CYST MORPHOLOGY AND SEROLOGICAL VARIATION IN CYSTIC ECHINOCOCCOSIS PATIENTS FROM TURKANA KENYA

A thesis submitted to the University of Salford for the degree of Doctor of Philosophy in the School of Environment and Life Sciences.

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ABSTRACT

Cystic echinococcosis (CE) is parasitic disease caused by cestode *E. granulosus*. Turkana in Northern Kenya carries one of the highest CE burdens in the world. Between 1983 and 2015 a control programme implemented there by African Medical Research Foundation (AMREF) using ultrasound screening for diagnosis evaluated CE prevalence. Several treatment options were available in the programme for those affected. This programme generated a vast amount of raw data as well as serum samples from infected patients was analysed in the first part of this project to assess the efficacy of treatments in Turkana. Data analysis showed that 7.9 % of untreated cases achieved spontaneous cure and 34% improved without intervention. Chemotherapy appeared to stabilise the cysts rather than cure and increasing dose to two was ineffective in inactive cysts. Surgical outcome was improved with chemotherapy from 85% to 91% cure.

The project also sought to undertake a detailed analysis of serum antibody responses of individual patients and correlate them to gross ultrasound pathologies. Five recombinant antigens identified from literature to have different properties were purified and optimised. These were *Echinococcus granulosus* antigen B *(Eg*AgB), *Echinococcus granulosus* elongation factor 1 beta/delta *(Eg*EF-1β/δ), *Echinococcus granulosus* fatty acid binding protein 1 *(Eg*FABP1), Echinococcus *granulosus* heat shock protein 70 *(Eg*HSP70) and *Echinococcus granulosus* thioredoxin peroxidase (*Eg*TPx). Crude hydatid cysts fluid was also used for comparison. The optimised antigens were then assessed for differential immunoreactive properties towards specific total IgG and sub-classes 1 and 4 serum antibodies.

Significant differences in expression of IgG1 and IgG4 was found in response to some antigens. In simple cysts (CE1), IgG4 was highly expressed in comparison to IgG1, in response to HSP70 and *Eg*EF-1β/δ while in cases where the characterised by cyst infiltration and

calcification (CE4) cysts, IgG1 was the prominent antibody in response to HSP70, *Eg*EF-1β/δ and HCF. The best diagnostic tool was found to be expression of IgG1 in response to TPx which showed sensitivity of 95%.

In follow-up, responses of total IgG towards AgB was found to be useful. It increased in all recurring cases, decreased in all improved cases and remained relatively constant in unchanging cysts.

Use of circulating antigens rather than antibodies in diagnosis showed better results in active cyst. In patients with CE1 cysts, antigen levels were seropositive for 91.1 % (31/34), in CE2 cases 81% (34/42) of the patients, in CE3 cases 42% (9/21) and in CE4 cysts and 59 % (16/27). Overall hydatid cyst antigens were detected in 72.6 % (90) of the cases.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning. All of the ultrasound scanning, clinical examinations and treatment (chemotherapy and surgery) was done by the AMREF Team. All the serum samples were collected by Mr. Eberhard Zeyhle of AMREF. These were initially stored at – 20^{o}C at AMREF, Lokichoggio and subsequently at AMREF, Nairobi. Samples were then transferred to Salford and stored at – 20^{o}C until use for diagnostic procedures. Ultra sound images and patient data (in a basic spreadsheet) was provided courtesy of Mr Eberhard Zeyhle, AMREF. Initial cloning and expression of recombinant antigens was done by Dr. Anthony Bodell. The stocks of *E.coli* cells harbouring the recombinant antigens were provided to the candidate for further expression of diagnostic antigens.

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GLOSSARY

AE	Alveolar echinococcosis
ALBZ	Albendazole
AMREF	African Medical Research Foundation
*Bam*H	1 Type II endonuclease obtained from *Bacillus amyloliquefaciens*
BCIP/NBT	(5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium)
cDNA	Complementary DNA
CE	Cystic echinococcosis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosophates xv
EDTA	Ethylenediaminetetraacetic acid
*Eg*AgB	*Echinococcus granulosus* antigen B
*Eg*EF-1β/δ	*Echinococcus granulosus* elongation factor 1 beta/delta
*Eg*FABP1	*Echinococcus granulosus* fatty acid binding protein 1
*Eg*HSP70	*Echinococcus granulosus* heat shock protein 70
*Eg*TPX	*Echinococcus granulosus* thioredoxin peroxidase
ELISA	Enzyme-linked immunosorbent assay
gDNA	Genomic deoxyribonucleic acid
HC	Hydatid cyst
HCF	Hydatid cyst fluid
IgG	Total immunoglobulin
IgG1	Immunoglobulin subclass-1
IgG	4 Immunoglobulin subclass-4
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside xvi

LB	Luria-Bertani
LB-amp	Luria-Bertani medium containing ampicillin
LL	Laminate layer
PAGE	Polyacrylamide gel electrophoresis
PAIR	Puncture, Aspiration, Injection and Re-aspiration
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PMBC	Peripheral blood mononuclear cells
PMSF	Phenylmethylsulfonyl fluoride
PNPP	p-nitrophenylphosphate
PSC	Protoscolex
PT	Percutaneous treatment
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis
TBE	Tris base, boric acid, and EDTA
TE	(Trishydroxymethyl) Aminomethane and EDTA
TEMED	Tetramethylethylenediamine
Th1	Subset helper-inducer T-lymphocyte 1 cells
Th2	Subset helper-inducer T-lymphocyte 2 cells
TRIS	(Trishydroxymethyl) Aminomethane
US	Ultrasonography
WHO	World Health Organisation

1 INTRODUCTION

A study in 1982 found that Turkana had the highest incidence of hydatid in the world. As a result, African Medical and Research Foundation (AMREF) (French, 1992) implemented a control programme to alleviate the problem. This programme focused on reducing the infection in dogs by regular praziquantel treatment and culling of stray, and on diagnosis of new cases for treatment via surgery of albendazole chemotherapy. Because of basic health care facilities surgery was often delayed for several months until surgeons from Nairobi could visit Kakuma hospital in Turkana. For this reason, many patients could be monitored by ultrasound scanning and serology during an "untreated" period. Many patients were also put on one or more courses of albendazole treatment without surgery and monitored for long periods of time. A final group of patients refused all forms of treatment because of personal beliefs, but were followed up by ultrasound and serology.

For over 30 years the programme generated data relating to variations in cyst morphology. Many patients were diagnosed and followed-up with ultrasound scans and serological sampling. Therapies included albendazole chemotherapy, surgery, PAIR. Patients were followed up after therapy and treatment outcomes evaluated over time. Other cases were followed up without treatment for periods of time due to logistical problems and clinical assessments. The programme generated a vast amount of raw data as well as serum samples from infected patients. The data base for full clinical information is the property of AMREF but a separate sub set with serological data base will be available under the university of Salford.

Throughout the study it was evident that there was great variation in both hydatid cyst size and morphology within individual patients. There was also great variation in how each patient

responded to albendazole treatment. The current study was therefore designed to investigate cyst morphological variation in both untreated and treated individuals. Different morphologies are associated with pathological categories (CE1 – CE5) but since most of our cases fell in the category of CE1 – CE4, our study was based on those. We analysed the association of cyst morphology with a) age of the patient b) size of the cyst c) length of follow-up d) outcome of therapy and other parameters.

Another major aim of the study was to improve field diagnosis of CE. The only method used in diagnosis and patient follow-up was ultrasound scanning. In this study, we assessed the value of recombinant antigens in detection of anti-hydatid antibodies in serum for immunodiagnosis and follow-up. The rationale behind this was serological studies are useful in confirming imaging where cystic lesions are morphologically unclear. Ultrasound imaging detects abdominal cysts and misses out on cysts in the lungs, bones and central nervous system. In community surveys, serological studies may provide partial diagnosis of the cases missed to allow follow-up better imaging techniques.

From literature we identified 5 recombinant antigens which may have differential immunoreactive properties towards serum antibodies in patients with different CE types. These were *Echinococcus granulosus* antigen B *(Eg*AgB), *Echinococcus granulosus* elongation factor 1 beta/delta *(Eg*EF-1β/δ), *Echinococcus granulosus* fatty acid binding protein 1 *(Eg*FABP1), *Echinococcus granulosus* heat shock protein 70 *(Eg*HSP70) and *Echinococcus granulosus* thioredoxin peroxidase (*Eg*TPx). The antigens optimised and then assessed for differential immunoreactive properties towards specific total IgG and sub-classes 1 and 4 serum antibodies in 100 patients all harbouring different cyst types.

Another possible route towards immunodiagnosis is the assessment of circulating *E. granulosus* antigens. These are only detectable in serum during active infection and levels

decrease continually after successful chemotherapy or surgical removal. In this study, we evaluated the relevance of *E. granulosus* antigens in diagnosis and long-term follow-up of CE.

The structure of this study is set out in the following diagram.

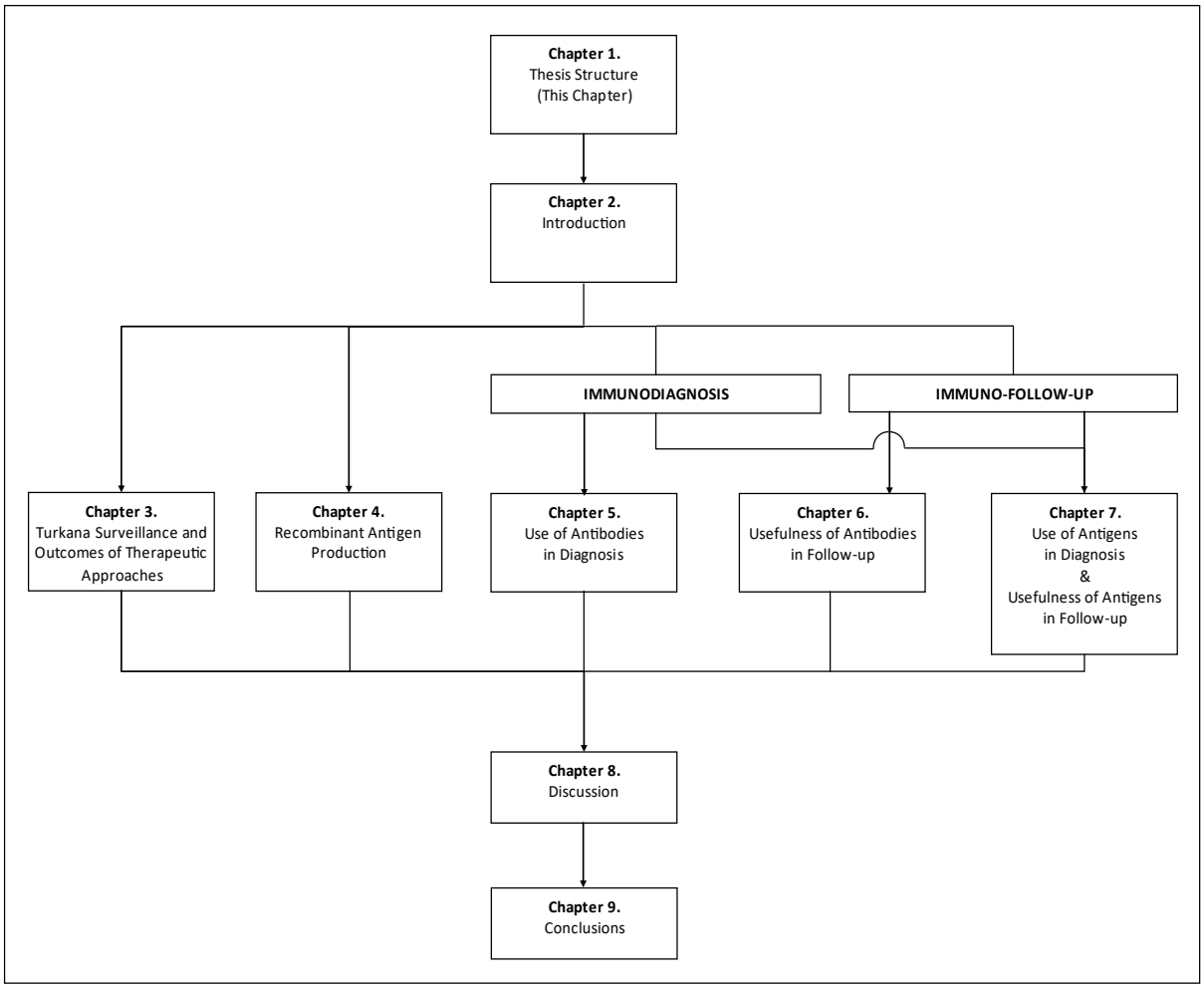


Figure 1-1: Thesis structure.

Chapter 2: Literature was reviewed from key workers in the field.

Chapter 3: We aimed at evaluating different human and cyst factors at first presentation. Human factors included age and sex while cyst factors included cyst locations, numbers and types. We also sought to relate cyst morphological changes to different treatment outcomes.

Look at how cyst morphology varied with age, gender, and what different cyst stages had to do with those

Chapter 4: Five antigens with a potential to show differential immunoreactivity towards serum antibodies in patients with CE were purified and optimised for immunodiagnostic testing. The rationale of this was that current serological methods show sub-optimal sensitivities and specificity. No single antigen has been found that is suitable for all cyst sizes and types in different organs. The answer might lie in using multiple antigens consecutively.

Chapter 5: With the antigens purified in chapter 4, we sought to compare the diagnostic value of different antigens and coordinate the immune responses to defined CE pathology.

Chapter 6: We sought to evaluate if there was a longitudinal link between antibody profiles of individual patients and morphological changes in cysts. We also sought to associate responses of total serum IgG and IgG sub-classes 1 and 4 to disease outcome during follow-up. Antibody levels fluctuate during the course of an infection depending on the rate of cyst development, antigenicity and sensitivity to chemotherapeutic agents. We evaluated different antigens and their association with those changes as they may be useful markers for cyst fertility, viability or instability.

Chapter 7: We evaluated the relevance of the level of circulating antigen to cyst morphology. We also aimed to also link changes in antigen profiles to morphological changes in cysts during follow-up. The rationale behind this chapter was that unlike circulating antibodies, circulating antigens are found in serum only when there is an active infection and continually decrease after successful treatment. We assessed their usefulness in diagnosis and follow-up of active cysts.

2 Literature Review

2.1 General Introduction

Echinococcosis is a cyclo-zoonotic parasitic disease caused by larval stages of cestodes of the genus *Echinococcus* the order cyclophillidea. The parasite has an indirect life cycle which requires two mammalian hosts. The definitive hosts are carnivores harbouring the egg-producing stages and intermediate hosts which include lagomorphs, rodents and ungulates harbouring infective metacestode stages. Infection to humans is accidental but not uncommon and the World Health Organisation (WHO) has classified it as a 'Neglected Zoonotic Disease' (NZD) (Brunetti et al., 2010; McManus et al., 2012; Molyneux, 2012). It affects wildlife, domestic animals as well as humans especially the marginalised poor in remote communities. Classification is generally based on human infection and as such, two main species are prevalent: *Echinococcus granulosus s*ensu lato (the cause of cystic echinococcosis) and *Echinococcus multilocularis* (the cause of alveolar echinococcosis). Besides the two main ones, other species have been shown to cause minor incidences of human disease like the polycystic *E.oligarthrus* (Zimmerman et al., 2009) and *E.vogeli* mostly found in South America (D'Alessandro et al., 2008).

2.2 Speciation and genetic variation within *Echinococcus*

Different strains or subspecies of *E. granulosus* have been confirmed by genetic studies with diversity in morphology, development and host specificity. They are genotypically named G1 – G10 according to the intermediate hosts in which they were first discovered (Eckert et al., 1997; Lavikainen et al., 2003; Romig et al., 2015). The variations in different strains include hook number and dimensions, host infectivity and specificity, biochemical composition, genetic makeup and infectivity to humans (Eckert et al., 1997). Sheep strain G1 is the most common cause of infection in humans and may be responsible for more than 88% of human cystic echinococcosis cases (Rojas et al., 2014). *Echinococcus multilocularis* which causes

alveolar echinococcosis in humans has long been recognised as a distinct species of Echinococcus. It is characterised by multi-vesicular lesions, primarily in the liver, although other organs, including the lungs, spleen and brain may be affected (Bakhsh et al., 2017; Gottstein et al., 1985). Intermediate hosts for *E. multilocularis* include small mammals including microtine voles *Arvicola terrestris*, *Microtus arvalis, Microtus limnophilus* (Duscher et al., 2006; Kotwa et al., 2019), Tibetan hares, *Lepus oiostolus* (Xiao et al., 2004) shrews, *Sorex jacksonii*, ground squirrels *Citellus undulatus lyratus*, and harvest mice, *Peromyscus gossypinus* (Smyth, 1968). The definitive hosts are usually canids *E.multilocularis* has been found in red fox, *Vulpes* (Duscher et al., 2006) grey wolves, *Canis lupus* (Al-Sabi et al., 2018; Hegglin et al., 2008), coyotes, *Canis latrans* (Luong et al., 2018) and domestic dogs, *Canis familiaris* (Budke et al., 2005). Three main isolates for *E. multilocularis* named the 'European', 'Asian' and 'North American' clades have been identified with the names describing their geographical location (Nakao et al., 2009).

Uni-cystic echinococcosis is caused by *E.oligarthrus.* In South America, rodents are the intermediate host and the final hosts include wild felids including pumas, jaguars and Pa*nthela onca* (Rausch et al., 1981). It is potentially zoonotic and human cases have been reported in Colombia and French Guiana (D'Alessandro et al., 2008; Debourgogne et al., 2017).

Polycystic echinococcosis is caused by *E. vogeli* and is characterised by growth and development of polycystic structures in visceral organs. Their major intermediate host is usually the neotropical rodent with bush dogs being the definitive host. It is also zoonotic with human incidents recorded in Paraguay and Argentina (do Carmo Pereira Soares et al., 2014). The human infection by *E. oligarthrus* and *E. vogeli* appear to be restricted to South America with twelve cases reported in 1979 rising to 106 cases by 2007 in 12 countries (D'Alessandro et al., 2008; Tappe et al., 2008).

2.3 General life cycle of *E. granulosus*

As a taeniid tapeworm, *Echinococcus* species have an indirect life cycle involving two mammalian hosts through predator-prey interactions (**Figure 2-1**).

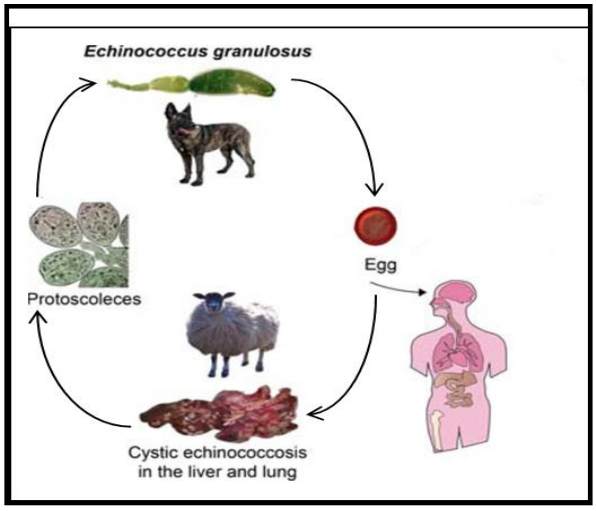


Figure 2-1: Lifecycles of *Echinococcus granulosus*. Adapted from (McManus et al., 2003).

The adult tapeworm ranges in length from 3 mm to 6 mm. Its anatomy include a head (scolex) and 3 segments (proglottids), an immature proglottid, a mature proglottid and a gravid proglottid**.** The scolex is the attachment organ and has a prominent rostellum with a double row of 30 - 36 hooks and 4 suckers. The gravid proglottid contains sexually mature organs and eggs. Inside each egg is a 6 hook-armed embryo hexacanth (**Figure 2-2**). The adult tapeworm lives in the small bowel of its definitive host, attached to the mucosa by its hooklets (Beyrouti et al., 2007).

Traditionally, domestic dogs are the main definitive host for *E. granulosus* whereas foxes are the definitive host of *E. multilocularis*. The definitive host acquires infection by ingestion of parasitised viscera. Mastication, as well as the action of pepsin releases the viable protoscoleces from the brood capsule (Smyth, 1968). The apical region evaginates and establishes by attaching to the host tissue with their suckers reaching maturity in the small

intestine es. Mature *E. multilocularis* is found in the posterior region of the small intestines whereas *E.granulosus* is found in the anterior quarter (Thompson, 2017), perhaps due to different physiological requirements (Macpherson et al., 1985). The parasite forms new proglottids and matures into a hermaphroditic worm. Egg production commences after self-insemination and the number of eggs produced per proglottid varies within species. *Echinococcus granulosus* has been shown to produce as many as 1500 eggs per proglottid whereas *E. multilocularis* produces up to 200 eggs per proglottid (Mehlhorn et al., 1983).Gravid proglottids pass out in the faeces of the definitive host. Each egg can survive in the atmosphere from between 2 days to 3 months depending on environmental conditions. The egg sizes can range between 30 to 40 µm in diameter and contain an outer vitelline layer, an impermeable granular layer and a thin cytoplasmic layer surrounding the oncosphere (Eckert et al., 1983; Sarwari, 2018).

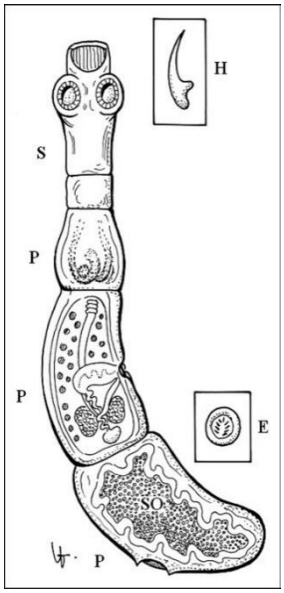


Figure 2-2: *Echinococcus granulosus* adult worm. Adult form of tapeworm. E egg; H hook; P proglottids; S head or scolex; SO sexual organs (Tagliacozzo et al., 2011)

Intermediate hosts acquire infection through oral ingestion of the infective viable eggs in contaminated soil, water, and plants. The eggs hatch in the stomach and small intestines of the intermediate host by the disintegration of the embryonic blocks and release of the oncosphere

from its membrane(Thompson, 2017). This disintegration and release may be assisted by proteolytic enzymes in the stomach or small intestines (Smyth, 1968). Once liberated, the oncosphere penetrates the microvilli and migrate across the intestinal mucosa to their predilection site. Oncospheric development then takes place through vascularisation, degeneration of the hooks, central cavity formation and development of the germinal and laminated layer (Heath, 1971) as shown in **Figure 2-3**. Various animal species including man are aberrant hosts and have no role in the parasite lifecycle.

