table 4.21 Allele combinations and genotypes of TgCatMx6 and TgGoatUS20 for the introns UPRT7, UPRT1, HP2, EF1 and GRA6.

	UPRT7							UPRT1							HP				
Position	136	164	294	368	469	573	Allele	57	111	132	136	142	269	Allele	215	302	304	428	Allele
Consensus	T	A	T	C	A	A		G	C	T	G	G	C		C	C	G	T	
GT1	G	G	C	.	G	G	I	.	A	C	.	C	T	I	.	.	.	.	I
ME49	.	.	.	.	.	.	II	.	.	.	.	.	.	II	.	.	.	.	II
VEG	.	.	.	.	.	.	III	.	.	.	.	.	.	III	T	A	A	C	III
TgCatMx6	.	G	.	T	.	.	u-1	C	A	C	C	C	.	u-1	.	.	.	.	I/II
TgGoatUS20	.	G	.	T	.	.	u-1	C	A	C	C	C	.	u-1	.	.	.	.	I/II
TgPigMx53	.	.	.	.	.	.	II/III	-	-	-		-	-	ND	-	-	-	-	ND

	GRA6						EF						
Position	113	143	178	234	243	Allele	145	254	349	427	487	574	Allele
Consensus	C	T	C	G	A		C	T	G	T	C	G	
GT1	.	G	.	.	.	I	.	.	.	C	.		I
ME49	T	.	.	.	G	II	A	C	A	.	T	A	II
VEG	.	.	T	A	.	III	.	.	.	.	.		III
TgCatMx6	T	.	.	.	G	II	.	.	.	.	.		III
TgGoatUS20	T	.	.	.	G	II	.	.	.	C	.		u-1
TgPigMx53	.	G	.	.	.	I	-	-	-	-	-		ND

For a better understanding of the relationship between the *T. gondii* isolates from Yucatan and those from North and South America it is necessary to compare the genotyping with a wider range of isolates. Therefore, sequencing data for the *T. gondii* representative

genotypes with North and South American origin were downloaded from ToxoDB and used to construct a phylogenetic three with the strains obtained in the present study. A total of 49 isolates obtained from animals and humans from the USA, Canada, Brazil, French Guyana, Uruguay, Costa Rica and Colombia were used (table 4.22). GRA6, UPRT1, UPRT7, EF1 and HP2 loci were concatenated for the 49 isolates downloaded from ToxoDB (including reference strains), TgCatMx6 and TgGoatUS20. TgPigMx53 was also included in the analysis but only with GRA6 and UPRT7.

Table 4.22 Origin, source and haplogroup of the 49 prototypic American isolates of *T. gondii*. ToxoDB.

Isolate	Country	Source	Haplogroup*
GT1	USA	Goat	1
ME49	USA	Sheep	2
VEG	USA	Human	3
ARI	USA	Human	12
B41	USA	Bear	12
B73	USA	Bear	2
CAST	USA	Human	7
CASTELLS	Uruguay	Sheep	4
COUG	Canada	Cougar	11
CtCo5	Colombia	Cat	15
GUY-KOE	French Guiana	Human	5
GUY-MAT	French Guiana	Human	5
GUY-DOS	French Guiana	Human	10
GUY-2003-MEL	French Guiana	Human	ND
GUY-2004-ABE	French Guiana	Human	ND
GUY-2004-JAG1	French Guiana	Jaguar	11
M7741	USA	Sheep	3
P89	USA	Pig	9
RAY	USA	Human	12
RH	USA	Human	ND
RH-88	USA	Human	ND
RH-JSR	USA	Human	ND
ROD	USA	Human	3
RUB	USA	Human	5
SOU	USA	Human	ND
TgCatBr1	Brazil	Cat	4
TgCatBr10	Brazil	Cat	9
TgCatBr15	Brazil	Cat	9
TgCatBr18	Brazil	Cat	4
TgCatBr10	Brazil	Cat	9
TgCatBr25	Brazil	Cat	4
TgCatBr26	Brazil	Cat	6
TgCatBr3	Brazil	Cat	ND
TgCatBr34	Brazil	Cat	4
TgCatBr44	Brazil	Cat	10
TgCatBr5	Brazil	Cat	15
TgCatBr64	Brazil	Cat	6
TgCatBr72	Brazil	Cat	6
TgCatBr9	Brazil	Cat	6
TgCkBr141	Brazil	Chicken	7
TgCkCr1	Costa Rica	Chicken	ND
TgCkCr10	Costa Rica	Chicken	ND
TgCkGy2	French Guiana	Chicken	3
TgDogCo17	Colombia	Dog	1
TgH18009	French Guiana	Human	ND
TgH18021	French Guiana	Human	ND
TgRsCr1	Costa Rica	Toucan	5
TgShUS28	USA	Sheep	6
VAND	French Guiana	Human	10

*ND not done.

In both Neighbor-Joining and UPGMA phylograms TgCatMx6 and TgGoatUS20 were clustered together with the atypical isolates GUY-2004-JAG1 and COUG. The bootstrap values obtained with the Neighbor-Joining approach for TgCatMx6, TgGoatUS20 and GUY-2004-JAG1 nodes suggested a strong clustering (74). In contrast, the cluster with COUG was much lower (35), indicating a weaker separation. COUG and GUY-2004-JAG1 are isolates with mixed type I, II and u-1 alleles which fell into the haplogroup 11 (Su *et al*., 2012). Haplogroup 11 did not correspond to a particular geographical area as it was composed only by these two strains of which one is from Canada and the other from French Guiana (figure 4.3). TgPigMx53 was clustered between the Brazilian atypical isolates atypical TgCkBr141 and TgCatBr9 characterized to have mixed type I, II, III and u-1 alleles. Nevertheless, TgPigMx53 was located on a long branch, indicating that although related, it still diverged from TgCkBr141 and TgCatBr9. The low bootstrap value obtained in the TgPigMx53 nodes (21, 32) supported the poor clustering. In addition, the branches in which TgPigMx53 and TgCatMx6 were clustered connected to the widespread genotypes, II and 12 found in USA (isolates ARI, B41, RAY, ME49, B73). Type I and III strains appeared more divergent from TgPigMx53 and TgCatMx6. Much higher divergence was noted between TgPigMx53, TgCatMx6 and atypical genotypes commonly found in South America (haplogroups 4, 5, 8, 9 10 and 15). The UPGMA tree showed identical clustering of TgCatMx6 and TgGoatUS20. In addition, the bootstrap values were slightly higher as TgCatMx6 and TgGoatUS20 had an internal boostrap value of 96 and the external nodes which connected these strains with GUY-2004-JAG1 and COUG had values of 76 and 50 respectively. In contrast, in the UPGMA tree, the strain TgPigMx53 was not clustered with any other strains and remained isolated with a bootstrap value of 17.

The construction of either of the phylogenetic trees based on the analysis of two loci lacked the capability to cluster TgPigMx53 with related genotypes. The alignment of the sequences showed that TgPigMx53 was a strain that was closely related to type I and III strains, however in the phylogenetic tree type III and I strains were considered divergent from TgPigMx53. Better resolution was observed for the isolate TgCatMx6 with which 5 loci were used. Nevertheless, the analysis of the SNPs within the alignment showed it to be closely related to type II strains. In the phylogram, although the branch with type II strains was connecting to TgCatMx6, the node showed a poor bootstrap value.

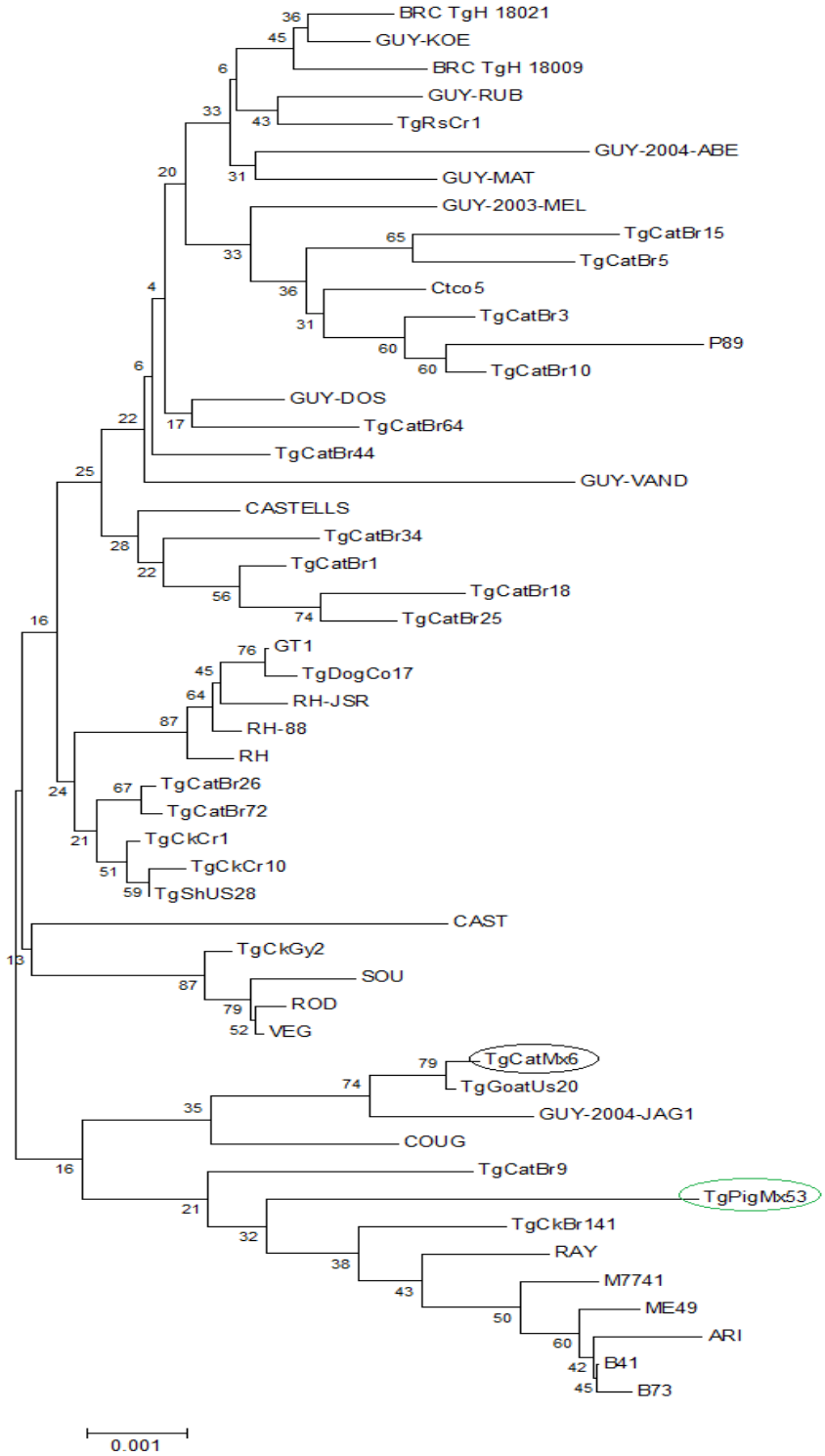

Figure 4.12 Clustering of *T. gondii* strains using the sequencing data of 5 loci. The phylogenetic tree was constructed in MEGA6 using the Neighbor-Joining method. The number on the branches indicate the bootstrap value (1000 replicates). The length of the branches was drawn to scale of evolutionary distance (number of base substitution per site), which was calculated using the Maximum Composite Likelihood Method. Blue and green circles shown the position of TgCatMx6 and TgPigMx53 in the tree.

In order to have a better understanding of the relationship between isolates from Yucatan and from North and South America, a deeper analysis was performed by using a greater number of loci. The 16 loci (SAG1, Alt-SAG2, 3'SAG2, 5'SAG2, SAG3, GRA6, BTUB, PK1, C22-8, C29-2, L358, Apico, UPRT1, UPRT7, EF1 and HP2) were concatenated and included in the phylogram. C22-8 and Apico could not be included in 32 of the 49 prototypic strains due to a lack of sequencing data. In addition, in this analysis, TgGoatUS20 was not included as data of the remaining 9 loci have not been yet generated. TgPigMx53 was included in the analysis without the UPRT1, EF1 and HP2 loci. The Neighbor-Joining and UPGMA phylograms obtained by analyzing sequencing data of the 16 loci (when available) allowed a much clearer segregation in clusters. Clusters were organized by haplogroups and therefore by geographical areas (figure 4.13 shows the Neighbor-Joining tree). Bootstraps values were in general higher which denotes a more robust analysis. Clusters A, B, C and D were composed almost exclusively of South American isolates (with the exception of P89) which fell into haplogroups, 4, 5, 6, 8, 9, 10 and 15. Cluster I comprised isolates exclusively from North America which had type 12 and II genotypes (ARI, B73, B41, ME49, RAY). Cluster H, located next to cluster I with the USA isolates, comprised the atypical isolates COUG and GUY-2004-JAG1 with mixed type I, II, III and u-1 alleles. Interestingly, TgCatMx6 occupied an intermediate position between those clusters (figure 4.13). The bootstrap value (78) of the branch which include both clusters I and H together with TgCatMx6 supported the close relationship between these genotypes. However, the bootstrap value of the node in which TgCatMx6 was grouped within the cluster I was moderate (45) indicating some divergence. Cluster G was composed exclusively of type I and related type I lineages which comprised isolates from North and South America. Cluster F was composed of isolates from North and South American origin with mixed type I and III genotypes. Finally, cluster E was composed of type III strains which are found in North and South America. TgPigMx53 was clustering between cluster E and F which are found in both North and South America. The bootstrap value obtained for the node which includes cluster E, F and TgPigMx53 was high (82) indicating a strong relationship among isolates. However, the bootstrap value obtained for the node which included TgPigMx53 in cluster E was low (18) indicating a poor clustering. The UPGMA tree showed identical topology to the Neighbor-Joining tree supporting the most robust clustering when using a larger number of loci. Boostrap values were however slightly higher when using Neighbor-Joining method.

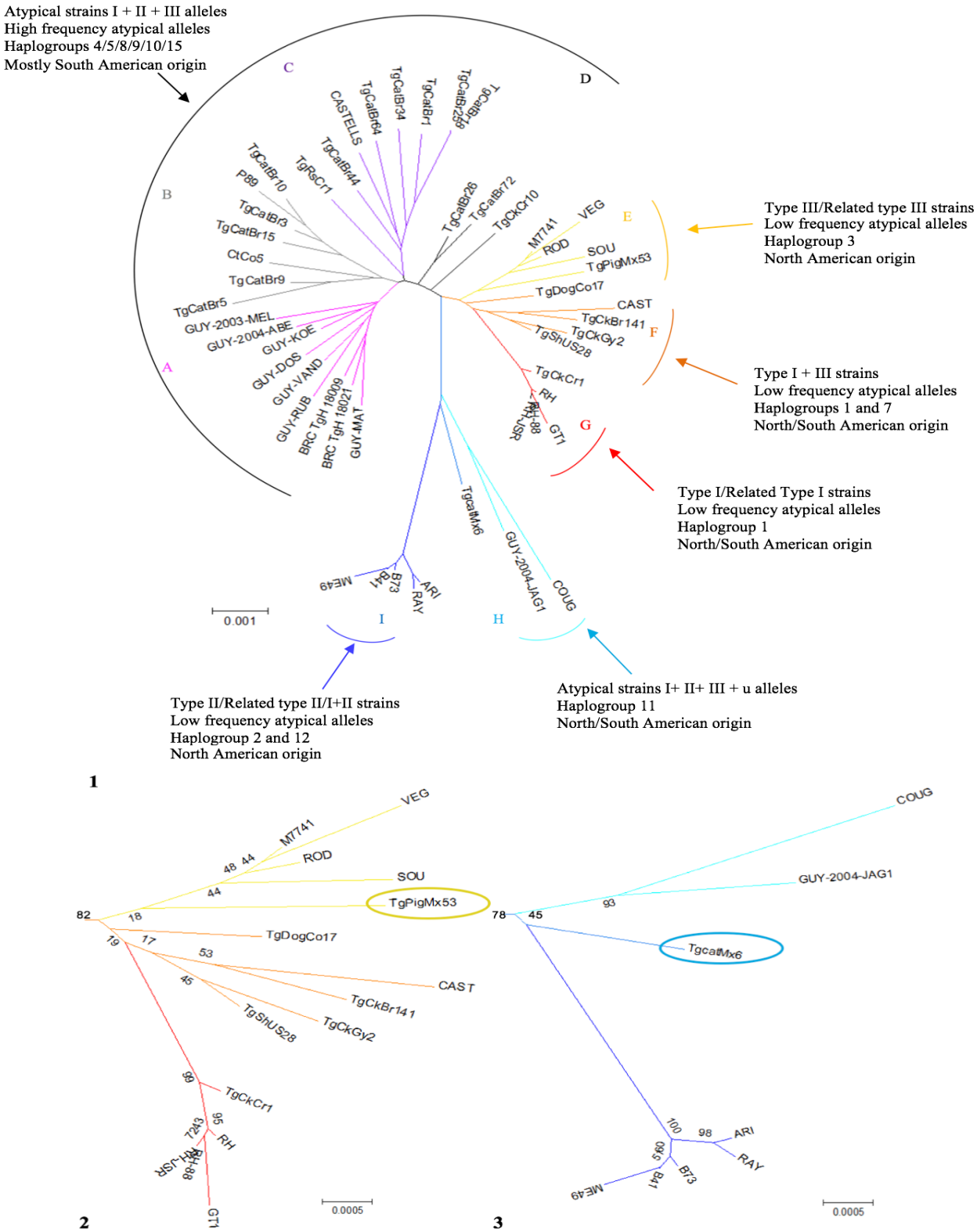

Figure 4.13 Clustering of *T. gondii* strains using the sequencing data of 16 loci. 1: phylogenetic tree constructed in MEGA6 using the Neighbor-Joining method. The length of the branches was drawn to scale of evolutionary distance (number of base substitution per site). The evolutionary distance was calculated using the Maximum Composite Likelihood Method. Color and letters A-I illustrate the segregation in clusters. 2 and 3: Detail of the E, F, G, H, I branch. The number on the branches indicate the bootstrap value (1000 replicates. Blue and yellow circles shown the position of TgCatMx6 and TgPigMx53 in the tree.

4.5 Discussion

In vitro isolation of *T. gondii* from pig tongues was attempted in the first instance as it was suggested as an alternative to bioassay to demonstrate the viability of *T. gondii* in tissue (Warnekulasurilla *et al.,* 1998, Zintl *et al.,* 2009). *In vitro* culture has the advantage of overcoming ethical considerations of using laboratory animals. Furthermore, results are obtained more quickly than in bioassay as there is no need to wait for seroconversion of the mice or for oocyst shedding from cats. In contrast, the number of studies in which *T. gondii* has successfully been isolated by *in vitro* culture are scarce and most of them have been performed in animals with high levels of MAT titres (Waap *et al*., 2012), with an active infection (Al-Qassab *et al*., 2009, Miller *et al.,* 2001) or experimentally infected (Zintl *et al.,* 2009, Hitt and Filice, 1992). Results of this study suggested that the use of this technique for *T. gondii* isolation from tissue is a challenge; sterility and cell viability was an issue and the recovery rate was a low as 0%. Perhaps a larger sample size could have improved the recovery rate. On the other hand, Warnekulasurilla *et al.,* (1998) obtained a recovery rate of 1.5% from 67 samples, although its low recovery was attributed to the curing process of the meat. Zintl *et al.,* (2009) used PCR positive placentomes from experimentally infected ewes and reported the low recovery rate of 28%. Warnekulasurilla *et al.,* (1998) and Zintl *et al.,* (2009) also reported high levels of loss of cells and cytopathic effect in monolayers. Results in this study showed the high percentage of 50% of loss of cultures due to bacterial and fungal overgrowth. Zintl *et al.,* (2009) reported lower contamination levels (22%). The organ used for isolation could have influence in the higher levels of contamination experienced in the present study as the surface of the tongue is in contact with high levels of microorganisms such as bacteria and fungus (Abrahamian *et al.,* 2011). This was one of the reasons which drove us to perform *in vitro* isolation from pig blood, since blood can easily be maintained as sterile. Conversely, similar contamination levels (55.5%) were obtained when blood was used as a source for *in vitro* isolation. Therefore, independently of the source of isolation, sterility was an issue. Hitt and Filice, (1992) performed *in vitro* isolation from blood and contamination issues were not reported. However, the animals used in this study were laboratory animals which are meant to be free from pathogens. Therefore, studies are not totally comparable as field animals can harbour other microorganisms. To my knowledge, there are no reports available of *T. gondii in*

vitro isolation from blood from naturally infected animals. Therefore, this study cannot be compared directly with other studies. Klun *et al.,* (2011) isolated *T. gondii* from blood of naturally infected pigs by mouse bioassay. In the Klun *et al.,* (2011) study, a total of five mice died after inoculation with blood from three pigs infected with the pathogen *Erysipelothrix rhusiopathiae*, which is a bacteria commonly present in pigs that may can be asymptomatic. Identification of the contamination was not performed in the present study but the proliferation of other pathogens which may be present in pigs is a possibility.

Due to the difficulty in identification of *T. gondii*, *T. gondii* isolation was confirmed only when PCR of the media was positive. Difficulty in *T. gondii* recognition was also noted by Zintl *et al.,* (2009) and Warnekulasurilla *et al.,* (1998). In the present work, even though a previous separation of the leucocytes by density gradient followed by several washes was performed, RBC and platelets were still present in some of the cultures. The presence of these cells increased the difficulty of examination since these were often covering the monolayer and even more, often the shape of RBCs resembled that of *T. gondii* when deformed. Zintl *et al.,* (2009) also relied on PCR to identify the parasite in cultures from experimentally infected ewes. In the present study, 20% of the cultures were PCR positive, of which 45% and 27% were positive with two (B1 and SAG1) and three (B1, SAG1 and SAG2 or SAG3) genetic markers respectively (TgPigMx56, 57, 66). Therefore, *T. gondii* was isolated from blood by *in vitro* isolation from 20% of the sampled pigs. This suggests high levels of *T. gondii* parasitemia in the farm 1. To the writer's knowledge this is the first time that *T. gondii* has been isolated *in vitro* from blood from naturally infected pigs. The cultures were inoculated into mice at the end of the experiment merely for isolate maintenance since other systems of parasite storage such a - 80 °C freezer or liquid nitrogen were not available in the Hideyo Noguchi Research Centre. Unfortunately, the surviving mice tested serum and PCR negative. The reason for death of these mice remains unknown and due to the mice being inoculated at the end of the research visit, the possibility of death related to *T. gondii* infection could not be ruled out. Inoculated tachyzoites produce illness or death with 1-3 weeks post inoculation. In contrast avirulent strains grow very slowly in mice.