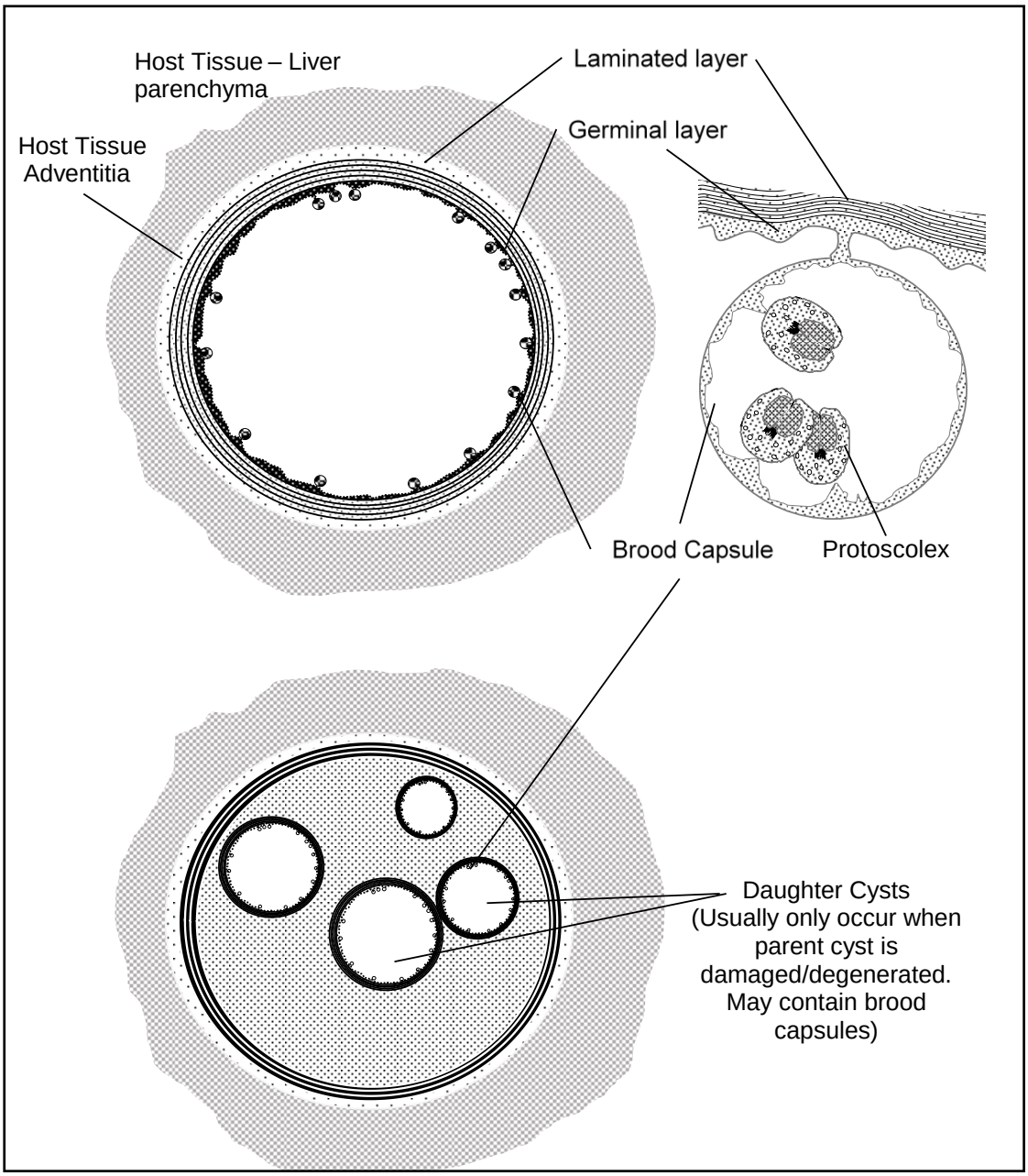


Figure 2-3: Schematic drawing of a liver hydatid cyst. BC brood capsule; DC daughter cyst; GL germinal layer; L liver; LL laminated layer; P pericyst; S protoscolex (Rogan, 2019 unpublished).

2.4 Cystic Echinococcosis in humans and its public health implication

2.4.1 Global distribution of Echinococcus granulosus and CE.

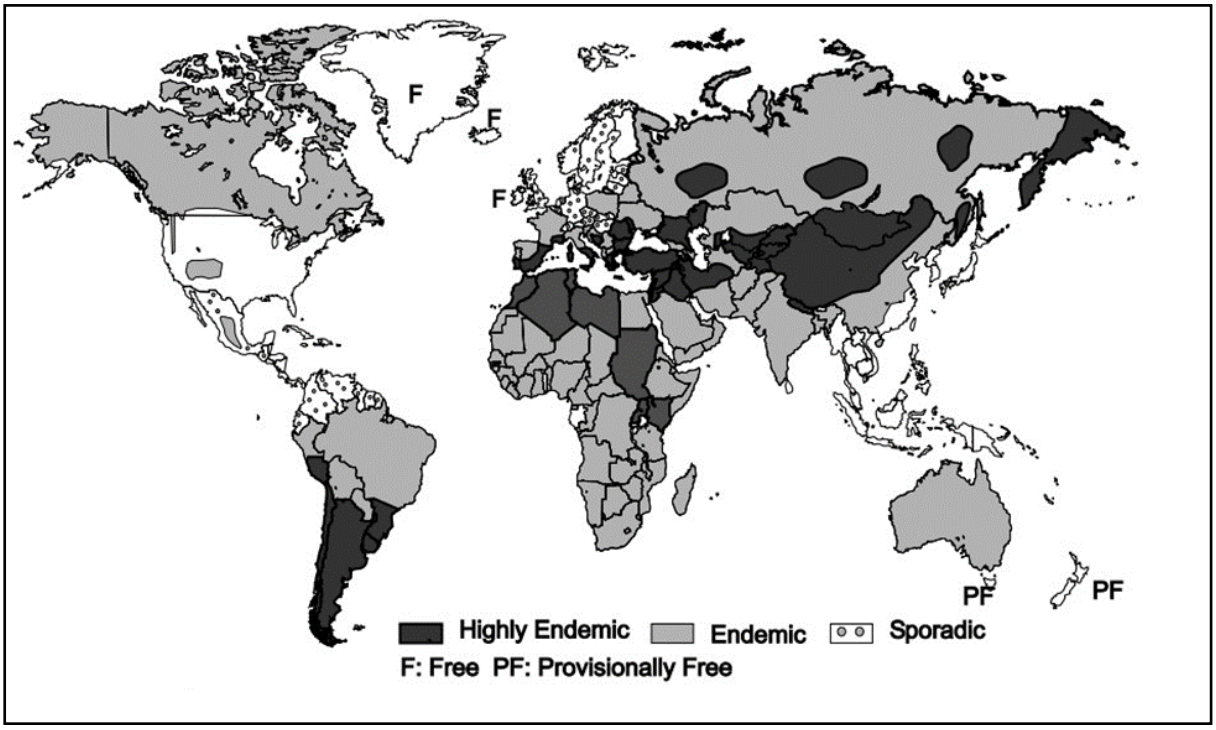


Figure 2-4: Geographical distribution of *E. granulosus.* (Adapted from WHO/OIE (2001a); (Eckert et al., 2004)

Echinococcus granulosus is the most geographically widespread of the Echinococcus species (**Figure 2-4**) occurring in Europe (Orsten et al., 2018), North America (Lavikainen et al., 2003), South America (Reyes et al., 2010), Africa (Rogan et al., 2006), Asia (Chakrabarti et al., 2012) and Australasia (Jenkins, 2005). The main life cycle is the domestic one which involves mainly domestic dogs and domestic animals namely cattle, sheep, goats and camels (Eckert et al., 2004). However, wild animals have also been found to be infected including jackals, hunting dogs, hyenas, lions and leopards (Rausch et al., 1981; Wassermann et al., 2015) and in herbivores wildebeest, impala, hartebeest, grants gazelle, buffalo, blue duiker (Eugster, 1978) waterbuck, warthog and giraffe (Wassermann et al., 2015).

It is predominant in areas where sheep rearing is a significant economic activity since sheep are the most susceptible intermediate hosts. Other factors that feed these transmissions include poor sanitation, close association between domestic animals and humans as well as inadequate knowledge about the transmission and life cycle. Dogs are the major definitive host for *E. granulosus* and proximity of dogs and humans especially in playful intimate contact is a driving factor for human infection (Chaâbane-Banaoues et al., 2016). Dogs shed the eggs which adhere to their hairs, muzzle and paws and accidental ingestion of those eggs causes human infection. Indirect contact with the eggs through contaminated arthropods, water sources and vegetables may also cause infection (MacPherson et al., 1983).

The sylvatic cycle of *E. granulosus* occurs between wild carnivores and wild herbivores and mainly involves the definitive hosts the wolf, moose and the intermediate hosts, caribou jackals and deer in Sri Lanka and macropods and dingoes in Australia (Himsworth et al., 2010)

2.4.2 Cystic Echinococcosis

Human infection with *E. granulosus* causes tissue cysts, a condition known as cystic echinococcosis, also called hydatid disease, *E. granulosus* echinococcosis and hydatidosis. Humans, though acting as intermediate hosts, are a 'dead end host' and rarely play a role in the natural life cycle following infection. Aetiology of infection involves per-oral infection with *E. granulosus* eggs known as primary echinococcosis after which individual encapsulated cysts may develop in any anatomical site in the human body (McManus et al., 2017). In the human host, many cysts remain sterile and may become calcified while others grow and produce protoscoleces and daughter cysts (MacPherson et al., 1983). Primary cysts are commonly hepatic, and most patients harbour solitary cysts with single organ involvement. Cysts may development in many internal organs mainly in the liver and the lungs, although *E. granulosus* can affect any organ including abdominal cavity, heart, bone, muscle, nervous system. Liver cysts account for 63 – 85% of the cases while lungs were 25 – 30%. Other locations include

muscles, bones spleen, kidneys, heart, thyroid, pancreas, breast, prostate and thyroid (Grosso et al., 2012; Kiresi et al., 2003).

Multiple cysts may be found either in one organ or multiple organs. They usually indicate secondary infection mostly resulting from primary cyst rupture. This can either be spontaneously or through induced trauma, releasing viable protoscoleces into the surrounding (Adewunmi et al., 2004; Pakala et al., 2016). In Turkana, of 369 patients, 72 % harboured solitary cysts, 12 % had 2 cysts while 16 % had 3 or more cysts (Romig et al., 1986). The right lobe of the liver is more commonly infected than the left lobe (60 – 85%) and multiple cysts are more likely to be in the liver (Mihaila et al., 2015). Symptoms appear when cysts exert mass effect on affected organ or adjacent organs.

2.4.2.1 Complications of hydatid cysts

Most uncomplicated hydatid cysts are asymptomatic. Nonspecific complications may be due to allergic reactions and occasional toxicity (Gelincik et al., 2007). Symptoms can be produced by mass effect or complications of loss of cyst integrity. They range from pain, jaundice and fever to more severe pathological manifestations including hepatomegaly, cholangitis, splenomegaly and portal hypertension in abdominal CE , chest pain, expectoration and coughing with pulmonary CE (Goumas et al., 2007; Mehta et al., 2016). Symptoms depend on locality and size of cysts, speed of growth and complications of rupture. Most cysts causing symptoms are larger than 5 cm in diameter except when they involve the brain or eyes (Ali et al., 2009). In the abdomen, where there is less restriction on growth through pressure from other organs, cysts may grow to several litres. Large cyst can cause complications due to mechanical pressure associated with a growing space-occupying and isolated mass compressing the adjacent organs, causing pain (Sayek et al., 2004). This may prove fatal especially if the pressure is on the diaphragm and lungs (Boudaya et al., 2014). Chronic inflammation of the liver may result as the cyst pushes against it in growth causing adhesion

to the adjacent organs, especially pronounced in infected cysts. Cysts have been shown to adhere to the hepatic hilum or the retro-hepatic vena cava (Lewall, 1998).

Secondary complications may occur due to infection, rupture or leakage of the cyst. This may be due to trauma or degeneration of the cyst membrane due to age or chemotherapy. Minor leaks may cause increased pain, flushing and urticaria while a major rupture can lead to life-threatening allergic reaction and secondary echinococcosis (Pakala et al., 2016)

Incidence of biliary communication in liver cysts varies between 2 % and 42 % (Ramia et al., 2014; Sakhri et al., 2004). It is mostly caused by intra-cystic pressure, rupture into the bile duct or due to erosion of the bile duct wall due to the cyst (Ramia et al., 2014). Cyst fluid or daughter cysts enter the ducts of the biliary tract leading to obstruction by daughter cysts resulting in cholangitis or into the bronchial tree causing expectoration of cyst fluid. (Yuksel et al., 2007). This usually leads to allergic reactions and complicates treatment as the use of scolecidal agents may cause damage to the ducts (Cooney et al., 2004).

Cysts may become infected with bacteria and *E. coli* is the most common infection. The symptoms range from mild fever to full-blown sepsis and this has been shown to reduce the effectiveness of albendazole and mebendazole (Prousalidis et al., 2008). Surgical interventions are cautious as there is a possibility of the bacteria killing off the cyst. Also, if the patient's health has declined significantly, there is an even higher chance of biliary communication (Pakala et al., 2016).

2.4.3 Public health implication of Echinococcosis

The disease burden of echinococcosis in endemic communities is considerable. Direct costs are due to mortality among the treated and untreated patients, morbidity, direct and indirect costs of hospitalisation and recovery period and any residual disability or clinical sequelae (Piseddu et al., 2017). Present estimates suggest that cystic echinococcosis results in the loss

of at least one million DALYs annually and possibly up to three million with about 200,000 new cases diagnosed each year (Hotez et al., 2014). Other costs are less tangible and difficult to calculate. The reduced capacity to function optimally during the prodromal period, anxiety attached to the knowledge that one is infected as well as the real fear it creates, not just for the infected person but also for the wider community. Treatment of CE requires either surgical interventions or long periods of chemotherapy and sometimes both (Santivanez et al., 2010). This can be costly and very often challenging because the most affected communities are usually behaviourally and/or geographically isolated from main stream health systems as they tend to be nomadic, semi-nomadic or transhumant communities.

Besides the health costs, economic losses are associated with CE due to losses in livestock Animal death or the viscera condemnation have a direct economic impact which is calculable. However losses due to reduction in carcass weight, fertility rate and milk production can have a high degree of variability (Torgerson, 2003; Torgerson et al., 2000; Torgerson et al., 2001).

Reports on the presence of hydatid disease in places where it had not been reported before has raised questions over the recent years as it appears to be increasing in global public health. It may be reflective of recent spread or just better surveillance (Hotez et al., 2014). In Turkana, clinical diagnosis is considerably underestimated and only reflects a small proportion of existing infections, usually due to variable incubation periods and misdiagnosis of clinical infections. The diagnostic facilities are meagre and dependent on faith-based hospitals and charitable organisation. As the rate of infection is very high, nearly every tumour seen by a doctor is suspected of being hydatid disease and the common consensus ‘is it's 70% correct’ (MacPherson et al., 1983)

Since most of the Turkana people do not hold down a formal job, it is difficult to calculate time lost from work, cost of convalescence, time taken to resume normal job and differences in cost due to treatment from different cyst locations.

2.4.4 CE in Kenya

East Africa loosely consists of 10 countries including Burundi, Rwanda, Tanzania, Uganda, Kenya, Somali, Southern Sudan, Eritrea, Ethiopia and Djibouti. Echinococcosis has been reported in 8 of them except for Rwanda and Burundi which may be down to under reporting rather than absence. It is endemic in pastoral nomadic communities but rare in agricultural ones. Kenya has one of the highest infection rates in the world, mostly confined to Turkana County. The Maasai have a similar lifestyle to the Turkana but have a much lower preverence of hydatid disease (Macpherson et al., 1989; Romig et al., 2011). However, there is a greater preverence of *Taenia saginata* among the Maasai and there is a probability that infection with *T. saginata* gives concomitant immunity, prevents superinfection and reduces parasite population pressure on the existing parasite (Lightowlers, 2010).

Assessments of surgical incidence estimated 96 per 100,000per annum in Turkana (O'Leary, 1976) and a report by French and Nelson's in 1982 estimated annual cases at 198 cases per 100 000 people in the north and 19 cases per 100,000 in the south of Turkana (French et al., 1982). It was also found that the north harboured 3 times the number of dogs per person compared to the south. Despite the extra-ordinarily high human incidence, the infection rate in domestic animals was much lower than would be expected. The opposite was true for Maasai land where human cases are much lower than would be expected given the high infection rates among domestic animals (French et al., 1982; Romig et al., 2011). This discrepancy may be explained by the fact that alongside domestic cycles, wild cycles have been reported involving jackals, wild dogs and lions as definitive hosts and wildebeests, and other herbivore species as intermediate hosts in Turkana communities (Macpherson et al., 1993).

Human factors may also play a role in influencing such variations including the fact that concentration of dogs in homes in Turkana is high as compared to those accompanying herds in Maasai land and therefore close interaction with women and children is much higher (Macpherson et al 1983).

2.4.4.1 The Turkana community

Turkana District lies on the floor of the Rift Valley in the North-West corner of Kenya. One of the most important features of the district is the variability of rainfall and this determines the whole landscape with the possibility of drought every 3 to 10 years (Mccabe, 1987). The Turkana people are nomadic pastoralists and are among the most disadvantaged of the Kenyan poor. Their way of life has remained unchanged and movements are influenced not only by rainfall and availability of fodder for their animals but also by traditional ceremonies, many of which are performed in the same initiation sites around the district (Mccabe, 1990). Rainfall usually falls on the higher grounds, mainly in storms, and all the seasonal rivers start flowing. The people move from the hills and higher grounds into the plains for celebrations, weddings and initiations. At the start of the dry season, they will all move again to the higher grounds and the water supply will mostly be from dug up wells and residues from the dried river beds (Best, 1980). The Turkana people are neighboured by the Karamojong in Uganda and the Toposa in Sudan. The political borders are however very vague as the way of life among the three tribes are quite similar regarding land use, economic and social interactions. Insecurity is a serious problem since all three groups are in constant conflict and are seriously armed. Healthcare initiatives by the government appear to neglect these nomadic populations because of the logistical requirements, geographic isolation, uncertain civil status, poor communications, possible hostility due to lack of trust and their perceived low priority.

On the other hand, even when there is provision of medical services, the Turkana have a distrust of government structures due to their possession of illegal firearms creating a barrier to the use

of available services. Constant movement makes access to dispensaries in villages difficult as groups with animals avoid farming areas. Visits to the village markets exclude the most vulnerable – women and children (Cohen, 2005).

2.4.4.2 Health care provision among the Turkana

As with most nomadic communities, the Turkana are a marginalised group due to lack of proper infrastructure, medical facilities as well as traditional and cultural barriers. Most Turkana live in small temporary dwellings made from wood, animal skins and palm leaves and inside the homes live a man, his wives, children and animals. Their movement and/or settlement is highly influenced by rainfall which is sporadic. Provision of continuous medical services is often hindered by language barriers, unpredictable relocations and low population densities resulting to neglect from the government and various agencies (Barkey et al., 2001). Medical provision in Turkana has fallen in the hands of charitable organisations as well as church groups. The largest provider of medical as well as surveillance services in Turkana has been African Medical Research Foundation, (AMREF), who set up a centre in Northwest Turkana to investigate the cases of CE which were the highest worldwide. In 1983 a control programme was set up in the most populated area of Turkana based around the settlement of Lokichokio. The main objectives of the programme were surveillance for the detection and treatment of hydatid patients, regulation of dog numbers, dosing with praziquantel_and education of the local community (Macpherson et al., 1984). Traditionally, the Turkana have two kinds of illnesses, one caused by "God", which is how they regard hydatid disease, the other caused by "witchcraft". Those illnesses caused by God are treated using herbs, usually boiled and the infusion drunk mostly to induce vomiting. Western medication is also treated as thus and is considered more potent if it induces vomiting (Loewenthal et al., 1991). The illnesses caused by a witch doctor on the other hand can only be treated by a diviner or medicine man. The line between the two is unclear and if a remedy does not work, the illness is re-categorised. While

the Turkana people have an acceptance for western medication especially that which works quickly, e.g. anti-malaria medication and surgical treatment, they will resort to the traditional medication if they deem the medication is taking too long or is not working (Harragin , 1994).

In general, efforts at reduction of incidence of echinococcosis in Turkana are confounded by

a) Community ignorance of the risk factors including dog association as well as adherence to chemotherapy.

b) The low priority given by the government to arid and pastoral communities due to their marginal contribution to the GDP

c) Complicated human treatment which requires complex surgery or continuous chemotherapy,

d) Unmanaged dog populations with many dogs being stray. Attempts to kill the strays, sterilise the females and deworm owned dogs have had short term results.

e) The nomadic nature of the people complicates follow – up and supervision on compliance to chemotherapy. Lack of continuous government funding as well as the community's failure to 'own' the eradication project also has negative effect on control.

The success of AMREF among the Turkana has been mostly due to their clinics being mobile, their respect of the cultural ethics as well as consistency. The local people seeing the results of treatment over time build trust and willingness for treatment. However, as the projects wound down, the success of their control measures was rapidly being reversed especially in control of stray dog population..

2.4.4.3 *E. granulosus* transmission and risk factors for human infection in Turkana Kenya

One of the greatest risk factors in human CE is the behaviour of man towards domestic dogs. Direct contact occupationally between dogs and livestock is an important driver (Craig et al., 1984; French et al., 1982). The chief risk factor for dog infection is access to raw infected offal either through purposeful feeding or lack of restraint (Moro et al., 1999). Lacks of knowledge about echinococcosis as well as the presence of other acute poverty-related diseases and conditions are a boost for canine infection (Buishi et al, 2006). Another significant risk factor for both human and animal CE are water sources, shared indiscriminately between animals and humans (Macpherson et al., 1985). Nomadic lifestyle dictates ownership of many dogs living in proximity women and children in the homestead, sometimes sleeping in the same room as the sheep and human giving access to intermediate hosts (French et al., 1984)

2.5 Variation in cyst morphology, pathology and natural history of cyst development

In many early studies in different parts of the world, patients with CE were simply listed as "hydatid patients". However, descriptions, diagnostic criteria, pathology and the outcomes of different treatments are highly variable (Rexiati et al., 2014). *E. granulosus* is an extremely slow growing parasite with cysts varying significantly in size and internal structure. The advent of portable ultrasound scanners has highlighted this and aided in the decision-making process in relation to surgical or chemotherapeutic treatment strategies. Variability in clinical features and treatment outcomes points to the need for a more personalised approach to therapy (Saylam et al., 2013). Ability to correctly diagnose and classify cysts allows the clinician to decide on whether to treat, choose the treatment option (surgery, chemotherapy or PAIR therapy), determine response to treatment and choose an alternative therapy where necessary and in some cases, identify any complications whether natural or induced (Macpherson et al., 2003).

Over the years, different types of classification of CE has been suggested. Garbi's classification (Gharbi et al., 1981) based it on the ultrasound morphology and structure of hepatic cysts in 121 patients. Five categories were proposed, from pure fluid collection in type I, fluid collection with split walls in type II, fluid collection with septa in type III, heterogenous echo patterns in type IV and thick walls in type V. These categories acted as a cornerstone for all other classifications and a few modifications have been added. They form basis for clinicians to perform or recommend the appropriate interventions. Ultrasound has become an increasingly useful tool in diagnosis as well as monitoring the progression or regression of cysts. The WHO-informal working group on Echinococcosis (WHO - IWGE) have also come up with a standardised classification to unify and simplify diagnosis (Eckert et al., 2001) summarised in **Table 2-1**

Ultrasound classification of CE cysts

Cyst status	Cyst type	Ultrasound Diagnostic Features
ACTIVE	**CL**	They contain uniform clear fluid and are indicative of cysts in the early stages of development and usually progress to other cystic lesions. Non-parasitic lesions were excluded during further observation and follow up.
	CE1	Fertile, unilocular and bear visible cyst walls. They exhibit snow flake signs due to movement of brood capsules referred to as hydatid sand. Sizes vary from small < 5cm, medium >5cm <10cm, large >10cm.
	CE2	Multivesicular and multiseptated with daughter cysts filling the unilocular mother cyst either partially or completely. The daughter cysts have 'honey comb' like structures inside the wheel-like septations and contain viable protoscoleces. The cell walls are visible and US pictures are pathognomonic.
TRANSITIONAL & ACTIVE	**CE3**	Transitional cysts with cell walls detached and appearing as a floating membrane ("water lily sign"), indicating residual cyst fluid. They may contain daughter cells anechoic in appearance that are less rounded due to reduced intra-cystic fluid pressure. These are signs of a cyst degrading but may be followed by daughter cell production, making it active again.
INACTIVE	**CE4**	Inactive cysts with heterogeneous hyperechoic degenerative contents and no daughter cysts. They have a ball of wool sign and US features are not pathognomonic in most cyst locations requiring a differential diagnosis.
	CE5	They are characterised by a thick wall and can been either fully or partially calcified. US features are not pathognomonic.

Table 2-1: Features of the WHO-IEWG Classification (WHO Working Group, 2003)

2.5.1 Anatomical/Morphological Variations

2.5.1.1 Cystic lesion

Cystic lesions (CL) may or may not be of parasitic origin. They are usually small or medium size without any internal echoes and septations and no obvious pathognomonic signs. Additional diagnostic techniques are necessary for differential diagnosis. They are circular or oval and contain uniform clear fluid and represent cysts in the early stages of development. They are active and will progress to other cystic lesions (**Figure 2-5**).

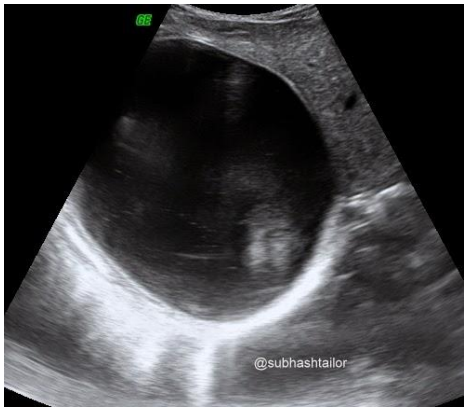


Figure 2-5: A hepatic sonogram shows a CL cyst.

The unilocular cystic mass has a smooth regular and slightly echogenic wall.

2.5.1.2 Cyst type 1 (CE1)

These are usually fertile, unilocular cystic lesions with visible cyst walls and visible anechoic content (**Figure 2-6**). They exhibit snowflake signs due to movement of brood capsules referred to as hydatid sand. High-Resolution sonogram demonstrates an echogenic layered appearance with a clear inner endocyst and outer ectocyst (arrows).

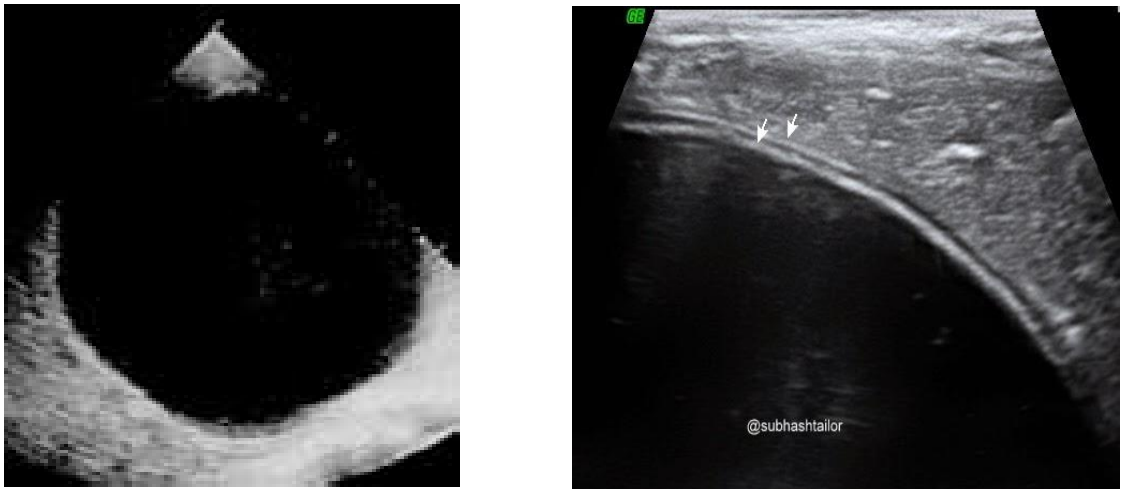


Figure 2-6: Sonogram of CE1 cysts.

(Subhash T., 2015,April 19) ultrasound in hydatid disease. Retrieved from http://sonodigest.blogspot.com/2015/04/ultrasound-in-hydatid-disease.html)

2.5.1.3 Cyst type 2 (CE2)

The CE2 cysts are multi-vesicular and multi-septate with daughter cells filling the unilocular mother cyst either partially or completely (**Figure 2-7**). The septations appear wheel-like with daughter cysts producing honey comb-like structures. They contain viable protoscoleces, the cell walls are visible and US pictures are pathognomonic. They have variable sizes Small < 5cm, medium >5cm <10cm, large >10cm.

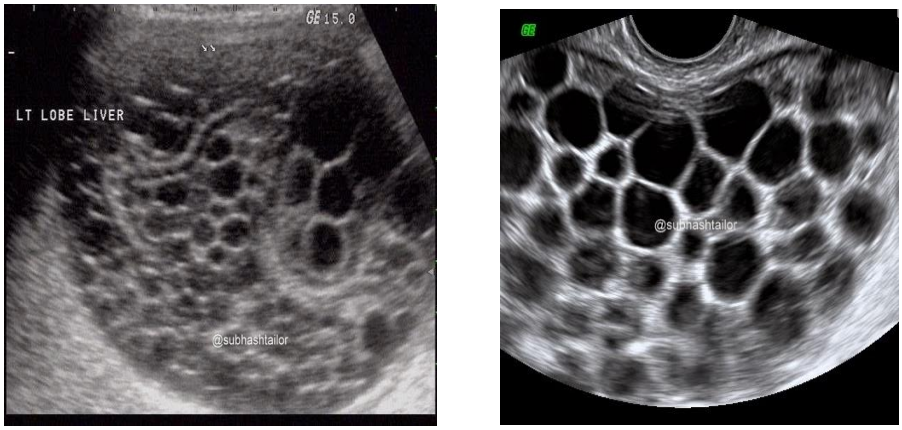


Figure 2-7: Pelvic scan showing a CE2 cyst. (Subhash T., 2015, April 19)

2.5.1.4 Cyst type 3 (CE3)

These are transitional cysts with cell walls detached and appearing as a floating membrane, indicating residual cyst fluid (**Figure 2-8**). They may contain daughter cysts and are less rounded due to reduced intra-cystic fluid pressure. These are signs of a cyst degrading but may be followed by daughter cell production, making it active again.

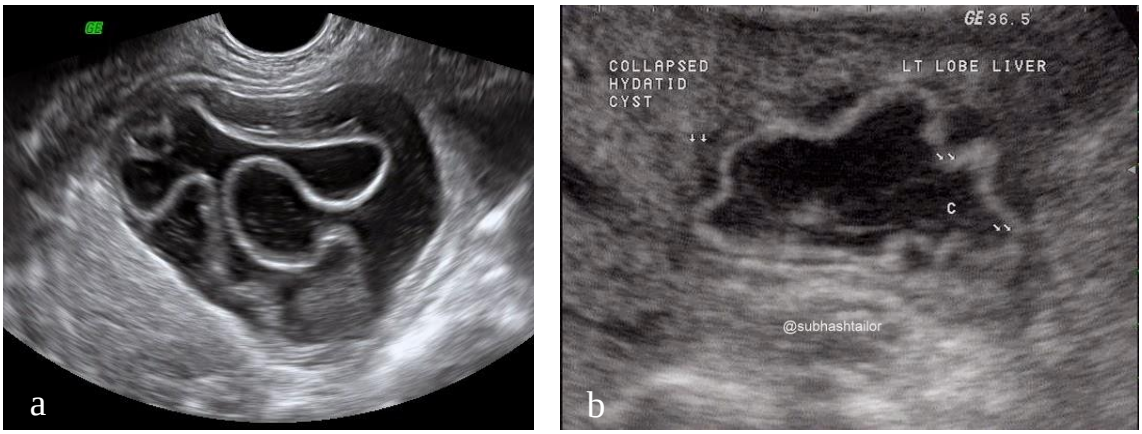


Figure 2-8: Sonogram of Cyst Type 3(CE3). The sonogram shows a large complex with detached endocyst layer echogenic structure b) collapsed hydatid cyst with detached endocyst.

In recent years it has been suggested that the C3 classification could be subdivided into C3a and C3b with C3a being as described above and C3b referring to collapsing daughter cysts in a semi-solid matrix (Brunetti et al. 2010). (Subhash., 2015,April 19) ultrasound in hydatid disease.

2.5.1.5 Cyst type 4 (CE4)

These are inactive cysts with heterogeneous hyperechoic degenerative contents and no daughter cysts (**Figure 2-9**). They have a ball of wool sign and US features are not pathognomonic in most cyst locations requiring a differential diagnosis.

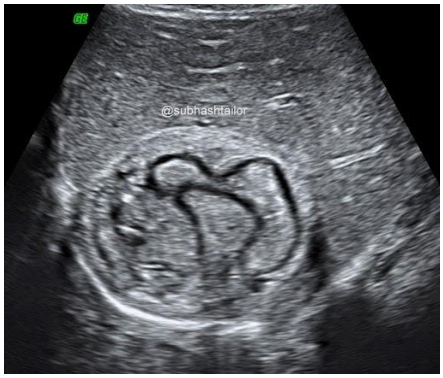


Figure 2-9: Sonogram of a CE4 cyst. The figure shows a solid mass with multiple hypoechoic structures with evidence of a fusing inactive cyst.

(Subhash, 2015,April 19)

2.5.1.1 Cyst Type 5 (CE5)

These are considered inactive calcified cysts (**Figure 2-10**). They are characterised by a thick arch shaped wall and can been either fully or partially calcified.

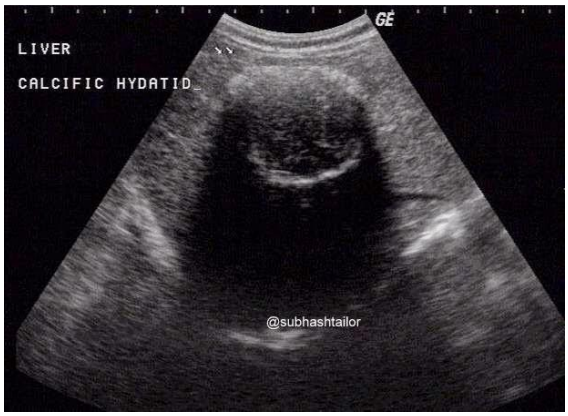


Figure 2-10: Sonogram of a CE5 cyst. It depicts a rounded mass with echogenic rim calcification casting distal shadowing.

(Subhash, 2015) ultrasound in hydatid disease.

Classifications are also made based on cyst size where Cyst size < 5cm is classed as small (s), 5-10cm as medium (m), 10cm – 15cm as large (l) and > 15 cm as very large (v.l) (Guarnera et al., 2004).

2.5.2 Natural history of CE and variation in cyst development

The period between oral egg infection and development of first symptoms in CE infection is difficult to establish due to the slow growing nature of hydatid cysts. The rate of growth may vary according to cyst location and age of patient. Cyst growth is slow thus initial primary infection is asymptomatic and may remain so for many years, sometimes never manifesting at all (Ammann et al., 1996) . Other cysts may develop and size may range between 1 cm and 15 cm (Gahukamble et al., 1991; Romig et al., 2011) though larger cysts have been reported (Nadeem et al., 2006). In Turkana, most cysts range between 1cm and 10 cm diameter in patients with an average size of 10.2 cm (SD ± 4.9) though Romig et al., (1986) found that 16 % measured ≥ 16 cm. The rates of growth of the cysts vary greatly in different host species and among different human populations. Slow growth is observed among indigenous people in Alaska compared to the Turkana in Kenya (MacPherson et al., 1983; Wilson et al., 1968)

Most initial infections involve unilocular hepatic cysts which may remain asymptomatic for many years whereas pulmonary cysts become symptomatic due to compression of the lung tissue which causes chest pains (Karaoglanoglu et al., 2001). Abdominal cysts are usually diagnosed incidentally during medical check-up for other illnesses, routine health check, or due to trauma/fall where the cysts rupture causing anaphylactic shock.

On diagnosis, it is difficult to establish when the infection happened. In children, the infection can only be as old as the child while in adults it is difficult to establish time of infection based on imaging alone. The cyst rate of growth can however be calculated in consequent follow-up though rates of growth have been known to vary among different communities and age groups. Rates of growth can therefore be used as an indicator of time of infection as well as disease prognosis if left untreated. There are few studies on untreated cysts due to ethical reasons but in some cases, patients have refused treatment or treatment was not readily available. Estimated growth in published studies vary between 0 and 2.9 cm per annum in hospital treated cases and

between 6.7 and 7.2 by mass ultrasound screening. Most of the cases show no cyst growth at all and in some cases even spontaneous cure/disappearance of the cyst (Romig et al., 1986).

From the many longitudinal ultrasound studies that have been carried out, it is clear that not only are there differences in the internal structure of cysts from individuals (WHO-IEWG classification), but also that the structure of cysts within the same individual can change in both untreated patients and those treated with albendazole. The natural history of cyst development therefore can involve both growth of the cyst and internal modifications which are usually perceived as progressively or degenerative. Cysts with a CE1 classification may progress to CE2 or CE3 and ultimately to CE4 or CE5 (classified as inactive). It is important to note that this progression is not necessarily linear, and cysts may revert back to previous forms or remain unchanged at each stage for long periods (Solomon et al., 2017). Whilst the changes in cyst morphology during chemotherapy are understandable, those that occur in untreated patients are less well understood but may have an immunological basis (Rogan et al., 2015) (**Table 2-2**).

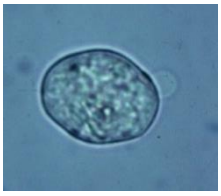
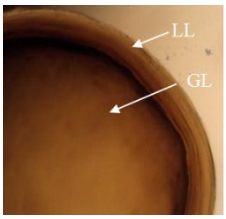
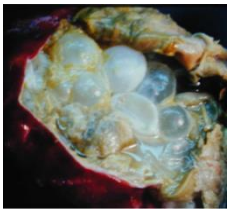
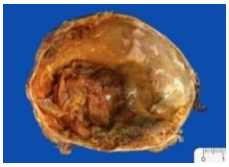
Phase of Development	Morphology	Developmental events	Approximate time post-infection	Immunological events
Establishment		Oncosphere to small hydatid cyst (<2mm). Surface membrane changes and formation of the laminated layer	0 - 1 month	Initiation of antibody response and cellular infiltration – highly effective at parasite killing
Established Maturing	 Sterile cyst with germinal and laminated layers	Growth - Increase in size of cyst (2-20 mm) and laminated layer. Cysts may become fertile (produce protoscoleces) at the end of this phase.	1 – 12 months	Detectable IgG to a range of antigens. Formation and thickening of host adventitial layer. Cytokine activity largely of Th2 type. Lack of fertility may be affected by antibody or cellular attack
Established Stable		Large fertile cysts greater than 1 cm. Growth rate increases variable 0.2 - 3cm/year. WHO Classification Type 1. Growth rate variable 0-3cm/year. Well-developed pericyst.	1 year +	Detectable IgG to a range of antigens. Antibody levels may fluctuate over time. Generally modulated cytokine response (Th2 or mixed). Well-developed adventitial layer.
Established Unstable		Cysts generally greater than 10cm. Internal reorganisation including collapse of cyst wall or formation of daughter cysts. WHO Classification Types 2 and 3.	3 years +	Antibody profile variable. May involve IgG1. Cytokine profile more Th1 or mixed. Cell population in adventitial layer may contain high numbers of macrophages (Th1) or Eosinophils (Th2).
Established Degenerative	 *	WHO Classification Types 4 and 5.	3 years +	Often reduction in antibody response in calcified cysts. Infiltration of cyst cavity with leucocytes may be accompanied by elevated antibody levels.

Table 2-2: Re-evaluated phases of parasite development/immunity (Rogan et al., 2015).

2.6 Diagnosis of CE

Human hydatid disease is usually diagnosed by the clinicians only when advanced and symptomatic cases present themselves requiring immediate intervention. Four major reasons for seeking medical help include, a) When large cysts exert mechanical pressure on the tissues and organs affecting general functions and maybe causing pain, b) When a trauma causes rupture of the cyst consequently resulting to an allergic reaction, c) when other symptoms including eosinophilia or other allergic phenomena are present and d) incidental finding during surgical, imaging or body scanning for other conditions. Many asymptomatic infections are diagnosed during active surveillance campaigns which are now conducted even in the very remote communities where CE is endemic.

2.6.1 Imaging

Imaging techniques that can identify space-occupying lesions in internal organs are available and many of them have been used in diagnosing CE. These include X rays, Magnetic Resonance Imaging/Computerised Tomography (MRI/CT) scans as well as ultrasound.

Ultrasound is the most common imaging technique used for diagnosing CE. It is safe, painless, non-invasive, easy to perform and relatively inexpensive after the initial cost. It can determine the number, size, location and condition of the cyst and also gives instant results which can be permanently recorded (MacPherson et al., 1983). Portable ultrasound machines are now particularly in use for diagnosis of asymptomatic cases during routine epidemiological mass screening. They give satisfactory results for abdominal localisations showing between 93–98% (Carmena et al., 2006; Macpherson et al., 2003). Imaging is also a useful tool in follow up of patients, allowing the clinician to continuously assess changes in size, morphology, development or regression which in turn influences the choice of treatment. (WHO WorkingGroup, 2003). Many asymptomatic patients stay symptom-free for many years requiring no otherwise risky intervention (Wang et al., 2003). Despite its great value as a

diagnostic tool, ultrasound is not always ideal for diagnosis of extra abdominal cyst locations, determining cyst complications for example infection or visualising liquids within the cyst (Golzari et al., 2013). Other imaging techniques are in use for diagnosis and follow-up of CE including computerised tomography, (CT) and magnetic resonance imaging (MRI. They are especially useful in the diagnosis of extrahepatic cysts especially lung and bone cysts, calcified cysts, monitoring therapy and recurrences (Gougoulias et al., 2010). They also give better clarity in small cysts (>1 cm in diameter) (Bukte et al., 2004) through differential diagnosis.

While these advanced techniques are precise and accurate in diagnosis of CE, they are very expensive and require technical expertise that is not readily available in the endemic areas. They are mostly useful in passive surveillance when the patient is already in hospital whereas portable ultrasound scanners allow active surveillance making screening campaigns possible even in the very remote communities where CE is endemic (MacPherson et al., 1987)

2.6.2 Serology

Early diagnosis of echinococcosis greatly improves treatment outcome (Wen et al., 2019). The asymptomatic nature of early infection necessitates large scale surveillance of populations at risk (Zhang et al., 2006). Today, primary diagnosis of hydatid disease is based on US images in line with the current classification system of hydatid cysts.

Serological studies are useful in confirming US imaging where cystic lesions presented are morphologically unclear and in community surveys since US mostly detects abdominal cysts and may miss out on lung cysts, bones or CNS. Serology may provide partial diagnosis for some of those cases missed and allow a follow-up with better imaging techniques (Rogan et al., 1997).

Any CE treatment requires long term follow up and monitoring to determine cure or relapse. Imaging provides some information on morphological changes in cyst structure, but these are

not pathognomonic in some cases. It is also not possible to predict disease outcome in heterogeneous cysts in terms of the parasite stability/ instability or in transition from non-viable to viable. Potential to link the US images to immunological markers is needed especially during the cyst stages that are "silent" post-treatment when the cyst changes may go undetected (von Sinner et al., 1991).

Many serologic techniques have been evaluated and reviewed for diagnosis of CE(Jiang et al., 2001; Liu et al., 1993; Mamuti et al., 2004; Mohammadzadeh et al., 2012; Santivanez et al., 2012), usually based on detecting host immune responses to CE infection especially antibodies, cellular responses and to a lesser degree circulating antigen.

2.6.2.1 Antibody detection

A wide range of specific antibody production against protoscoleces, the germinal layers and the transforming oncosphere have been investigated for their diagnostic value. Different classes and subclasses of antibodies have been assessed for serodiagnosis of CE using various parasite antigen preparations. The most frequently used source of antigen is *E.granulosus* hydatid cyst fluid (HCF). It is antigenically rich and has been shown to contain more than ten immune-precipitable antigens derived from the parasite (Li et al., 2010; Siracusano et al., 1991). Hydatid cyst fluid (HCF) is a complex mixture of glycoprotein, lipoproteins, carbohydrates and salts (Oriol et al., 1971). About 60 % of this is host material and the rest is from metacestodes metabolism (Zhang et al., 2003). Crude extracts of native antigens exhibit poor diagnostic performance due to variability in antigen quality from different sources, locations and parasite genotypes (Zhang et al., 2011). Crude HCF is highly immunogenic with sensitivity between 75 to 95 % but is difficult to standardise. It also has limitations in specificity as it crossreacts with other cestode infections (89%), nematode infections (39 %)

and trematode infections (30%) (Eckert et al., 2004). To improve its diagnostic value, crude HCF has been purified into antigen 5 (Ag5) and antigen B (AgB) for ELISA and immunoblotting for detection of total IgG / IgG subclasses or other Ig isotypes (IgM, IgE, IgA) in human sera (Khabiri et al., 2006; Virginio et al., 2003).

2.6.2.2 Recombinant technology

Other parasite tissues including protoscolex and adult somatic antigens have been investigated for their potential use in serodiagnosis in human CE. In their crude form, they have shown little promise due to poor sensitivity and specificity (Jiao et al., 2013; Severi et al., 1997). Some of the antigens explored are associated with adult excretory and secretory products(Carmena et al., 2005) oncosphere antigens (Chow et al., 2004) as well as carbohydrate-rich laminated layer of the *E.granulosus* metacestode (Diaz et al., 2011; Taherkhani et al., 2007). Diagnostic performance of CE antigens has been greatly improved by recombinant proteins (Kordafshari et al., 2015; Zhang et al., 2007) .

Antigenicity and the immune responses against immunodiagnostic antigens are complex and trying to establish a "one fits all" situation is difficult. In virtually all studies evaluating the effectiveness of different antigens in diagnosis, each preparation has been screened against "clinically proven cases" but as stated previously, clinical cases represent an extensive range of possible cyst morphologies. In addition, in the case of recombinant antigens, different authors have used different expression systems making comparisons more difficult.

In this thesis we assess the relative antibody profiles of clinically distinct CE cases against a panel of *E.granulosus* recombinant antigens produced in the same expression vector.

2.6.2.2.1 Recombinant antigen production and standardization

Sensitive diagnostic tools are important in clinical diagnosis, epidemiological surveillance and infection control. Current serological methods in CE diagnosis show sub-optimal sensitivity and specificity and standardising the use of available antigens has proved difficult.

Antibody levels have been shown to fluctuate in the course of an infection. Changes in immune responses depend on the cysts rate of development, antigenicity, transmission dynamics and sensitivity to chemotherapeutic agents (Barnes et al., 2012; Daeki et al., 2000). Different antigens have been associated with those changes and their use as markers for cyst fertility, viability or instability assessed by different authors (Carmena et al., 2004; Jiao et al., 2014; Ortona et al., 2004). However, no single diagnostic antigen has been found that is suitable for all cyst sizes and classifications in different organs.

Hydatid fluid and its components such as antigen B have been the main antigens used in diagnosis. Crude hydatid fluid has high sensitivity but limitations arise with specificity while antigen B has good specificity but limited sensitivity (Zhang et al., 2011). In addition to cyst fluid antigens, other studies have looked at other parasite tissues such as protoscoleces, laminated layer and excretory-secretory products for use as diagnostic antigens but optimum sensitivities and specificities have not been achieved thus far (Carmena et al., 2006; Sarkari et al., 2015). Shortcomings of using native antigens include variability in antigen quality when sourced from different locations and sources. Hydatid fluid from sheep, cattle and horses all vary in antigenicity and even cysts fluid from the same genotype and host species can show variations (Gottstein et al., 1987).

Many molecules from *E. granulosus* have been cloned and produced as recombinant proteins. Use of recombinant antigens reduces the variability in antigenicity although even they can show variation depending on the expression system used (Sadjjadi et al., 2009). We identified 4 antigens from literature which may have different properties and appeared to have differential immunoreactive properties towards serum antibodies in patients with CE (Carmena et al., 2006; Zhang et al., 2003). Elongation factor 1 (EF-1β/δ) is a 24 – 31 kDa protein found in both the protoscoleces and hydatid cyst fluid (Siracusano et al., 2012; Vuitton, 2004; Zeghir-Bouteldja et al., 2017) and is thought to be useful in parasite translation and elongation (Kamaishi et al.,

1996). Its expression is reported to be highly correlated to presence of allergic reactions with a higher response to IgE than IgG and may therefore play a role in allergic reactions in CE cases. Ortona et al. (2004) and Rigano et al. (1995) reported a correlation to IgG4. Presence of EF-1β/δ human auto-antibodies in CE patients was demonstrated by Mori et al. (1986)and the homology to EF-1β/δ may suggest their possible role in parasite immune evasion. High levels of serum antibodies in response to EF-1β/δ antigen for example have been recorded in patients with calcified cysts while lower titres were recorded in patients with active cysts (Margutti et al., 1999). Elevated levels of this protein in hydatid cyst fluid mayn be indicative of a degenerating cyst. The higher percentage of humoral immune responses to EF-1β/δ observed in CE patients with calcified cysts than in patients with active cysts suggests that the protein is released into the hydatid fluid after the degeneration of PSC and indicates its possible use in immune-surveillance of CE. Furthermore, EF-1β/δ may play a key role in the allergic disorders (urticaria, itching, and anaphylactic shock) that often complicate the course of CE (Margutti et al., 1999; Siracusano et al., 2008).

Thioredoxin peroxidise (TPx) has been isolated from protoscoleces and hydatid cyst wall but is absent in cyst fluid (Manzano-Román et al., 2015; McManus et al., 2003). In the early stages of development, the cyst is surrounded by cells from host immune responses including macrophages, eosinophils and neutrophils (Margutti et al., 2008). This is accompanied by production of highly reactive oxygen species (ROC) not just from the host but also from the metabolic activities of the cyst. These ROCS can cause protein oxidation and lipid peroxidation leading to cyst breakdown (Wang et al., 2018). TPx is thought to be an enzymatic scavenger helping the parasite escape oxidative damage by removing H_2O_2 (Salinas et al., 1998). Similar levels are expressed throughout the parasite lifecycle in both active and inactive cysts (Li et al., 2004) found making it a valid antigen for diagnosis.