Although the sample size was not comparable, *T. gondii in vitro* isolation, was more efficient from blood than from tissue. One of the reasons for the small sample size in tissue

isolation was feasibility. The protocol to process the tongues is tedious, time consuming and only a limited number of samples can be performed at a time by any one person, whereas the blood can be processed in batches. Furthermore, the sampling of tissue samples was dependent on availability while blood samples were easier to obtain.

Given the low success obtained by *in vitro* isolation from tissue, bioassay in mice was preferred as the isolation methodology for the rest of the isolation study. Higher isolation rates were obtained by mouse bioassay, with which *T. gondii* was isolated from three pigs (8.8%). This is the first time that *T. gondii* has been isolated from pigs in Mexico and the first report of *T. gondii* isolation in Yucatan. Galván-Ramírez *et al.,* (2010a) noted that one mouse seroconverted following bioassay of chops of pork purchased in local stores in Jalisco, however the infection was not confirmed. Interestingly, tongues from the two pigs from which *T. gondii* was isolated by bioassay, were negative by *in vitro* isolation (pig 3, 10 table 4.7, 4.9). Hence, bioassay in mice proved to be more sensitive than *in vitro* isolation. In addition, two of the pigs from which *T. gondii* was isolated were seronegative by MAT test (pig 9, 10 table 4.7, 4.9). The *T. gondii* isolation in these mice was also confirmed during the genotyping experiment by further amplification with two of the SAG2, GRA6, or SAG3 loci (TgPigMx61, 64, 65). Therefore, confirmation of the positivity of these mice was confirmed by 4 genetic markers (SAG1, B1, SAG2 and GRA6 or SAG3). These results are supported by other studies in which *T. gondii* was isolated from seronegative pigs (Dubey *et al*., 1996b, Dubey *et al*., 2002a). In Dubey *et al.,* (1995b) 15.8% of the 170 isolates were obtained from seronegative pigs (MAT titres <20). It has been estimated that overall, 3.8% of isolates are obtained from seronegative pigs (Dubey *et al*., 2002a). These results emphasise that screening based only on serology would give a false underestimation of the infection levels in pigs.

T. gondii bioassay allowed the isolation of *T. gondii* from pigs that did not react immunologically, nevertheless bioassay of *T. gondii* was not successful in isolating *T. gondii* from four of five seropositive pigs. Three of the four mice inoculated with tissues from the pig with high antibody titres (MAT 1:32000) (pig 1 table 4.7, 4.9) died before screening and the surviving mouse resulted seronegative. Two of the mice inoculated with tissues from the pig with MAT titres 1:100 died before screening and the remaining two did not seroconvert (pig 2 table 4.7, 4.9). Infection with mouse virulent strains was not suspected as mice did not die within

the observational period. *T. gondii* isolates are classified as virulent when mice die within a month p.i. (Pena *et al*., 2008) and these mice died within a period of 2 to 10 months p.i. Nevertheless, independently of the cause of death, whether the mice that died before screening were *T. gondii* positive or not remains unknown as the body was never recovered. However, there is a strong agreement between the diagnosis method to confirm that pigs 1 and 2 were *T. gondii* positive as they were positive with three serological techniques (MAT, DT and ID Screen®) and amplified with 13 (TgPigMx53) and 3 (TgPigMx59) genetic markers respectively. The other four mice inoculated with tissues from two ELISA (ID Screen®) positive pigs survived but did not seroconvert (pigs 4, 33 table 4.7, 4.9). Mouse bioassay of pig tissue is considered a highly sensitive method. Dubey *et al.,* (1996b) recovered 93% of isolates by using tongue from experimentally infected pigs as a source of inoculum. However, field studies which used tissues from seropositive pigs obtained percentages of isolation ranging from 9.8% (n=233) to 47.2% (n=36) (Wang *et al*., 2016, Cademartori *et al*., 2014). Dubey *et al*., (1995b) observed that the percentage of isolates obtained from naturally infected sows was correlated with the antibody titre. In this way, the isolation success was >70% when pigs had MAT antibody titre ≥ 200 while the isolation success dropped to 38%-37% for pigs with MAT antibody titers of 20-40. Pig 4 and 33 had low antibody titres in MAT (1:4 and 0 respectively) and ELISA (OD 0.28 and 0.42 respectively). The reason why Dubey *et al*., (1995b) showed an increase in isolation success with the antibody titre in pigs is not clear. The level of antibody titre is related to the timing of infection, being the highest in the acute phase of the infection (Dubey *et al*., 1996b). Perhaps in Dubey *et al.,* (1995b) higher parasite burdens were more frequent in pigs with high anybody titer but further studies on this subject have not been published so far. Further studies could include quantification of the parasite burden in pork with bioassay and serological studies. Furthermore, Dubey *et al.,* (1995b) used a combination of mouse and cat bioassays and the cat bioassay is believed to be more sensitive than bioassay in mice (Dubey, 2009b, Dubey, 2010a, Dubey and Beattie, 1988). In addition, the genotype of the parasite also is a factor that can influence the isolation success. The conversion rate from tachyzoite to encysted bradyzoites vary between strains and strains with lower or inefficient number of cysts may show lower recovery rate as tachyzoites are more sensitive to pepsin digestion (Fux *et al.,* 2007). Despite testing negative with the MAT test, the seropositivity of

pigs 4 and 33 obtained with ID Screen® was confirmed by PCR, not only using SAG1 N-PCR but also with SAG2 and SAG3 (TgPigMx60) or SAG2, SAG3 and GRA6 (TgPigMx62).

In contrast, isolation was not confirmed in one seropositive mouse as the parasite was not demonstrated in the brain either by SAG1 N-PCR or B1 N-PCR (pig 6, table 4.7, 4.9). Three possibilities exist as to why the parasite could not be demonstrated in the mouse brain. Firstly, the mouse could have been exposed to *T. gondii* present in the inoculum but this was not viable or it may have failed to establish the infection. The genotype of the parasite can also be involved in this failure as the capacity to infect mice can vary between *T. gondii* strains (Fux *et al.,* 2007). Another factor that may vary between different *T. gondii* strains is the ability to form cysts. For example, some type I strains are non-cystogenic in mice (Ferreira *et at.,* 2006). Therefore, the demonstration of a type I *T. gondii* strains in mouse tissues may be unsuccessful. Other possible explanations could be that the number of cysts in the brain was very low and the PCR methods used in the present work were not sensitive enough or the amount of brain used for DNA extraction (25-50mg) did not contain enough parasites. The genotype of the parasite can also influence the density of parasites in mouse brain (Bontell *et al.,* 2009, Dubey, 2010a). Finally, another scenario is that the ELISA used in mice was not specific enough and this mouse was a false positive. In addition, the bodies of three mice which tested positive by ELISA could not be recovered and therefore the establishment of the infection was not confirmed in these mice either. These three mice were infected with tissues of two pigs which were seronegative by MAT and IDscreen®. However, it is noteworthy to remark that one of these pigs (15, table 4.7, 4.9) was SAG1 N-PCR positive in tissue and this result was confirmed by SAG2 (TgPigMx52) but the other was PCR negative (pig 13, table 4.7, 4.9). Therefore, overall the three bioassayed seropositive mice from which *T. gondii* was not recovered either because the brain tested negative by PCR (from pig 6) or because mice died before PCR screening (from pig 13) were inoculated with tissues from PCR and seronegative pigs (MAT and IDscreen). Whether those mice were infected or not, it is not totally clear. A few studies have reported the same issue. For example, Galván-Ramírez *et al.,* (2010a) investigated *T. gondii* viability in 70 chops of pork purchased in Mexico and one mouse seroconverted but *T. gondii* could not be found in brain, lungs and heart. In another similar study in Brazil, Dias *et al.,* (2005) bioassayed 149 fresh pork sausages of which 13 (8.7%) produced mice seroconversion but *T. gondii* only was found in mice from one sample (0.7%). Galván-Ramírez *et al.,* (2010a) attributed the failure of the

parasite recovery to factors related to the host such as mouse susceptibility and the parasite dose and strain. For example, interferon gamma gene knock-out (KO) mice are more susceptible to *T. gondii* infection, allowing the parasite to replicate faster and to produce a greater number of cysts. In this case, BALB/c mice were chosen due to availability and lower price. In addition, the number of brain cysts varied between *T. gondii* strains and the dose used (Ferreira *et al.,* 2001), although this factor can be mitigated by using KO mice or immunosuppressants when using other mouse breeds (Dubey, 2010a). The use of corticosteroids or KO mice can be considered for future isolation studies. In addition, the validity of the ELISA used in the screening of the mice is questionable. The IgG ELISA kit (Human Toxo IgG Human-GmbH, Wiesbaden, Germany) was used because the serum could not be imported to England. This ELISA has been shown to be unreliable in pigs (section 2.4.1). The use of DT or MAT would have been used as a first choice in other circumstances. An optimisation of the ELISA test was performed before the screening of this animal species. The chessboard titration showed that this test was more specific in mice than in pigs as the OD of the negative control remained low for all the dilutions (figure 4.5 and 2.8). In addition, the BR was much higher when mouse serum was used instead of pig serum which can be extrapolated to giving a better sensitivity and specificity. Even though these differences in BR values between the two animal species were considerable, BRs still did not have an optimal value according to Crowther, (2000). The interplate variance (CV) of the ELISA performed during the mice screening showed extremely high values for both the P1 (45%) and P2 (38%). Nevertheless, the OD was corrected for each sample according to Lind *et al.,* (1997) and the values obtained produced a clear bimodal distribution (figure 4.6). Despite the optimization performed, further validation is recommended for a more robust diagnostic test as the infection was not confirmed by other diagnostic methods both in the two pigs used for bioassay and in the three mice which resulted as positive by serology. To perform a ROC curve with a panel of known positive and negative samples to calculate an optimum cut off is the best option in the cases when the MAT or DT are not available. In the present study, validation of this ELISA in mice could not be performed due to the lack of a panel of known positive samples. In addition, to evaluate the specificity of a serological test an evaluation of cross-reactivity against other pathogens is necessary and a panel of serum samples from mice infected with other pathogens was not available. A validation of a serological test with serum from non-infected laboratory mice is not recommended as they

would lack on concurrent infections that inoculated mice may harbour.

In contrast to the bioassay of pig tissue, the bioassay performed from blood was less efficient than *in vitro* isolation. Of 17 samples obtained from PCR positive pigs, only two seroconverted mice (11.7%) of which in only one, the infection was confirmed by PCR (5.8%). Hitt and Filice, (1992) obtained 62% of isolation success using blood from experimentally infected rabbits. However, isolation results are not comparable as in this study, animals were experimentally infected with higher doses (10,000 tachyzoites) of the parasite which may be reflected the higher parasite burdens. In addition, susceptibility to the parasite could also influence the parasite burden. All pigs were asymptomatic in the present study; in contrast, in Hitt and Filice (1992) 90% of the rabbits from which *T. gondii* was recovered had clinical symptoms. Interestingly, all rabbits which were negative by mouse bioassay remained asymptomatic (Hitt and Filice, 1992). Klun *et al.,* (2011) used naturally infected pigs and obtained a slightly higher isolation success than in the present study but much lower than in Hitt and Filice (1992). In Klun *et al.,* (2011) seven (30%) of the swine blood samples used for bioassay produced seroconversion in mice. However, mice seropositivity was confirmed by PCR only in 3 (13%) of the 22 bioassays performed. Klun *et al.,* (2011) used a very different strategy: samples were collected from pigs at the abattoir (old pigs) and the isolation was performed only in seropositive animals. Nevertheless, no correlation was found between success of isolation from blood and antibody titres in pigs. This is not surprising as the presence of tachyzoites in blood (acute infection) is not immediately preceded by the immune response. In the present study, the rationale was to sample young pigs (8-12 weeks) as these showed the higher PCR prevalences in blood (figure 3.2). The higher success obtained in Klun *et al.,* (2011) could be related to a closer monitoring of the mice. In Klun *et al.,* (2011), only 5.6% of mice died before screening while in the present work the level of losses reached the high percentage of 36.2%. Likewise, in the bioassay of tissue, the cause of death was not attributed to *T. gondii* virulent strains as mice did not die within a month p.i.

A total of 13 of 34 slaughtered pigs for human consumption tested positive to *T. gondii* DNA in tongue tissue (38.2%, 95% CI: 22%-56.4%). Of these 13 PCR positive animals in tissue, only 4 showed anti-*T. gondi* antibodies. Of the remaining 9 PCR positive pigs, *T. gondii* was successfully isolated from 2 pigs and one pig produced mouse seroconversion. Therefore,

in 54% of the 13 PCR positive pigs in tissue, *T. gondii* infection was confirmed by other methodologies of a different nature (antibody detection, mice bioassay, mice seroconversion). Of the remaining 6, PCR positivity was confirmed with three additional genetic markers in one sample (TgPigMx58) and with two additional genes in three samples (TgPigMx54, TgPigMx60, TgPigMx63). Therefore, a total of 11 samples (85%) which tested SAG1 N-PCR positive were confirmed by other methods. Different possibilities can be attributed to the two SAG1 N-PCR positive pigs in which *T. gondii* infection could not be demonstrated by other methods. In the present work the presence of tissue cysts in pigs did not concur with the immunological response neither by PCR nor by mouse bioassay. Higher sensitivity in PCR than bioassay may be attributed to non-viable parasites in pig tissue (Oliveira *et al*., 2004). The discrepancy between PCR and bioassay has also been suggested to be due to the sparse distribution of the parasite in tissue (Yai *et al*., 2003). Yai *et al.,* (2003) did not isolate *T. gondii* from two experimentally infected pigs which conversely, were PCR positive in tissue. Similar results were obtained by Owen *et al*., (1992) in which positivity was determined by PCR in the tissue of experimentally infected sheep in three animals in which *T. gondii* was not isolated. Moreover, the parasite burden in pig has been estimated to be 0.5-2 cysts in 50 g of tissue (Dubey, 2006, Dubey, 2009b). Approximately one tenth of this homogenate was used for each mouse, hence it is not surprising that very few cysts (or single cyst) were not present in the inoculum. In contrast, slightly higher volumes were used for the DNA extraction since to make 25-50 mg more sediment (not diluted in this case) was needed.

PCR prevalence in tongue was much higher than the 0% obtained in 70 cuts of pork in Jalisco (Galván-Ramírez *et al.,* 2010a). The reason for this discrepancy could be attributed to a lower incidence of the parasite in Northern areas of the country. The origin of the cuts of pork could not be determined in the Galván-Ramírez *et al.,* (2010a) study. Intensive farming in Northern Mexico has shown lower prevalences in market age pigs raised in intensive farming (0%) than in the present study, while in pigs raised in backyard systems in Northern Mexico had comparable prevalence (12.7%) to that obtained in Yucatan. Other reasons for the absence of *T. gondii* DNA in pork in the Galván-Ramírez *et al.,* (2010a) study could be related to a lower sensitivity of the DNA extraction method. In the present study, DNA extraction was performed from a sediment obtained after digesting 50g of tongue while in Galván-Ramírez *et al.,* (2010a) only 1g of pork was digested. In the present study, detection of DNA was much higher when

50g were used as the starting material (32.3%) than when 25-50 mg was used (17.6%). Although no statistical difference supported this difference (p=0.208), studies which used larger quantities of starting material for DNA extraction (15g-100g) reported high sensitivities in PCR (Wang *et al*., 2012, Opsteegh *et al*., 2010, Yai *et al.,* 2003). A combination of both, digested and non-digested methods, produced higher levels of detection of *T. gondii* DNA (38.2%) than by using only digested (32.3%) or non-digested (17.6%) samples. Oliveira *et al*., (2004) also noted high PCR prevalence by using both methods (47.1%) than using only digested (24.2%) on non-digested (36.4%). Conversely, although results were not supported statistically, in Oliveira *et al*., (2004) higher prevalence was obtained when using non-digested (low starting material) than digested samples (higher starting material) which could be attributed to the uneven distribution of the parasite in pig tissue.

PCR protocols showed different amplification rates in the present study. Of 74 samples sequenced with the SAG1 locus 11%, 27% and 26% were confirmed with 3 (SAG2, SAG3 and GRA6), 2 (SAG2 and SAG3 or SAG2 and GRA6 or SAG3 and GRA6) or one (SAG2 or SAG3 or GRA6) additional genetic markers, respectively. Thus, 65% of SAG1 positive samples were confirmed at least with one other locus. 35% of the samples which tested positive with SAG1 did not amplify with SAG2, SAG3 or GRA6. However, taking into account the B1 screening performed in farm 2 (section 3.4.2), the number of these samples which only amplified with one marker dropped to 22%. It is interesting that 1 of the samples which was not confirmed with B1 in farm 2 was positive with SAG2 (table 3.7 and 4.7, TgPigMx10). SAG2 showed the higher percentage of amplification (53%), followed by SAG3 (50%) and GRA6 (38%). SAG1 has been shown to be a reliable genetic marker as it was sensitive (figure 3.1) and specific (table 4. 14). All the sequences had 100% identity with *T. gondii,* in addition, cross contamination was not suspected as DNA sequences differed from each other and strict precautions were adopted during pre and post amplification procedures. Mason *et al.,* (2010) performed a rigorous comparison of the performance of B1, SAG1, 5' SAG2, 3'SAG2 and SAG3 in the presence of a low copy number of tachyzoites (10-0.01 parasites). After six replicates, Mason *et al.,* (2010) noted that when the number of parasites was low, the results were inconsistent (between and within markers) as amplification was only present from 0 to one or few replicates, this effect was named as the 'pond dipping effect' (Mason *et al.,* 2010). The disagreement between the amplification of the different genetic markers obtained in the present work was suggested to be

result of a combination of low copy number of the parasite in blood and tissue with differences in sensitivity of SAG1, SAG2, GRA6 and SAG3 genetic markers. Sensitivity was only evaluated in SAG2 which was slightly lower than that for SAG1 (figure 3.1).

Higher seroprevalence of anti *T. gondii* antibodies was found in slaughtered pigs (14.7%, 95% CI: 5%-31%) in comparison with the main stratified epidemiological study (1.4%, 95%CI: 0.6%-2.7%). This is the first report in Yucatan in which the seroprevalence of pigs at market age has been investigated using a validated serological test. The prevalence obtained in this age group was relatively high although much lower than the overestimated prevalence of 96.8% obtained in pigs sampled in the same slaughterhouse (Hernández-Cortazar *et al*., 2016a). Although the results obtained in the present study have shown a more optimistic picture, pork still could be a potential source of *T. gondii* infection to the population in this locality. Furthermore, mouse bioassay has shown that pigs are not only exposed, but also the parasite remained infective in at least 8.8% of the pigs bioassayed. The tissue cyst is extremely robust, it can survive for weeks at temperatures ranging from 1 to 48ºC (Dubey *et al*., 1990). Epidemiological studies in abattoirs are also scarce in the rest of Mexico. There is another study in which a seroprevalence of 0% was noted among slaughtered pigs raised in intensive farms in Oaxaca (Alvarado-Esquivel *et al.,* 2015). However, in the Alvarado-Esquivel *et al.,* (2015) study the sample size was only 17 pigs. Larger sample sizes were used in the Alvarado-Esquivel *et al*., (2011a) study, in which a prevalence of 12.7% was found among 1074 slaughtered pigs in Durango and Sonora, however, the animals in this study were pigs raised in backyard systems. The lack of knowledge of the incidence of this pathogen in the pig industry of this country is concerning. Future studies should be implemented in abattoirs for a better knowledge of the risk derived from pork consumption. Overall, results of this work have shown that bioassay is expensive, tedious, time-consuming and requires rigorous monitoring. Thus, although it is an extremely valuable method to demonstrate viability in meat, bioassay is not recommended for large scale studies. A combination of PCR and serology is proposed for intensive studies as results can be obtained as fast as in one day and samples can be tested in batches of large numbers. A combination of serology and PCR in abattoir samples has been shown to provide a more robust procedure to evaluate *T. gondii* incidence in pork destined for human consumption than one method itself.

Results from the sympatric study suggested that rodents could be a source of infection of *T. gondii* in pigs from farm 1. The SAG1 N-PCR prevalence was as high as 42.8% (95%CI: 17.6%-71%) in tissues from the rats captured in the farm. Therefore, rodent controls should be implemented. Several studies have demonstrated that rodents can play an important role as a reservoir of *T. gondii* in pig farms. These can be a direct and indirect source for pigs, by being either a prey for pigs or for cats which cohabit within the farm (Lubroth *et al* 1983, García *et al*., 1999, Weigel *et al*., 1995b). Either way, *T. gondii* prevalence has been seen to decrease dramatically in farms when rodent control was applied (Kijlstra *et al*., 2008). The genotyping study was not intensive enough to reveal the direct source of infection of pigs by tracking genotypes, due to the failure to obtain isolates. Genotyping performed directly on the samples was challenging and only some loci could be amplified.

Due to financial reasons, only some sites were chosen for the genotyping study. Farm 1 and farm 2 were chosen in the first instance due to their proximity, access and coexistence with other animal species that could lead to future sympatric studies in the area. Two farms from the second environment were chosen randomly to compare *T. gondii* genotypes among the two different environments. Swine tissue samples were also genotyped as they were expected to have higher parasite burdens than blood samples. Although there are no studies in pigs which compared parasite burden in both blood and tissue, in murine models, higher concentrations of parasites have been reported in tissue than in blood (Djurković-Djaković *et al.,* 2012). Genotyping of rat samples was performed as an attempt to understand the transmission dynamics of *T. gondii* in pig farms.