E. granulosus fatty acid binding protein 1 (EgFABP1) is a developmentary regulated protein characterised in the larval stages of *E. granulosus.* Their roles may be provision of fatty acids from the host to the parasite for membrane synthesis and metabolic pathway (Alvite et al., 2001). The homology to other protein families like retinol binding protein and interstitial binding proteins suggest their role in transport and storage of lipids (Jones 1994). Strong immune responses of FABP1 to larval stages of *E. granulosus* have been recorded (Porfido et al., 2012) an indication that they may be an early marker of asexual reproduction (Alvite et al., 2001)

Heat shock protein 70 (HSP70) are highly conserved proteins whose roles include stabilising misfolded proteins, stress responses and protein translocation (Mayer et al., 2005). Increased levels of HSP70 are usually a sign that an organism is exposed to stress (Hogervorst et al., 1992; Kaufmann, 1990). It has been found to be a dominant immunogen in parasitic infections including leishmaniasis, oncocerciasis and schistosomiasis (Maresca et al., 1994).

EgAgB is a 120–160 kDa polymeric protein that dissociates in to 8, 16, and 20–24 kDa subunits under reducing conditions. The biological role of EgAgB includes inhibition of neutrophil chemotaxis, inducing apoptosis of immune cells and triggering of non-protective Th2 responses (Zhang et al., 2011). EgAgB belongs to a multigenic family with five other defined genetic groups: EgAgB1, EgAgB2, EgAgB3, EgAgB4, and EgAgB5 (Chemale et al., 2001; Fernández et al., 1996). These subunits share sequence identity of between 44% and 81%. Similar antigens have been found in *E.multilocularis* species and genus Taenia (Carmena et al., 2006), giving rise to cross-reactivity. Native EgAgB has sensitivity of between 60% to 85% in ELISA test (Carmena et al., 2006). In one study using ELISA test, EgAgB recombinant antigen showed a sensitivity of 74%, 96%, 90%, and 56% in patients with CE1, CE2, CE3, and CE4/CE5 cyst stages, respectively(Li et al., 2010).

After purification, the antigens showed a good immune response towards a pool of positive sera and poor responses towards negative sera. This was a good basis for the next two chapters where these antigens were used for diagnosis and follow-up of confirmed CE patients.

2.6.2.3 Antigen detection

Many inconsistencies are associated with serodiagnosis based on antibody detection due to crossreactivity with other helminths producing false positives (Craig, 1986). Other limitations include low responses in patients (Macpherson et al., 2003; Siracusano et al., 2008) and variation in responses with cyst type, cyst size and location (Hernandez-Gonzalez et al., 2008; Piccoli et al., 2013) and failure to distinguish between past and present infection, making it difficult to assess the efficacy of a treatment.

Circulating antigens are only found in sera during an active infection and decline rapidly on disease resolution either through surgery or effective chemotherapy (Chandrakesan et al., 2003; Sadjjadi et al., 2009) and may be a better method of immunodiagnosis of CE as well efficacy of treatment. Sensitivity of assays detecting circulating immune complexes and circulating antigens vary between 33 – 85% with better results obtained from a field coagulation test (Ghorbanpoor et al., 2006; Parija et al., 1997). Just like in antibody detection various factors affect sensitivity and specificity. These include nature of cysts where intact and small cysts do not release as much antigen and are therefore more difficult to detect whereas large, rapidly growing and fertile cysts have shown superior sensitivity (Lightowlers et al., 1995). In one experiment, the assay also showed superior results in liver cysts compared with kidney and lung but also showed crossreactivity with visceral leishmaniasis (Sadjjadi et al., 2009).

2.7 Treatment of echinococcosis

Although it is estimated that 1 million people suffer CE at any time and 3.6 million DALYs are lost (Hotez et al., 2014), it is a disease of the neglected poor communities and as such

receives very little attention in developed countries. This explains why clinical management procedures over the years still have inadequate evaluation on efficacy, effectiveness, rate of relapse, rate of adverse reaction and cost. Also, why there is no advancement in development of new drugs.

At infection, the patients are asymptomatic, and the cysts are small. The rate of growth has been shown to be different in different communities, organs as well as age groups (Mufit et al., 1998; Romig et al., 1986). Onset of clinical symptoms depends on size of cyst, organ infected, localisation of cyst in that organ, number of cysts and the pressure on surrounding tissue (Eckert et al., 2004). Abdominal cysts can remain asymptomatic for long periods of time whereas lung and bone cysts cause pain quite early after infection and are diagnosed earlier in the infection process. There is also the possibility of cyst rupture due to trauma which can cause anaphylactic shock or may lead to dissemination of protoscoleces resulting in secondary hydatidosis (Svrckova et al., 2018)

At present, four treatment modalities are used namely Surgery, PAIR, Chemotherapy and watch and wait. Evidence in choosing any of the treatment options remains controversial due to insufficient carefully designed clinical studies.

2.7.1 Surgery.

Surgery remains the treatment of choice and is performed on a high proportion of patients because of its potential to cure completely. It has been shown to be very successful in cases of simple cysts located in less risky organs. Surgery is indicated for solitary, large, superficial cysts that are likely to rupture, infected cysts, those in vital organs and the ones exerting substantial mass effect (Brunetti et al., 2010). Open surgical procedures may be conservative, involving removing the parasite foci and leaving the pericystic membrane in situ while a more

radical surgical approach sees the complete removal of both cystic materials along with the pericystic membrane (Akbulut et al., 2010)

It is counter-indicated in multiple cysts in multiple organs, in patients with high surgical risk and may prove impractical in areas with inadequate expertise and facilities in which situations alternative options of treatment are considered. Surgical patients experience more risk of complication but lower relapse rate about 2% to 25% and this is greatly reduced by pre and post operational chemotherapy (Khuroo et al., 1997).

2.7.2 Puncture Aspiration Injection Re-aspiration (PAIR)

Puncture Aspiration Injection Re-aspiration (PAIR) was introduced in the mid-80S. It involves percutaneous puncture of the cyst under ultrasonographic guidance, aspiration of a substantial portion of the cyst fluid, injection of a parasiticidal solution and re-aspiration of the cyst fluid content after 5 - 20 min (Macpherson et al., 2003). A few ml of aspirate is first tested biochemically for sodium, potassium, chloride, calcium, proteins and glucose (Filice et al., 1997; Solbiati et al., 1985) and bilirubin in case of liver cysts and also parasitologically to assess for cyst viability (Filice et al., 1997). A parasiticidal solution, 95% ethanol is then injected into the cyst, approximately an equivalent of one- third of the amount aspirate, and re-aspirated after 5 - 20 min (Ormeci, 2014). Ethanol has been shown to be effective a few min after contact (Bean et al., 1985)

It minimally invasive technique usually preceded and followed up by chemotherapy, with albendazole (Park et al., 2009). Complications with PAIR can range from 28% when used alone and reduced to 5-10% when used alongside chemotherapy (Filice et al., 2000).

There exists a risk of anaphylactic shock and/or spillage leading to dissemination of parasite and consequent implantation and secondary CE. This can be minimised by advances in imaging techniques as well as use of finer needles and catheters and about one per cent of the patients experience anaphylaxis or spillage (Dziri et al., 2004). Used together, PAIR and chemotherapy

showed efficiency as good as peri-cystectomy in hepatic CE with shorter hospital stay (Akhan et al., 2014; Filice et al., 1997). A shortcoming of PAIR may be incomplete evacuation of the cyst content which may become an abscess, become infected or recur (Sayek et al., 2004).

Modified forms of PAIR, percutaneous evacuation (REVAC) and percutaneous puncture drainage and curettage which include concomitant removal of entire cyst content have been described (Gabal et al., 2005; Zerem et al., 2005). It involves insertion of a bore catheter into the cyst, infusing scolecidal agents and aspirating again until there complete wash out of the contents with the cyst is completely collapsed and consequently removing the entire endocyst. These techniques are indicated for multivesicular cysts, infected cysts, patients with several small cysts as well as pregnant women(Gabal et al., 2005). Protoscolecidal agents include hydrogen peroxide, cetrimide, chlorhexidine, hypertonic water and alcohol (Filice et al., 1997). The guidelines on scolicidal concentration are different in different setting and achieving optimal concentrations is difficult (Belghiti et al., 1986). Contraindications for PAIR are cysts communicating with the biliary tree, cysts in a risky or inaccessible location and calcified or solid cysts, young patients under 3 years old and inaccessible and ruptured cysts (Sayek et al., 2004).

2.7.3 Chemotherapy

Chemotherapy with benzimidazole compounds was initially recommended for inoperable cysts and patients with multiple cysts (John, 2003). Today it is routinely used as a drug of choice for pre and post-surgery as well as an alternative to surgery in uncomplicated cysts (Macpherson et al., 1986) and its use has increased over the years. Benzimidazole anthelmintic (BZD) including methylcarbamate compounds mebendazole (MBZ) and albendazole (ABZ) have been used for treatment of CE in humans for decades (Davis et al., 1986).

However, problems persist in determining the optimal administration period either as an adjunct perioperative therapy or on their own as this is still not clearly defined. Other limitations include the high cost of the drugs, side effects as well as efficacy not being optimum. Benzimidazole anthelmintic are not readily soluble in water and this influences absorption and limits their bio-availability leading to erratic therapeutic success (Lanusse et al., 1993). To achieve high plasma concentration of these compounds, they need to be dissolved in gastro-intestinal fluids (GI fluids), pass through the intestinal mucosa into the bloodstream and sustain an effective concentration around the parasite. Poor GI absorption is problematic in sustaining systemic availability in enterally administered BZD (Davis et al., 1986; Sadjjadi et al., 2009). Albendazole has a better GI absorption rate than mebendazole after oral administration and has replaced mebendazole as the drug of choice (John, 2003; Vuitton, 1997).

The benzimidazole compounds work by inhibiting tubulin polymerization, cellular transport, energy metabolism and waste excretion in the parasite. As a consequence, the formation of new microtubules is stopped disrupting cell division, the energy reserves are progressively depleted and the waste products accumulate inside the cells eventually resulting in parasite death (Gottschall et al., 1990).

Effective chemotherapy is still lacking, and extensive studies are still being done on the drugs available. Mebendazole was the first drug tested for efficacy in echinococcosis and it is still in use as an alternative drug to albendazole which is now the “first intent” drug (Nakaya et al., 1998). The recommendation for the use of mebendazole is a daily dosage of 40 – 50mg/kg body weight divided in 3 doses and given postprandially during a fat rich diet. Absorption of mebendazole is suboptimal and average blood levels of < 10 µg/ml does not produce therapeutic effects invivo (Macpherson et al., 1986; Marriner et al., 1986). Severe adverse effects are relatively frequent due to the high dose. They include disturbance of hepatic

function, depression of the bone marrow and glomerunephritis (Bekhti et al., 1987; Bryceson et al., 1982; Kung'u, 1982). Therefore, a shift from mebendazole to albendazole has resulted from good commercial availability in most countries, better bioavailability, easier administration as well as overall better efficacy (Brunetti et al., 2009; John, 2003).

Albendazole, given in two divided doses a day has shown to be more efficient than mebendazole. Variabilities exist in duration of treatment and is determined by ultra sound based follow-up which may result in repetitive treatment (Stojkovic et al., 2009). Cyclic albendazole treatment at the dosage of 10 – 20 mg/ kg of body weight /day, divided in to 2 doses is administered continuously for 28 days postprandially. The patients are then given a "washout period" of 10 – 15 days when no medication is administered before starting a second dose when applicable (Steiger et al., 1990). Effectiveness of albendazole is still quite low and only 8 – 20 % of patients show complete cure or significant decrease of cyst size(Davis et al., 1986; Todorov et al., 1990). Factors affecting therapeutic results of BZD may include age of patient, location of cyst, age of cyst, the morphology of cyst and the immune responses of the patient (Horton, 2018; Stojkovic et al., 2009).

Benzimidazole compounds are generally well tolerated but their prolonged use in treatment of echinococcus causes adverse reactions. When they occur, adverse effects include gastrointestinal disturbances, hepatotoxicity, alopecia and leukopenia. Monitoring aminotransferases as well as blood counts is recommended during chemotherapy (Nahmias et al., 1994). Both drugs are contraindicated for early stages of pregnancy due to high risk of embryo toxicity and teratogenicity (Bradley et al., 2001). There are great intra and inter-individual variations in BZD pharmacokinetics and ideally levels of albendazole sulfoxide and mebendazole in plasma should be monitored (Eckert et al., 2001) though this is not always possible in the endemic areas.

Praziquantel (PZQ), a derivative of pyrazinoisoquinolone is an effective antihelmintic which has been routinely used against a wide range of parasite in both humans and animals (Cioli and Picamattocia, 2003). It has better bioavailability than albendazole and mebendazole and peak plasma levels reached after 1–3 hours after oral administration (Mandour et al., 1990). Relative bioavailability of PZQ increases if administered of food (Castrol et al., 2000).

Praziquantel is generally well tolerated with incidences of adverse effects around 10–15% King and Mahmoud., 1989). Side effects include abdominal pain, nausea, dizziness, diarrhoea, anorexia and headache. Studies of PZQ in monotherapy have shown unsatisfactory results. A study by Piens et al. (1989) where 30 patients were treated with PZQ, 9 CE patients showed no difference in cyst viability after two 10-day courses of 75 mg/kg/day separated by between 3 weeks and a control group untreated group. However, it was found to be effective in preventing relapses.

The benefit in using PZQ in combination with ABZ in chemotherapy for CE has been evidenced (Cobo et al., 1998) particularly where there has been a cyst content spillage during surgery. It may also reduce the risk of disease recurrence and the dosage needed therefore reducing adverse effects. (Kern., 2003).

2.7.4 Watch and wait

Mass screening has proved that cysts do spontaneously disappear, rupture, collapse or remain unchanged for many years. In transitional cysts (CE3), degenerating cysts (CE4, CE5) and also in small cysts, watch and wait may be recommended with careful follow up every 3-6 months for a minimum of 2 years (Piccoli et al., 2014).

2.8 Follow – up and evaluation of treatment and disease progression

Follow up - on patients either after treatment or for monitoring untreated cases is commonly done using imaging and serology. This is important in treated cases because spillage during surgical removal or PAIR is common and recurrence causing secondary echinococcosis and may not become apparent until months or years after treatment. For patients with uncomplicated inactive cysts, expert consensus is that they are left untreated and are closely monitored using the ‘‘watch-and-wait’’ approach as there is a possibility of self-cure or remaining unchanged (Piccoli et al., 2014).

2.9 Clinical and epidemiological significance of cyst variation

The WHO-IGWE cyst classification, relating to variation in cyst structure was produced to evaluate treatment options. The complex nature of cyst location, size and internal structure means that no one intervention approach is suitable for all cyst types. Brunetti et al. (2010) recommended a stage-specific approach as shown in **Table 2-3** below. Variation in cyst morphology and classification has also been deemed to be useful for epidemiological purposes. The predominant cyst type in a community may be indicative of when the infection occurred. According to natural history, it is assumed that CE4 and CE5 cysts are older than CE1 cysts. In communities where CE1 cysts dominate, transmission from dogs is likely to be more recent than in communities where CE4 and CE5 cysts predominate (Craig et al., 2007; Polat et al., 2003).

WHO	Surgery	Percutaneous	Drug	Suggested	Resources
CE1				<5 cm ABZ PAIR	Optimal Minimal
		✓	✓	>5 cm PAIR + Albendazole	Optimal
CE2			✓	Albendazole	Optimal
	✓	✓			Minimal
CE3a				<5 cm Albendazole	Optimal
		✓	✓	PAIR	Minimal
				>5 cm PAIR + Albendazole	Optimal
				PAIR	Minimal
CE3b				Non-PAIR	Optimal
	✓	✓	✓	Non-PAIR PT	Minimal
CE4				Watch and Wait	Optimal
CE5				Watch and Wait	Optimal

Table 2-3: Suggested approaches to uncomplicated hepatic cysts.

2.10 Aims and Objectives

In the information presented above shows that the morphology of hydatid cysts can vary considerably between patients, requiring different approaches to treatment. However, the variation in cyst morphology can also impact on diagnosis of CE about specific antibody detection tests which are frequently used to confirm the parasitic nature of the lesion in community screening. The observed transitions from one cyst type to another are indicative of disease progression or regression both during albendazole therapy and in untreated patients. However, the changes observed by ultrasound scanning represent gross morphological events and do not reflect underlying immunological variation that may be of use in the follow-up of patient progress.

The Echinococcus intervention programme in Turkana, Kenya run by AMREF generated over 30 years of data relating to cyst morphologies in different age and sex classes. It followed up

many patient's therapy with sequential ultrasound scans and serological sampling, with and without albendazole. The main objective of the project was to establish a better understanding of the factors that influence morphological variation in cysts. The specific aims of this project were therefore:

1. To evaluate age and sex differences in cyst presentation at first detection.

2. To relate morphological changes to different treatment options.

3. To evaluate the relevance of cyst morphology to antibody profiles in relation to immunodiagnostic antigens.

4. To link morphological changes in cysts to antibody profiles during long term follow-up of individual patients with and without albendazole therapy

3 TURKANA SURVEILLANCE AND OUTCOMES OF THERAPEUTIC APPROACHES

The surveillance, the laboratory and clinical teams sometimes accompanied by the University of Salford personnel, created a contemporaneous log of data between 1983 and 2015. This was during the active years of African Medical Research Foundation (AMREF) hydatid control programme in Northern Turkana. In this chapter, we aimed to analyse and interpret the surveillance data. We also assessed the outcomes of different interventions and evaluated responses to treatment for CE patients.

3.1 Introduction

A study in the 1970s revealed that surgical incidence of hydatid disease was an estimated 96 per 100,000 per annum in Turkana (O'Leary, 1976). Another by study estimated annual cases at 19 per 100,000 in the south of Turkana 198 cases per 100 000 people in the north making it the highest in the world (French et al., 1982). Bases on these findings, AMREF started a hydatid control programme in the north of Turkana in 1983.

Morbidity attributable to CE occurs in all age-groups and disease have been described in patients below 1 year old (Tanki et al., 2018) and a man over 80 years (Lopez-Marcano et al., 2017). At infection, patients are asymptomatic and the cysts small. The rate of growth varies in different communities, organs as well as age groups.(Sarkar et al., 2016). Onset of clinical symptoms depends on size of cyst, organ infected, localisation of cyst in that organ, number of cysts and the pressure on surrounding tissue (Usluer et al., 2010). Abdominal cysts can remain asymptomatic for long periods of time whereas lung and bone cysts cause pain quite early after infection and are diagnosed earlier in the infection process (Song et al., 2007). There is also the possibility of cyst rupture due to trauma which can cause anaphylactic shock or may lead

to dissemination of protoscoleces resulting in secondary hydatidosis (Castanares-Zapatero et al., 2009)

In humans, hydatid cysts develop with great variability in size, location and among different human populations (Moro et al., 1999). Slow growth is observed among indigenous people in Alaska compared to the Turkana in Kenya, (Gottstein et al., 1985; MacPherson et al., 1983). Cysts undergo different rates of growth and development or degeneration and calcification sometimes remaining dormant for long periods of time. The factors influencing these variations are not well understood but they lead to complicatios in diagnosis and clinical management (Stojkovic et al., 2016).

3.2 Treatments and interventions

Four treatment modalities were used in the patients diagnosed in Turkana namely Surgery, PAIR, Chemotherapy and watch and wait. Evidence in choosing any of the treatment options remains controversial and the clinician decided what treatment was suited for each patient. Factors influencing these decisions included symptoms, patient history as well as availability of resources (Horton, 2018). In the absence of written guidelines, the interventions in Turkana were explained by the medical officer (Dr. Wangomb'e, personal communication, June 2018).

3.2.1 Surgery

Cystectomy or endo-cystectomy was performed on the patients who had large cysts, cysts with daughter cysts and the cysts with complications. It involved open surgery where the cyst was opened and the hydatid material removed including laminated layer and germinal layer. A protoscolicide (95% ethyl alcohol) was then used to treat the pericyst cavity before suturing the patient. The tissue around the cyst was neither entered nor resected as the operations were carried out by a general surgeon in a rudimentary hospital. Hospital stay was on average 9 days (range 6 – 17 days).

3.2.2 PAIR (Puncture – Aspiration-Injection-Re-aspiration),

A less invasive form of surgery was also used for treatment of large cysts. The procedure involved, (i) per-cutaneous puncture of the cyst under ultrasonographic guidance (ii) aspiration of a substantial portion of the cyst fluid, (iii) injection of a parasiticidal solution (95% ethanol; approximately an equivalent of one- third of the amount aspirated), (iv) re-aspiration of the cyst fluid content after 5 min. It was sometimes preceded and followed up by chemotherapy with albendazole.

3.2.3 Chemotherapy (Albendazole)

Treatment with albendazole was used on patients with inoperable cysts, multiple cysts in two or more organs, high-risk surgical cases, those who refused invasive treatment. In some situations, surgical treatment was not possible due to logistical problems and chemotherapy was offered as an alternative till surgery was possible. Where deemed necessary, cyclic chemotherapy with albendazole was given. The dosage of 10 – 20 mg/ kg of body weight /day divided into 2 doses and administered continuously for 28 days postprandially. The patients were then given a "washout period" of 10 – 15 days when no medication was administered before starting a second dose when applicable. Some patients came to the medical centre to have their medication administered to prove compliance. At the beginning of the study, guidelines on the number of courses to be administered were lacking and some patients received up to 8 courses of albendazole. The guidelines provided some limits where inoperable cases received a maximum of 4 doses, pre-surgical cases had one dose 6 week before surgery and post-surgical prophylaxis cases received up to 3 courses. Some patients had more than one cyst and the treatment regime given was determined by this among other factors including age, weight as well as willingness of the patient to adhere to long term treatment.

3.2.4 Watch and wait

Many patients were followed up with no interventions either indefinitely or until it became necessary and possible to intervene. This was due to limitations in term of resources, personnel and patient reluctance to treatment and comorbidities. Other reasons included adverse effects of treatment, the fact that some cysts remained unchanged either in size or nature while others self-cured. Some people remained untreated for prolonged periods of time as they were not available for treatment. Cases that had received intervention in the past were considered watch and wait if enough time had elapsed from previous intervention. In this study, this was 6 months since the previous chemotherapy treatment and 1 year since the last surgical procedure.

3.2.5 Follow - up

Follow-up on patients either after treatment or for monitoring untreated cases was done via imaging. This was to assess efficacy or cure as well as disease progression or regression. In surgical patients, it was used to monitor healing and residual cavity reabsorption after endo-cystectomy. Post-chemotherapy, patients were followed up to monitor cyst changes in size and morphology. In untreated patients, follow – up was important in monitoring the natural history of the disease, which included cyst type and growth rate.

3.3 Study design

The research and medical team from AMREF carried out a surveillance and treatment programme between 1983 and 2015. All residents of North Turkana were invited to participate in a community-based CE screening programme, which incorporated abdominal ultrasonography and a questionnaire. The participants who received a CE-positive diagnosis upon abdominal examination had a blood sample taken for testing and were requested to complete an epidemiological questionnaire. They were then directed to the local mission hospital where a hydatid treatment unit had been established and partly funded by AMREF.

A second assessment was done at the hospital to determine the best therapeutic pathway for each patient in accordance with the guidelines set by African Medical Research Foundation and the Ministry of Health for Turkana County. The questionnaire was completed by everyone who participated in the study with parents giving consent for children below 18 years of age. The general information gathered included age, gender, location and medical history. In addition, potential factors influencing infection including occupation, previous infection, frequency of contact with dogs and source of water were collected. A Turkana – English translator was employed where necessary though most people spoke Swahili.

3.4 Study area and Climatic Factors

The study area (Figure 3-1) covered 40,000 km^2 which stretched from the Uganda Rift Valley escarpment east of the 34°E to 35°E longitude. To the north, it bordered the Kenya/Sudan boundary and to the south the Lokichar main road The area is hot, dry and has an annual rainfall of 575mm. The rainfall peaks are found in November and March-April (data from the Meteorological Station, Lokichokio). The surveillance was done during the dry season when most people were found in an accessible area.