A total of 11 animals showed double peaks in the chromatogram at one or two loci. The presence of more than one allele for a given locus is characteristic of a mixed infection with two different *T. gondii* strains (Ajzenberg *et al.,* 2002). Infections with multiple strains have been reported in sheep (Ajzenberg *et al.,* 2002), humans (Aspinall *et al*., 2003) in pork, lamb, beef (Aspinall *et al*., 2002), chickens (Dubey *et al*., 2002b, Lindström *et al*., 2008, Dubey *et al*., 2006), mice (Bajnok *et al*., 2015), cats (Dubey *et al.,* 2009, Dubey *et al.,* 2007) and marsupials (Pan *et al*., 2012). The coinfection with mixed strains of *T. gondii* has been considered a rare event for many years. Nevertheless, several studies have suggested that this could result from an underestimation rather than an unusual event due to differences in resolution techniques

(Villena *et al.,* 2004, Aspinall *et al*., 2002, Sreekumar *et al*., 2005 Pan *et al.,* 2012). Sreekumar *et al*., (2005) noted that sequencing techniques were more clear and resolve identities better than RFLP. Conversely, currently RFLP is still the most commonly used technique for genotyping. Villena *et al.,* (2004) suggested that the isolation of the parasite prior to genotyping could lead to the loss of strains in the case of multiple infections. Lindström *et al.,* (2008) noted multiple infection in chicken tissues from Uganda by direct genotyping, however, when tissues were bioassayed in mice it produced the recovery of one single strain (Bontell *et al.,* 2009). Aspinall *et al*., (2003) found by direct genotyping on clinical human samples a similar frequency of infection with multiple type I + II strains (31%) than for infection with either only type I (31%) or type II (34%) strains. Pan *et al.,* (2012) found infections with multiple strains in 69% (11/16) of Australian marsupials also by direct sequencing in clinical samples. In contrast, isolation of multiple strains has been reported on rare occasions (Ajzenberg *et al.,* 2002, Dubey *et al*., 2009). Results of this study showed that infections with multiple *T. gondii* strains are frequently found in pigs from Yucatan. Dubey *et al*., (2009) reported a mixed infection in a cat from Durango, Mexico. Infection with multiple strains have been reported mostly in tropical areas which present higher diversity of *T. gondii* genotypes (Dubey *et al*., 2002b, Lindström *et al*., 2008, Dubey *et al*., 2006, Dubey *et al.,* 2009, Dubey *et al.,* 2007). Mixed infections were mostly found in swine blood (20%) rather than in tissue (6.6%). This presence of multiple genotypes during acute infection could mean that most of the coinfection occurred in a short period of time or that during the acute infection an activation of the latent stage was aroused. The first scenario is connected to high levels of the parasite in the surrounding environment, which is a possibility as all the farms had free access to cats, birds and rodents (Appendix III).

MLST of the SAG1, SAG2, SAG3 and GRA6 loci showed that *T. gondii* genotypes in Yucatan were characterized by a high frequency of type I (43%) and atypical alleles (46%) while type II (3%) and III (8%) were less predominant. For the samples in which sequencing data were generated for more than two loci, genotypes were represented by 1 to 2 samples each. Therefore, *T. gondii* strains circulating in Yucatan showed high levels of diversity. Genotyping studies in Mexico also noted high diversity as 85% of the genotypes had one or two isolates each. In contrast, lower frequency of atypical alleles and higher frequency of type II and III alleles than in the present study was noted elsewhere (Dubey *et al*., 2004b, Dubey *et al.,* 2009, Alvarado-Esquivel *et al*., 2011b, Dubey *et al*., 2013, Rico-Torres *et al*., 2015). In addition,

although clonal types were not generally the rule, Dubey *et al.,* (2009) found 100% of clonal type III in isolates obtained from 4 free range chickens. Nevertheless, diversity in the other Mexican studies could be underestimated as RFLP was used which is less good at resolving identities than the MSLT used in the present work.

SAG1 and SAG2 loci are genes which encode for surface antigens (Manger *et al.,* 1998a). These sequences play an important role during cell invasion and are highly immunogenic. Kim and Boothroyd, (2005) suggested they may have a function in the persistence of infection by reducing the host immune response. SAG3 is also another gene of the SAG family which encodes for surface proteins; however, this one is also involved in the pathogenicity of the parasite (Dzierszinski *et al*., 2000). In addition, GRA loci are genes that encode for dense granule proteins which are excretory/secretory antigens. 20 GRA genes have been identified and named as GRA1-20. The group of GRA proteins are shed during and/or after invasion of the parasited cell. The GRA6 protein plays an important role in the antigenicity and pathogenicity of *T. gondii*. GRA6 is also involved in stabilising the tubular network with the aid of the GRA2 protein after invasion (Nam, 2009). Due to the importance of SAG and GRA genes in parasite survival, these are considered conserved sequences in which mutation may happen under selective pressure (Manger *et al.,* 1998b). Here, overall, a total of 71 novel SNPs were noted among SAG1, SAG2, GRA6 and SAG3. The frequency of novel SNPs suggested that these genotypes were divergent from the classic type I, II and III lineages. However, although PCR techniques are highly sensitive diagnostic methods, there is a risk of error during elongation which could lead to mutations. The estimated error for the type of Taq polymerase used was ~10^{-5} (McInerney *et al*., 2014). This error rate may not seem very high but novel SNPs should be interpreted with caution as a high number of PCR cycles were used (65). SNPs which were parsimony informative are considered more robust than unique SNPs as these are shared by two or more sequences. In order to provide more robust genotyping data, sequences with non-parsimonious SNPs should be amplified independently and sequenced in triplicate. The confirmation of these unique SNPs was not performed due to time and financial constraints; thus, their validity remains questionable.

Of the 71 novel SNPs, seven were shared by two samples each (SAG1, GRA6 and SAG2 loci), one was shared by three samples (SAG2 locus), one was shared by 11 samples

(SAG2) and the remaining SNPs belonged to one sample each. The novel SNP located at position 466 of the SAG2 locus (figure 4.13) was shared by 11 animals. Special attention should be given to this novel mutation which was shared by such a high number of animals. In addition, this mutation was non-synonymous leading to a change from lysine to glutamic acid (Appendix IV). This mutation could be indicative of positive selection (Bontell *et al.,* 2009). In addition, the fact that this mutation is shared by 11 animals reinforces this hypothesis. Thus, the SAG2 allele named as u-4 in the present study could be a successful genotype which maybe frequent in Yucatan. The clonal expansion of new atypical genotypes has been reported in Brazil (BrI-IV) and USA (type 12).

The phylogenetic tree based on the Neighbor-Joining analysis built with the concatenated loci SAG1, SAG2, SAG3 and GRA6 had low bootstrap values as some of the loci were not available for some of the strains (tree A). Nevertheless, strains were segregated into five main groups (A, B, C, D, E). Bootstrap values improved when only samples with four loci successfully sequenced were used (Tree B). Strains were different from the type I, II and III reference strains. *T. gondii* strains from Yucatan were seen to be more closely related (by sequencing of these 4 loci) to the type I lineage than to type II and III except for two strains from the second environment (TgPigMx46, TgPigMx50) and the cat isolate (TgCatMx6) which were clustered in group C. MLST applied to three or four loci was enough to distinguish between samples, apart from TgPigMx21 and TgPigMx54 which were indistinguishable. However, use of the 3 or 4 loci was not powerful enough to find similarities between the genotypes from Yucatan and prototypic strains with an American origin. It was challenging to obtain genotyping data by using clinical samples since several PCRs were negative for some loci. This issue could be improved by using isolates where the DNA concentration of the parasite was higher. The challenges of direct genotyping have been reported by others (Cañón-Franco *et al*., 2013, Bajnok *et al*., 2015, Prestrud *et al*., 2008 Calero-Bernal *et al*., 2015).

Genotyping data obtained with the set of 12 loci (SAG1, Alt-SAG2, 3'SAG2, 5'SAG2, SAG3, GRA6, BTUB, PK1, C22-8, C29-2, L358, Apico) provided greater resolution and allowed more accurate classification of the genotypes (TgPigMx53 and TgCatMx6) than by using only 4 or less loci. TgCatMx6 had an atypical genotype with a mix of alleles (Type I, II, III and u). The use of the 12 loci revealed high a level of type II background. The percentage of

type I, II, III and novel SNPs by using four loci was 39%, 32%, 28% and 0% respectively while when using 12 loci it was 21%, 50%, 28% and 1% respectively. With the strain TgPigMx53, the percentage of the type I, II, III and novel SNPs was 25%, 0%, 75% and 0% while when using the 12 loci the percentage of SNP origins changed slightly to 36%, 0, 64% and 0% respectively.

The isolate TgCatMx6 had an identical RFLP pattern to the isolate TgGoatUS20 obtained in USA from a goat (Dubey *et al*., 2011b). Additionally, sequencing data of the TgGoatUS20 for GRA6, UPRT1, UPRT7, HP2 and EF1 showed that this was closely related to the isolate obtained in this study TgCatMx6. This relationship was supported in the Neighbor-Joining and UPGMA trees with a robust bootstrap value (79 and 96 respectively) (figure 4.12). In contrast, TgPigMx53 had a unique genotype that has not been reported before. The other studies in Mexico, also isolated strains with identical RFLP patterns to those obtained in the USA. Of the 7 genotypes published in Mexico two were reported in the USA, one was reported in USA, Colombia and Brazil, one was reported in USA, Colombia, Brazil, China, Sri Lanka and Vietnam, one was distributed world-wide and two were unique (Dubey *et al*., 2004b, Dubey *et al.,* 2009, Alvarado-Esquivel *et al*., 2011b, Dubey *et al*., 2013, Rico-Torres *et al*., 2015).

The phylogram constructed using 16 loci (SAG1, Alt-SAG2, 3'SAG2, 5'SAG2, SAG3, GRA6, BTUB, PK1, C22-8, C29-2, L358, Apico, UPRT1, UPRT7, EF1 and HP2) showed a robust clustering of TgPigMx53 and TgCatMx6 with related genotypes. TgCatMx6 was clustered with type II and 12 which represent the highly extended genotypes in USA. In addition, the cluster was closely related to the atypical isolates COUG and GUY-2004-JAG which have been isolated in Canada and French Guiana. In contrast, TgPigMx53 was clustered with genotypes III and related type III strains represented by North American strains. This cluster was also connecting with type I and III genotypes which had North and South America origin. Hence, TgPigMx53 and TgCatMx6 were closely related to *T. gondii* genotypes which are representative of North America (haplogroups 12, 1, 2, 3) and to genotypes which are found in both North and South America (haplogroups 11, 7). In contrast, TgPigMx53 and TgCatMx6 were high divergent from those genotypes which are representative of only South America (haplogroups, 4, 5, 6, 8, 9, 10 and 15).

In conclusion, this study has demonstrated the practical difficulties of isolation and identifying strains and genotypes of *T. gondii*. It has also shown high frequency of *T. gondii* in pork meat intended for human consumption (38.2%, 95% CI: 22%-56.4%). However, the percentage of parasite recovery was substantially lower and therefore the viability (8.8%, 95% CI: 1.8%-23%). The viable *T. gondii* present in pork meat seems low, but existent. Therefore, it is important that consumers should be aware that there is a risk of *T. gondii* transmission from ingestion of undercooked pork. Whether this percentage of viability is biased due to the failure of the isolation technique needs further research. Additional work is needed for a better assessment of the risk derived from pork consumption since there is a possibility of bias due to the origin of the positive samples belonging to the same farm. Thus future studies are recommended which involve assessment of pork from pigs raised in a greater number of farms. Finally, it has shown that the strains isolated in pigs from Yucatan are related to other *Toxoplasma* strains in both South and North America. It is not therefore a generically isolated region. Further work is required to get a wider understanding of the relationship of these strains to the global profile.

CHAPTER 5

5.1 General discussion

Yucatan represents one of the Mexican states with higher seroprevalence of *T. gondii* antibodies among the human population. Recent surveys reported seroprevalences of 55-59% among women undergoing recent abortion (Hernández-Cortazar *et al.,* 2016b, Vado-Solís *et al.,* 2013) and 91% among humans who resided in rural communities (Ortega-Pacheco *et al.,* 2015). 23% of the death caused by foodborne pathogens has been attributed to this parasite (Guo *et al.,* 2015). Pork is the most highly consumed meat as an integral part of the culinary culture of Yucatan (Arroyo *et al.,* 1999, Ponce, 2004). A large number of studies have reported a high seroprevalence of *T. gondii* among pigs raised in intensive farms. These elevated seroprevalences were opposite to the generalised low *T. gondii* disease levels found in pigs raised in similar intensive farming system elsewhere (figure 1.6B). Therefore, I considered it important to investigate what factors were 'breaking the pattern' in this geographical area.

In order to study the risk factors affecting the seroprevalence of *T. gondii* in pigs, a cross-sectional age stratified study was carried out across intensive farms in Yucatan. Sample size was calculated statistically by using the software Epi-info 7.1.3 (CDC, Atlanta, USA, 2007) and using as a reference the prevalence data obtained in the pilot study. Additional intensive farms were sampled in a second environment known as tropical sub-deciduous medium forest to compare the prevalence in those pigs with the pigs raised in the first environment known as a tropical deciduous low forest. Pig sample size in the second environment was calculated using Lot Quality Assurance Sampling (LQAS). Overall, a total of 632 pigs were sampled. Swine serum samples were screened to detect specific IgG antibodies against *T. gondii* using a commercially available ELISA kit for detection of human anti-*T. gondii* IgG (Human Toxo IgG Human-GmbH, Wiesbaden, Germany). This was combined with the serological test considered as a gold standard in pigs which is the widely used MAT test (Toxo-Screen DA, bioMerieux) (Dubey, 1997c, Dubey *et al.,* 1995c, World Organization of Animal Health, 2008). Results were confirmed with another gold standard test, the DT (Reiter-Owona *et al.,* 1999, World Organization of Animal Health, 2008) by sending the samples to a *Toxoplasma* reference

laboratory in Swansea and with a commercially available ELISA test validated in swine serum the ID Screen ® (IDVet, Montpellier, France). Results of these tests were compared statistically between each other by using the Receiving Operating Characteristic (ROC), calculating sensitivity, specificity and the Cohen's kappa coefficient (Chapter 2). DNA was extracted from the leucocyte fraction of the swine blood in order to increase the concentration of *T. gondii* DNA from the blood samples (Unno *et al.,* 2008). The sensitivity of three widely used N-PCR genetic markers, B1, SAG1 and SAG2, was measured by titration of RH DNA in the presence of a high density of host cells (Chapter 3). Swine blood was screened for detection of *T. gondii* DNA by a highly sensitive SAG1 N-PCR protocol as a measure of prevalence of animals with *T. gondii* active infection. Specificity and sensitivity of SAG1 N-PCR was confirmed by B1 N-PCR, SAG2 N-PCR and by DNA sequencing (Chapter 3 and Chapter 4). Individual farm and pig characteristics were collected by interviewing farmers, vets and by direct observation. Those characteristics were used in standard univariate methods such as the binomial logistic regression, $\chi 2$ test and Fisher's Exact test using SPSS statistic software to analyze the risk factors associated with *T. gondii* transmission in pigs (Chapter 2 and Chapter 3). A previous study examined the viability of tachyzoites circulating during the active infection in pigs by mouse bioassay (Klun *et al.,* 2011). Building on this pioneering study, viability of tachyzoites present in swine blood was explored by *in-vitro* isolation and by mouse bioassay (Chapter 4). The frequency of *T. gondii* in pigs destined for human consumption and its viability was investigated indirectly by using the MAT and directly by using the SAG1 N-PCR protocol proposed in the present work on tongue extracts and isolation protocols. Tongue muscle was the main target tissue of parasite distribution in pigs (Dubey *et al.,* 1996a, Dubey *et al.,* 1986a). Sensitivity of SAG1 N-PCR was compared between DNA extracted directly from muscle tissues and DNA extracted from digests of 50 g of tissue. The presence of viable parasites was examined by a gold standard which is the mouse bioassay (Dubey *et al.,* 2010a, Gangneux and Dardé, 2012) in meat samples intended for human consumption by sampling pigs at the abattoir (Chapter 4). An optimization of the ELISA test used during the mouse screening was performed by chess-board titration and calculating binding ratios (Crowther, 2000) followed by a correction of the OD for each sample (Lind *et al.,* 1997). Mouse seroconversion was confirmed by detection of *T. gondii* DNA in brain tissue as a target organ in this animal species (Dubey *et al.,* 2010a). *T. gondii* infection was studied in rodents and cats which cohabited with pigs in

order to find evidence of a transmission source (Chapter 4). I used my results along with the previous publications to consider whether monitoring and farm management should change in swine pig farms in this locality. Finally, the high resolution genotyping technique MLST was used for first time to produce genetic data of the *T. gondii* strains circulating in the area (Chapter 4).

This is the first epidemiological study performed in pigs from Yucatan with a validated serological test. The MAT test showed that anti *T. gondii* antibodies were virtually absent in the multistage stratified study reaching the low prevalence of 0.5% (95%CI: 0%-1.8%) among 8 to 20 week old pigs. MAT results were confirmed with the DT and ELISA kit ID Screen®. These prevalence data are closer to the published data in pigs raised in intensive farming systems in Mexico and other countries (figure 1.6B). The use of the IgG ELISA kit (Human Toxo IgG Human-GmbH, Wiesbaden, Germany) proved not to be suitable for the monitoring of anti *T. gondii* antibodies in pigs. The MAT test showed that in abattoir samples the seroprevalence reached 10.7% (95% CI: 2.2%-28.3%) when pigs were older than 20 weeks. In addition, an evaluation and improvement of the sensitivity and specificity of the commercial multispecies ELISA kit ID Screen® was performed. The combination of ELISA (IDScreen®) and MAT test lead to a slight increase in the seroprevalence to 1.5% (95%, CI: 0%-3.3%) in the multistage stratified study and to 17.8% in slaughtered pigs older than 20 weeks. A statistically significant age pattern (p=0.003) was found for seroconversion (Chapter 2). The age pattern is exacerbated when looking at farm 1 which had 0% (95%, CI: 0%-0.5%) prevalence in the multistage stratified study by using both serological tests, but in the oldest slaughtered pigs the presence of antibodies reached 20.8% (95% CI: 7%-42%) using both serological tests. This increase in anti *T. gondii* specific antibodies with age has been noted before in this animal species (García-Bocanegra *et al.,* 2010a, Villari *et al.,* 2009) and other animal species such as sheep (Katzer *et al.,* 2011, Van der Puije *et al.,* 2000), goats (Van der Puije *et al.,* 2000), cats (Hammond-Aryee *et al.,* 2015), horses (Boughattas *et al.,* 2011), otters (Conrad *et al.,* 2005), bears (Jensen *et al.,* 2010) seals (Cabezón *et al.,* 2011), rabbits, mice (Afonso *et al.,* 2007) and humans (Dubey *et al.,* 2012b). The increase in seroprevalence with age, has been related to an increased accumulation of the chance of being in contact with the parasite.

SAG1 and B1 were the most sensitive N-PCR protocols (figure 3.1 and 3.2). They had a detection limit of 5.7 and 0.7 tachyzoites with the presence of a high number of host cells. Therefore, these protocols were used as a diagnostic tool and I would propose their use as a robust direct detection method for *T. gondii* DNA in pig tissue and blood. PCR results in blood showed high levels of *T. gondii* DNA of 21.2% (CI: 18%-24.6%) which were not predicted by the animals serological status. In addition, PCR in pig tissue showed a high frequency of 38.2% of *T. gondii* DNA which again, was not predicted by the serological status. Although a higher number of seropositive animals tested PCR positive in tissue (80%) than seronegative animals (31%), this distribution was not statistically significant (Fisher test, p= 0.056). The presence of *T. gondii* in seronegative pigs, was confirmed by isolation of *T. gondii* from blood in 20% of samples (n=54) by using *in vitro* culture, in 6 % (n=17) by using mouse bioassay and by using mouse bioassay from their tissues in 6.9% (95% CI: 0.8%-22%) of the seronegative pigs (n=29). The presence of *T. gondii* DNA in pig tissue from seronegative animals (9) was confirmed in 78% of the animals by other methods (56% by genotyping and in 22% by bioassay) (figure 5.1). Similar levels of isolation and PCR detection in tissues of seronegative pigs have been reported in other studies (Slany *et al.,* 2016, Opsteegh *et al.,* 2016). Hence, seronegative pigs could be infected with *T. gondii* and therefore consumption of its meat and offal could lead to a *T. gondii* infection. A combination of direct and indirect methods should be used for a correct diagnosis of *T. gondii* in pigs. As illustrated in figure 5.1, the use of only one of the serology, PCR or bioassay methods by itself could underestimate the presence of *T. gondii* in pigs. In addition, the use of a combination of multiple diagnostic methods allows the production of the most robust data as positives can be confirmed with other additional method and therefore the possibility of false positives is reduced.

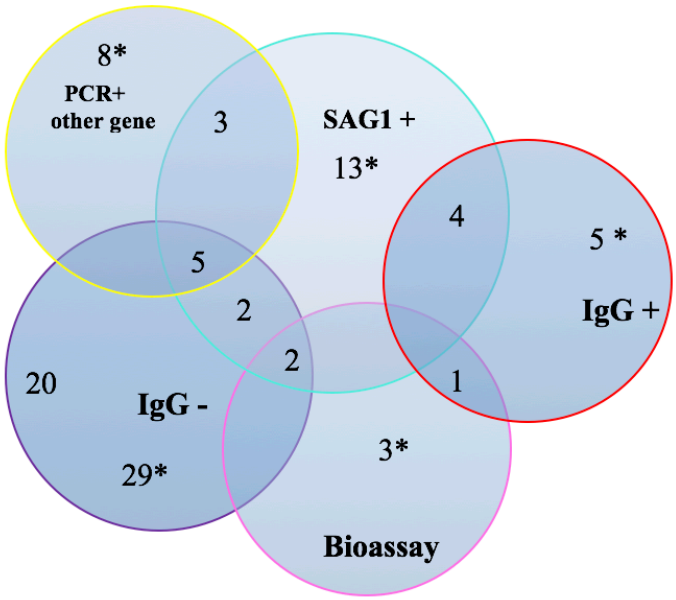

Figure 5.1 Relationship between techniques among the 34 pigs sampled at the abattoir. * represent the total number in each category, IgG+ represents the seropositive pigs, IgG – represents the seronegative pigs, SAG1 + represents the PCR positives in tissue, PCR+ other genes represents PCR positives in tissue using SAG2, SAG3 or GRA6 genetic markers. Shared numbers with other circles shows the positive and negative animals which were confirmed by the other diagnostic methods. The aim of this figure is to illustrate the advantage of using a combination of diagnostic methods rather than one.

Recent studies have found also discordant results between PCR and serological methods. Slany *et al.,* (2016), compared PCR in tissues by RT-PCR and ELISA (ID Screen®) in pigs raised in different farming systems and in wild boars in the Czech Republic. Slany *et al.,* (2016), noted that pigs raised in organic farms had a seroprevalence of 18.8% while PCR in tissues was 29.2%. Similar results were observed when pigs were raised in intensive farming; seroprevalence was as low as 3.3% but the *T. gondii* DNA was present in 13% of the pigs (Slany *et al.,* 2016). In contrast, Slany *et al.,* (2016) observed the opposite scenario in backyard pigs and wild boars; pigs raised in backyards showed a 27.6% of seroprevalence while PCR in pig tissues was 20.7% and wild boars had a seroprevalence of 15.4% while PCR in tissue was 8%. In the last EFSA external report, this disagreement was also observed, showing that 37% of seronegative animals with a cut off of 1:6 in the MAT (which is much lower than the cut off recommended of 1:25) tested positive by direct methods (PCR and bioassay). The specific percentage related to PCR was not specified in the report, but the percentage of bioassay success in pigs with a dilution in serum <1:6 was 6% and in animals with antibody titres < 1:25, 10%

(Opsteegh *et al.,* 2016). The agreement between any direct methods and MAT results was poor (k=0.18, 95%CI: 0-39) (Opsteegh *et al.,* 2016). Furthermore, the parasite was isolated from a seronegative animal which tested also PCR negative in tissue (Opsteegh *et al.,* 2016). This disagreement between direct and indirect methods, has not only been observed in pigs, it is observed in a wide range of animal species. Ruffolo *et al.,* (2016) reported a 4.9% of seroprevalence by IFAT in urban rats captured in Brazil. Bioassay was positive (IFAT) for 2 of the seropositive rats (1%) and from 7 seronegative rats (4%) (Ruffolo *et al.,* 2016). Aroussi, *et al.,* (2015) noted low agreement between PCR in horse meat and MAT results (k= 0.1-0.17, OD cut off 1:0-1:20) and between PCR on horse meat and ID Screen® results (k= 0.02-0.12, OD cut off 0.06-0.1) among horses from different countries (Argentina, Mexico, Germany, Canada, France and Uruguay). Epidemiological studies in sheep from UK have reported a seroprevalence rate of 30% among ewes (Van der Puig *et al.,* 2000), while results using direct methods found a prevalence among new-born lambs of 61% by PCR (Duncanson *et al.,* 2001). In a more recent study, using PCR diagnosis, persistent prevalence ranging from 41% to 69% was reported in three different flocks during 3 years of follow up (Williams *et al.,* 2005). These differences in outcome of PCR and serological assays are known to exist. In some species such as mice, rabbits and rats this discrepancy between diagnosis tests was suggested, in part, to be a result of immune tolerance after congenital transmission of *T. gondii* (Dubey *et al.,* 1997b, Rejmanek *et al.,* 2010, Araujo and Remington, 1974, Suzuki and Kobayashi, 1990, Owen and Three, 1998, Araujo and Remington, 1975). However, it is still unknown why this is happening in another animal species. In pigs, it is believed that congenital transmission is not epidemiologically important however this assumption is based on that congenital clinical toxoplasmosis being rarely found in pigs and that in experimental infected sows, it was difficult to transmit the parasite to the offspring (Dubey and Urban, 1990, Jungersen *et al.,* 2001). In Dubey and Urban, (1990), 17 sows were experimentally infected during pregnancy of which only 6 sows delivered congenitally infected piglets. However, congenital transmission was confirmed in piglets by using only mouse bioassay and histology. Piglets were sacrificed before term or three months after birth, therefore, no follow up was performed for further testing. This 'difficulty' of vertical transmission could be biased by the methods used, as histology is a method with limited sensitivity and bioassay measures the viability of the parasite. Venturini *et al.,* (1999) noted the low seroprevalence of 2% to anti-*Toxoplasma* IgG antibodies among 738

stillborn piglets in Argentina. Venturini *et al.,* (1999) concluded that the frequency of congenital infection in pigs was low due to the scarce levels of anti-*T. gondii* antibodies among aborted and stillborn piglets, however congenital infection was not studied among the liveborn piglets. Studies on congenital toxoplasmosis in pigs are very limited and these are based only on clinical cases of which most of them have been reported in outbreaks. Further work is needed in order to estimate how important this transmission is in pigs. The use of the combined methodology of PCR and serology is proposed. Another possible explanation is that the host's immunological response is being tolerant to selected *T. gondii* strains. For example, Hosseininejad *et al.,* (2014) noted that cats experimentally challenged with the *T. gondii* ME49 strain developed IgG antibodies later (19 d.p.i) than cats challenged with the VEG or RH strains (7 d.p.i). The immunological response to ME49 not only was delayed but also weaker in comparison to cats infected with VEG and RH strains. The titre for cats infected with the ME49 strain was 1:128 by IFAT in 3 cats and 1:256 in one cat. In contrast, the 8 cats infected with VEG or RH strain had titres ≥1:512 (Hosseininejad *et al.,* 2014). Initiation of an immune response depends on the host and on the ability of *Toxoplasma* to modulate immune mediators such as interleukins. Verhelst *et al.,* (2015), compared interleukins levels (IL-4 and IL-10) and antibody responses against different antigens in sheep and pigs after being experimentally infected with the strains PRU and IPB-G respectively. Verhelst *et al.,* (2015) noted that in sheep IL-4 and IL-10 levels increased two weeks post infection (w.p.i.) and remained elevated until the end of the experiment at 7 w.p.i. In pigs, however, the levels of these interleukins did not increase during the study. In addition, IgG antibodies were measured against TLA, rGRA1, rGRA7, rEC2 and rMIC3 antigens, sheep showed similar immune response against the 5 antigens which was characterized by a high increase of the IgG antibodies (against TLA, rGRA1, rGRA7, rEC2 and rMIC3 antigens) at 4 w.p.i. followed by a decrease at 6 w.p.i. whereafter IgG levels increased and remained elevated until the end of the experiment at 8 w.p.i. Interestingly in pigs, IgG antibodies were observed only against the antigen rGRA7 which fluctuated in a similar pattern to that in sheep (Verhelst *et al.,* 2015). Different immune responses between sheep and pigs could not be attributed to the different animal species as different *T. gondii* strains were also used in this study. Miranda *et al.,* (2015) found that pigs experimentally infected with the ME49 strain experienced an increase in neutrophils which did not occurr when pigs were infected with the RH strain. Hence, it seems that immune response

may depend on the animal species, *T. gondii* strains or both. Different strains of *T. gondii* can initiate different patterns of humoral responses. Whether a weaker immune response is associated with less virulent strains for a given animal species as an innate response or whether this tolerance is result of a coevolution of host with the local parasitic strains of a given area also remains to be assessed.

The fact that *T. gondii* has been isolated and detected using PCR in seronegative animals, raises the question, of whether the epidemiological data based solely on serological methods are perhaps underestimating the frequency of the parasite in animals and humans. The majority of epidemiological data have been based solely on serological methods for years since these have been considered as gold standards. Perhaps it is time for an update of this approach and to use a combination of direct and indirect methods in future epidemiological studies.

Not many studies in animals have made use of PCR on blood for diagnosis of *T. gondii*. The present study is pioneering in using PCR screening of pig blood in animals of different ages. Diagnostic procedures are usually based on using tissues or/and serological tests. The reason for this is probably because the chance of detecting the parasite during the chronic phase of the infection (IgG and tissues cysts) is higher than during the acute phase of the infection as it persists for longer. Here, results have shown that PCR in blood can be a very useful diagnostic tool of the active infection of *T. gondii.* PCR in blood is a fast, sensitive and specific method; in addition, blood samples are much easier to collect than tissue samples. When the positivity obtained in blood is compared between ages among animals sampled in farm 1 within the same year, the highest level of acute infection is obtained among 8 week-old pigs (44%), the levels of tachyzoites circulating dropped drastically in the next age group of 12 week-old pigs (19%). The PCR prevalence increased steadily for the next two age groups, 16 week old pigs had a PCR prevalence in blood of 25% which increased to 38% in 20 week old pigs. Among the slaughtered pigs in the same year raised in farm 1 and ≥ 24 weeks old (n=6), the PCR in blood dropped slightly to 33% and prevalence by PCR in tissue was as high a 66%. This changes in prevalence could be a direct method to monitor the parasite's journey during the life of the pig host. Experimental infection in pigs showed that the parasite can be detected in the bloodstream up to 28 d.p.i. (Wang *et al.,* 2013). Because in the present study, the age groups comprised different individuals, a decrease in the PCR prevalence in an older age group may not be related

to parasite clearance from the blood flow but it could be an indicative of lower levels of *T. gondii* transmission in a particular pen or farm area and vice versa. In farm 1, pigs were moved twice in their lives, first from farrowing (where pigs were kept since birth until the pig was weaned at 4-6 weeks old) to the weaning or nursery area (where pigs remained in general for 4-6 weeks) and second from the weaning to the fattening area where pigs remained in the same pen until slaughter (in some farms pigs were moved a third time to the finishing pen and were kept there during the last month of production cycle). The highest PCR prevalence in blood was found in 8 week old pigs, suggesting that those pigs become infected within approximately the previous 4 weeks (Wang *et al.,* 2013). This could indicate that the pigs became infected 1-before being moved to the weaning area, 2- in the weaning area, 3- both. Different sources of infection could be present in those pigs. For example, infection during suckling, weaning food contaminated with oocysts, or ingestion of intermediate or paratenic host are all possible scenarios. Other possibilities are a reactivation of the chronic infection where cleared parasites could have returned to the bloodstream after the maternal protection disappeared in the piglets (maternal derived antibodies are detectable up to 3-6 weeks of age). In contrast, the increase in the PCR prevalence in blood in the 20 week old pigs can be caused by higher levels of *T. gondii* transmission in a particular pen or due to a reactivation of a chronic infection, for example due to increased stress level in animals at that age. It is interesting that the multiple infections identified in animals from farm 1 were observed in blood of 8 week old pigs (TgPigMx20, 25) and 20 week old pigs (TgPigMx35, 37, 38) which were the age groups with higher PCR prevalence in blood. These could be suggestive of higher levels of *T. gondii* transmission in those particular pens or perhaps higher levels of stress present at those ages (for example, due to weaning in piglets or animal movements to other corrals) leading to a reactivation of dormant infections. The multiple infections noted in farm 2 also followed this pattern, two were found in 16 week old pigs (TgPigMx15, TgPigMx16) and one in a 20 week old pigs (TgPigMx19). Farm 2 had the highest PCR prevalence level in 16 week old pigs (44%). Hence, PCR of blood is proposed as a diagnostic method to monitor the disease at farm level. A follow up of the piglets since birth to slaughter to track the disease could be beneficial to identify 'hot spots' of transmission on a particular farm. In addition, PCR can also be used to confirm the presence of *T. gondii* on 'suspected sources' of contamination such as maternal milk, soil, water, food, cat faeces, trapped rodents or other intermediate hosts as a routine. Results of the present work

suggest that the monitoring by PCR of blood in farm 1 was a better predictor of the presence of tissue cysts in abattoir samples than serology. Thus the prevalence of PCR in blood could be used to 'predict' the prevalence of tissue cysts in later ages. It seems logical that if one pig is PCR positive in tissue, this should have been PCR positive in the blood at some point in its life. However, further efforts should be performed in order to standardize PCR protocols.

The variable farm was not an explanatory factor for *T. gondii* infection, neither by using serological methods (p=0.24 and p=0.153) or blood PCR screening (p = 0.21, p=0.1) in any of the environments. However, results from the questionnaire along with the sympatric study suggest that farms should improve sanitary standards. Factors such as high cat density, lack of rodent control, presence of rodents, open access to birds or other wild animals or open feeders and warehouses to the environment could increase the possibility of *T. gondii* infection. *T. gondii* was isolated from a cat from farm pilot A and *T. gondii* DNA was detected in tissues from 43% (96%CI = 17%-71%) of the 14 rats trapped in farm 1. We can not assume that the rat infestation in farm 1 or the high cat density in farm pilot A were directly related to *T. gondii* infection in pigs raised in those farms, however it can be said that the parasite had many opportunities to succeed in such farm environments. Rodent controls should be more strictly implemented on pig farms in this geographical area. In fact, rodent control has been shown to be a much faster and effective method to control the transmission of this parasite in pig farms (Kijlstra *et al.,* 2008) than control of oocyst shedding (Mateus-Pinilla *et al.,* 1999). In addition, rodent control could also help to control other diseases which can be transmitted to pigs through rodents and therefore from pigs to humans such as salmonellosis, trichinosis, tularaemia and leptospirosis (CDC, 2011). Rodent control could also help to control diseases which could be transmitted directly to humans by direct contact from rodents such leptospirosis, salmonellosis, Hantavirus or rat-bite fever (CDC 2011, 2012). In addition, rodents in Yucatan have been identified to be reservoirs of endemic pathogens such as *Rickettsia typhi* (Peniche-Lara *et al.,* 2015), *Rickettsia phelis* (Panti-May *et al.,* 2015), *Bartonella vinsonii* (Schulte-Fischedick *et al.,* 2016), *Leptospira spp* (Torres-Castro *et al.,* 2014)*, Borrelia burgdorferi* (Solís-Herández *et al.,* 2016), *Leishmania mexicana* (Andrade-Narvaez *et al.,* 2003) and *Trypanosome cruzi* (Lopez-Cancino *et al.,* 2015).

Currently, due to the lack of control of stray animals in Yucatan, the density of feral and stray cats is very high in rural and urban areas. Farms are attractive to cats as these can provide various sources of food. A large body of literature suggested that having cats in farms was the main source of *T. gondii* transmission in pigs (Weissenböck and Dubey, 1993, Dubey, 1994, García *et al.,* 1999, Matos *et al.,* 1999; Tsutsui *et al.,* 2003, Meerburg *et al*. 2004. García-Bocanegra *et al.,* 2010b), however it has not been demonstrated that the extermination of cats will lead to the elimination of the disease. In contrast, having a good sanitary management and control of the population of existing cats (neutering and spaying) could be more beneficial than elimination of them. Having their own farm cats would be probably safer as these are territorial and therefore could prevent stray and feral cats with unknown heath status from moving to the farm. A vaccine in cats to prevent oocyst shedding is currently not commercially available (Mateus-Pinilla *et al.,* 1999) and according to the research this advance may still not eradicate the disease as the definitive host is not strictly necessary to maintain the parasite (Owen and Trees, 1998, Hide *et al.,* 2009, Rengifo-Herrera *et al.,* 2012, Prestrud *et al.,* 2008). However, the implementation of vaccination in cats as a routine sanitary procedure could reduce substantially the parasite burden in the environment.

As a critique of the project design, collaboration with more research groups could have been beneficial for the project as it would have expanded the possibilities of obtaining other diagnostic methods which were unavailable in Yucatan. The use of a validated test during the pilot study could have changed the whole body of this thesis. Results of the present work represent a good example of how the use of serological tests without a previous validation can trigger the misinterpretation of the infectious status of a disease in a whole geographical area. In this case the *T. gondii* seroprevalence in pigs was being highly overestimated in the locality of Yucatan. The serological data generated for the general human population of Yucatan was generated during the National surveys (NSS, NHS and NSHS) by either non-species specific tests such IFAT or ELISAs which were validated by using a commercial ELISA kit for humans and by western blot (Caballero-Ortega *et al.,* 2012, Caballero-Ortega *et al.,* 2014). Prevalences in humans in other studies in Yucatan were obtained by using commercial kits for humans which are validated by the commercial company (Hernández-Cortazar *et al.,* 2016b). Therefore, although the DT (which is considered the gold standard in humans) was not employed in this area, prevalence data in humans can be considered fairly robust in this population as it was

generated by strict validation (Caballero-Ortega *et al.,* 2012, Caballero-Ortega *et al.,* 2014). In contrast, regarding seroprevalence data of *T. gondii* in animal species standardized tests have only been used in pigs (Chapter 2). Seroprevalences in other animals such as lambs (42%), cats (100%) and dogs (98%) were obtained exclusively with the ELISA kit Human Toxo IgG (Human-GmbH, Wiesbaden, Germany) by mixing the human and the secondary antibody anti-species and without a previous validation (Jimenez-Coello *et al.,* 2013, Hernández-Cortazar *et al.,* 2014, Ortega-Pacheco *et al.,* 2015). Therefore, further testing with standardised methods or validation of the ELISA is critical in order to have robust data of the disease levels for a wider number of species which inhabit this geographical area. Currently, of the overall total of 76 seroepidemiological studies in pigs recorded since 2009, 11% have published data using either an in-house ELISA or a commercial ELISA kit for humans without mentioning a previous validation (table 1.1). On the other hand, it can be said that the remaining 89% of the studies produced accurate data obtainined by using commercial ELISA kits for swine use (25%), MAT (22%), DT (1%), IFAT (13%), HIAT (9%), LAT (2%) or an in-house ELISA validated with a gold standard (17%) (table 1.1), which means that at least 89% of the serological data produced in the last 5-6 years can be considered accurate. Although it is a hopeful start, greater work should be accomplished in order to improve the quality of the public data.

As a critique of the bioassay experiments, stricter monitoring could have provided more successful results. The number of deaths of inoculated mice ranged from 40% to 75% depending on the experiment and this represents a high loss of very valuable material, some of which could have been harbouring potential *T. gondii* isolates. These unusually high levels of mouse deaths with unknown cause reflects the poor management of the animal house. In fact, there was a lack of personnel for veterinary/animal health assistance under care of the research animal unit. Records of presumptive illness or cause of death were not performed by the animal house for any animal. Furthermore, the animal house does not keep any records of animal losses. There was also a lack of a notification system, consequently when animals were ill, moribund or dead usually no action was performed. Therefore, due to the poor management of the animal house and the lack of monitoring during my absence, precise dates and biological samples were not available outside my research trips. It is difficult to establish a rigorous comparison between methods since not all the samples had the same chance of being positive in bioassay due to loss of the mice (for some of the samples no mice were screened). An improvement of the

regulations of laboratory animal management and care is suggested, at least in this institution (I am not aware of the regimen in other Mexican research institutes), in order to increase standards of both animal welfare and the quality of science.

The general geographical distribution of *T. gondii* genotypes shows the predominance of clonal types in North America and Europe with higher diverse genotypes in South America. Su *et al.,* (2012) (whose study remains the most extensive in this subject so far) clustered the *T. gondii* genetic population structure into clades which were accepted to show a strong geographical separation. However, this classification was not definitive as some genotypes still remained unclassified such as genotypes which fell into haplogroups 7, 11 and 13 which were located at the boundaries between two haplogroups (Su *et al.,* 2012). Similar patterns were followed by the isolates obtained in this study, TgPigMx53 and TgCatMx6 which presented a mixture of genes found in both North and South America. This suggests that these genotypes were a result of genetic crosses among strains from those geographical areas. In the present study, this admixture could have been enhanced by geographical proximity as Mexico borders between these two geographical areas creating therefore diffused boundaries between the predominant genotypes from the USA and South/North America.

The genotypes found in the present study may also reflect the existence of independent domestic and wild *T. gondii* life cycles in Mexico. *T. gondii* has a lower genetic diversity with a predominance of clonal types in anthropized ecosystems such Europe or the USA in comparison with wild populations such as Amazonian and French Guiana where genotypes are highly diverse (Lehman *et al.,* 2003). Interestingly, as shown in figure 4.13, TgCatMx6 was clustered in the middle between atypical genotypes obtained from wildlife (COUG, GUY-2004-JAG1, B41,) and type II genotypes found mostly in anthropized areas. This intermediate position could suggest that these genotypes were the result of hybridization between 'wild' and 'anthropized' strains. The existence of these wild-domestic hybrids has been noted in French Guiana, Canada, and USA (Dubey *et al.,* 2011b, Mercier *et al.,* 2011, Khan *et al.,* 2014). This genetic exchange is likely to happen in countries where large territories are still non-anthropized and therefore a co-existence between anthropized and wild ecosystems can occur. Recombination or genetic exchange between strains can only occur during the sexual cycle. Thus, this genetic exchange will occur in nature when a felid ingests multiple *T. gondii* strains

either as a result of a single event (example, a prey with a multiple infection) or multiple events within a short time span (example, more than one prey harbouring one or more strains each). The presence of genetic exchange in this geographical area is supported by the existence of multiple infections in animals from farm 1 and farm 2. The presence of multiple *T. gondii* strains in an intermediate host gives an excellent opportunity for genetic exchange if the host is consumed by a feline predator. Considering the high levels of *T. gondii* PCR positive animals, the presence of different genotypes within the small area of sampling and the co-existence of cats with animals which could be their prey such as rodents or birds within the same farm, it is not surprising that a cat had opportunities to become infected with different *T. gondii* strains. The fact that all the farms sampled in Yucatan were open to the environment offers the opportunity of hybridization between *T. gondii* strains circulating in the wild and domestic life cycle. The result of this genetic exchange could eventually lead to the creation of novel recombinant strains. The discovery of a novel recombinant type I and III in the present study in one pig TgPigMx53 supports this theory of sexual recombination. New recombinant genotypes were also reported in Mexico (Dubey *et al.,* 2009, Dubey *et al.,* 2013,) and USA (Dubey *et al.,* 2011a, Velmurugan *et al.,* 2009, Dubey *et al.,* 2011b) some of which were also found in domestic animals (Velmurugan *et al.,* 2009, Dubey *et al.,* 2011b, Dubey *et al.,* 2009).

5.2 Future studies

The design of the study was based on previous publications which reported a false and extremely high seroprevalence in pigs (Ortega-Pacheco *et al.,* 2011, Ortega-Pacheco *et al.,* 2013, Jiménez-Coello *et al.,* 2013). A different approach to monitor the disease in the area is proposed now that the present study has shown an alternative picture. Seroepidemiological surveys should be focused in old pigs (>20 weeks) as results have shown higher seroprevalences. Sampling at abattoirs is a good choice as high number of blood samples can be collected rapidly. Abattoirs which collaborate to facilitate information exchange related to the farms would be beneficial in order to study the risk factors associated with *T. gondii* transmission between farm management systems. In addition, abattoirs receive animals from farms located in separated geographical areas, this provides an additional advantage as seroprevalence can be compared between different environments without the need to travel long

distances. A combination of PCR in tissues and serological diagnosis in abattoir samples is recommended to monitor the incidence of *T. gondii* in pork destined for human consumption. The use of a validated serological test is critical, MAT is suggested as it has shown to be highly specific and sensitive. In addition, this study has shown that although a validated, highly sensitive and specific assay is used such as MAT, DT or IDscreen®, still there is a low percentage of pigs that were false negative. Therefore, a combination of two serological diagnostic tests is recommended, preferably MAT and IDscreen as the DT is only available in some reference centres. The use of the N-PCR protocols optimized in the present work is also proposed as they have been shown to be very sensitive and specific. B1 N-PCR has been shown to be the most sensitive method, although SAG1 N-PCR and SAG2 N-PCR have the advantage of allowing genotyping of the *T. gondii* isolates involved. As a critique of the SAG1 locus, this had less discriminatory power than SAG2. Thus, a combination of SAG1 and SAG2 and B1 is suggested when genotyping is also one of the aims of the study. When the purpose of the study is only diagnosis, at least two loci are recommended (SAG1 and B1) to avoid false negatives due to a failure of complete DNA extraction and/or amplification of one of the genes.