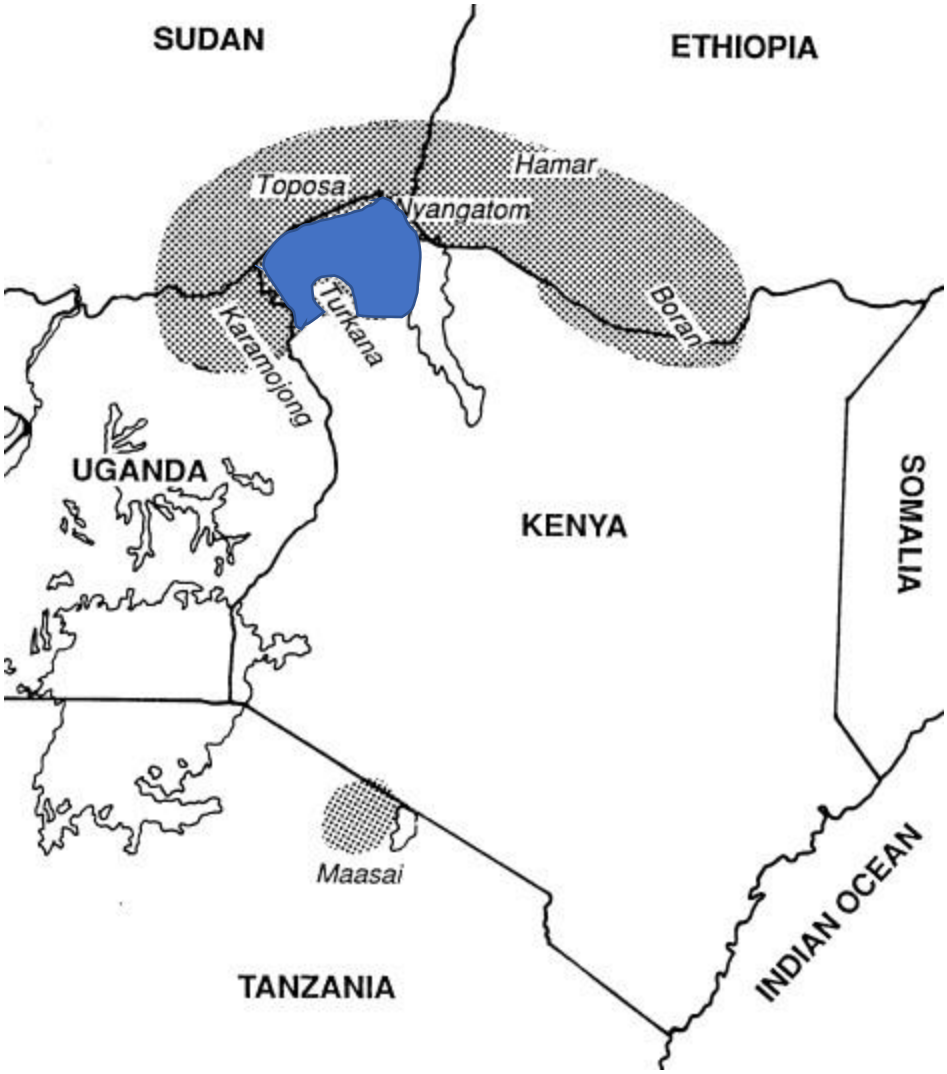


Figure 3-1: Study Area.

The area shaded blue shows the North of Turkana where the surveillance took place (Romig et al., 2011)

3.5 Ethical considerations

At the beginning of each screening session, informed consent was obtained from all adults participating in the survey and from a parent for each participating child. To maintain confidentiality, each participant was assigned a unique identification number which was also used in follow-up. Participation was voluntary, and individuals were not obligated to remain in the study. The study was reviewed and approved by the African Medical Research Foundation Hydatidosis Control Program and the Kenya Ministry of Health. With regards to

this PhD project, written consent was obtained from AMREF authorising the use of the database and serum samples for further research. This was submitted for ethical approval through the University of Salford.

3.6 Screening for hydatid cysts

In the field surveillance of CE, diagnosis and classification was carried out using a portable 3.0 MHz real-time linear ultrasound scanner (Siemens Healthcare Limited, Surrey UK). This was powered by a 1 kW portable electric generator equipped with an accurate measuring gauge. Most patients were screened standing up using liquid paraffin as a transducing medium (). The cysts were categorised accordig to the guidelines of World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE). They were classified as active (CL, CE1 and CE2), transitional (CE3), or inactive (CE4 and CE5) based on their appearance (**Figure 3-2**). Due to the long term nature of this study, C3 cysts were not sub-divided into C3a and C3b as this only became standard many years into the study.and is now considered to be a useful approach. Where possible, cyst sizes and numbers were recorded and monitored in subsequent visits even after completion of therapy, cure or untreated cases.

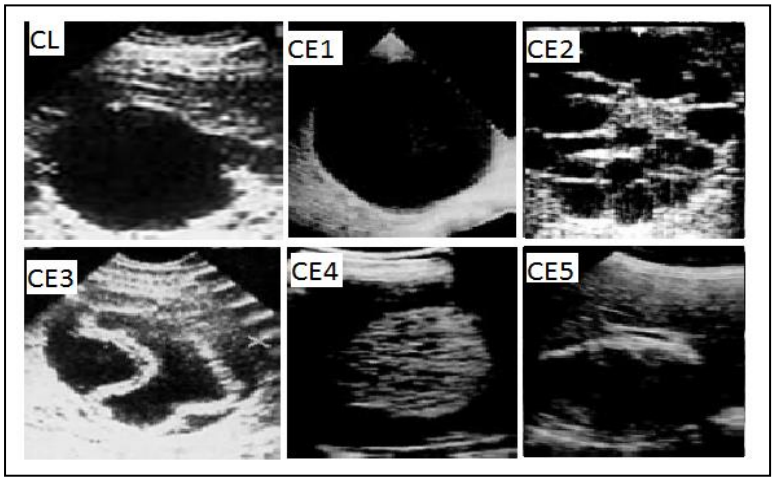


Figure 3-2: Classification of Hydatid Cysts:

Key: CL – Active, uniform nuclear fluid, no clear cyst walls; CE1 – Active, unilocular fluid-filled cyst; CE2 – Active, with daughter cysts; CE3 – Transitional cyst with collapsing wall; CE4 – degenerative contents and inactive with hyperechoic fluid; CE5 – inactive calcified cyst (WHO-IWGE, 2003).

3.7 Statistical Analysis

In community surveys, data from interviews and ultrasound scans were entered in Excel and analysis and figures made using SPSS statistical package version 23 (SPSS Inc Chicago, IL), R statistical software version 2.15.2 (R Development Core Team, 2012) and (GraphPad Prism version 6, SanDiego - CA USA). Descriptive statistics were used to assess the frequencies in both demographic, human factors and follow-up periods.

Associations between the demographic and human factors and cyst morphologies were done using Chi square test. Where these associations were significant, ordinal multiple regression was done to describe them.

3.8 Results

Over 50,000 people were screened during the AMREF programme which ran from 1983 to 2015. A simple flow chart showing how patients were recruited in the study is shown **in Figure 3-3**

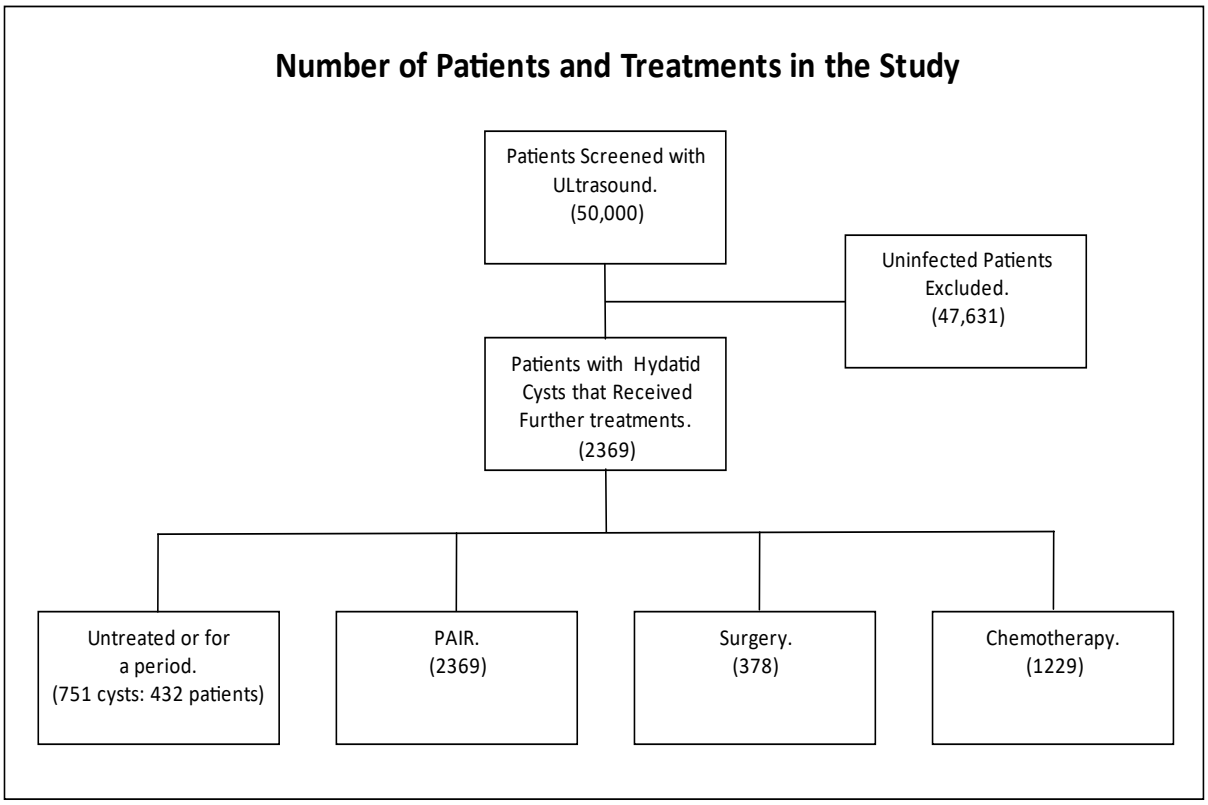


Figure 3-3: Patient recruitment by AMREF in Turkana

3.8.1 Screened Population

During the running of the programme, 2369 patients from a cross-section of locations in the North of Turkana County were diagnosed as having CE using ultrasound imaging. During the running of the programme, 2369 patients from a cross-section of locations in the North of Turkana County were diagnosed as having CE using ultrasound imaging. Majority of patients were identification and recruitment from patient files supplied from AMREF surveillance study. These patients were seen and treated in Kakuma Mission Hospital. Other patients were from Kakuma main hospital and Lopading sub-County hospital. Of these, 299 had had a previous surgical procedure to treat CE and were considered as a re-infection or a recurrence.

The Mean ± SE age of patients at diagnosis was 28.53 ± 0.325 and a male: female ratio of 1: 2.3. Other demographic factors are summarised in **Table 3-1** below.

Attribute	Characteristic	n	Percentage
Gender	Male	606	29.3
	Female	1463	70.7
Previous Surgery	Yes	299	17.2
	No	1740	82.8
Cyst Number	Single	1431	60.7
	Multiple	931	39.3
	2 cysts	611	25.8
Multiple Cysts	3 - 5 cysts	222	9.4
	6 - 8 cysts	61	2.6
	> 8 cysts	28	1.2
Cyst Type	CE1	777	33.4
	CE2	604	26
	CE3	240	10.3
	CE4	677	29.1
	CE5	29	1.2

Table 3-1: Characteristics of hydatid patients at first diagnosis.

3.8.1.1 Proportion of Infections during the years of surveillance

The surveillance programme was run in phases of 3 years beginning in 1983 to 2015. There was a break in active surveillance between 2004 and 2011 where only clinical diagnosis was done on self-referring symptomatic patients. To assess longitudinal changes in infection proportion for different age groups during the 25 years of active surveillance, the number primary infections was analysed, and the cases stratified in to 8 age groups. Primary infection was assumed if there was no previous history of CE infection. The hypothesis explored was that the infection dynamics did not change with time and were there was no variation in infection proportions in different age groups. **Figure 3-4** shows the proportion of new cases divided up according to age groups during each phase of the surveillance.

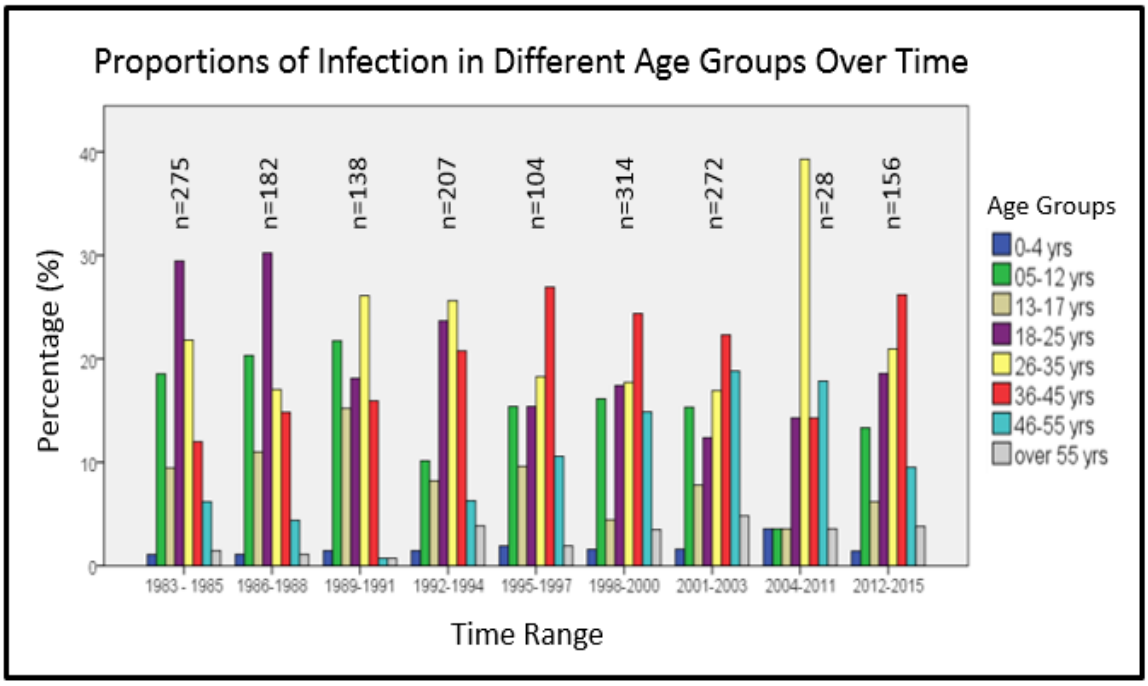


Figure 3-4: Changes in proportion of infection in different age groups over the years

Throughout the years of active surveillance, the children below 4 years did not show much fluctuation in proportions of infection 1.6 ± 0.7 %. This was also the case with those over 55 at 2.76 ± 1.46 %. The proportion of infection in other age groups varied at diagnosis over the

years. The proportion of infection in older children in the age-bracket 5 – 12 showed marked fluctuations and declined from between 19 - 21% at the start of the surveillance to between 10 -15 % by the end. The proportions of infection in this age bracket continued to fall during the years of passive surveillance.

Infection proportions in the 5 -12 years age group was 18.5% at the start of the program, gradually increasing to 21.7 by the 9th year of surveillance. This trend reversed in the following years with fluctuations of between 10 and 16%. There was only one symptomatic case during the years of passive surveillance and when the field diagnosis resumed, the proportions come down slightly from 15.3% to 13.3%.

Among the 13 – 17-year olds, there was gradual increase in infection proportions in the first 9 years of surveillance. These decreased sharply by the 12th year and remained relatively low at around 7%. In similarity to the younger groups, there was only one symptomatic case in the years of passive surveillance and infection proportions had declined further when active surveillance was resumed.

The group with the highest number of cases at the start of the program was in the 18 – 25 years age group. There was 29.5% in proportion in this age group, rising to 30.2% 6 years after the start of the programme. By the ninth year, the number had declined sharply to 18% and despite fluctuations over the years, the proportion declined to 12.4% by the end of the first phase of active surveillance. Four symptomatic cases were diagnosed and treated during the 7 years period of passive surveillance. This age group appeared to be the most susceptible group and on restarting the field diagnosis, the proportion had increased from 12.4% to 18.6% in the asymptomatic population. The proportion of infection fluctuation was also high 19.96 ± 6.44 %.

The second highest proportion of infection at the start of the surveillance programme was among the 26 – 35-year olds at 21.8%. unlike the younger group, there was a decline in the proportion of cases in the first 6 years of study. This was followed by an increase in proportions in the years that followed with the highest proportion being in the 7^{th} -12^{th} year at which this group had the highest proportion of new cases. B There was gradual decline and by the end of active surveillance, this group accounted for 17% of all the cases. The break in active study had the greatest negative impact in this age group as on restart there was a 5% increase in proportion of cases affected. The highest number of symptomatic self-referring cases (39.3%) during the passive years was also found in this group. This group had highest number of cases and greatest variation in infection proportion at 22.63 ± 7.16%.

Age group 36 – 45 years saw the proportion of new cases increase gradually for 15 years in a row. At the start of surveillance, the proportion was 12% increasing gradually to 27% in 15 years. By the 13th year of the program the proportions the highest number of new cases (26.9%) was in the 36 – 45 years age group. With slight fluctuations, it remained in between 22 – 27% throughout, even after the surveillance break. There was 14% proportion of symptomatic cases needing treatment during the inactive years

The 46 – 55 years age group had few cases at the start of study and the proportion of infection declined for the first 9 years of active surveillance from 6.2% to 0.7%. The proportions then increased gradually to 18.8% in the next 12 years at the end of active surveillance. During passive surveillance, 18 % of the cases seen were in this age group. On return to field diagnosed, the proportion had declined to 9.5%. A similar trend was seen in those over 46 years old.

The difference in infection over the years was significant among the 13 – 17-year olds ($p = 0.016$), 18 – 25-year olds ($p < 0.001$), 36 – 45-year olds ($p = 0.002$) and 46 – 55 age group ($p < 0.001$). There was increase in mean age at first diagnosis from 25.4 to 31 years during the years of active surveillance between 1983-85 and 2010-2015.

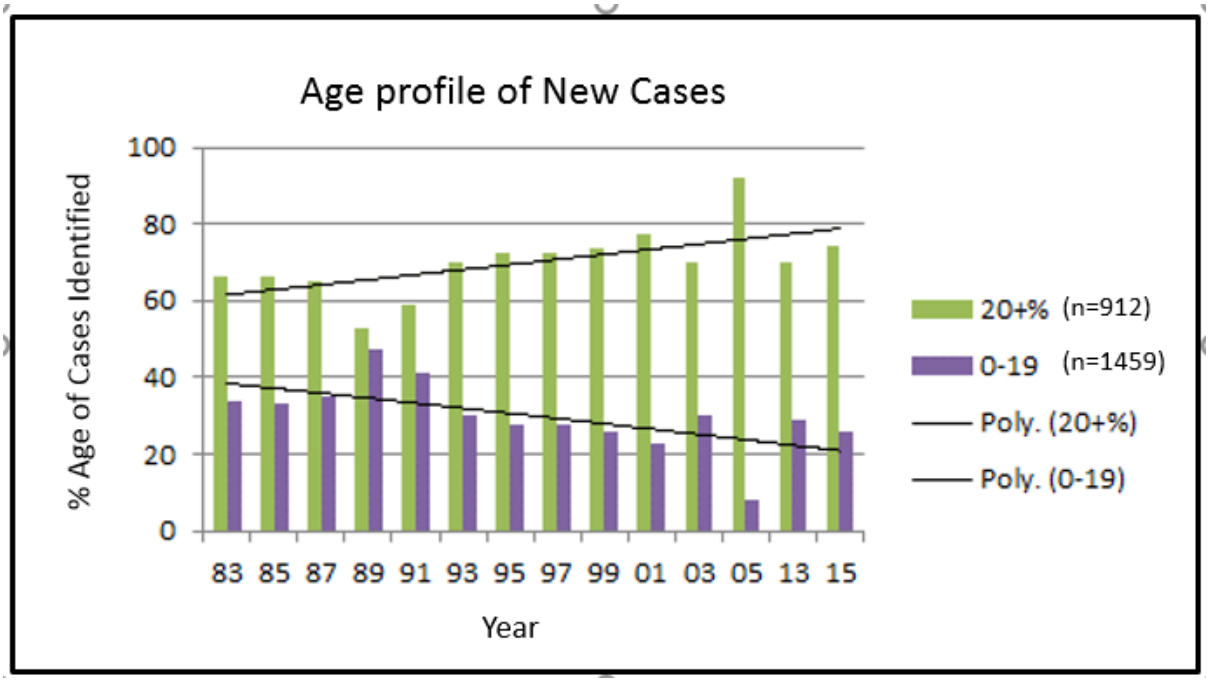


Figure 3-5: Change in infection proportion in years of Surveillance

There was an opposite trend in infection proportions among the cases under 20 and those over 20 years (**Figure 3-5**). At the start of the program, infection proportion between people under 20 and those over 20 was 35% and 65%. The trend showed an initial increase in persons under 20 and decrease for persons older than 20 years. This trend peaked in 1988-1989 with almost similar rates of infection. The number of younger infections started declining in younger cases compared to the older cases to 8% and 92% by 2005. After a 7-year break from active surveillance, the rate of infection among the young had increased to 29% and reduced to 26% in the next 3 years of surveillance.

3.8.1.2 Differences in infection between genders at diagnosis

The participants diagnosed as infected with CE were categorised according to gender as well as age groups. The differences between male and females at diagnosis is shown in. **Figure 3-6**.

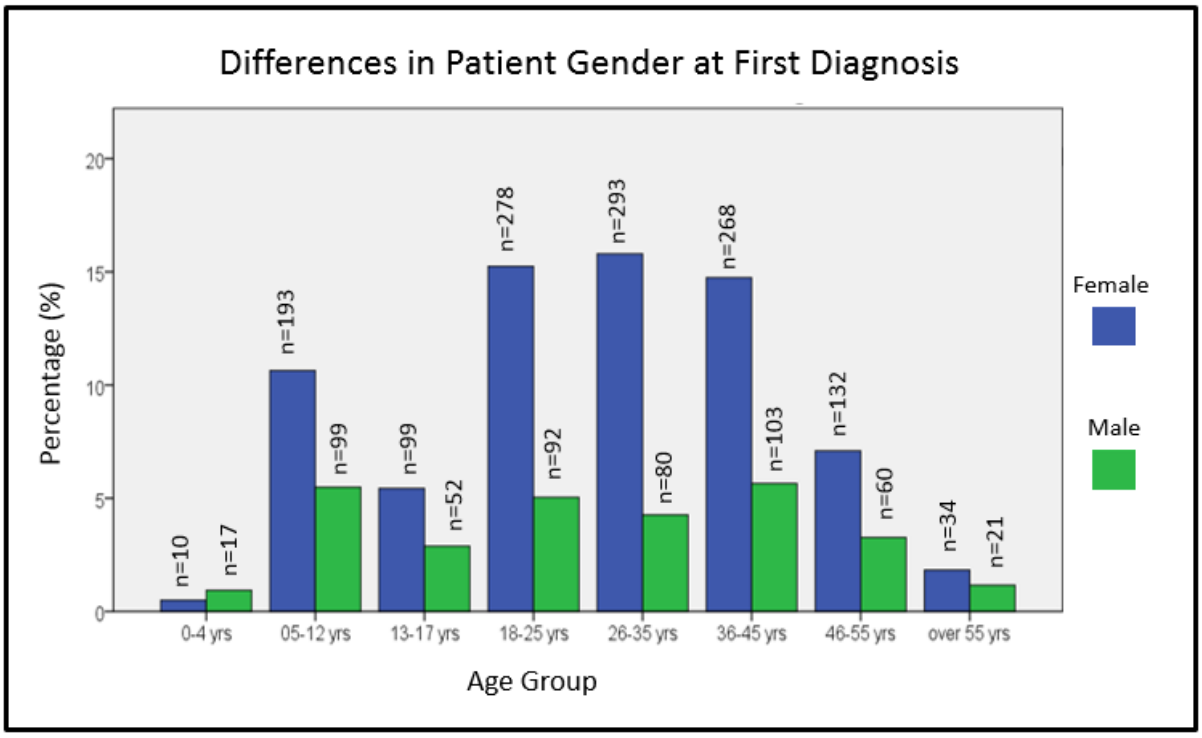


Figure 3-6: Gender differences according to age group at first diagnosis.

There was a great variation between genders in different age groups. In children below 5 years, more males than females were infected, but the trend changed in older cases. In the pre-pubescent age group of 6 – 15, the gap in infection between male and female became eminent and increasingly so with the child bearing age of 16-25 years and child rearing years of 26-45 years. This difference reduced in the later years of 46 – 65 and almost returned to similarity with childhood years later in life, in the over 65 years.