One of the limitations of this work was to find a large slaughterhouse which received pigs from a large number of farms. An attempt was made to collaborate with bigger abattoir, but efforts were unsuccessful. Results of the present study could be biased as only one abattoir was sampled and nearly all the positive pigs were raised in farm 1. The frequency of *T. gondii* found in this abattoir should not be generalised to other farms or abattoirs in Yucatan. Future studies should find more abattoirs distributed in different locations or sampling in a large scale abattoir which receives samples from several farms.

Interestingly, recent studies found that MAT on cardiac fluid had better concordance with *T. gondii* isolation and PCR on cardiac tissue in comparison to MAT on serum and diaphragm fluid (Opsteegh *et al.,* 2016). Whether the reason for this was that the serology and the direct method (PCR and bioassay) were performed in the same matrix (heart) is unknown. Future studies could assess the repeatability of this finding by also using additional organs. This finding could imply a sampling advantage as the matched tissue with blood from the same animal can easily be obtained by abattoir samplings. This could be a good method to monitor *T. gondii* in abattoirs. Although PCR on tissue has been shown to be faster and cheaper than

isolation assays, the use of mouse bioassay is still the only method to examine the presence of the viability of the parasite in pork. Data from the present study has shown a higher recovery rate of *T. gondii* tissue cysts using the mouse bioassay than *in vitro* isolation. However, due to the tediousness of the assay, this can be simplified by sampling hearts and performing isolation only in a set of selected hearts with seronegative and seropositive cardiac fluid (seropositive and seronegative pigs) for each of slaughtered batch of pigs. Following this, pigs with positive bioassayed hearts can be tracked to the farm of origin to improve sanitary management related to *T. gondii.* Using this isolation strategy will also reduce the number of mice/cats used in the bioassay which is important from an ethical and animal welfare point of view.

Screening of the active infection by PCR in blood extracts is proposed as a future study to monitor the active disease at farm level. This could be useful to identify 'hot spots' of *T. gondii* transmission in a particular farm and consequently implement adequate strategies to minimise the source of infection in the particular area. A combination of two or more PCR protocols is recommended to increase the robustness of the surveillance.

Future research could be addressed at studying congenital transmission in pigs as the knowledge on this topic is very limited. A proposed strategy is to undertake a follow up study of the offspring derived from seronegative and seropositive dams by both serology and PCR techniques. Thus it may reveal whether in new-born piglets from *T. gondii* infected sows, the detection of circulating tachyzoites can be detected earlier than in new-born piglets from seronegative sows (this could be an indicative of congenital transmission during pregnancy). Serological follow up of sucking piglets derived from seropositive and seronegative dams to find out whether in later life, after challenge, pigs which were passively immunized through colostrum experience a longer time gap before seroconversion in comparison with pigs which were not passively immunized.

The name 'atypical' was used to classify those genotypes that were 'rare' and did not fit in the initial classification of the three clonal types. The intensive genotyping of *T. gondii* isolates performed during the last 20 years has slowly broken down the theory that *T. gondii* genetic structure is essentially comprised of the three clonal types I, II and III. Studies have shown that atypical or recombinant strains are not rare anomalies, but rather key genotypes of the gene pool providing a much better picture of the genetic population structure of this parasite.

Although *T. gondii* genetic diversity is higher in South America and Central America than in the Northern hemisphere (Dubey and Su, 2009, Lehmann *et al.,* 2006, Pena *et al.,* 2008, Rajendran *et al.,* 2012); atypical genotypes are not exclusively from South or Central America as over time new atypical genotypes are continuously being discovered in North America (Verma *et al.,* 2016, Dubey *et al.,* 2011b), Europe (Verma *et al.,* 2015), Australia (Brennan *et al.,* 2016) and Africa (Bamba *et al.,* 2016). Further work is needed for a much clearer classification of genotypes as the term 'atypical' is currently including a large proportion of the known genotypes.

Further genotyping studies should also include intensive sampling of isolates in more geographical areas and hosts as the current genotyping research could still be biased. One of the reasons for the underestimation of *T. gondii* genetic diversity in the early genotyping studies was probably the limited origin of the isolates since the vast majority where collected from humans and domestic animals in Europe and North America (Howe and Sibley, 1995, Ajzenberg *et al*., 2002). A better picture is available now since genotyping studies have also been performed on isolates from wild animal species and from additional geographical areas such as South/Central America and other remote places (Rajendran *et al*., 2012, Dubey *et al.,* 2011a, Dubey *et al.,* 2010). However, there are still many places that have not been explored. For example, currently there is a lack of intensive studies in the European, Asian and Australian forests which, perhaps harbour higher diversity than in the parallel domestic environments. In addition, genotyping studies performed on isolates from humans, are limited to clinical cases. Therefore, whether the same genotypes are found in asymptomatic people is unknown. In addition, some studies attempt the isolation of *T. gondii* still only in seropositive animals which is a good strategy to save time and expenses. However, here and other studies have shown that there is a proportion of isolates obtained from seronegative animals. These strategies raise the question of whether isolates are still being selected when these are obtained only from seropositive animals, acutely infected animals or human clinical cases.

5.3 General conclusions

I conclude that the prevalence of *T. gondii* in pigs from Yucatan is much lower than previously thought and correlates well with studies located in other Mexican states (Alvarado-Esquivel *et al*., 2011a, Alvarado-Esquivel *et al*, 2012c, Alvarado-Esquivel *et al*., 2014 Alvarado-Esquivel *et al*., 2015). I report the current need to make new epidemiological surveys in this area by using serological methods validated in this animal species because the IgG ELISA kit (Human Toxo IgG Human-GmbH, Wiesbaden, Germany) has been shown not to perform well in pigs. The SAG1 N-PCR protocol proposed in the present work has been shown to be highly sensitive and specific for the diagnosis of *T. gondii* in swine blood and tissue samples. I also conclude that the consumption of pork can be a risk to humans when undercooked as *T. gondii* was isolated from 8.8% of the pigs sampled at the abattoir. For an accurate evaluation of the presence of *T. gondii* in pork, a combination of serology and direct methods (PCR or/and bioassay) is suggested as the use of one by itself can lead to the assumption of false negatives. The reason behind such a relatively high percentage of seronegative pigs were PCR and bioassay positive remains unclear. These finding are common to recent studies and need further assessment. I also conclude that the IgG ELISA kit (Human Toxo IgG Human-GmbH, Wiesbaden, Germany) needs further validation for use in mice as it was outside of my scope to test a wider panel of positive and negative controls. Another conclusion is that stricter rodent controls should be applied on farms in this geographical area. Implementation of neutering and spaying campaigns is suggested to control the population of cats. I also conclude that the genotypes in Mexico seem to share common characteristics with genotypes from both North and South America. Whether this genetic pool is caused by geographical proximity or/and hybridization between *T. gondii* strains which circulate in the wild and domestic cycle needs further assessment. Finally, I conclude that reinfection of an intermediate host with a second *T. gondii* strain seems a relatively common event in this geographical area.

5.4 The impact of this research

Based on my research and my analysis of other studies, there are some measures which could be useful for the Mexican Authorities of Yucatan to improve public health in relation to *T. gondii:*

1-Implementation of rodent control on pig farms.

2-Implementation of neutering and spaying campaigns to control the population of stray and feral cats across urban and rural areas.

3-Public education of the possible risks derived from *T. gondii* transmission and how to prevent transmission of the parasite. For example, the importance of cooking meat well, the use of gloves when working with soil or disposing of cat litter, washing vegetables before consumption, etc.

4-The use of validated serological tests in combination with PCR and bioassay in abattoirs to monitor the viability and presence of the parasite in pork meat.

5-The use of validated serological tests in combination with PCR on pig farms in order to monitor the disease.

6-Epidemiological studies on additional animal species such as cats, chickens, sheep, goats and cattle which could be a potential source of *T. gondii* transmission.

7-To monitor the viability of the parasite in meats from other animal species such lamb, goat, chicken, cattle and horse.

8-Stricter regulation of the management and care of laboratory animals.

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APPENDIX I

AI.1 Summary of seroepidemiological studies in pigs (*Sus scrofa domesticus*)

Table 1.1 Global seroprevalence of IgG antibodies anti *T. gondii* in the domestic pig from 1990 to 2016. Updated from Dubey, 2009b and Guo *et al*., 2014. Last updated14/10/16.

Country	Test *	Sample size	Source **	Prevalence	Reference
Argentina	IFAT	109	Abattoir [f]	63.3%	Omata *et al.,* 1994
	MAT	230	Abattoir [s]	37.8%	Venturini *et al.,* 2004
	MAT	88	Intensive farm [a]	4.5%	Venturini *et al.,* 2004
	MAT	112	Free range farm [a]	40.2%	Venturini *et al.,* 2004
	IFAT	300	Free range and intensive farms [f, s]	41%	Pardini *et al.,* 2012
	ELISA [h]	300	Free range and intensive farms [f, s]	44.3%	Pardini *et al*., 2012
	ELISA [h]	149	Free range farms	75.2%	Basso *et al.,* 2013
	ELISA [p]	149	Free range farms	83.2%	Basso *et al.,* 2013
	IFAT	149	Free range farms	80.5%	Basso *et al.,* 2013
	WB	149	Free range farms	83%	Basso *et al.,* 2013
	ELISA [h]	148	Intensive farms	4.7%	Basso *et al.,* 2013
	ELISA [p]	148	Intensive farms	8.8%	Basso *et al.,* 2013
	IFAT	148	Intensive farms	8.1%	Basso *et al.,* 2013
	WB	148	Intensive farms	23%	Basso *et al.,* 2013
Austria	CFT	3917	Abattoir	3.8%	Quehenberger *et al.*, 1990
	IFAT	2300	Abattoir [f]	0.8%	Edelhofer., 1994
	MAT [b]	1316	Intensive farms	4.3%	Steinparzer *et al*., 2015
	ELISA [p]	1316	Intensive farms	4.2%	Steinparzer *et al*., 2015
	ELISA [q]	1316	Intensive farms	2.3%	Steinparzer *et al*., 2015
	ELISA [i]	1316	Intensive farms	1.8%	Steinparzer *et al*., 2015
	MAT [b]	52	Organic farms	63.5%	Steinparzer *et al*., 2015
	ELISA [p]	52	Organic farms	69.2%	Steinparzer *et al*., 2015
	ELISA [q]	52	Organic farms	67.3%	Steinparzer *et al*., 2015
	ELISA [i]	52	Organic farms	67.3%	Steinparzer *et al*., 2015
Bangladesh	IHAT	200	Abattoir [f]	20%	Biswas *et al*., 1993
Brazil	IFAT	198	Abattoir	90.4%	Guimarães *et al*., 1992
	IHAT	200	Intensive farms	18%	Grünspan *et al*., 1995
	IFAT	267	Backyard	24%	García *et al*., 1999
	ELISA [h]	300	Abattoir [f]	9.6%	Suárez-Aranda *et al*., 2000
	MAT	286	Free range farms [f]	17%	Dos Santos *et al*., 2005
	MAT	759	Intensive and free range farms	1.3%	Caporali *et al*., 2005
	IFAT	395	Abattoir [f]	2.6%	Carletti *et al*., 2005
	IFAT	29	Abattoir [s]	20.7%	Carletti *et al*., 2005
	IFAT	38	Backyard	65.8%	Bonna *et al*., 2006
	MAT	80	Backyard	37.5%	Cavalcante *et al*., 2006
	IFAT	80	Backyard	43.7%	Cavalcante *et al*., 2006
	MAT	550	Backyard	20.2%	Oliveira *et al*., 2007
	IFAT	117	Abattoir	8.5%	Moura *et al*., 2007b
	MAT	262	Abattoir	0%	Pezerico *et al*., 2007
	MAT	304	Abattoir	7.2%	Da Silva *et al*., 2008
	IFAT	290	Intensive farms [f]	32.1%	Correa *et al*., 2008
	IFAT	408	Backyard	25.5%	Millar *et al*., 2008

	IHAT	110	Backyard	50%	Freitas *et al*., 2009
	ELISA [h]	371	Intensive farms [f]	14.8%	Bezerra *et al*., 2009
	ELISA [h]	94	Backyard [f]	32.9%	Bezerra *et al*., 2009
	MAT	606	Intensive farms [s]	14.1%	Piassa *et al*., 2010
	IFAT	130	Abattoir	36.2%	De Acevedo *et al*., 2010
	IFAT	708	Intensive farms [s]	12.8%	Muraro *et al*., 2010
	IFAT	200	Backyard	48.8%	Villalobos *et al*., 2011
	ELISA [h]	34	Free range farms	20.6%	Teixera and De Oliveira, 2011a
	ELISA [h]	27	Intensive farms	11.5%	Teixera and De Oliveira, 2011a
	IFAT	305	Abattoir	12.5%	Samico-Fernandes *et al*., 2012
	ELISA [h]	143	Extensive and intensive farms	25.5%	De Sousa *et al*., 2014
	IFAT	100	Free range farms	36%	Cademartori *et al*., 2014
	IFAT	190	Free range and intensive farms	19.5%	Feitosa *et al*., 2014
Bolivia	IHAT	291	Backyard	48.5%	Orellana *et al.,* 2004
Burkina Faso	ELISA [i]	300	Free range farms [a]	29%	Bamba *et al*., 2016
Canada	MAT [b]	1443	Abattoir [s]	9.4%	Smith, 1991
	LAT [t]	2800	Abattoir [f]	8.6%	Gajadhar *et al*., 1998
	ELISA [s]	6048	Intensive farms [f]	0.74%	Poljak *et al*., 2008
Chile	DT	1474	Abattoir [a]	28.1%	Tamayo *et al*., 1990
	ELISA [s]	340	Abattoir	8.8%	Muñoz-Zanzi *et al*., 2012
China	IHAT	816	Not stated	10.4%	Lin *et al*., 1990
	IHAT	525	Not stated	20.19%	Huang, 1991
	IHAT	831	Abattoir	17%	Zou *et al*., 2009
	ELISA [i]	1022	Abattoir	27%	Zhou *et al*., 2010
	IHAT	605	Intensive farms [s]	14.4%	Huang *et al*., 2010
	ELISA [c]	813	Backyard [a]	53.4%	Yu *et al*., 2011
	ELISA [h]	3558	Intensive farms [f]	24.5%	Tao *et al*., 2011
	IHAT	1164	Abattoir	12%	Liu, 2012
	IHAT	908	Abattoir	30.6%	Wu *et al*., 2012
	ELISA [h]	2277	Intensive farms	28.8%	Du *et al*., 2012
	IHAT	1014	Intensive farms [f]	4.6%	Chang *et al.,* 2013
	IHAT	1129	Intensive farms [s]	31.3%	Xu *et al*., 2014
	IHAT	1232	Intensive farms [a]	22.9%	Jiang *et al*., 2014
	ELISA [c]	70	Free range [pm]	70%	Li *et al*., 2015
Costa Rica	CIA	300	Abattoir	26%	Torres *et al*., 1991
	IFAT	496	Farms [a]	43.8%	Arias *et al*., 1994
Czech Republic	DT	2616	Abattoir	5.9%	Hejlíček and Literák, 1993
	DT	259	Abattoir	0.4%	Vostalová *et al*., 2000
	IFAT	20	Not stated	20%	Sedlák and Bártová, 2007
	ELISA [i]	551	Abattoir	36%	Bártová and Sedlák, 2011
	ELISA [i]	48	Organic farms	18.8%	Slany *et al.,* 2016
	ELISA [i]	121	Intensive farms	13%	Slany *et al.,* 2016
	ELISA [i]	29	Backyard	20.7%	Slany *et al.,* 2016
Egypt	ELISA [h]	180	Abattoir	52.2%	Moghazy *et al*., 2011
	MAT	180	Abattoir	56.6%	Moghazy *et al*., 2011
	IHAT	180	Abattoir	42.7%	Moghazy *et al*., 2011
	DT	180	Abattoir	35.5%	Moghazy *et al*., 2011
Ethiopia	MAT [b]	402	Intensive farms	32.1%	Gebremedhin *et al*., 2015
France	ELISA [i]	49	Abattoir	2%	Roqueplo *et al*., 2011
	MAT	207	Free range farms [f]	6.3%	Djokic *et al*., 2016a
	MAT	1342	Intensive farms [a]	2.7%	Djokic *et al*., 2016a
	MAT	3600	Intensive [a]	7%	Djokic *et al*., 2016b
Germany	ELISA [h]	1005	Abattoir [f]	20.5%	Fehlhaber *et al*., 2003
	ELISA [h]	2041	Intensive farms [s]	18.5%	Damriyasa *et al*., 2004

	IFAT	2041	Intensive farms [s]	16.5%	Damriyasa *et al*., 2004
	ELISA [h]	1500	Intensive farms [s]	9.3%	Damriyasa *et al*., 2005
	ELISA [h]	300	Intensive farms [f]	5.2%	Schulzig *et al*., 2005
	ELISA [h]	4721	Intensive farms [f]	3.8%	De Buhr *et al*., 2008
	ELISA [h]	180	Free ranged farms [f]	2.8%	De Buhr *et al*., 2008
	ELISA [h]	60	Organic farms [f]	11.7%	De Buhr *et al*., 2008
	ELISA [h]	38	Abattoir [s]	31.6%	De Buhr *et al*., 2008
Ghana	ELISA [h]	641	Free range farms [a]	40.6%	Arko-Mensah *et al*., 2000
Greece	ELISA [i]	206	Intensive farms [s]	4.3%	Papatsiros *et al*., 2016
Honduras	ELISA [h]	150	Intensive farms	32%	Zúniga and Lorca, 2009
Indonesia	LAT [l]	208	Abattoir [f]	6.3%	Inoue *et al*., 2001
	ELISA [h]	205	Not stated	14.6%	Ichikawa *et al.,* 2015
	MAT [b]	100	Not stated	44%	Hartati, 2015
Italy	IFAT	90	Intensive farms [a]	64.4%	Genchi *et al*., 1991
	ELISA [p]	2160	Intensive farms [a]	16.3%	Villari *et al*., 2009
	IFAT	960	Intensive farms [f]	16.1%	Veronesi *et al*., 2011
	ELISA [i]	21	Organic farms	95.2%	Bacci *et al*., 2015
Japan	LAT [l]	155	Intensive farms [f]	5.2%	Matsuo *et al*., 2014
Latvia	ELISA [h]	269	Intensive farms [f]	0.4%	Deksne *et al.,* 2013
	ELISA [h]	543	Free range farms [f]	6.2%	Deksne *et al.,* 2013
Malaysia	IHAT	122	Not stated	15.6%	Rajamanickam *et al*., 1990
	IFAT	100	Intensive farms [s]	0%	Chandrawathani *et al*., 2008
Mexico	ELISA [h]	1203	Not stated	8.9%	García-Vázquez *et al*., 1993
	ELISA [m]	79	Intensive farms	100%	Ortega-Pacheco *et al*., 2011
	MAT	1074	Backyard	16%	Alvarado-Esquivel *et al*., 2011a
	MAT	519	Backyard	9.1%	Alvarado-Esquivel *et al*., 2011a
	MAT	188	Backyard	17.2%	Alvarado-Esquivel *et al*., 2012c
	MAT	337	Intensive farms	0.5%	Alvarado-Esquivel *et al*., 2012c
	ELISA [m]	429	Intensive farms	95.8%	Ortega-Pacheco *et al*., 2013
	ELISA [m]	30	Backyard	75%	Jimenez-Coello *et al*., 2013
	MAT	402	Backyard	45.3%	Alvarado-Esquivel *et al*., 2014
	MAT	17	Intensive farms	0%	Alvarado-Esquivel *et al*., 2015
	MAT	291	Backyard	13.7%	Alvarado-Esquivel *et al*., 2015
	ELISA [m]	81	Backyard [a]	53%	Dzib-Paredes *et al.,* 2016
	ELISA [m]	60	Abattoir [f]	96.6%	Hernández-Cortazar *et al.,* 2016a
Netherlands	ELISA [h]	23348	Abattoir [f]	2.1%	Berends *et al*., 1991
	ELISA [h]	1009	Abattoir [s]	30.9%	Van Knapen *et al*., 1995
	ELISA [h]	994	Abattoir [f]	1.8%	Van Knapen *et al*., 1995
	LAT	635	Free range farms	4.7%	Kijlstra *et al*., 2004
	LAT	660	Organic farms	1.2%	Kijlstra *et al*., 2004
	LAT	621	Intensive farms	0%	Kijlstra *et al*., 2004
	ELISA [h]	2796	Organic farms	3%	Meerburg *et al*., 2006
	ELISA [h]	265	Intensive farms	0.4%	Van der Giessen *et al*., 2007
	ELISA [h]	402	Organic farms	2.7%	Van der Giessen *et al*., 2007
	ELISA [h]	178	Free range farms	5.6%	Van der Giessen *et al*., 2007
	ELISA [h]	406	Organic farms	10.9%	Kijlstra *et al*., 2008
	ELISA [p]	120666	Intensive farms	2%	Swanenburg *et al*., 2015
	ELISA [p]		Organic farms	3.6%	Swanenburg *et al*., 2015
Nigeria	ELISA [i]	302	Abattoir [a]	29.14%	Onyiche and Ademola, 2015
Panama	IFAT	290	Intensive farms	32.1%	Correa *et al*., 2008
Peru	ELISA [h]	96	Abattoir [f]	32.3%	Suaréz-Aranda *et al*., 2000
	WB	137	Abattoir	27.7%	Saavedra and Ortega, 2004
Poland	ELISA [h]	925	Farms (type not stated)	36.4%	Bartoszcze *et al*., 1991
	MAT [b]	106	Abattoir	26.4%	Sroka *et al*., 2008

	ELISA [h]	1754	Not stated	19.2%	Holec-Gasior *et al*., 2010
	MAT [b]	861	Abattoir	14.3%	Sroka *et al*., 2011
	ELISA [h]	861	Abattoir	15.4%	Sroka *et al*., 2011
Portugal	MAT	333	Free range and intensive farms [s, f]	15.6%	De Sousa *et al*., 2006
	MAT [b]	254	Free range and intensive farms [a]	9.8%	Lopes *et al*., 2013
	MAT [b]	127	Intensive farms [a]	7.9%	Esteves *et al*., 2014
	MAT [b]	254	Free range farms [a]	6.7%	Esteves *et al*., 2014
	MAT [b]	337	Intensive farms [f, s]	0%	Caiado *et al*., 2015
Romania	ELISA [i]	1700	Intensive farms [a]	0.8%	Hotea *et al*., 2010
	IFAT	2564	Backyard	30.2%	Pastiu *et al*., 2013
	IFAT	371	Intensive farms [s]	12.4%	Pastiu *et al*., 2013
	IFAT	660	Intensive farms [f]	0%	Pastiu *et al*., 2013
Serbia	MAT	323	Intensive farms and abattoirs [s]	40.9%	Klun *et al*., 2006
	MAT	282	Intensive farms and abattoirs [f]	15.2%	Klun *et al*., 2006
	MAT	212	Intensive farms	3.8%	Klun *et al*., 2011
	MAT	276	backyard	13.4%	Klun *et al*., 2011
Slovakia	ELISA [p]	923	Abattoir [f]	2.2%	Turčecová *et al*., 2013
	ELISA [p]	47	Abattoir [s]	4.3%	Turčecová *et al*., 2013
Spain	MAT	880	Intensive farms [f]	20.2%	García-Bocanegra *et al*., 2010a
	MAT	322	Intensive farms [s]	15.5%	García-Bocanegra *et al*., 2010a
	MAT	1400	Intensive farms [s]	24.2%	García-Bocanegra *et al*., 2010b
	MAT	1570	Intensive farms [f]	9.7%	García-Bocanegra *et al*., 2010b
	ELISA [i]	709	Free range farms	73.4%	Hernández *et al*., 2014
	IFAT [f]	1200	Intensive farms [f]	24.5%	Herrero *et al*., 2016
Sweden	MAT	60	Farms (type not stated)	50%	Ljungström *et al*., 1994
	ELISA [h]	110	Abattoir [s, b]	17.3%	Lundén *et al*., 2002
	ELISA [h]	695	Abattoir [a]	3.3%	Lundén *et al*., 2002
	ELISA [i]	340	Intensive farms	1%	Wallander *et al.,* 2016
	ELISA [i]	340	Organic farms	8%	Wallander *et al.,* 2016
Switzerland	ELISA [h]	120	Intensive farms [s]	36%	Berger-Schoch *et al*., 2011
	ELISA [h]	100	Free range farms [f]	13%	Berger-Schoch *et al*., 2011
	ELISA [h]	50	Intensive farms [f]	14%	Berger-Schoch *et al*., 2011
Taiwan	LAT	3880	Not stated	27.7%	Chang *et al*., 1990
	LAT [l]	111	Abattoir	28.8%	Fan *et al*., 2004
	LAT	395	Abattoir	10.1%	Tsai *et al*., 2007
Uruguay	DT	601	Abattoir [a]	70.2%	Freyre *et al*., 1991
UK	LAT [r]	317	Abattoir [a]	4.7%	Halová *et al*., 2013
	DT	626	Abattoir [a]	7.4%	Powell *et al*., 2016
	MAT	2074	Abattoir	3.6%	Opsteegh *et al.,* 2016
USA	ELISA [h]	2613	Not stated [a]	7.4% [NAHMS]	Zimmerman *et al*., 1990
	MAT	11229	Abattoir [f]	23% [NAHMS]	Dubey *et al*., 1991
	MAT	613	Abattoir [s]	42% [NAHMS]	Dubey *et al*., 1991
	MAT	509	Farms (type not stated)	48.5%	Dubey *et al*., 1992
	MAT	273	Intensive farms [s]	14.3%	Smith *et al*., 1992
	MAT	3841	Intensive and extensive farms [s]	36%	Assadi-Rad *et al*., 1995
	MAT	4252	Intensive farms [f]	2.3%	Dubey *et al*., 1995a
	MAT	2617	Intensive farms [s]	15.1%	Dubey *et al*., 1995a
	MAT	5080	Intensive farms [s]	20.8%	Weigel *et al*., 1995a
	MAT	1885	Intensive farms [a]	3.1%	Weigel *et al*., 1995a
	MAT	3479	Intensive farms [s]	20% [NAHMS]	Patton *et al*., 1996
	MAT	3473	Not stated	19% [NAHMS]	Hu *et al*., 1997
	MAT	4712	Intensive farms [a]	3.2% [NAHMS]	Patton *et al*., 1998
	MAT	3236	Intensive farms [s]	15% [NAHMS]	Patton *et al*., 1998
	MAT	2238	Intensive and extensive farms	0.58%	Davies *et al*., 1998

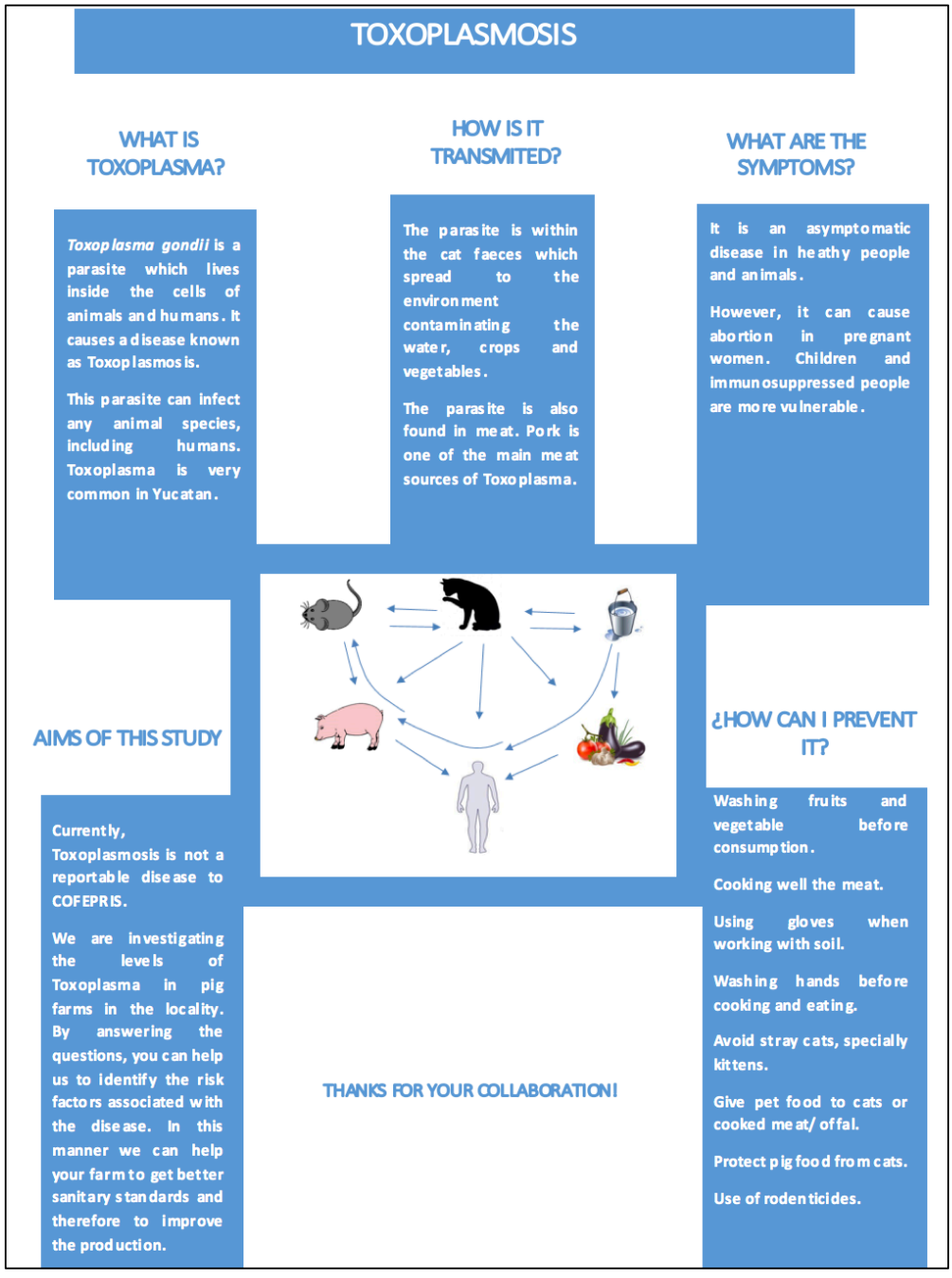
	MAT	1897	Intensive and extensive farms	47.4%	Gamble *et al*., 1999
	MAT	8086	Intensive farms [s]	6% [NAHMS]	Patton *et al*., 2000
	MAT	5720	Intensive farms [a]	0.9% [NAHMS]	Patton *et al*., 2000
	MAT	55	Free range farm [pm]	92.7%	Dubey *et al*., 2002a
	WB	152	Abattoir	16.4%	Saavedra *et al*., 2004
	ELISA [s]	48	Free range farm	25%	Dubey *et al*., 2008
	MAT	48	Free range farm	70.8%	Dubey *et al*., 2008
	ELISA [h]	324	Free range farms	6.8%	Gebreyes *et al*., 2008
	ELISA [h]	292	Intensive farms	1.1%	Gebreyes *et al*., 2008
	ELISA [s]	6238	Intensive farms [a]	2.6% [NAHMS]	Hill *et al*., 2010
	MAT	33	Organic farms [f]	90.9%	Dubey *et al*., 2012c
	ELISA [s]	5688	Intensive farms [f]	3.8% [NAHMS]	Hill, 2014
Venezuela	IHAT [w]	425	Farms (type not stated)	12.5%	Romero *et al*., 2007
Vietnam	MAT	587	Intensive farms [s, f, b]	27.7%	Huong and Dubey, 2007
West indies	MAT	247	Not stated	23.1%	Chikweto *et al*., 2011
	ELISA [i]	185	Farms (type not stated) [s, f, b]	24.3%	Sharma *et al*., 2015
Zimbabwe	MAT	97	Not stated	9.3%	Hove and Dubey, 1999
	IFAT	238	Intensive farming [f]	19.8%	Hove *et al*., 2005
	IFAT	70	Backyard [s]	35.7%	Hove *et al*., 2005

* IFAT: indirect fluorescent antibody test, [t]: commercial IFAT kit by ® bioMerieux, MAT: modified agglutination test, [b]: Toxo-Screen DA® bioMerieux, ELISA: enzyme-linked immunosorbent assay, [h]: in home ELISA, [p]: PrioCHECK® Toxo ELISA, [q]: PIGTYPE® Toxoplasma Ab Qiagen, [i]: ID Screen® IDVet, [s]: commercial ELISA kit SafePath Laboratories, [c]: commercial ELISA kit Haitai (Biological Pharmaceuticals), [p]: commercial ELISA kit by Institut Pourquier, [m]: commercial ELISA kit Human-GmbH, WB: western blot, CFT: complement fixation test, IHAT: indirect hemagglutination antibody test, [w] Toxo-test HAI® (Wiener), [y]: commercial kit NY/T 573-2002, LAT: latex agglutination test, [t]: Toxo-test MT® (Tanabe), [l]: Lat ToxocheckMT® (Eiken), [r]: commercial kit Toxoreagent RST701, (Mast Group), DT: Dye test, CIA: carbon immunoassay.
** [f]: finisher, pig around 5-6 months old, [s]: sow, female pig between 2-5 years old, [b]: boar, male pig between 2-5 years old, [a]: pigs sampled at different ages, [pm]: farm with poor management system.
[NAHMS]: National Animal Health Monitoring System.

APPENDIX II

AII.1 Informative leaflet

This document was used before the sampling to approach the subject of toxoplasmosis to the farmers. This also helped towards their understanding of the study. This is a translation of the original (in Spanish) which can be found in the next page.



LA TOXOPLASMOSIS

¿QUÉ ES TOXOPLASMA?

Toxoplasma gondii es un parasito que produce una enfermedad llamada Toxoplasmosis. El hospedador definitivo es el gato, pero cualquier animal, incluido el ser humano puede infectarse. De hecho, es una enfermedad muy común en la población mejicana.

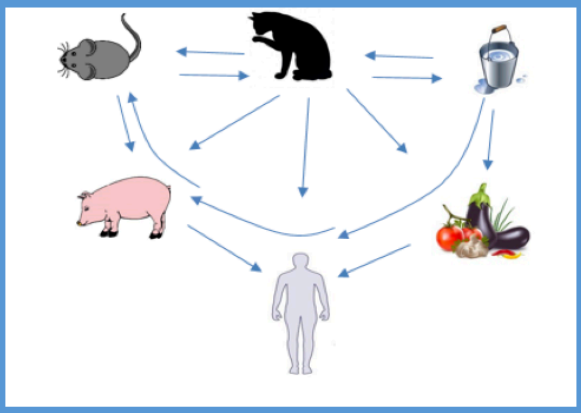
¿CÓMO SE TRANSMITE?

Las heces de los gatos son el principal transmisor, infectando agua, hortalizas y suelo. La ingestión de cualquier carne poco cocinada es una ruta de transmisión muy común. La carne de cerdo juega un papel muy importante, ya que forma parte de la dieta diaria

¿QUÉ SINTOMAS PRODUCE?

Generalmente, es asintomática en personas sanas. Para mujeres embarazadas si presenta más riesgo, así como en bebes o inmunodeficientes.

No es una enfermedad peligrosa para los cerdos pero puede producir abortos, o/y retraso del crecimiento o/y pérdida de peso y por tanto menor rendimiento económico



OBJETIVOS DEL ESTUDIO

Actualmente, la ausencia de Toxoplasmosis en la producción porcina no es requisito determinado por COFEPRIS. En este estudio, se están analizando diferentes factores de riesgo (cerdos, agua ratones, gatos) para identificar el problema si lo hay e implantar medidas correctoras para mejorar el estado sanitario de la explotación para satisfacer futuros estándares sanitarios.

¿CÓMO PREVENIR LA TRANSMISIÓN?

Lavando los vegetales antes de la ingestión.

Cocinando bien la carne

Prevenir el exceso de gatitos jóvenes y callejeros y dar carne bien cocida a los gatos propios. Evitar la presencia ratones y pájaros; y resguardar la comida de éstos

Monitoreo con su veterinario para detectar cargas elevadas del parasito circulante y adoptar las medidas correctoras sugeridas

GRACIAS POR SU COLABORACIÓN

AII.2 Description of the farm questionnaire

The farm questionnaire was filled in the day of sampling by interviewing the veterinary of the farm (when present) or the farmers. All farms were intensive and full cycle.

1- Farm characteristic and infrastructure
   - Area of the farm (in ha)
   - Description of the feeders
   - Description of the drinkers
   - Origin of the water
   - Is the water receiving any kind of treatment? If yes, description and how often.
   - Type of floor: is the same for all the production areas? specify
2- General farm management
   - What type of food is used for pigs? Is the food the same for all ages? if not, when the food is changed from one age group to another? How many types of food?
   - Where and how the pig food is stored? Example, open warehouse, close warehouse, in bags, loose
   - Are all pigs confined or some free range? If yes, age and how many
   - Are the pigs moved to other corrals? If yes, how many times?
3- About the hogs
   - Total of: sows: boars: suckling piglets: weaners: growers: finishers: Overall:
   - Breed of the pigs:
   - Do you observe tail biting or ear chewing behavior?
   - Do you observe cannibalism (eat carcasses)?
   - Do you observe pigs eating other animals? Example rodents
   - Do you buy pigs: if yes, how often?
   - Do you buy semen or use your own boars?
4- Contact with other animals
   - Does the farm raise other animal species? Description
   - Do you have cats? If yes, how many?
   - Do you feed the cats with commercial food?

- Do you see stray cats? if yes how many
- Do you see rodents?
- Do you see birds or other wild animals? Do birds have access to the farm?

5- Pig health
   - Please indicate the percentage of abortions:
   - Does the farm practice a rodent control? How often and what is used? Example rodenticides, cats, traps
   - Does the farm practice an insect control? How often and what is used?
   - Are pig carcasses removed from the corrals?
   - Pig sanitary program:
     vaccines:
     deparasitation:
     cleaning routine:
6- Comments

APPENDIX III

AIII. 1 Description of farm management and characteristics

Table III.1 Description of the farm management and characteristics of farm 1-6 and pilot A.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6	Pilot A
Owned cats	0	3	2	20	15-20	10	30
Stray cats	yes	yes	no	yes	yes	yes	yes
Herd size	>600	<600	>600	<600	>600	>600	>600
Farm size	2ha	1.5ha	100ha	4ha	7ha	3.5ha	10ha
Cats/ha	0	2	0.02	5	3	3	3
Rodents	yes	no	no	yes	yes	yes	yes
Rodent-control [a]	yes	no	yes	yes	yes	yes	yes
Drinkers	automatic	automatic	automatic	automatic	automatic	automatic	automatic
Feeders [b]	automatic	open	open	automatic	open	open	automatic
% Aborts year	1.5	0	2	3	1	2	3
Water origin	Well	Well	Well	Well	Well	Well	Well
Types of food [c]	3	4	8	6	1	4	4
Water treatment	No	No	Yes quicklime	No	Yes chlorine	No	No
Pig movement [d]	2	2	3	2	2	2	2
Warehouse	open	open	closed	open	open	open	outside
Tail biting/ear chewing	yes	no	no	yes	yes	yes	no
Cannibalism [e]	no	no	no	no	no	no	no
Carnivorism [f]	no	no	no	yes	no	no	no
Wildlife access [g]	yes	yes	yes	yes	yes	yes	yes
Another livestock	yes	yes	no	yes	no	no	no
Nursery floor	High perforated	High perforated	High perforated	High perforated	High perforated	Low concrete	ND

Note farm pilot B is not included as is the same farm as farm 1

[a] Rodent control: farm 4 and 6 used cats, farm 1, 3 and 5, pilot B rodenticides.

[b] All automatic feeders were open to the environment (see figure A1)

[c] Types of food refers to how many types of food are used to feed the pigs depending on age and use (breeding, fattening…).

[d] Pig movement refers to how many times pigs are moved to another corral during the production cycle.

[e] Pig carcasses are removed by the workers.

[f] Farmers of farm 4 reported have observed the pigs eating rodents.

[g] All farms were open as in the example (figure A1).

Floors were concrete in all farms except in the nursery area.

[h] Farm1: cattle, sheep and goats; farm 2: free range chickens and turkeys; farm 4: turkeys and zebus.

Table AIII.2 Description of farm management and characteristics of farm 7-12.

	Farm 7	Farm 8	Farm 9	Farm 10	Farm 11	Farm 12
Owned cats	0	4	5	3	2	2
Stray cats	no	yes	yes	yes	yes	yes
Herd size	<600	<600	>600	>600	<600	<600
Farm size	2ha	2ha	1ha	2ha	2ha	8ha
Cats/ha	0	2	0.02	5	3	3
Rodents	yes	yes	no	no	yes	yes
Rodent-control [a]	yes	yes	no	yes	no	yes
Drinkers	automatic	automatic	automatic	automatic	automatic	automatic
Feeders [b]	open	automatic*	automatic*	open	automatic	automatic
% aborts	1	0	4	0	2.5	2
Water origin	Well	Well	Well	Well	Well	Well
Water treatment	No	Yes chlorine	No	No	No	No
Types of food [c]	1	5	5	4	6	6
Pig movement [d]	1	2	2	2	2	3
Warehouse	open	open	open	open	open	open
Tail biting/ear chewing	yes	no	no	no	yes	yes
Cannibalism [e]	no	no	no	no	no	no
Carnivorism [f]	no	no	no	no	no	yes
Another livestock [g]	yes	yes	yes	no	yes	no
Wildlife access [h]	yes	yes	yes	yes	yes	yes
Nursery floor	ND	ND	ND	ND	ND	ND

[a] Rodent control: farm 8 used cats for rodent control farm 7, 10 and 12 rodenticides.
[b] automatic feeders were open when * on the other cases were automatic and close to the environment.
[c] Types of food refers to how many types of food are used to feed the pigs depending on age and use (breeding, fattening…).
[d] Pig movement refers to how many times pigs are moved to another corral during the production cycle.
[e] Pig carcasses are removed by the workers.
[f] Farmers of farm 12 reported have observed the pigs eating rodents
[g] Farm 7: zebus, farm 8: chickens and zebus, farm 9: caged chickens and cattle, farm 11: cattle.
[h] All farms were open as in the example (figure A1).
Floors were concrete in all farms. Nursery floor was not a variable to analyse in these farms as only 20 week old pigs were sampled.

AIII.2 Example pictures of farm 1 and farm 2

Figure A1 Picture of farm1 (1). Details of the fattening area: feeders, concrete floor and free access to wildlife.

Figure A2 Picture of farm 1 (2). Example of the concrete floor and the presence of a rat in a pen in the fattening area (red arrow).

Figure A3 Picture of farm 1 (3). Example of the rat infestation (Red arrows).

Figure A4 Picture of farm 2 (1). Example of one of the cats of farm 2.

Figure A5 Picture of farm 2 (2). Example of open feeders.

APPENDIX IV

AIV.1 Translated protein sequence Alt-SAG2

Blue color highlights the samples which present the novel amino acid substitution (from lysine to glutamic acid in blue page 294) shared by 11 animals.

Red highlights amino acid substitutions derived from the archetypal types I, II and III.

Green color highlights novel, but not shared amino acid substitutions.