Children less than 4 years old had a female to male ratio of 1:1.88. This was the only age group where males diagnosed were significantly more than female ($p < 0.000$). All other cases had a higher number of female infections than male. The age groups that showed significant differences were 5 – 12 years old, ($p = 0.05$), 26 – 35 age group, ($p<0.001$).

The highest number of infections was among women between 36 and 45 years, also the highest number of males diagnosed belonged to this age group. Just under 18% of the patients were

children under 13 years of age with the youngest being 15 months old and 13.4 % were over the age of 46 with the oldest in this study was 63 years old.

3.8.1.3 Cyst locations at first diagnosis

Overall, 3119 cysts were detected via ultra sound imaging over 32 years of surveillance. Of these, 2775 cysts were in what are generally referred to as common sites and they accounted for 93 % of all the cysts. Most liver and kidney cysts were solitary but in all other organs, more than 50 % of the cases had multiple organ involvement ranging from 2 to 5 organs. **Table 3-2** shows the differences in single and multiple infections according to organs. In patients diagnosed with cysts in the right liver lobe, 72.1% had solitary cysts, 18% cysts in a different lobe or organ concurrently, 6.5% harboured cyst in 3 different organs etc.

Affected Organ	n	Single cyst n (%)	Cysts in 2 organs n (%)	Cysts in 3 organs n (%)	Cysts in 4 organs n (%)	Cysts in 5 organs n (%)
R Liver	1458	1051 (72.1)	262 (18.0)	95 (6.5)	40 (2.7)	10 (0.7)
L Liver	387	167 (43.2)	130 (33.6)	39 (10.1)	41 (10.6)	10 (2.6)
Kidney	66	44 (70.6)	4 (6.1)	7 (10.6)	1 (1.5)	10 (15.5)
Spleen	128	60 (46.9)	29 (22.6)	18 (14.1)	12 (9.3)	9 (7)
Omentum/Mesentery	409	133 (32.5)	144 (24.1)	80 (21.4)	42 (12.1)	10 (3.1)
L Abdomen	330	131 (39.7)	80 (24.2)	70 (21.2)	39 (11.8)	10 (3.0)

Table 3-2: Predilection sites of cysts at first diagnosis in common organs

The most common organ involved was the liver harbouring 66.5 % (n = 1885) with the right lobe harbouring 52.6 % (n=1458) and the left lobe 14.9% (n=387). Most of the cysts in the right liver lobe were single cysts (72.1%, n=1051) but those in the left lobe were mostly coinfections with other organs. Apart from the right liver lobe and the kidneys, less than half of the cysts found in other organs were single cysts.

Most multiple organ infection was found to involve both lobes of the liver with or without any other organ involvement (187 cases). In 2 cases both lobes and the kidneys were involved, in 15 patients both lobes and the spleen, in 68 patients both lobes and the omentum and in 52

cases both lobes and the lower abdomen. In other cases, only the right lobe was involved in multi-organ infection. Coinfection between right lobe and the omentum/mesentery was found in 212 cases while with the lower abdomen with was found in144 cases. In cases where 2 organs were involved, only 43 patients did not have hepatic cysts and in infection involving 3 organs only 4 did not have liver involvement. All cases with more than 3 organs infected had a hepatic cyst.

The mesentery/omentum had the highest number of extra hepatic cysts with 409 (14.7 %). Unlike the liver, single cysts accounted for only 32% (n=133) with high coinfection with the liver and the lower abdomen. Coinfection without hepatic involvement was found in 29 cases where the other organ was the lower abdomen, 3 cases with the spleen and 1 with the kidney. The kidneys had the second highest percentage of single cysts (70.6) though the number of patients was small compared to the 3 main organs. There were 42 cases of right kidney infection while the left kidney had only 11 cases. In 8 cases, the cysts in the left kidney were solitary.

Between them, the lower abdomen, the liver and the omentum/mesentery accounted for 92.8 % of all the cysts found during imaging to have multiple organ involvement.

3.8.1.3.1 Cysts in rare locations

Non-common locations of hydatid cysts infections are considered to be cysts found in other body organs apart from the ones described above. The cases in this study are summarised in **Figure 3-7** below.

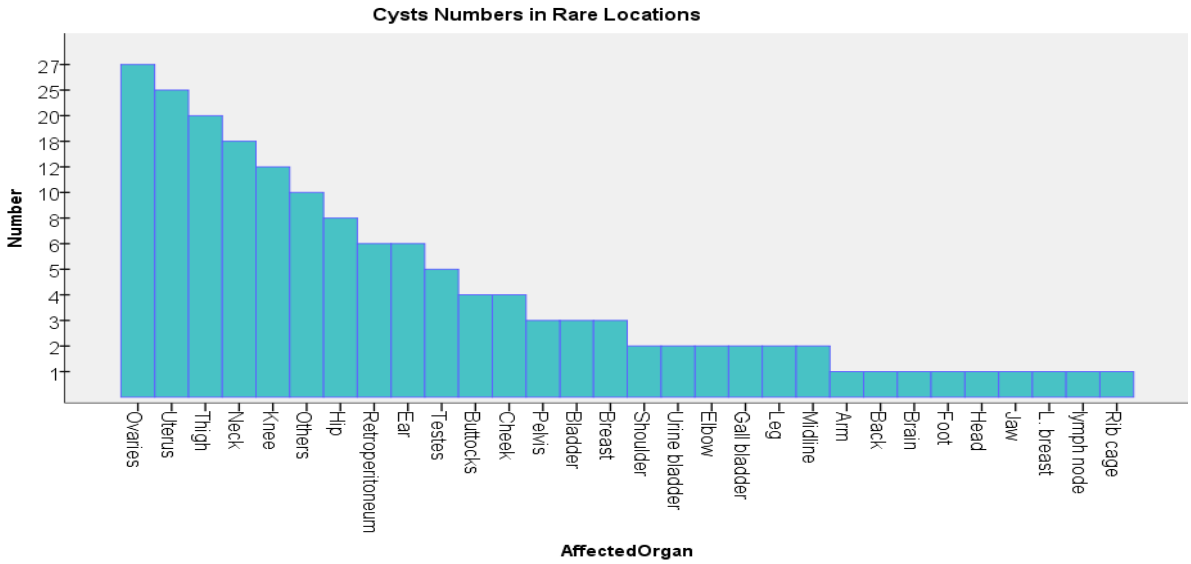


Figure 3-7: Rare locations of hydatid cysts

In Turkana, 175 cysts fell in this category with the highest number being found in the ovaries/uterus 29.9% (n = 52), thigh 11% (n = 20), neck 10.3% (n = 18) and knee 6.9% (n = 12). Of interest in this group was the number of cases where the rare location was the primary cyst. In 86% (152) of the cases, there no other organ involved, 18 cases had 2 organ involvements, 1 case had 3 organs infected and 4 cases the patients had 4 or more cysts elsewhere in the body.

3.8.1.4 Factors associated with cyst type at diagnosis

Data from patient history and questionnaire was compiled to find associations between gender, age and pervious treatment with cyst morphology. A summary of these demographic and human factors and their association to different cyst types at diagnosis were analysed and described in **Table 3-3**.

Factor	Characteristic	χ^2	df	p value
Age	CE1	195.534	108	0.000***
	CE2	144.916	108	0.031**
	CE3	135.649	108	0.000***
	CE4	188.741	108	0.000***
	CE5	171.736	108	0.001**
Gender	CE1	0.034	1	0.448
	CE2	0.714	1	0.215
	CE3	1.275	1	0.149
	CE4	1.933	1	0.091
	CE5	0.149	1	0.442
Previous Surgery	CE1	18.429	1	0.000***
	CE2	23.308	1	0.000***
	CE3	4.655	1	0.031**
	CE4	2.644	1	0.062
	CE5	0.865	1	0.275

***significant at 1% and ** at 5%

Table 3-3: Factors associated with cyst types at diagnosis

Age was associated with CE1, CE3, CE4 and CE5 cyst types at 1% level and with CE2 cyst type at 5% level. Gender was not while previous surgery showed significant association with CE1 and CE2 cysts at 1% level, CE3 at 5% level and CE4 at 10 % level. The relationships were further investigated and reported below.

3.8.1.4.1 Relationship between age and cyst types at first diagnosis

At primary diagnosis, cysts were described according to cyst type. The differences in cyst types at diagnosis with age were assumed to be indicative of length of time from infection. A total of 2757 cysts were characterised according to cyst type and age at diagnosis (**Figure 3-8**). Of these, 826 (31.2 %) were CE1 cysts, 782 (28.3 %) CE2 cysts, 270 (9.8 %) were CE3, 779 (28.2) were CE4 and 30 (1.1 %) were CE5.

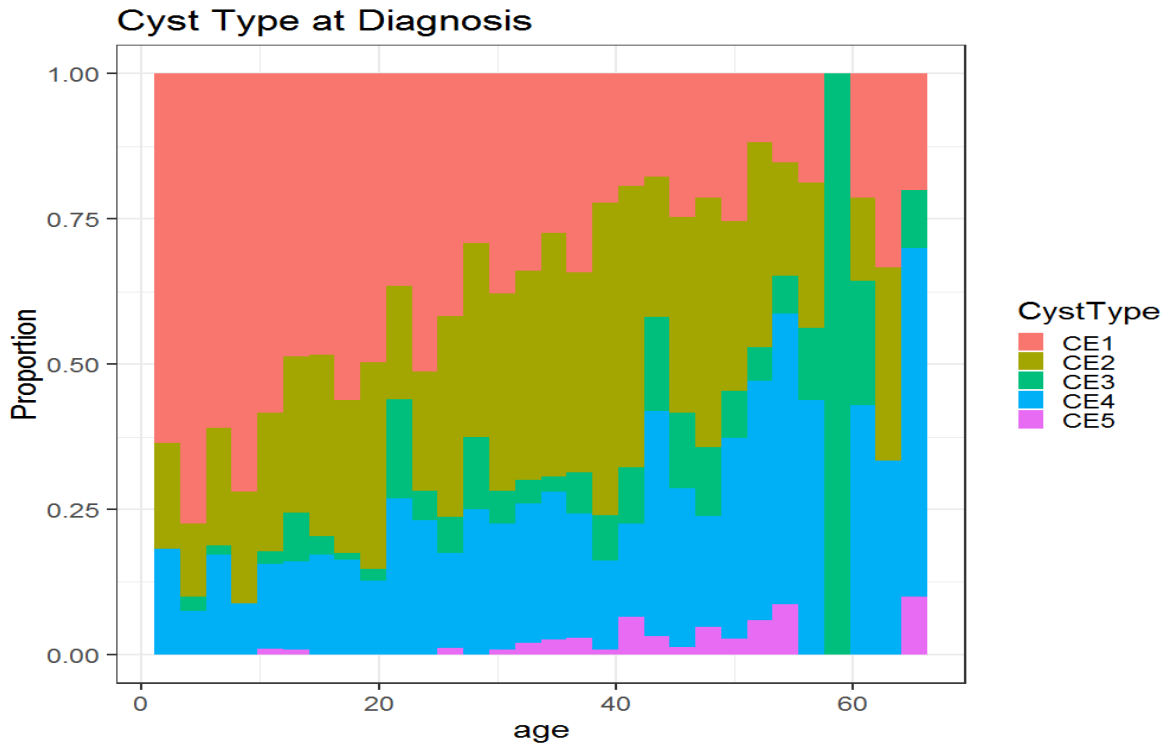


Figure 3-8: Cyst types at first diagnosis with age

Key: Red is representative of CE1 cysts, olive of CE2 cysts, light green of CE3 cysts, blue represents CE4 cysts and pink represents CE5 cysts. 1 rectangle represents 2 yrs

In children below 12 years, the predominant cyst type was CE1 accounting for 63.4 % of all cysts in this age group. This was the highest proportion in any age group and accounted for 26.1 % of all CE1 cysts. There was a gradual decrease with little fluctuations in proportion of patients with CE1 cysts with age. Among those between 46 and 55 years CE1 cysts accounted for 21% of all the cyst types. The proportion of CE2 cysts was lowest among children under

12 years and adults over 55 years old. This was significantly different to all other age groups which had a mean proportion of 33.7% and SD = 4.3. Unlike CE1 whose proportion reduced with age, CE2 cysts appeared stable with few fluctuations. The proportion of CE2 cysts fluctuated very marginally and remained very similar in for the ages between 13 and 24 years old. This number increased again between 26 and 45 coming down again from 45 to the oldest age group. Infection with CE2 was similar in the very young and the very old.

Compared to the numbers seen with the rest of the cysts, very few CE3 cysts were diagnosed. Over 87 % were found in the cases older than 18 years old with a third in the those 36 – 45-year olds. Recent classification methods have divided CE3 and some CE4 cysts into CE3a and CE3b and there may have been mis-categorisation. In the cases diagnosed as CE4 cysts, 94% were over 18 years old. The youngest child with a CE4 cyst was 3 years old indicating that the rate of cyst change from CE1 to CE4 can be less than 3 years. Over 40% of newly diagnosed cases in the over 55 age-group had CE4 cysts a complete reversal of what was seen in the children under 4. Similarly, the highest percentage of those in the age group 45 – 55 had a disproportionately large number of CE4 cysts compared to other types as well as other groups.

Overall, age was shown to be associated with cyst type according to the regression model shown in **Table 3-4**.

Cyst Type	Estimate	Std Error	df	sig	95% Confidence Interval	
					Lower Bound	Upper Bound
CE1	-0.012	0.003	1	0.000***	-0.017	-0.006
CE2	0.015	0.003	1	0.000***	0.009	0.020
CE3	0.002	0.004	1	0.673	-0.006	0.010
CE4	0.005	0.003	1	0.062	0.000	0.010
CE5	0.042	0.012	1	0.000***	0.019	0.066

***significant at 1% and ** at 5%

Table 3-4: Contribution of age to cyst morphology at diagnosis

The results showed that there was a strong association between age and cyst type. There was a significant and negative relationship between CE1 cyst types with age and older cases were less likely to be diagnosed with CE1 cases. Conversely there is a strong significant positive coefficient CE2 and CE5 cyst types indicating that older cases were highly likely to be diagnosed with CE2 and CE5 than were younger cases. The association with CE4 was and CE3 were not significant.

3.8.1.4.2 Relationship between age and cyst numbers at first diagnosis

Cyst numbers at first diagnosis varied with age. The number of cysts per person ranged from 1 to more than 8. The cyst numbers increased with age though most cases harboured solitary cysts (**Figure 3-9**). All children below 3 years had a single cyst. The youngest case with 6 – 8 cysts was 10 years old and the oldest person was 50 years old. The highest number of infections with 8 or more cysts was seen among 43 – 50 years old with the youngest being 19 years old and the oldest 54 years old.

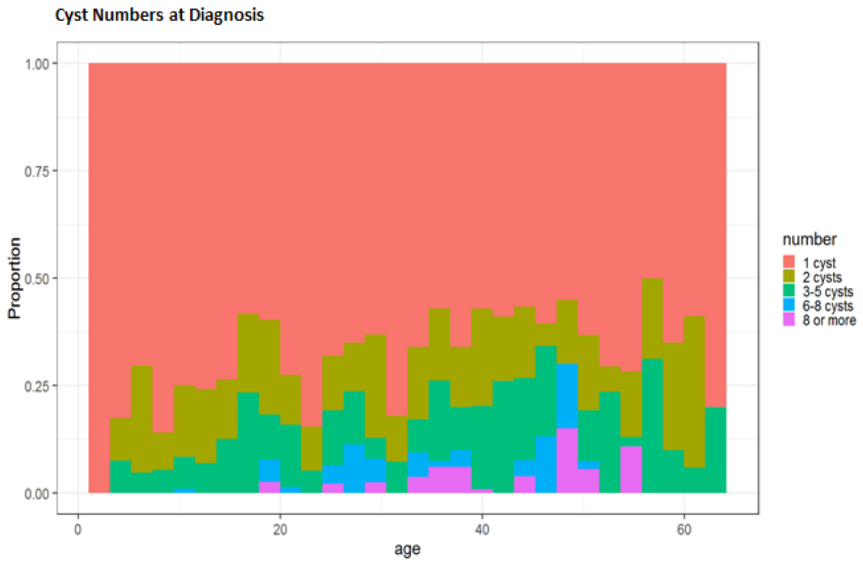


Figure 3-9: Number of cysts with age group

Key: red represents 1 cyst, olive depicts cases with 2 cysts, Light cases with 3 - 5 cysts, blue depicted cases with 4 – 8 cysts and pink more than 8 cysts was shown in colour.

Overall, cyst numbers varied significantly with age (χ^2= 595.69, p < 0.000). Infection with multiple cysts increased gradually with age with though most of the patients only ever harboured one cyst. The highest number of cysts recorded in one individual was 14 in 5 different organs and 3 different cyst types. Overall, age was shown to be associated with cyst type according to the regression model shown in **Table 3-5**.

				95% Confidence Interval	
Cyst Type	Estimate	Wald	Sig.	Lower Bound	Upper Bound
1 Cyst	0.473	0.452	0.501	-0.907	1.854
2 cysts	2.060	8.513	0.004**	0.676	3.444
3-5 cysts	3.786	27.935	0.000***	2.382	5.190
>8 cysts	-4.641	40.411	0.000***	-6.072	-3.210

***significant at 1% and ** at 5%

Table 3-5: Contribution of age to cyst sizes at diagnosis

The results showed that there was a strong association between age and cyst numbers for multiple cysts. There was a significant and positive relationship between for 2 to 5 cysts with age and older cases were more likely to be diagnosed with multiple cysts. Conversely there is a strong significant negative coefficient with more than 8 cysts indicating that older cases were less likely to be diagnosed multiple infections than were younger cases. The association with CE1 cysts was not significantly associated with age.

3.8.2 Evaluation of therapeutic approaches

Patients diagnosed with CE during surveillance were directed to the local mission hospital. The hospital had a working relationship with the surveillance project and free treatment was offered. A second assessment was done at the hospital to confirm field analysis. Confirmed CE patients received one of four approaches, chemotherapy, surgery, PAIR or watch and wait. Due to the fragmented nature of the data in terms dosage, cyst locations and duration of treatment, analytical parameters were established to assess a vast amount of data. Descriptions of outcomes were appropriated to provide enough differentiation for analysis and summarised as below.

a) Cysts not operated on

Cured was recorded where

- Cyst contents had degenerated from active and transitional cysts (CE1, CE2, CE3) to inactive and calcified cyst types (CE4 or C5)
- The cyst was clinically assessed as disappeared.
- There was complete detachment of cyst membrane

Improved cases were registered where

- There was partial degeneration of cyst content and the classification moved from active to transitional cyst type (CE1 or CE2 to CE3).
- There was reduction in cyst diameter by 10 % or more
- There was partial detachment of cyst membrane, loss of daughter cysts or appearance of calcification

No change was recorded where there was no visible change in appearance, or the size had reduced by less than 10 % of the size of the original cyst

A *deteriorated* cyst described a cyst which had

- Grown bigger in size,
- Reverted to an active cyst (CE1 or CE2) from transitional and inactive (CE3 and CE4)
- Had not changed in size or morphology but had become infected usually bacterial infection and causing secondary abscess.

b) Pair Cases

Cured was recorded for those that showed cysts disappearance or complete obliteration of the internal structures of the cyst mostly leaving just a thin echogenic linear scar

Inactive was recorded where cysts showed partial obliteration or calcification of the cyst contents. Also, for cysts that showed a folding of the endocyst with significant size reduction and absence of daughter cysts

Regenerating Was recorded where cysts appeared to be regaining their original size and morphology or progressing to active types from inactive.

c) Cysts operated on

Cured was recorded where

- Cysts were no longer visible
- Cysts had collapsed completely.

c) ***Recurrence*** was recorded where a cyst was found in the residual cavity of one that had been previously operated.

Notes

- All patients whose compliance to chemotherapy was inadequate were excluded from data analysis.
- Analysis excluded lung cysts which could not be assessed by ultrasound. In patients harbouring multiple cysts, each cyst was treated individually

- Time was measured from the end of the last dose of chemotherapy or the date of surgery.

3.8.2.1 Results in followed-up untreated patients

A total of 432 patients harbouring 751 cysts were followed up for between 6 and 38 months with 1788 observations being made. A survivorship curves for different outcomes are described **Figure 3-10**

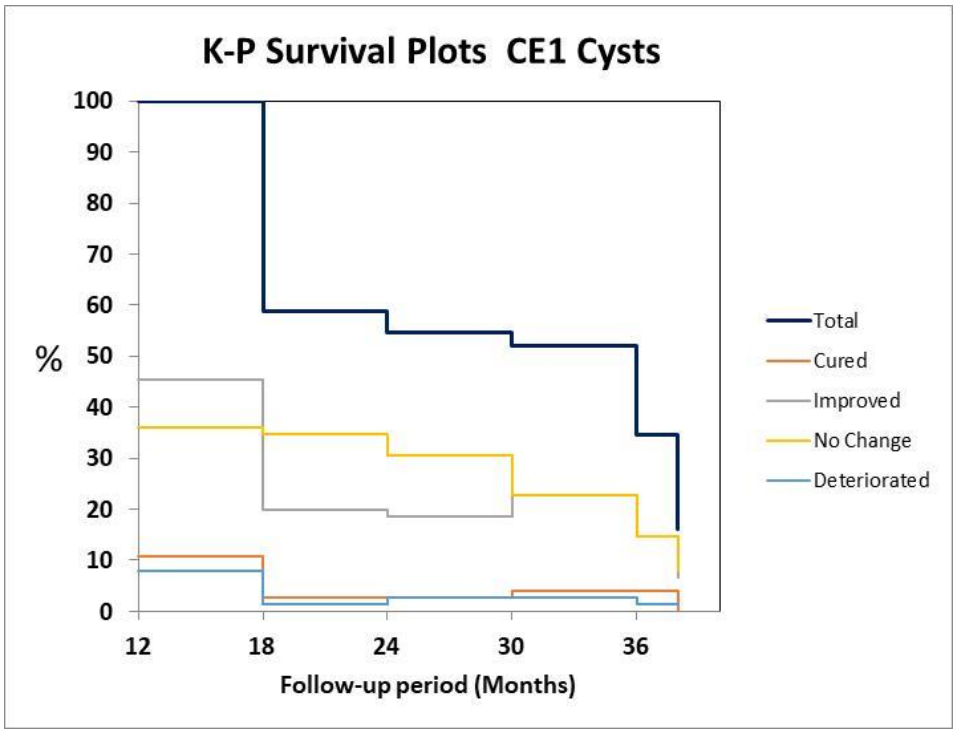


Figure 3-10: A survivorship curve for untreated patients.

The age groups of the followed-up patients varied between 3 years and 55 years with a mean of 31.8 years and a standard deviation of 14.2 years. Other factors are described in **Table 3-6**.

Patients were considered untreated if they did not receive any intervention after diagnosis, had received chemotherapy but a period of 6 months had lapsed since the last dose or had received a surgical intervention but a period of 1 year had lapsed since the last surgical procedure.

Factor	Characteristic	n	%
Gender	Female	577	76.80
	Male	174	23.20
Previous Surgery	Yes	29	0.90
	No	722	99.10
Cyst Nature	Multiple cysts	480	63.91
	single cyst	271	36.09
Cyst Type	CE1	237	31.60
	CE2	136	18.13
	CE3	156	20.80
	CE4	206	27.47
	CE5	15	2.00
Follow - up	06-12 months	191	25.47
	13 - 18 months	163	21.73
	19 - 24 months	136	18.13
	25 - 30 months	124	16.53
	31 - 36 months	104	13.87
	37 - 38 months	32	4.27
Cyst Size	Large	204	27.2
	Medium	348	46.4
	Small	21	2.8
	Very Large	177	23.6

Table 3-6: General description and follow-up period of patients that were untreated

The time elapsed in months between a paired first and last observation either before treatment or patient's last attendance was recorded. Cyst type at the start and the end of follow up were also noted. The outcome of cured, improved, no change or deteriorated was recorded at the end of follow-up (**Table 3-7**).

Cyst Type	Change	n (%)	Mean and (S.D) of Follow-up Period	95 % Confidence of Follow-up Period
CE1	Cured	18 (7.6)	18.44 (10.6)	13.15 – 23.74
	Improved	89 (37.6)	19.09 (10.1)	18.01 -26.49
	No change	110 (46.4)	20.26 (8.9)	18.57 – 21.96
	Deteriorated	20 (8.4)	22.25 (9.05)	18.01 – 26.49
CE2	Cured	4 (2.9)	18.44 (10.6	13.15 – 23.74
	Improved	61 (44.9)	19.01 (10.1)	16.8 – 21.44
	No change	65 (47.8)	20.26 (8.9)	18.57 – 21.96
	Deteriorated	6 (4.4)	22.5 (9.8)	18.01 – 26.49
CE3	Cured	25 (16.1)	20.42 (9.7)	16.52 – 24.33
	Improved	28 (18.1)	23 (9.3)	19.38 – 26.62
	No change	89 (57.4)	20.38 (8.6)	18.56 – 22.21
	Deteriorated	13 (8.4)	20.46 (6.9)	16.24 – 24.68
CE4	Cured	10 (4.9)	16.2 (8.8)	9.85 – 22.55
	Improved	62 (30.1)	19.68 (9.4)	17.29 – 22.04
	No change	98 (47.6)	20.91 (9.0)	19.1 – 22.72)
	Deteriorated	36 (17.5)	24.08 (7.9)	21.42 – 26.75
CE5	No change	11 (78.6)	24.55(7.8	19.26 – 29.83
	Deteriorated	3 (21.4)	21.67 (10.2)	-3.83 – 47.16

Table 3-7: Outcomes of untreated patients over time

The highest proportion of cured cases was seen in CE3 cysts where 16.1 % of the cases were cured in an average of 20 months. The second-best cure results was in the CE1 group with 7.6 % of the cases considered cured in the shorter period of time on average 18.4 months. Noteworthy is the fact that CE3 cases achieved better cure rate than CE4 cysts. Patients with CE2 cysts showed the lowest rate of cure without intervention at 2.9 % but greatest rate of improvements at 45%. The greatest proportion of cysts remained unchanged both in morphology and size (49.7%) and few cysts deteriorated compared to other outcomes (10.1%).

The association between outcome, follow-up period and cyst type were analysed and tabulated in **Table 3-8**. We sought to find out if there was any association between the cyst type and the cyst condition at the end of follow-up otherwise named outcome.

Factor	Characteristic	χ^2	df	p value
Outcome	CE1	17.434	3	0.001***
	CE2	8.444	3	0.038**
	CE3	33.224	3	0.000***
	CE4	17.536	3	0.001***
	CE5	7.996	3	0.046**
Follow-up Period	CE1	10.244	5	0.069
	CE2	2.155	5	0.827
	CE3	5.948	5	0.311
	CE4	2.221	5	0.818
	CE5	11.197	5	0.048**

***significant at 1% and ** at 5%

Table 3-8: Association between outcome and cyst type in untreated patients.

Overall, paired before and after results showed that 10.4 % (n=78) of the cysts got worse by either progressing to CE1 or CE2 cyst from CE3 or CE4 cysts or by growing larger or causing clinical symptoms. Another 32% (n = 240) improved with no intervention either by regressing from an active CE1 or CE2 to a CE3, CE4 or CE5 cyst. Nearly half of all the cases (49.7 %, n = 373) showed no clinical or morphological difference. In 7.9 % (n=59) of the cases, cysts were not visible or were calcified CE5 inactive cysts in consequent imaging. Association between disease outcome and the cyst type at the start of follow-up was analysed using chi square and the results tabulated in **Table 3-8**. There was significant association in disease outcome at 5% level for CE2 and CE5 and at 1% level for CE1, CE3 and CE4. There was also an association with CE5 at 5% levels.

To assess the type of relationship this association had an ordinal regression model was done and the results tabulated in **Table 3-9**.

Cyst Type	Results	Estimate	Std Error	sig	95% Confidence Interval	
					Lower Bound	Upper Bound
CE1	Follow-up	-0.009	0.009	0.310	-0.026	0.008
	Cured	0.038	0.305	0.902	-0.560	0.636
	Deteriorated	-0.726	0.325	0.025**	-1.362	-0.090
	Improved	0.476	0.174	0.006**	0.135	0.817
CE2	Follow-up	-0.009	0.011	0.417	-0.029	0.012
	Cured	-1.077	0.536	0.044**	-2.127	-0.026
	Deteriorated	-0.042	0.334	0.901	-0.695	0.612
	Improved	0.311	0.207	0.132	-0.094	0.716
CE3	Follow-up	0.006	0.010	0.553	-0.014	0.026
	Cured	0.930	0.289	0.001***	0.362	1.497
	Deteriorated	-0.458	0.328	0.163	-1.100	0.185
	Improved	-0.819	0.233	0.000***	-1.275	-0.363
CE4	Follow-up	0.005	0.009	0.565	-0.013	0.023
	Cured	-0.551	0.367	0.133	-1.270	0.167
	Deteriorated	0.871	0.256	0.001**	0.369	1.373
	Improved	-0.018	0.189	0.924	-0.388	0.352
CE5	Follow-up	0.038	0.029	0.190	-0.019	0.096
	Cured	-0.534	1.055	0.613	-2.602	1.535
	Deteriorated	0.229	0.666	0.731	-1.075	1.533
	Improved	-21.345	0.000		-21.345	-21.345

***significant at 1% and ** at 5%

Table 3-9: Contribution of cyst type to outcome in untreated cases

Cured cases were significantly likely to be CE3. There was a reverse relationship between cured cases and CE2 cysts. Improved cases were positively related to CE1 and negatively related to CE3. Deterioration was positively related to CE3 and CE4 but negatively related to CE1. All other outcomes had no significant relationship to cyst type.

3.8.2.1.1 Changes in cysts sizes in untreated patients

Data from 32 patients, harbouring 49 cysts whose sequential ultrasound images were available was analysed for changes in cyst size over 12 months. A paired first and last observation of cyst diameter was taken. The difference in cyst sizes was summarised in **Table 3-10**

Change in Size	Number	Mean ± SD of Cyst Diameter (mm)	
		Start	Finish
Reduced 5 - 24 %	7	109 ± 27	96 ± 15
Reduced 5% to increased 6%	13	100 ± 14	103 ± 16
7 - 30% increase	12	92 ± 7	111 ± 8
>31 % increase	10	48 ± 28	113 ± 19
Collapsed	5	56 ± 71	72 ± 84
Disappeared	2	------	

Table 3-10: Changes in cyst size in untreated patients over 12 months of follow-up

After 12 months of follow – up, 7 cysts showed reduction in size of between 5 and 24 %, 13 cysts remained within ±5 % of the size at diagnosis, 12 cysts increased by between 7 – 30 % and 10 cysts increased over 20 %. A further 5 cysts collapsed, and 2 cysts were no longer visible on ultrasound scans. The greatest increase was in a 12-year-old girl who had a cyst that grew from 4 to 135mm in 12 months but overall average growth of 24 mm in diameter. There was no significant difference between sexes or age-groups. Although 22 cysts (46 %) of the cysts grew by 7% or more, other cysts remained the same, reduced in size, collapsed or disappeared without any intervention.

3.8.2.2 Chemotherapy results

Chemotherapy with albendazole was administered to patients harbouring 1229 cysts. The mean age of the patients that underwent chemotherapy was 27.4 years, SD = 14.5 years range 2 – 63 years. Other attributes recorded for the patients that received chemotherapy are summarised in **Table 3-11**.

Attribute	Characteristic	n	Percentage
Gender	Male	260	21.2
	Female	969	78.8
Previous Surgery	Yes	198	16.1
	No	1031	83.9
Cyst Number	Single	536	43.6
	Multiple	680	55.3
Cyst Type	CE1	248	20.2
	CE2	319	26.0
	CE3	204	16.6
	CE4	452	38.8
	CE5	6	0.50
Follow-up Period	3-6 months	429	34.9
	6 -9 months	441	35.9
	9 - 12 months	359	29.2
Number of Courses	1 Course	928	75.5
	2 Courses	194	15.8
	3 or more Courses	107	8.70

Table 3-11: Attributes of the patients that underwent chemotherapy

To analyse the chemotherapeutic outcome, a paired first and last observation of cyst morphology either before treatment or patient's last attendance was recorded and all the observations between the two disregarded. The factors above were assessed for association with outcomes of chemotherapy and the results tabulated in **Table 3-12**.

Factor	χ^2	df	p-value
Age	1041.654	174	0.000***
Gender	7.819	3	0.050**
Cyst Number	6.473	6	0.372
Number of courses	44.305	6	0.000***
Follow-up Period	18.618	6	0.005**
Cyst Type	23.890[a]	15	0.067

***significant at 1% and ** at 5%

Table 3-12: Factors associated with outcome of chemotherapy

The attributes that had significant associations with chemotherapeutic outcome were age and number of courses of treatment at 1% level and gender and follow-up period at 5% level.

CE1 Cysts

Albendazole treatment was given to 248 cases of CE1. The patients were then followed up for 3 – 12 months post treatment and the outcome summarised in **Figure 3-11** to Error! Reference source not found.below. With one course of treatment the most significant changes were seen in the first 6 months post-treatment. In this period, cure was achieved in 7.6% of the cases, 15% of the cysts got worse, 31% improved and 46% remained the same. In the subsequent months of follow-up, over 70% of the cysts did not change further but a further 4 cases were cured. Overall, cure was achieved in 11 (4.5%) of all CE1 cases that had 1 dose of chemotherapy. In 63.6%of the cured cases, it was achieved in the first 3 months after the end of treatment while another 27.3% achieved cure within 9 months of treatment. Of the 58 CE1 cases that improved 29 (50%) were recorded 3 months post-treatment, 21 (36.2%) between 6 and 9 months and 8 (13.8) improved between 9- and 12-months post-treatment. In the cases of deteriorating cysts, 22 were recorded of which 63.6 % (n=14) were recorded in 3 – 6 months post-treatment, n=4 (9.1%) deteriorated 6-9 months post-treatment and another 4 (9.1%) got worse 9 – 12 months post-treatment . Overall, 1 dose of chemotherapy caused changes shortly after administration and a few patients were cured. but majority of the cysts returned to original status within a year. The outcome of 1 dose of chemotherapy in patients with CE1 cysts was highly significant ($p<0.001$) as was follow-up period ($p<0.001$).

A total of 22 CE1 cysts received 2 courses of chemotherapy and followed up after the second dose over 12 months. Seven were followed up for 6 -9 and 4 for 9 – 12 months. In this group, 1 case was considered cured after 9 months. Overall, there were 9.1 % cured cysts, 36.4% had shown improvement and 45.5% had not changed. One cyst showed deterioration.

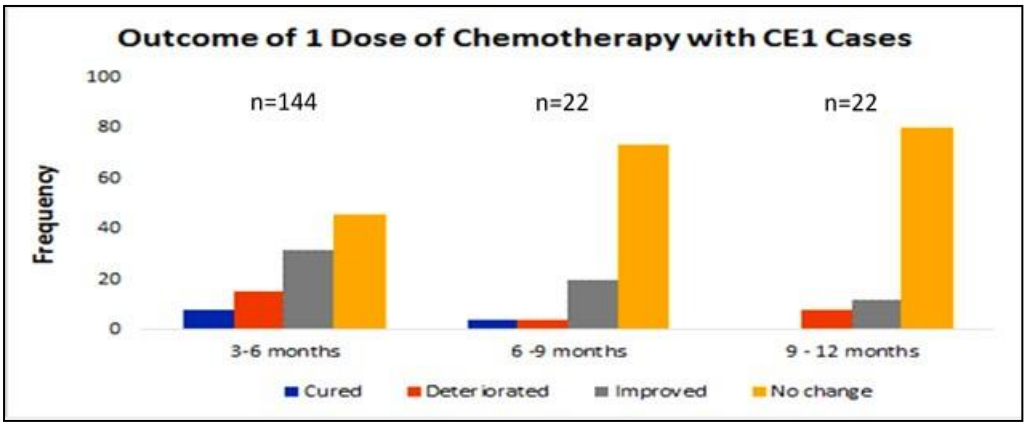


Figure 3-11: Outcome of 1 dose chemotherapy on CE1 cysts

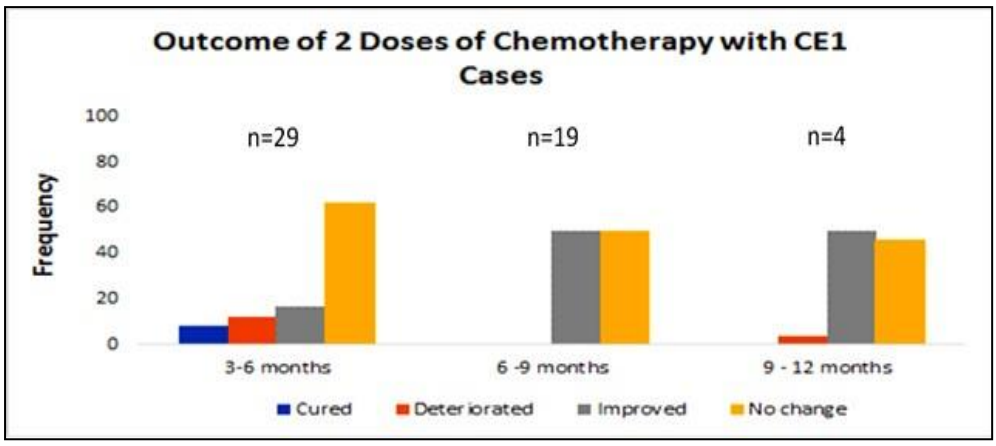


Figure 3-12: Outcome of 2 doses chemotherapy on CE1 cysts

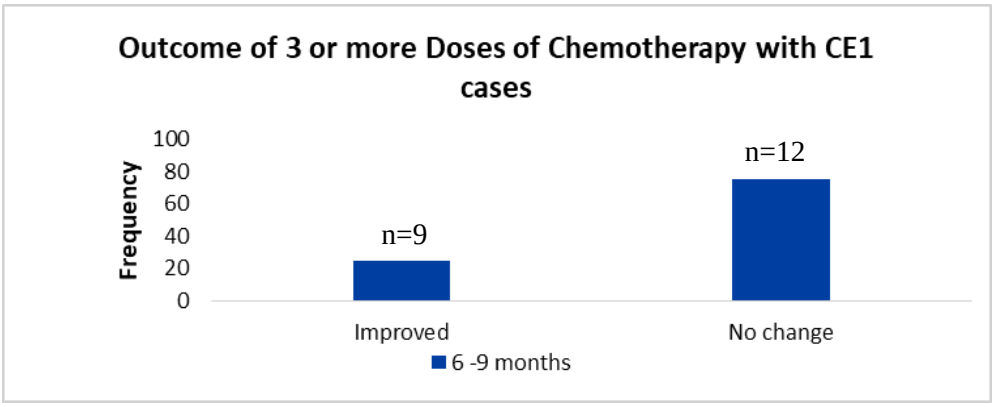


Figure 3-13: Outcome of 3 or More doses of chemotherapy on CE1 cysts

The outcome of 12 CE1 cases that received 3 or more doses of chemotherapy, followed up from the date of their last dose was assessed for a year. All were followed up for 6 - 9 months. Overall, 3 cases improved and 9 showed no change. In this group, there were no cure or deterioration of cysts.

CE2 Cysts

CE2 cyst responses to different courses of chemotherapy were analysed and the outcomes are summarised in **Figure 3-14 to Figure 3-16** below.

A total of 222 cases of CE2 cases treated with one dose of albendazole and followed up for 3 – 12 months post treatment. Of these, 100 cases were followed up for 6 months. In this time, 9 cysts (9 %) were cured, 9 (9 %) of the cysts improved, 70 (70 %) cysts showed no changes and another 12 (12%) got worse. In the next three months, another 50 cysts were followed up. In this group, 9 (18%) cysts were cured, and 9 (18%) cysts improved, a third of the cysts 31 (62%) did not change and 1 cyst got worse. A further 72 cysts were followed up for between 9 - 12 months. In this group, there was 2 (2.8%) other cysts got cured, 9 (12.5%) improved, 59 (81.9%) did not change and 2 (2.8%) deteriorated. A total of 48 cases of CE2 cysts received 2 courses of chemotherapy and were followed up for 12 months after they completed the second dose. Ten for were followed up for 6 months, 33 for 6 -9 months and 5 for 9 - 12 months. The greatest improvement was seen 3 -6 months post-treatment with 50 % of the patients showing improvement, a higher number than the ones that did not change who were at 30%. Only one cyst got worse in this group. A year after chemotherapy, 7 (50%) of the cases were considered improved, 6 out of 14 cases remained the same and 1 cyst got worse.

Sixteen CE2 cases treated with 3 or more doses of chemotherapy were followed up for 12 months. In the first 3-6 months, there were no cured cases but 2 (12.5%) deteriorated and 2 (12.5%) improved. Two thirds (75%) showed no change. Another 23 cases were followed up

for 6 – 9 months. In this group, 1 (4.3%) was considered cured, 2 (8.7%) deteriorated, 5 (21.7%) improved and 15 (65.2%) did not change. A further 9 cases were followed up for 9 – 12 months and every case in this group showed no change.

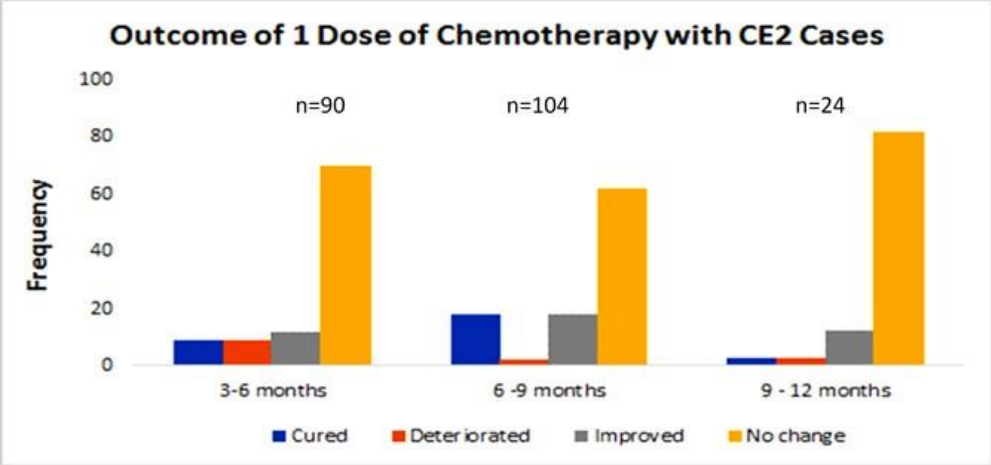


Figure 3-14: Outcome of 1 dose of chemotherapy CE2 cysts

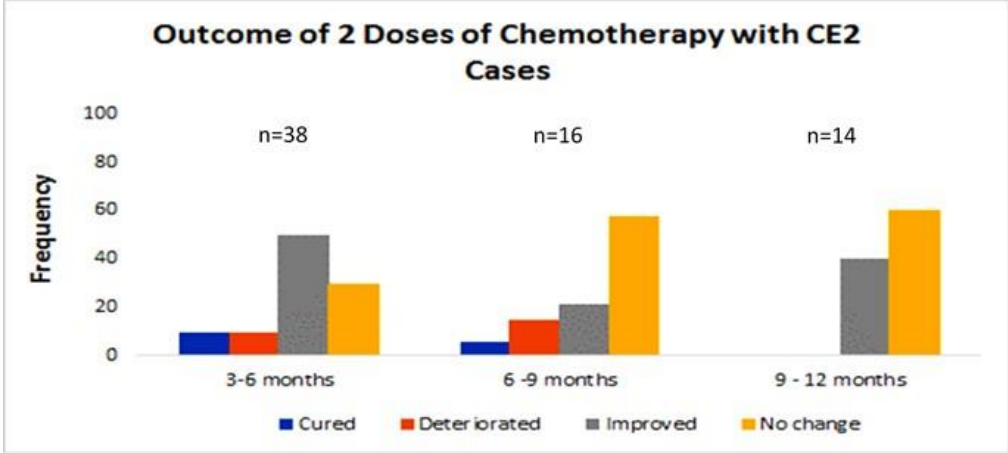


Figure 3-15: Outcome of 2 doses of chemotherapy

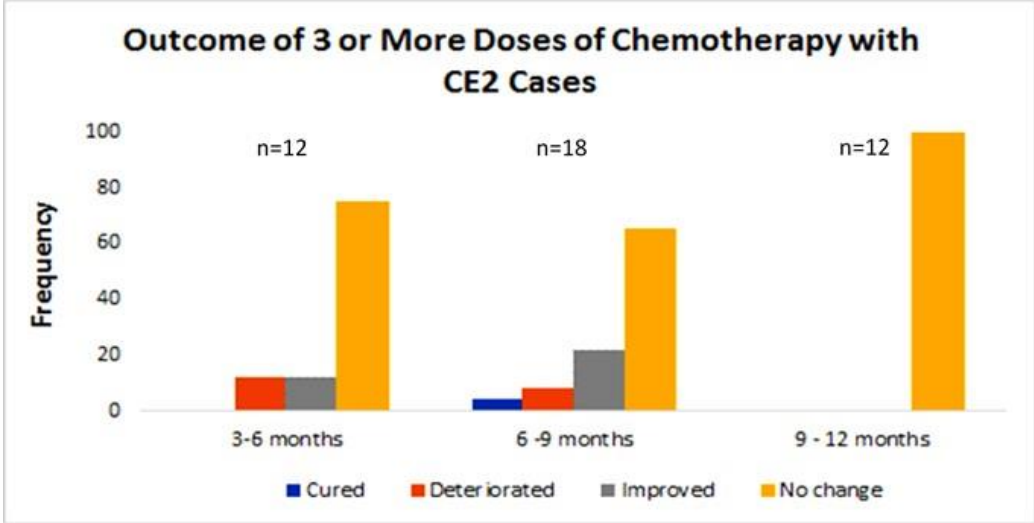


Figure 3-16: Outcome of 3 or More doses of chemotherapy

CE3 cysts

A total of 131 cases with CE3 cysts were treated with one dose of albendazole and followed up for 3 – 12 months. Of these, 37 cases were followed up for 3 – 6 months, 37 were followed up for 6-9 months and 57 cases for 9 – 12 months. In the cases followed up 3-6 months, 5 (13.5%) were cured, 1 deteriorated and 1 improved while 30 (81.1%) did not change. After 6-9 months of follow-up, there were no further cured cases and 1 (2.7%) case deteriorated. Improvement was recorded in 11 (29.7%) of the cases and 25 (67.6%) did not change. A further 57 cases were followed up for 9 – 12 months. In this group, 1 (1.8%) case was cured, 1 case deteriorated 5 (8.8%) of the cases improved and 50 (87.7%) did not change.

A total of 58 cases of CE3 cysts received 2 courses of chemotherapy. A total of 24 were followed up for 3 – 6 months, 8 were followed up for 6 – 9 months and 26 were followed up for 9 – 12 months. Of the 24 followed up for up to 6 months, cure was achieved in 2 (8.3%) cases, another 3 (12.5%) cases deteriorated, 4 (16.7%) improved and 15 (62.5%) did not change. In the cases followed up for 9 – 12 months, there were no cured cases, 1 case

deteriorate, 13 (50%) improved and 12 (46.2%) did not change. Overall, 31 (53.4%) of the cysts did not change, 56.6% improved or were cured and 6.9% of the cysts got worse.

Fifteen cases of CE3 cysts received 3 doses of chemotherapy. Six cases were followed up for 3 – 6 months and none of them changed. Nine cases were followed p for 9 – 12 months and only one improved. The other 8 did not change. No cysts were found to have cured or deteriorated.

A summary of the outcomes to different doses is shown in **Figure 3-17 to Figure 3-19**.