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GT1          ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
M49          ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
VEG          ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgRatMx4     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgRatMx5     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgcatMx6     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx2     ILCVVS RVAALGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx4     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx5     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx7     ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx10    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx14    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx15    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx19    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCVVGT MSFSKTTSLA SLALTGLFVV
TgPigMx20    ILCVVS RVAVLGTEL* LCTIAV*HNL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx25    ILCVVS RVAVLGTEL* LCTIAV*HNL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx28    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx29    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx30    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx35    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA TLALTGLFVV
TgPigMx37    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx38    NNNNVN NVAVLGTEL* LCTIAV*HNL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx39    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx40    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx44    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx46    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx47    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx50    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx52    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
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TgPigMx54    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
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TgPigMx59    ILCVVS RVAVLGTEL* SCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx60    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx61    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx62    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx63    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx64    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx65    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx66    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
TgPigMx72    ILCVVS RVAVLGTEL* LCTIAV*HLL SRSNLCLVGT MSFSKTTSLA SLALTGLFVV
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GT1          FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
M49          FKFALASTTE TPAPIECTAG ATKTVEAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
VEG          FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgRatMx4     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgRatMx5     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgCatMx6     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx2     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx4     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx5     FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVAFQCGDK LTISPSGEGD VFYGKECTDS
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TgPigMx25    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx28    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
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TgPigMx30    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx35    FKFALASTTE TPAPINCTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx37    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
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TgPigMx63    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
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TgPigMx65    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx66    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
TgPigMx72    FKFALASTTE TPAPIECTAG ATKTVDAPSS GSVVFQCGDK LTISPSGEGD VFYGKECTDS
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GT1          RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
M49          RKLTTVLPGA VLKAKVEQPP KGPATYTLSY DGTPEK
VEG          RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgRatMx4     RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgRatMx5     RKLTTVLPGA VLTANVEQPP KGPATYTNNN DGNNNN
TgCatMx6     RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx2     RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx4     RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx5     RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx7     RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx10    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
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TgPigMx15    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx19    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx20    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx25    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx28    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx29    RRLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx30    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx35    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPNK
TgPigMx37    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx38    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx39    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx40    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx44    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx46    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx47    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx50    RKLTTVLPGA VLTAKVEQPP KGPATCTLSY DGTPEK
TgPigMx52    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx53    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx54    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx55    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx57    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx58    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx59    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx60    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx61    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx62    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx63    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx64    RKLTTVLPGA VSTAKVEQPP KGPATYTLSY DGTPEK
TgPigMx65    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
TgPigMx66    RKLTTVLPGA VLTAKVEQPP KGPATYTLSY DGTPEE
TgPigMx72    RKLTTVLPGA VLTAKVQQPA KGPATYTLSY DGTPEK
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APPENDIX V

AV.1 List of pig samples with serological and PCR results

Pig	Age[1]	Farm	MAT[2]	IDScreen[3]	DT[3]	PCR Blood[4]	PCR Tongue[4]	Bioassay[5]	Culture[6]	Chapter 2, 4 Tables [7]	Genotype
1	16	Pilot A	–	np	np	–	–	np	nd/c	1a	np
2	16	Pilot A	–	np	np	–	–	np	nd/c	2a	np
3	16	Pilot A	–	np	np	–	+	np	nd/c	3a	TgPigMx40
4	17	Pilot A	–	np	np	–	–	np	np	4a	np
5	16	Pilot A	–	np	np	–	+	np	np	5a	TgPigMx41
6	16	Pilot A	–	np	np	–	–	np	np	6a	np
7	24	Farm 1	–	np	np	–	–	–	–	7a/9b	np
8	24	Farm 1	–	np	np	–	+	+/-	–	8a/10b	TgPigMx64
9	25	Farm 1	1:25	+	np	+	–	+/nd	–	1/9a/3b	TgPigMx61
10	12	Pilot A	–	np	np	–	np	np	np		np
11	12	Pilot A	–	np	np	–	np	np	np		np
12	10	Pilot A	–	np	np	–	np	np	np		np
13	14	Pilot A	–	np	np	–	np	np	np		np
14	10	Pilot A	–	np	np	–	np	np	np		np
15	10	Pilot A	–	np	np	–	np	np	np		np
16	14	Pilot A	–	np	np	+	np	np	np		np
17	12	Pilot A	–	np	np	–	np	np	np		np
18	14	Pilot A	–	np	np	–	np	np	np		np
19	14	Pilot A	–	np	np	–	np	np	np		np
20	12	Pilot A	–	np	np	–	np	np	np		np

21	14	Pilot A	–	np	np	–	np	np	np		np
22	8	Pilot A	–	np	np	+	np	np	np		np
23	10	Pilot A	–	np	np	–	np	np	np		np
24	12	Pilot A	1:1000	+	np	–	np	np	np	2	np
25	8	Pilot A	–	np	np	–	np	np	np		np
26	8	Pilot A	–	np	np	–	np	np	np		np
27	10	Pilot A	–	np	np	–	np	np	np		np
28	8	Pilot A	–	np	np	–	np	np	np		np
29	8	Pilot A	–	np	np	–	np	np	np		np
30	26	Farm 1	–	np	np	–	np	np	np		np
31	26	Farm 1	–	np	np	+	np	np	np		np
32	26	Farm 1	–	np	np	–	+	+/-	np	11b	TgPigMx65
33	26	Farm 1	–	np	np	–	–	nd/–	np	12b	np
34	16	Farm 1	–	np	np	–	np	np	np		np
35	16	Farm 1	–	np	np	–	np	np	np		np
36	16	Farm 1	–	np	np	–	np	np	np		np
37	16	Farm 1	–	np	np	–	np	np	np		np
38	16	Farm 1	–	np	np	–	np	np	np		np
39	20	Farm 1	–	np	np	–	np	np	np		np
40	20	Farm 1	–	np	np	–	np	np	np		np
41	20	Farm 1	1:10	+	np	–	np	np	np	3	np
42	20	Farm 1	–	np	np	–	np	np	np		np
43	20	Farm 1	–	np	np	–	np	np	np		np
44	25	Farm 1	1:10	–	np	–	np	np	np		np
45	25	Farm 1	–	np	np	–	np	np	np		np
46	25	Farm 1	1:10	–	np	–	np	np	np		np
47	25	Farm 1	1:10	–	np	–	np	np	np		np

48	25	Farm 1	1:10	–	np	–	np	np	np
49	25	Farm 1	1:10	–	np	–	np	np	np
50	25	Farm 1	–	np	np	–	np	np	np
51	25	Farm 1	–	np	np	–	np	np	np
52	25	Farm 1	1:4	–	np	–	np	np	np
53	25	Farm 1	1:4	–	np	–	np	np	np
54	25	Farm 1	–	–	np	–	–	S	13b	np
55	25	Farm 1	–	–	np	–	–	S/nd	14b	np
56	8	Farm 1	–	np	np	–	np	np		np
57	8	Farm 1	–	np	np	–	np	np		np
58	8	Farm 1	–	np	np	–	np	np		np
59	8	Farm 1	–	np	np	+	np	np		TgPigMx21
60	8	Farm 1	–	np	np	+	np	np		TgPigMx20
61	8	Farm 1	–	np	np	–	np	np		np
62	8	Farm 1	–	np	np	–	np	np		np
63	8	Farm 1	–	np	np	+	np	np		TgPigMx22
64	8	Farm 1	–	np	np	+	np	np		TgPigMx23
65	8	Farm 1	–	np	np	–	np	np		np
66	8	Farm 1	–	np	np	+	np	np		TgPigMx25
67	8	Farm 1	–	np	np	–	np	np		np
68	8	Farm 1	–	np	np	–	np	np		np
69	8	Farm 1	–	np	np	+	np	np		TgPigMx26
70	8	Farm 1	–	np	np	+	np	np		TgPigMx27
71	8	Farm 1	–	np	np	–	np	np		np
72	12	Farm 1	–	np	np	–	np	np		np
73	12	Farm 1	–	np	np	–	np	np		np
74	12	Farm 1	–	np	np	–	np	np		np

75	12	Farm 1	–	np	np	–	np	np	np	np
76	12	Farm 1	–	np	np	–	np	np	np	np
77	12	Farm 1	–	np	np	+	np	np	np	TgPigMx24
78	12	Farm 1	–	np	np	–	np	np	np	np
79	12	Farm 1	–	np	np	–	np	np	np	np
80	12	Farm 1	–	np	np	–	np	np	np	np
81	12	Farm 1	–	np	np	+	np	np	np	TgPigMx28
82	12	Farm 1	1:4	–	np	–	np	np	np	np
83	12	Farm 1	–	np	np	+	np	np	np	TgPigMx32
84	12	Farm 1	–	np	np	–	np	np	np	np
85	12	Farm 1	–	np	np	–	np	np	np	np
86	12	Farm 1	–	np	np	–	np	np	np	np
87	12	Farm 1	–	np	np	–	np	np	np	np
88	16	Farm 1	–	np	np	+	np	np	np	TgPigMx30
89	16	Farm 1	–	np	np	–	np	np	np	np
90	16	Farm 1	–	np	np	+	np	np	np	TgPigMx29
91	16	Farm 1	–	np	np	+	np	np	np	TgPigMx31
92	16	Farm 1	–	np	np	–	np	np	np	np
93	16	Farm 1	–	np	np	–	np	np	np	np
94	16	Farm 1	–	np	np	–	np	np	np	np
95	16	Farm 1	–	np	np	+	np	np	np	TgPigMx33
96	16	Farm 1	–	np	np	–	np	np	np	np
97	16	Farm 1	–	np	np	–	np	np	np	np
98	16	Farm 1	–	np	np	–	np	np	np	np
99	16	Farm 1	–	np	np	–	np	np	np	np
100	16	Farm 1	–	np	np	–	np	np	np	np
101	16	Farm 1	–	np	np	–	np	np	np	np

102	16	Farm 1	–	np	np	–	np	np	np		np
103	16	Farm 1	–	np	np	–	np	np	np		np
104	20	Farm 1	–	np	np	–	np	np	np		np
105	20	Farm 1	–	np	np	+	np	np	np		TgPigMx34
106	20	Farm 1	–	np	np	–	np	np	np		np
107	20	Farm 1	–	np	np	–	np	np	np		np
108	20	Farm 1	–	np	np	–	np	np	np		np
109	20	Farm 1	–	np	np	–	np	np	np		np
110	20	Farm 1	–	np	np	+	np	np	np		TgPigMx35
111	20	Farm 1	–	np	np	–	np	np	np		np
112	20	Farm 1	–	np	np	+	np	np	np		TgPigMx36
113	20	Farm 1	–	np	np	+	np	np	np		TgPigMx37
114	20	Farm 1	–	np	np	–	np	np	np		np
115	20	Farm 1	–	np	np	–	np	np	np		np
116	20	Farm 1	1:4	–	np	+	np	np	np		TgPigMx38
117	20	Farm 1	–	np	np	–	np	np	np		np
118	20	Farm 1	–	np	np	–	np	np	np		np
119	20	Farm 1	–	np	np	+	np	np	np		TgPigMx39
120	25	Farm 1	–	–	np	–	+	S/nd	np	15b	TgPigMx52
121	25	Backyard	–	–	np	–	–	–	np	16b	np
122	8	Farm 2	–	np	np	–	np	np	np		np
123	8	Farm 2	–	np	np	–	np	np	np		np
124	8	Farm 2	–	np	np	–	np	np	np		np
125	8	Farm 2	–	np	np	+	np	np	np		TgPigMx1
126	8	Farm 2	–	np	np	–	np	np	np		np
127	8	Farm 2	–	np	np	+	np	np	np		TgPigMx3
128	8	Farm 2	–	np	np	+	np	np	np		TgPigMx4

129	8	Farm 2	–	np	np	–	np	np	np		np
130	8	Farm 2	–	np	np	+	np	np	np		TgPigMx6
131	8	Farm 2	–	np	np	–	np	np	np		np
132	8	Farm 2	–	np	np	–	np	np	np		np
133	8	Farm 2	–	np	np	–	np	np	np		np
134	8	Farm 2	–	np	np	–	np	np	np		np
135	8	Farm 2	–	np	np	–	np	np	np		np
136	8	Farm 2	–	np	np	–	np	np	np		np
137	8	Farm 2	–	np	np	–	np	np	np		np
138	12	Farm 2	–	np	np	–	np	np	np		np
139	12	Farm 2	–	np	np	–	np	np	np		np
140	12	Farm 2	–	–	–	–	np	np	np		np
141	12	Farm 2	–	–	–	–	np	np	np		np
142	12	Farm 2	–	np	np	–	np	np	np		np
143	12	Farm 2	–	+	–	–	np	np	np	4	np
144	12	Farm 2	–	np	np	–	np	np	np		np
145	12	Farm 2	–	np	np	–	np	np	np		np
146	12	Farm 2	–	–	–	–	np	np	np		np
147	12	Farm 2	–	–	–	–	np	np	np		np
148	12	Farm 2	–	np	np	–	np	np	np		np
149	12	Farm 2	–	–	–	+	np	np	np		TgPigMx5
150	12	Farm 2	–	np	np	+	np	np	np		TgPigMx7
151	12	Farm 2	–	np	np	–	np	np	np		np
152	12	Farm 2	–	–	–	+	np	np	np		TgPigMx8
153	12	Farm 2	–	np	np	+	np	np	np		TgPigMx9
154	16	Farm 2	–	–	–	–	np	np	np		np
155	16	Farm 2	–	+	–	+	np	np	np	5	TgPigMx10

156	16	Farm 2	–	–	–	+	np	np	np		TgPigMx12
157	16	Farm 2	–	np	np	–	np	np	np		np
158	16	Farm 2	–	–	–	+	np	np	np		TgPigMx11
159	16	Farm 2	–	np	np	–	np	np	np		np
160	16	Farm 2	–	np	np	+	np	np	np		TgPigMx13
161	16	Farm 2	–	np	np	–	np	np	np		np
162	16	Farm 2	–	np	np	–	np	np	np		np
163	16	Farm 2	–	np	np	+	np	np	np		TgPigMx14
164	16	Farm 2	–	np	np	+	np	np	np		TgPigMx15
165	16	Farm 2	–	np	np	–	np	np	np		np
166	16	Farm 2	–	np	np	–	np	np	np		np
167	16	Farm 2	–	np	np	+	np	np	np		TgPigMx16
168	16	Farm 2	–	–	–	–	np	np	np		np
169	16	Farm 2	–	np	np	–	np	np	np		np
170	20	Farm 2	–	–	–	–	np	np	np		np
171	20	Farm 2	–	np	np	–	np	np	np		np
172	20	Farm 2	–	–	–	+	np	np	np		TgPigMx18
173	20	Farm 2	–	–	–	–	np	np	np		np
174	20	Farm 2	–	np	np	+	np	np	np		TgPigMx19
175	20	Farm 2	–	–	–	–	np	np	np		np
176	20	Farm 2	–	–	–	–	np	np	np		np
177	20	Farm 2	–	–	–	+	np	np	np		TgPigMx17
178	20	Farm 2	–	–	–	+	np	np	np		TgPigMx2
179	20	Farm 2	–	np	np	–	np	np	np		np
180	20	Farm 2	–	np	np	–	np	np	np		np
181	20	Farm 2	–	np	np	–	np	np	np		np
182	20	Farm 2	–	np	np	–	np	np	np		np

183	20	Farm 2	–	–	–	np	np	np		np
184	20	Farm 2	–	np	np	np	np	np		np
185	20	Farm 2	–	np	np	–	np	np		np
186*	16	ng	–	+	np	–	–	np		np
187	16	ng	–	np	np	–	–	np		np
188	16	ng	–	–	np	–	+	np		np
189	16	ng	–	np	np	–	–	np		np
190	16	ng	–	np	np	–	–	np		np
191	16	ng	–	–	np	+	–	np		np
192	25	Backyard	–	np	np	–	+	np	17b	TgPigMx63
193	25	Farm 1	–	np	np	+	–	np	18b	np
194	25	Farm 1	–	–	np	–	–	np	19b	np
195	8	Farm 3	–	np	np	–	np	np		np
196	8	Farm 3	–	np	np	–	np	np		np
197	8	Farm 3	–	np	np	–	np	np		np
198	8	Farm 3	–	np	np	–	np	np		np
199	8	Farm 3	–	np	np	–	np	np		np
200	8	Farm 3	–	np	np	–	np	np		np
201	8	Farm 3	–	np	np	–	np	np		np
202	8	Farm 3	–	np	np	–	np	np		np
203	8	Farm 3	–	np	np	–	np	np		np
204	8	Farm 3	–	np	np	–	np	np		np
205	8	Farm 3	–	np	np	–	np	np		np
206	8	Farm 3	–	np	np	+	np	np		np
207	8	Farm 3	–	np	np	–	np	np		np
208	8	Farm 3	–	np	np	–	np	np		np
209	8	Farm 3	–	np	np	–	np	np		np

210	8	Farm 3	–	np	np	+	np	np	np	np
211	12	Farm 3	–	np	np	–	np	np	np	np
212	12	Farm 3	–	np	np	–	np	np	np	np
213	12	Farm 3	–	np	np	–	np	np	np	np
214	12	Farm 3	–	np	np	–	np	np	np	np
215	12	Farm 3	–	np	np	–	np	np	np	np
216	12	Farm 3	–	np	np	–	np	np	np	np
217	12	Farm 3	–	np	np	–	np	np	np	np
218	12	Farm 3	–	np	np	–	np	np	np	np
219	12	Farm 3	–	np	np	–	np	np	np	np
220	12	Farm 3	–	np	np	–	np	np	np	np
221	12	Farm 3	–	np	np	+	np	np	np	np
222	12	Farm 3	–	np	np	–	np	np	np	np
223	12	Farm 3	–	np	np	+	np	np	np	np
224	12	Farm 3	–	np	np	+	np	np	np	np
225	12	Farm 3	–	np	np	–	np	np	np	np
226	12	Farm 3	–	np	np	+	np	np	np	np
227	16	Farm 3	–	–	–	–	np	np	np	np
228	16	Farm 3	–	np	np	–	np	np	np	np
229	16	Farm 3	–	np	np	–	np	np	np	np
230	16	Farm 3	–	np	np	–	np	np	np	np
231	16	Farm 3	–	–	–	–	np	np	np	np
232	16	Farm 3	–	np	np	–	np	np	np	np
233	16	Farm 3	–	np	np	+	np	np	np	np
234	16	Farm 3	–	np	np	–	np	np	np	np
235	16	Farm 3	–	np	np	–	np	np	np	np
236	16	Farm 3	–	np	np	+	np	np	np	np

237	16	Farm 3	–	np	np	–	np	np	np			np
238	16	Farm 3	–	np	np	–	np	np	np			np
239	16	Farm 3	–	np	np	–	np	np	np			np
240	16	Farm 3	1:4	–	4 IU/ml	–	np	np	np	6		np
241	16	Farm 3	–	np	np	–	np	np	np			np
242	16	Farm 3	–	np	np	–	np	np	np			np
243	20	Farm 3	–	np	np	–	np	np	np			np
244	20	Farm 3	–	np	np	–	np	np	np			np
245	20	Farm 3	–	–	–	–	np	np	np			np
246	20	Farm 3	–	np	np	–	np	np	np			np
247	20	Farm 3	–	–	np	–	np	np	np			np
248	20	Farm 3	–	np	np	–	np	np	np			np
249	20	Farm 3	–	np	np	–	np	np	np			np
250	20	Farm 3	–	np	np	–	np	np	np			np
251	20	Farm 3	–	np	np	–	np	np	np			np
252	20	Farm 3	–	np	np	–	np	np	np			np
253	20	Farm 3	–	np	np	–	np	np	np			np
254	20	Farm 3	–	np	np	–	np	np	np			np
255	20	Farm 3	–	np	np	–	np	np	np			np
256	20	Farm 3	–	np	np	–	np	np	np			np
257	20	Farm 3	–	np	np	+	np	np	np			np
258	20	Farm 3	–	np	np	–	np	np	np			np
259	25	Farm 1	1:4	–	np	–	–	nd/–	np	8b		np
260	24	Farm 1	1:32000	+	500 IU/ml	+	+	nd/-	np	7,1b		TgPigMx53
261	25	Farm 1	1:100	+	32 IU/ml	–	+	nd/–	np	8,2b		TgPigMx59
262	8	Farm 4	–	np	np	–	np	np	np			np
263	8	Farm 4	–	np	np	+	np	np	np			np

264	8	Farm 4	–	np	np	–	np	np	np		np
265	8	Farm 4	–	np	np	+	np	np	np		np
266	8	Farm 4	–	np	np	–	np	np	np		np
267	8	Farm 4	–	np	np	–	np	np	np		np
268	8	Farm 4	–	np	np	–	np	np	np		np
269	8	Farm 4	–	np	np	–	np	np	np		np
270	8	Farm 4	–	np	np	–	np	np	np		np
271	8	Farm 4	–	np	np	–	np	np	np		np
272	8	Farm 4	–	np	np	–	np	np	np		np
273	8	Farm 4	–	np	np	–	np	np	np		np
274	8	Farm 4	–	np	np	+	np	np	np		np
275	8	Farm 4	–	np	np	–	np	np	np		np
276	8	Farm 4	–	np	np	–	np	np	np		np
277	8	Farm 4	–	np	np	–	np	np	np		np
278	12	Farm 4	–	–	–	–	np	np	np		np
279	12	Farm 4	–	np	np	–	np	np	np		np
280	12	Farm 4	–	np	np	+	np	np	np		np
281	12	Farm 4	–	–	–	–	np	np	np		np
282	12	Farm 4	–	–	–	+	np	np	np		np
283	12	Farm 4	–	np	np	–	np	np	np		np
284	12	Farm 4	1:4	–	2 IU/ml	+	np	np	np	9	np
285	12	Farm 4	–	–	–	–	np	np	np		np
286	12	Farm 4	–	np	np	+	np	np	np		np
287	12	Farm 4	–	np	np	+	np	np	np		np
288	12	Farm 4	–	np	np	–	np	np	np		np
289	12	Farm 4	–	np	np	–	np	np	np		np
290	12	Farm 4	–	np	np	–	np	np	np		np

291	12	Farm 4	–	–	–	–	np	np	np		np
292	12	Farm 4	1:25	–	8 IU/ml	–	np	np	np	10	np
293	12	Farm 4	–	np	np	+	np	np	np		np
294	16	Farm 4	–	np	np	–	np	np	np		np
295	16	Farm 4	–	–	–	–	np	np	np		np
296	16	Farm 4	–	np	np	+	np	np	np		np
297	16	Farm 4	–	np	np	+	np	np	np		np
298	16	Farm 4	–	–	–	+	np	np	np		np
299	16	Farm 4	–	np	np	–	np	np	np		np
300	16	Farm 4	–	np	np	–	np	np	np		np
301	16	Farm 4	–	–	–	–	np	np	np		np
302	16	Farm 4	–	np	np	–	np	np	np		np
303	16	Farm 4	–	np	np	–	np	np	np		np
304	16	Farm 4	–	np	np	–	np	np	np		np
305	16	Farm 4	–	np	np	+	np	np	np		np
306	16	Farm 4	–	np	np	–	np	np	np		np
307	16	Farm 4	–	np	np	–	np	np	np		np
308	16	Farm 4	–	np	np	+	np	np	np		np
309	16	Farm 4	–	np	np	–	np	np	np		np
310	20	Farm 4	–	np	np	–	np	np	np		np
311	20	Farm 4	–	–	–	–	np	np	np		np
312	20	Farm 4	–	–	–	–	np	np	np		np
313	20	Farm 4	–	np	np	–	np	np	np		np
314	20	Farm 4	–	np	np	–	np	np	np		np
315	20	Farm 4	–	np	np	–	np	np	np		np
316	20	Farm 4	1:10	–	np	+	np	np	np		np
317	20	Farm 4	–	np	np	–	np	np	np		np

318	20	Farm 4	–	np	np	–	np	np	np		np
319	20	Farm 4	–	–	–	–	np	np	np		np
320	20	Farm 4	–	np	np	–	np	np	np		np
321	20	Farm 4	1:10	–	np	–	np	np	np		np
322	20	Farm 4	–	np	np	+	np	np	np		np
323	20	Farm 4	–	np	np	–	np	np	np		np
324	20	Farm 4	–	np	np	+	np	np	np		np
325	20	Farm 4	–	np	np	–	np	np	np		np
326	8	Farm 5	–	np	np	–	np	np	np		np
327	8	Farm 5	–	np	np	–	np	np	np		np
328	8	Farm 5	–	np	np	–	np	np	np		np
329	8	Farm 5	–	np	np	–	np	np	np		np
330	8	Farm 5	–	np	np	–	np	np	np		np
331	8	Farm 5	–	np	np	–	np	np	np		np
332	8	Farm 5	–	np	np	–	np	np	np		np
333	8	Farm 5	–	np	np	–	np	np	np		np
334	8	Farm 5	–	np	np	–	np	np	np		np
335	8	Farm 5	–	np	np	–	np	np	np		np
336	8	Farm 5	–	np	np	–	np	np	np		np
337	8	Farm 5	–	np	np	–	np	np	np		np
338	8	Farm 5	–	np	np	–	np	np	np		np
339	8	Farm 5	–	np	np	+	np	np	np		np
340	8	Farm 5	–	np	np	+	np	np	np		np
341	8	Farm 5	–	np	np	+	np	np	np		np
342	12	Farm 5	–	np	np	–	np	np	np		np
343	12	Farm 5	–	np	np	+	np	np	np		np
344	12	Farm 5	–	np	np	–	np	np	np		np

345	12	Farm 5	–	np	np	+	np	np		np
346	12	Farm 5	–	np	np	–	np	np		np
347	12	Farm 5	–	np	np	–	np	np		np
348	12	Farm 5	–	np	np	+	np	np		np
349	12	Farm 5	1:10	–	np	–	np	np		np
350	12	Farm 5	–	np	np	+	np	np		np
351	12	Farm 5	–	np	np	+	np	np		np
352	12	Farm 5	–	np	np	+	np	np		np
353	12	Farm 5	–	np	np	–	np	np		np
354	12	Farm 5	–	np	np	+	np	np		np
355	12	Farm 5	–	np	np	–	np	np		np
356	12	Farm 5	–	np	np	–	np	np		np
357	12	Farm 5	–	np	np	–	np	np		np
358	16	Farm 5	–	np	np	+	np	np		np
359	16	Farm 5	–	np	np	+	np	np		np
360	16	Farm 5	–	np	np	–	np	np		np
361	16	Farm 5	–	np	np	–	np	np		np
362	16	Farm 5	–	np	np	–	np	np		np
363	16	Farm 5	–	np	np	–	np	np		np
364	16	Farm 5	–	np	np	+	np	np		np
365	16	Farm 5	–	np	np	+	np	np		np
366	16	Farm 5	–	np	np	–	np	np		np
367	16	Farm 5	–	np	np	–	np	np		np
368	16	Farm 5	–	np	np	–	np	np		np
369	16	Farm 5	–	np	np	–	np	np		np
370	16	Farm 5	–	np	np	–	np	np		np
371	16	Farm 5	–	np	np	–	np	np		np

372	16	Farm 5	–	np	np	–	np	np	np		np
373	16	Farm 5	–	np	np	–	np	np	np		np
374	20	Farm 5	–	np	np	+	np	np	np		np
375	20	Farm 5	–	–	–	–	np	np	np		np
376	20	Farm 5	–	np	np	–	np	np	np		np
377	20	Farm 5	–	np	np	–	np	np	np		np
378	20	Farm 5	–	np	np	–	np	np	np		np
379	20	Farm 5	–	–	–	–	np	np	np		np
380	20	Farm 5	–	np	np	–	np	np	np		np
381	20	Farm 5	–	np	np	–	np	np	np		np
382	20	Farm 5	–	np	np	–	np	np	np		np
383	20	Farm 5	–	np	np	–	np	np	np		np
384	20	Farm 5	–	np	np	–	np	np	np		np
385	20	Farm 5	–	np	np	–	np	np	np		np
386	20	Farm 5	–	np	np	+	np	np	np		np
387	20	Farm 5	–	np	np	–	np	np	np		np
388	20	Farm 5	–	–	–	–	np	np	np		np
389	20	Farm 5	–	–	–	–	np	np	np		np
390	18	Farm 7	–	–	–	+	np	np	np		TgPigMx43
391	18	Farm 7	–	np	np	+	np	np	np		TgPigMx42
392	18	Farm 7	–	np	np	–	np	np	np		np
393	18	Farm 7	–	–	–	+	np	np	np		TgPigMx44
394	18	Farm 7	–	np	np	+	np	np	np		TgPigMx45
395	20	Farm 7	–	np	np	–	np	np	np		np
396	20	Farm 7	–	np	np	–	np	np	np		np
397	20	Farm 7	–	np	np	–	np	np	np		np
398	20	Farm 7	–	np	np	+	np	np	np		TgPigMx46

399	20	Farm 7	–	np	np	+	np	np		TgPigMx47
400	20	Farm 8	–	np	np	–	np	np		np
401	20	Farm 8	–	np	np	+	np	np		TgPigMx48
402	20	Farm 8	–	np	np	–	np	np		TgPigMx50
403	20	Farm 8	–	np	np	–	np	np		np
404	20	Farm 8	–	–	–	–	np	np		np
405	20	Farm 8	–	np	np	–	np	np		np
406	20	Farm 8	–	np	np	+	np	np		TgPigMx49
407	20	Farm 8	–	np	np	+	np	np		np
408	20	Farm 8	–	np	np	–	np	np		np
409	20	Farm 8	–	np	np	–	np	np		np
410	20	Farm 9	–	np	np	–	np	np		np
411	20	Farm 9	–	np	np	+	np	np		np
412	20	Farm 9	–	np	np	–	np	np		np
413	20	Farm 9	1:4	–	np	–	np	np		np
414	20	Farm 9	–	np	np	–	np	np		np
415	20	Farm 9	–	np	np	–	np	np		np
416	20	Farm 9	–	np	np	+	np	np		np
417	20	Farm 9	–	np	np	–	np	np		np
418	20	Farm 9	–	np	np	–	np	np		np
419	20	Farm 9	–	np	np	–	np	np		np
420	16	Backyard	–	np	np	–	nd	np	20b	np
421	16	Backyard	–	np	np	–	–	np	21b	np
422	16	Backyard	–	np	np	–	–	np	22b	np
423	20	Farm 10	–	np	np	–	np	np		np
424	20	Farm 10	–	np	np	–	np	np		np
425	20	Farm 10	–	np	np	–	np	np		np

426	20	Farm 10	–	np	np	–	np	np	np		np
427	20	Farm 10	–	np	np	–	np	np	np		np
428	20	Farm 10	–	np	np	–	np	np	np		np
429	20	Farm 10	–	np	np	–	np	np	np		np
430	20	Farm 10	–	np	np	–	np	np	np		np
431	20	Farm 10	–	np	np	–	np	np	np		np
432	20	Farm 10	–	–	–	–	np	np	np		np
433	20	Farm 11	–	np	np	–	np	np	np		np
434	20	Farm 11	1:25	–	np	–	np	np	np	11	np
435	20	Farm 11	–	np	np	–	np	np	np		np
436	20	Farm 11	–	np	np	–	np	np	np		np
437	20	Farm 11	1:25	–	np	+	np	np	np	12	np
438	20	Farm 11	–	np	np	–	np	np	np		np
439	20	Farm 11	–	np	np	+	np	np	np		np
440	20	Farm 11	–	np	np	–	np	np	np		np
441	20	Farm 11	–	np	np	–	np	np	np		np
442	20	Farm 11	–	np	np	+	np	np	np		np
443	20	Farm 1	–	–	np	–	+	nd/–	np	23b	TgPigMx58
444	16	Backyard	–	np	np	–	–	–	np	24b	np
445	8	Farm 6	–	np	np	–	np	np	np		np
446	8	Farm 6	–	np	np	+	np	np	np		np
447	8	Farm 6	–	np	np	–	np	np	np		np
448	8	Farm 6	–	np	np	–	np	np	np		np
449	8	Farm 6	–	np	np	–	np	np	np		np
450	8	Farm 6	–	np	np	–	np	np	np		np
451	8	Farm 6	–	np	np	–	np	np	np		np
452	8	Farm 6	–	np	np	–	np	np	np		np

453	8	Farm 6	–	np	np	–	np	np	np		np
454	8	Farm 6	1:50	–	np	–	np	np	np	13	np
455	8	Farm 6	–	np	np	–	np	np	np		np
456	8	Farm 6	–	np	np	–	np	np	np		np
457	8	Farm 6	–	np	np	–	np	np	np		np
458	8	Farm 6	–	np	np	–	np	np	np		np
459	8	Farm 6	–	np	np	–	np	np	np		np
460	8	Farm 6	–	np	np	–	np	np	np		np
461	12	Farm 6	–	np	np	–	np	np	np		np
462	12	Farm 6	–	np	np	–	np	np	np		np
463	12	Farm 6	–	np	np	–	np	np	np		np
464	12	Farm 6	–	np	np	–	np	np	np		np
465	12	Farm 6	–	np	np	–	np	np	np		np
466	12	Farm 6	–	np	np	–	np	np	np		np
467	12	Farm 6	–	np	np	–	np	np	np		np
468	12	Farm 6	–	np	np	–	np	np	np		np
469	12	Farm 6	–	np	np	–	np	np	np		np
470	12	Farm 6	–	np	np	–	np	np	np		np
471	12	Farm 6	–	np	np	+	np	np	np		np
472	12	Farm 6	–	np	np	–	np	np	np		np
473	12	Farm 6	–	np	np	+	np	np	np		np
474	12	Farm 6	–	np	np	+	np	np	np		np
475	12	Farm 6	–	np	np	–	np	np	np		np
476	12	Farm 6	–	np	np	–	np	np	np		np
477	16	Farm 6	–	np	np	–	np	np	np		np
478	16	Farm 6	–	np	np	–	np	np	np		np
479	16	Farm 6	–	np	np	–	np	np	np		np

480	16	Farm 6	–	np	np	–	np	np	np		np
481	16	Farm 6	–	np	np	–	np	np	np		np
482	16	Farm 6	–	np	np	+	np	np	np		np
483	16	Farm 6	–	np	np	–	np	np	np		np
484	16	Farm 6	–	np	np	+	np	np	np		np
485	16	Farm 6	–	np	np	–	np	np	np		np
486	16	Farm 6	–	np	np	–	np	np	np		np
487	16	Farm 6	–	np	np	–	np	np	np		np
488	16	Farm 6	–	np	np	–	np	np	np		np
489	16	Farm 6	–	np	np	–	np	np	np		np
490	16	Farm 6	–	np	np	–	np	np	np		np
491	16	Farm 6	–	np	np	–	np	np	np		np
492	16	Farm 6	–	np	np	–	np	np	np		np
493	20	Farm 6	–	np	np	–	np	np	np		np
494	20	Farm 6	–	np	np	–	np	np	np		np
495	20	Farm 6	–	np	np	–	np	np	np		np
496	20	Farm 6	1:4	–	np	–	np	np	np		np
497	20	Farm 6	–	np	np	+	np	np	np		np
498	20	Farm 6	1:4	+	np	–	np	np	np	14	np
499	20	Farm 6	–	np	np	–	np	np	np		np
500	20	Farm 6	1:4	+	np	+	np	np	np	15	np
501	20	Farm 6	–	np	np	–	np	np	np		np
502	20	Farm 6	–	np	np	–	np	np	np		np
503	20	Farm 6	–	–	–	–	np	np	np		np
504	20	Farm 6	1:10	–	np	–	np	np	np		np
505	20	Farm 6	–	np	np	–	np	np	np		np
506	20	Farm 6	–	np	np	–	np	np	np		np

507	20	Farm 6	–	np	np	–	np	np	np		np
508	20	Farm 6	–	np	np	–	np	np	np		np
509	20	Farm1	1:4	np	np	–	–	np	np		np
510	20	Farm 12	–	np	np	+	np	np	np		np
511	20	Farm 12	–	np	np	–	np	np	np		np
512	20	Farm 12	–	np	np	–	np	np	np		np
513	20	Farm 12	–	np	np	–	np	np	np		np
514	20	Farm 12	–	np	np	+	np	np	np		np
515	20	Farm 12	–	np	np	–	np	np	np		np
516	20	Farm 12	–	np	np	–	np	np	np		np
517	20	Farm 12	–	np	np	–	np	np	np		np
518	20	Farm 12	–	np	np	–	np	np	np		np
519	20	Farm 12	–	np	np	–	np	np	np		np
520	25	Farm 1	–	np	np	–	+	nd/–	np	25b	TgPigMx54
521	25	Farm 1	–	np	np	–	–	nd/–	np	26b	np
522	25	Farm 1	–	np	np	–	–	nd/–	np	27b	np
523	25	Farm 1	–	np	np	–	–	nd/–	np	28b	np
524	25	Farm 1	–	np	np	–	–	–	np	29b	np
525	25	Farm 1	–	np	np	–	–	–	np	30b	np
526	25	Farm 1	–	np	np	+	+	nd	np	31b	TgpigMx51
527	25	Farm 1	–	np	np	–	–	nd	np	32b	np
528	25	Farm 1	–	+	np	–	+	–	np	16, 33b	TgPigMx62
529	8	Farm 1	–	np	np	+	np	nd/–	nd/c	1d	np
530	8	Farm 1	–	np	np	–	np	np	nd/c		np
531	8	Farm 1	–	np	np	–	np	np	nd/c		np
532	8	Farm 1	–	np	np	+	np	nd/+	nd/c	2d	np
533	8	Farm 1	–	np	np	–	np	np	nd/c		np

534	8	Farm 1	–	np	np	–	np	nd/c	
535	8	Farm 1	–	np	np	–	np	nd/c	
536	8	Farm 1	–	np	np	–	np	nd/c	
537	8	Farm 1	–	np	np	–	np	nd/c	
538	8	Farm 1	–	np	np	–	np	nd/c	
539	8	Farm 1	–	np	np	–	np	nd/c	
540	8	Farm 1	–	np	np	–	np	nd/c	
541	8	Farm 1	–	np	np	+	nd	nd/c	3d
542	8	Farm 1	–	np	np	+	np	nd/–	nd/c	4d
543	8	Farm 1	–	np	np	–	np	np	nd/c	
544	8	Farm 1	–	np	np	–	np	np	nd/c	
545	8	Farm 1	–	np	np	+	np	nd/–	nd/c	5d
546	8	Farm 1	–	np	np	+	np	nd	nd/c	6d
547	8	Farm 1	–	np	np	–	np	np	nd/c	
548	8	Farm 1	–	np	np	–	np	np	nd/c	
549	9	Farm 1	–	np	np	–	np	np	nd/c	
550	9	Farm 1	–	np	np	–	np	np	nd/c	
551	9	Farm 1	–	np	np	–	np	np	nd/c	
552	9	Farm 1	–	np	np	–	np	np	nd/c	
553	9	Farm 1	–	np	np	–	np	np	nd/c	
554	9	Farm 1	–	np	np	–	np	np	nd/c	
555	9	Farm 1	–	np	np	–	np	np	nd/c	
556	9	Farm 1	–	–	np	–	np	np	nd/c	
557	9	Farm 1	–	np	np	–	np	–	nd/c	
558	10	Farm 1	–	np	np	–	np	np	nd/c	
559	10	Farm 1	–	np	np	+	np	nd/–	nd/c	7d
560	10	Farm 1	–	np	np	+	np	–	np	8d

561	10	Farm 1	–	np	np	–	np	np	np	np
562	10	Farm 1	–	np	np	–	np	np	np	np
563	10	Farm 1	–	np	np	–	np	np	np	np
564	10	Farm 1	–	np	np	+	np	–	–	1c/9d
565	10	Farm 1	–	np	np	–	np	np	+	2c
566	10	Farm 1	–	np	np	–	np	np	+	3c
567	10	Farm 1	–	np	np	+	np	–	+	4c/10d
568	10	Farm 1	–	np	np	–	np	np	+	5c
569	10	Farm 1	–	np	np	+	np	nd/–	+	6c/11d
570	10	Farm 1	–	np	np	+	np	nd/–	–	7c/12d
571	10	Farm 1	–	np	np	–	np	np	+	8c
572	12	Farm 1	–	np	np	+	np	nd/–	+	9c/13d
573	12	Farm 1	–	np	np	+	np	–	–	10c/14d
574	12	Farm 1	–	np	np	+	np	nd/–	+	11c/15d
575	12	Farm 1	–	np	np	–	np	np	+	12c
576	12	Farm 1	–	np	np	–	np	np	–	13c
577	12	Farm 1	–	np	np	–	np	np	–	14c
578	12	Farm 1	–	np	np	–	np	np	–	15c
579	12	Farm 1	–	np	np	+	np	-	+	16c/16d
580	12	Farm 1	–	np	np	+	np	S/-	+	17c/17d
581	12	Farm 1	–	np	np	–	np	np	–	18c
582	23	Farm 1	1:10	+	np	–	+	nd	np	17, 4b
583	25	Farm 1	–	np	np	–	+	nd	np	34b
584	9	Farm 1	–	np	np	–	np	np	–	19c
585	9	Farm 1	–	np	np	–	np	np	–	20c
586	9	Farm 1	–	np	np	–	np	np	–	21c
587	9	Farm 1	–	np	np	–	np	np	–	22c

np
np
np
np
np
np
np
np
np
TgPigMx68
np
np
TgPigMx56
TgPigMx57
np
np
np
TgPigMx66
np
TgPigMx60
TgPigMx67
np
np
np
np

588	9	Farm 1	–	np	np	–	np	np	–	23c	np
589	9	Farm 1	–	np	np	–	np	np	–	24c	np
590	9	Farm 1	–	np	np	–	np	np	np		np
591	10	Farm 1	–	np	np	–	np	np	np		np
592	10	Farm 1	–	np	np	–	np	np	np		np
593	12	Farm 1	–	np	np	–	np	np	np		np
594	12	Farm 1	–	np	np	–	np	np	np		np
595	12	Farm 1	–	np	np	–	np	np	np		np
596	12	Farm 1	–	np	np	–	np	np	np		np
597	12	Farm 1	–	np	np	–	np	np	np		np
598	12	Farm 1	–	np	np	–	np	np	np		np
599	16	Farm 1	–	np	np	–	np	np	np		np
600	16	Farm 1	–	np	np	–	np	np	np		np
601	16	Farm 1	–	np	np	+	np	np	np		np
602	16	Farm 1	–	np	np	–	np	np	np		np
603	16	Farm 1	–	np	np	–	np	np	np		np
604	16	Farm 1	–	np	np	–	np	np	np		np
605	16	Farm 1	–	np	np	–	np	np	np		np
606	16	Farm 1	–	np	np	–	np	np	np		np
607	16	Farm 1	–	np	np	–	np	np	np		np
608	16	Farm 1	–	np	np	–	np	np	np		np
609	16	Farm 1	–	np	np	–	np	np	np		np
610	16	Farm 1	–	np	np	–	np	np	np		np
611	16	Farm 1	–	np	np	–	np	np	np		np
612	16	Farm 1	–	np	np	–	np	np	np		np
613	16	Farm 1	–	np	np	–	np	np	np		np
614	16	Farm 1	–	np	np	–	np	np	np		np

615	20	Farm 1	–	np	np	–	np	np	np		np
616	20	Farm 1	–	np	np	–	np	np	np		np
617	20	Farm 1	–	np	np	+	np	np	np		np
618	20	Farm 1	–	np	np	–	np	np	np		np
619	20	Farm 1	–	np	np	–	np	np	np		np
620	20	Farm 1	1:4	–	np	–	np	np	np		np
621	20	Farm 1	–	np	np	+	np	np	np		np
622	20	Farm 1	–	–	–	–	np	np	np		np
623	20	Farm 1	–	np	np	–	np	np	np		np
624	20	Farm 1	1:50	+	np	–	np	np	np	18	np
625	20	Farm 1	–	np	np	–	np	np	np		np
626	20	Farm 1	–	np	np	–	np	np	np		np
627	20	Farm 1	–	np	np	–	np	np	np		np
628	20	Farm 1	–	np	np	–	np	np	np		np
629	20	Farm 1	–	np	np	–	np	np	np		np
630	20	Farm 1	–	np	np	–	np	np	np		np
631	23	Backyard	1:10	–	–	–	+	nd/–	np	5b	TgPigMx55
632	25	Backyard	1:10	–	np	–	–	nd//–	np	6b	np

[1]Ages in weeks

[2] MAT results, –: negative, positives are given as antibody titres (agglutination in the higher dilution).

[3] ELISA IDScreen® results, np: not performed, -: negative, +: positives.

[4] Dye Test results, np: not performed, -: negative, positives results are given as IU.

[5] PCR in blood and tongue: SAG1 N-PCR only, +: positive, -: negative, np: not performed. Positives are accumulative for the samples tested more than once (Chapter 2 and Chapter 4).

[6] Mouse bioassay from blood or tissue as appropriate: -: negative mouse (seronegative and PCR negative or seronegative and PCR not performed due to the loss of the animal), nd: not done (loss of the mice before any screening), np: isolation was not performed, S: seroconversion and PCR negative or seroconversion with PCR not performed due to the loss of the animal, +: positive bioassay (seropositive mouse and PCR positive in brain). Note that the cultures which were inoculated into mice are not considered in the mouse bioassay as it was not a bioassay experiment. These were inoculated into mice as an attempt to maintain the parasites alive until the next visit.

[7] Culture: *in vitro* isolation for tissue or blood: np: not performed, nd/c= not done because of contamination issues, -: PCR negative media, +: PCR positive media for B1 N-PCR or/and SAG1 N-PCR.
[8] Number of the samples described in Chapter 2 and 4 as follows: 1-18 = table 2.24, 1-9 a = table 4.6, 1-34b = table 4.7, 1-24c= table 4.10, 1-17d= table 1.11.
* Samples 186-191 (grey cells) were not included in the study due to a mismatch. These samples were collected in a large-scale abattoir from which the farm of origin was not provided. Furthermore, the serum and tongues were collected from different animals. These sampling was performed during an attempt to find an additional abattoir in order to increase the sample size. However, the collaboration did not work and it was not possible to continue sampling in that abattoir.

APPENDIX VI

AVI.1Memorandum of the ethical approval

Academic Audit and Governance Committee

College of Science and Technology Research Ethics Panel (CST)

University of Salford MANCHESTER

MEMORANDUM

To **Ana Cubas (and Prof Judith Smith)**

cc: **Prof Judith Smith, Head of School of ELS**

From **Nathalie Audren Howarth, College Research Support Officer**

Date **4th March 2014**

Subject: **Approval of your Project by CST**

Project Title: **Epidemiology of Toxoplasma gondii in pigs**

REP Reference: **CST 13/72**

Following your responses to the Panel's queries, based on the information you provided, I can confirm that they have no objections on ethical grounds to your project.

If there are any changes to the project and/or its methodology, please inform the Panel as soon as possible.

Regards,

Nathalie Audren Howarth
College Research Support Officer

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College Research Support Officer
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Telephone: 0161 295 5278
Email: n.audren@salford.ac.uk