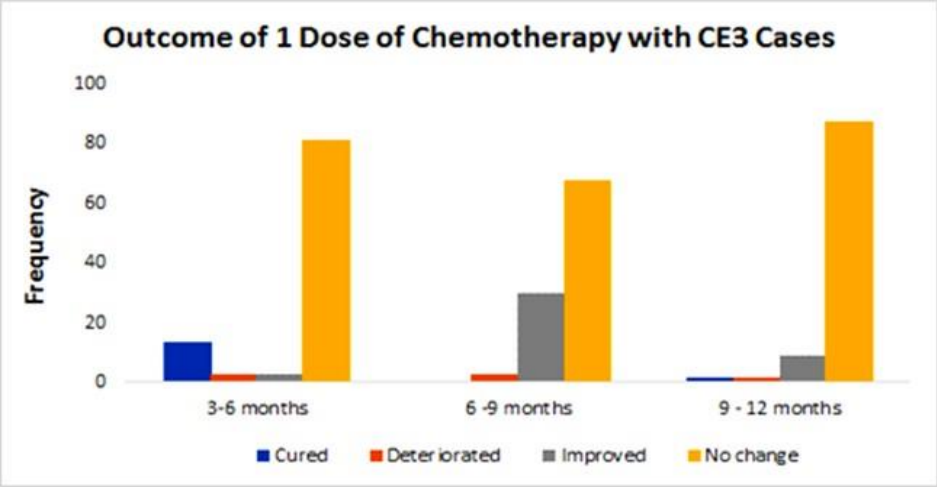


Figure 3-17: Outcome of 1 dose of chemotherapy on CE3 cysts

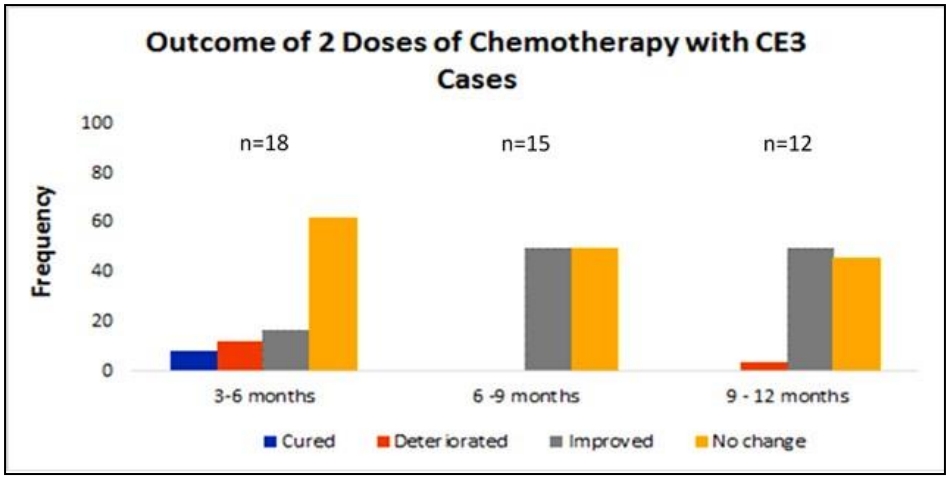


Figure 3-18: Outcome of 2 doses of chemotherapy on CE3 cysts

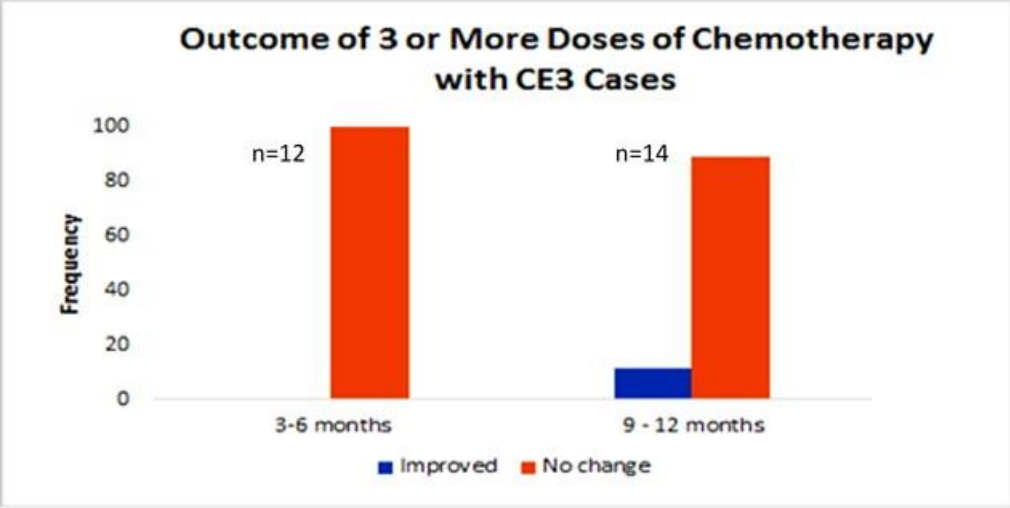


Figure 3-19: Outcome of 3 or More doses of chemotherapy on CE3 cysts

CE4 Cysts

A total of 346 cases of CE4 cysts, treated with one dose of albendazole were followed up for 3 – 12 months post treatment. One hundred and eighteen were followed up for 3 - 6 months. In this time, 7 (5.9 %) of the cysts were cured, 6 (5.1%) of the cysts improved 102 (86.4%) showed no changes and 3 cysts (2.5%) got worse. Another 159 cysts were followed up for 6 - 9 months and in this group, a further 5 (3.1 %) of the cysts were cured, 25 (15.7%) of the cysts improved, 122 (76.7%) did not change and 7 (4.4%) cysts deteriorated. Another 69 cysts were followed up for 9 -12 months. I this group, 6 (8.7%) of them were cured, 21 (30.4%) improved, 1 (1.4%) got worse and 41 (59.4%) remained the same. Overall, 18 (5.2%) cysts were cured by the end of the follow-up, 52 (15%) improved, 265 (76.6%) had not changed and 11 cysts (3.2%) had got worse.

A total of 74 cases of CE4 cysts received 2 courses of chemotherapy. Twenty-three were followed up for 3 – 6 months, 16 for 6 – 9 months and 35 for 9 - 12 months. Two (8.7%) cysts were cured in 3 – 6 months, 10 (43.5%) improved, 10 did not change and 1 cyst deteriorated. In 6 – 9 months post-treatment, there was no cured cyst nor was there deteriorating cyst. Six (37.5%) improved and 16 (62.5%) did not change. In 9 -12 months post-treatment, there were

no cured cysts, 5 (14.3%) deteriorated, 10 (28.6%) improved and 20 (57.1%) did not change. The sample size was small, and this may explain some of the variations, but the trend was similar to the CE3 cases after 2 doses of chemotherapy. The number of cases that got worse increased with time from 1 at 6 months to 5 in 12 months. Overall, 37.8% showed cure or improvement, 54.1 % of the cysts did not change and 8.1 % got worse.

Thirty-two CE4 cysts received 3 doses or more of chemotherapy before follow-up. Two were followed up for 3 – 6 months and of these, one was cured and one improved. Another 10 were followed up for 6 – 9 months. In this group, 2 more achieved cure, 1 deteriorated, 1 improved and 6 (60%) did not change. A further 20 cysts were followed up for 9 – 12 months. There was no 10% cure rate, 10% improvement and 80% did not change. Overall, 28.1% were cured or improved, 3.1% got worse and 68.8% did not change.

A summary of the outcomes to different doses is shown in **Figure 3-20 to Figure 3-22** below.

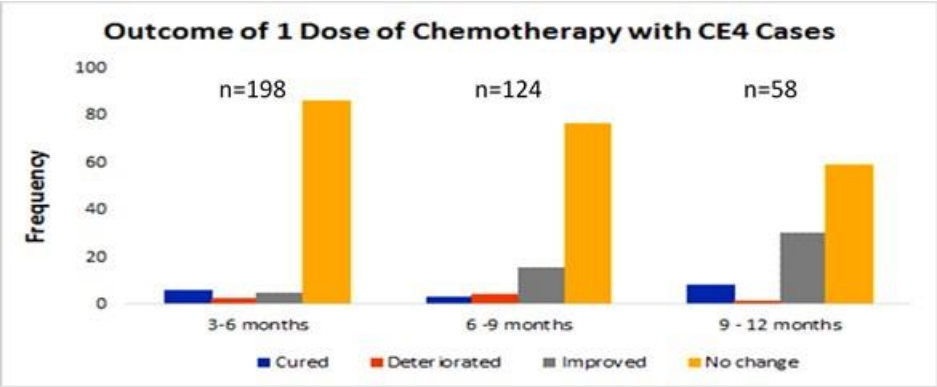


Figure 3-20: Outcome of 1 dose of chemotherapy on CE4 cysts

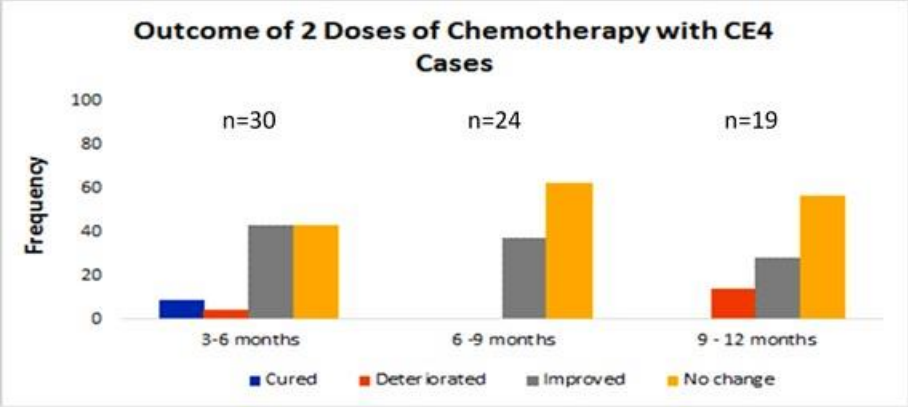


Figure 3-21: Outcome of 2 doses of chemotherapy on CE4 cysts

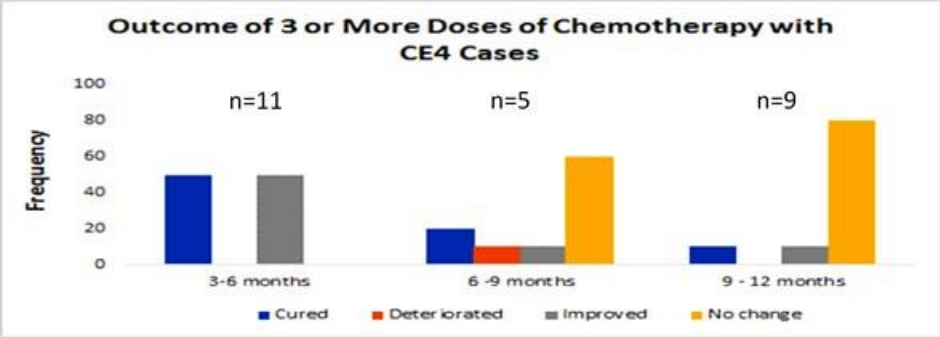


Figure 3-22: Outcome of 3 or More doses of chemotherapy on CE4 cysts.

Overall assessment

Overall, 928 cysts were treated with one dose of albendazole and the outcome assessed for periods between 3 – 12 months and results summarised in **Figure 3-23 to Figure 3-25**

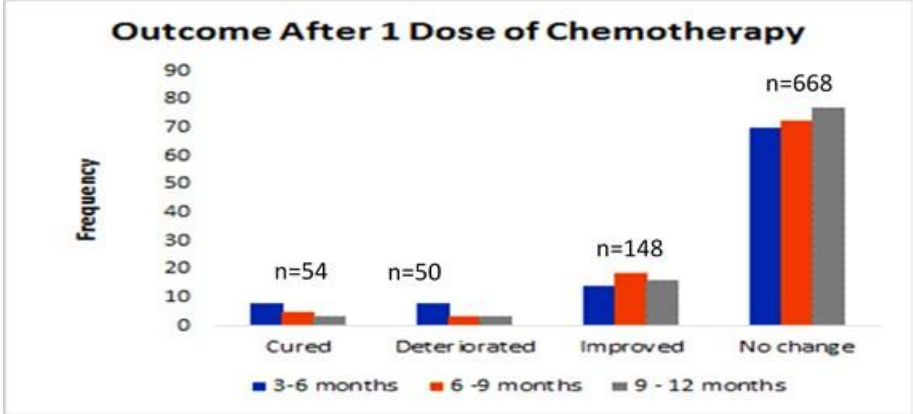


Figure 3-23: Outcome of 1 dose of chemotherapy overall

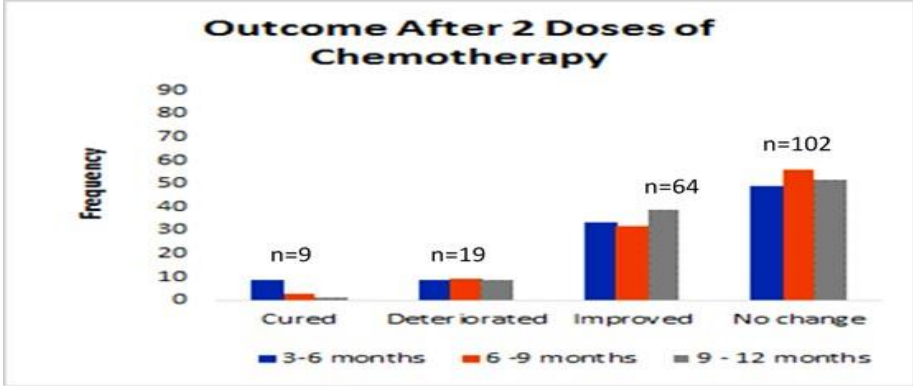


Figure 3-24: Outcome of 2 doses of chemotherapy overall

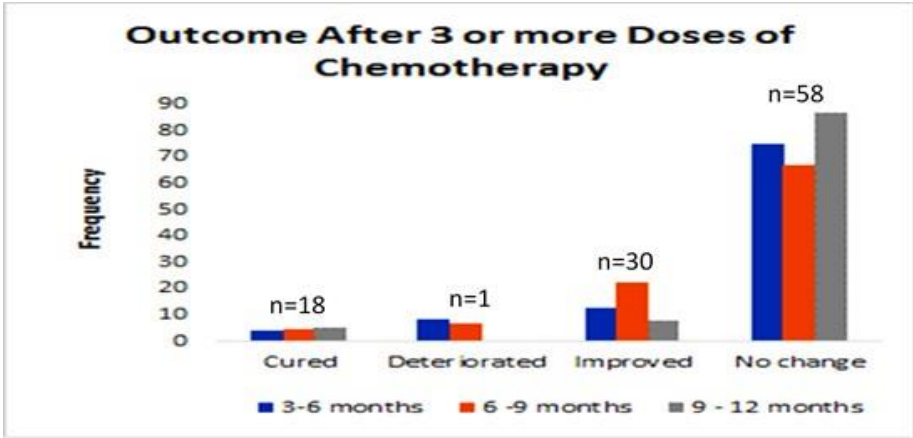


Figure 3-25: Outcome 3 or more doses of chemotherapy overall

Of those, 347 were followed up for 6 months post treatment. Cure was realised in 28 cases (8%), improvement in 49 cysts (14.1%), 243 cysts remained the same (70%) and 27 cysts got worse (7.8 %). Another 330 cysts were followed up for 6 months. Less cured cases 17 (5.2%) but number of improved cases increased to 18.5% (n = 61). A similar proportion to those in the 3 month group did not change (72.7%) and fewer cysts deteriorated at 3.6 %. Nine months post treatment, 98 cysts were followed up. In this group, 1 more cysts was cured, 17 (17.3 %) improved, there was no change in 72 (73.5 %) cysts and 8 (8.2 %) cysts deteriorated. A further 153 cysts were followed for 12 months. There was no deterioration of cysts in this group and 121 (79 %) did not change. Of the 21 % that got better, 5.2% (n = 8) were cured and 15.8% (n=24) had improved. Overall, 54 cysts (5.8 %) were cured, 151 cysts (16.3%) improved, 676 cysts (72.8%) did not change and 47 cysts (5.1%) got worse.

One hundred and ninety three patients received 2 doses of chemotherapy and follow-up began after completion of the second dose. Among them, 58 cases were followed up for 3 months during which 6 cysts (10.3 %) were cured, 19 cysts (32.7 %) improved, 28 (48.2 %) cysts remained the same and 5 (8.8%)cysts got worse. In the next three months, 66 cysts were followed up. In the six months post treatment, 2 cysts were cured, 21 (32.7%) improved, 37 (48.2%) did not change and 6 (8.6%) cysts got worse. In the group that was followed up for 6 months, there was an overall cure rate of 4.7%, improvement rate of 34.7 %, unchanging cysts were 51.8% and 8.8% were cysts that got worse. Among 107 cysts that received 3 doses of chemotherapy, 24 were followed up for 3 months. In this time, 1 case was cured, 3 cases improved 18 cases did not change (75.5%) and 2 cases got worse. Six months post treatment, of the 45 cysts followed, 2 were cured, 10 improved, 3 got worse and 30 did not change. There was cure in another 2 cysts after 9 months from chemotherapy and none at 12 month timepoint. The general outcome of the cases that received 3 courses of chemotherapy was 4.7 % cured, 15 % improvement, 75.7% that did not change and 4.7 % that got worse.

Giving a second dose of chemotherapy in CE1 cases improved the outcome from 25% to 43.5 % and a third had even better outcome where 73% of the cases were either cured or improved. A similar outcome was seen in CE3 cysts though the percentage was lower where 1 course achieved 24.2 % improvement or cure, 2 courses achieved 39% and 3 courses showed 52% cure or improvement. A second dose also showed improvement of outcome in CE3 and CE4 cysts, but a third dose showed none

Outcome (%)	Number	Cured	Improved	No Change	Deteriorated
Untreated	(n = 75)	10.7	45.3	36.0	8.0
1Course of Chemotherapy	(n = 928)	5.8	16.3	72.8	5.1
2 Courses of Chemotherapy	(n = 193)	4.7	34.7	51.8	8.8
3 or more Courses	(n = 107)	4.7	15.0	75.7	4.7
Overall	(n = 1229)	5.5	19.0	69.7	5.6

Table 3-13: Comparison of Outcomes.

Disregarding the number of courses of therapy, length of follow-up and cyst type, 68 out of 1229 (5.5 %) were cured, 234 (19%) improved, 857 (69.7 % did not change and 69 (5.6 %) deteriorated.

An example of a survivorship curve for outcome of 1 dose of chemotherapy over time is described below (**Figure 3-26)**

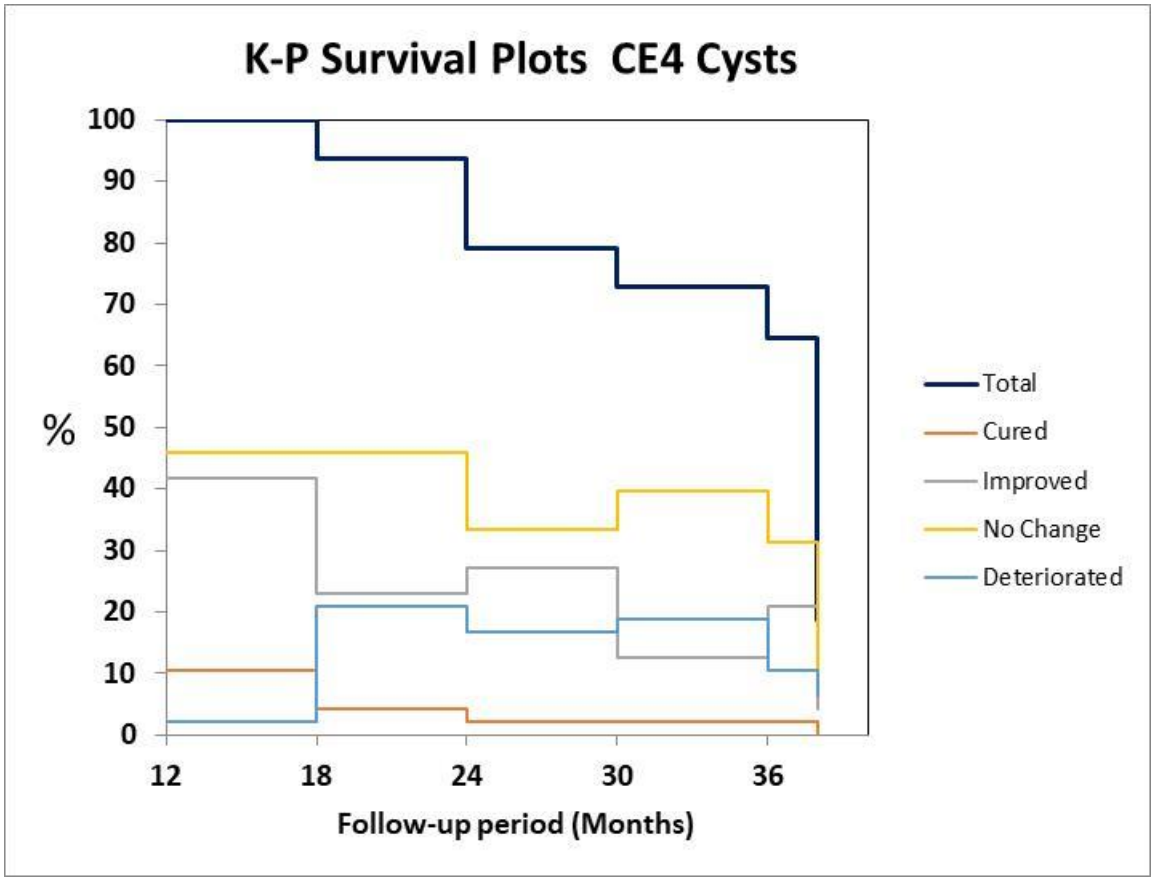


Figure 3-26: A survivorship curve for outcome of 1 dose of chemotherapy on CE4 cysts

Comparing this outcomes with the untreated cases in the first 12 months of follow-up, there was a better outcome overall among the untreated cases compared to the ones that received chemotherapy **(Table 3-13)**. Almost twice of untreated cases self-cured compared to those that received any amount of chemotherapy and almost three times in the ones that showed improvement . It is however noteworthy that the clinicians recommended therapy based on the changes in the cysts and the tendency was to treat those cysts that show signs of deterioration and leave those are stable or improving untreated. On the basis of this, the results of chemotherapy may indicate that deteriorating cysts may become stabilised by chemotherapy thus stopping further regression into active and larger cysts. The number of courses of albendazole given was at the discretion of the clinicians. The results of 3 or more courses were effective on CE1 and CE2 cysts but showed no further benefit in CE3 and CE4 cysts. In

summary, increasing the number of courses given had beneficial effects on cases that had CE1 and CE2 cysts but not CE3 and CE4 cysts (**Figure 3-27**).

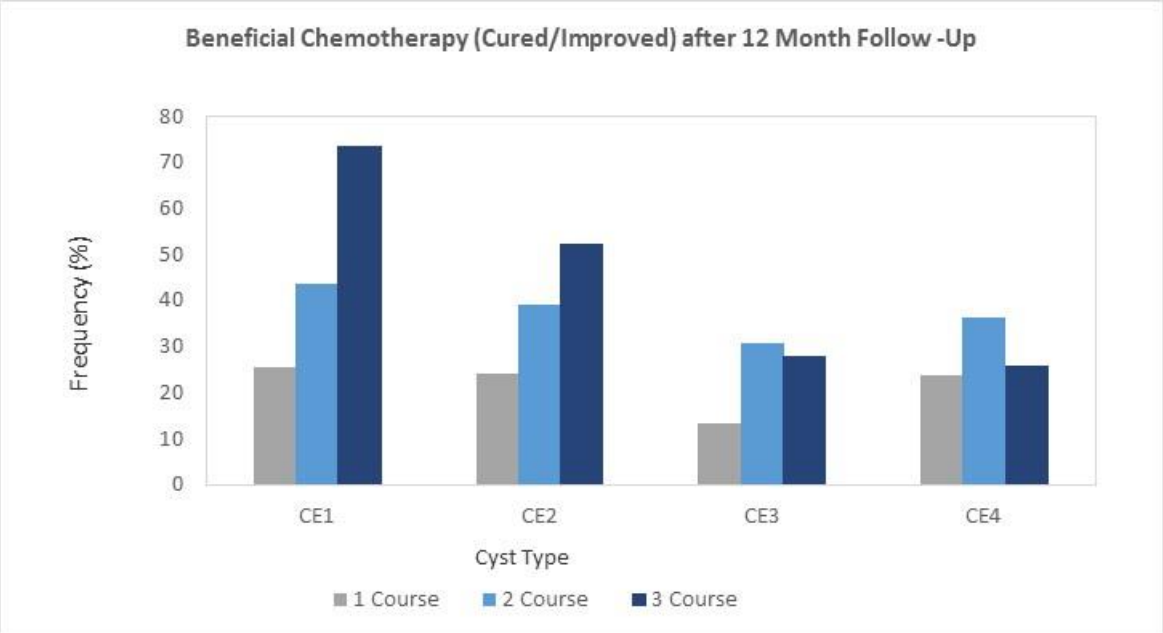


Figure 3-27: Beneficial outcomes of different doses of chemotherapy

Giving one dose of chemotherapy to CE1 patients achieved improvement in 25.4% of the cases. In patients that received 2 courses of chemotherapy, this improvement was seen in 43.5% and in those that received 3 courses, the number of improved cases increased further to 73.5%. A similar trend was found in patients harbouring CE2 cysts although these improvements were better in CE1 and CE2 case. This beneficial outcome with increase in dosage was not found in CE3 and CE4 cases where the rate of cure decreased after the second dose.

3.8.2.3 Responses to PAIR

Three hundred and sixty cysts underwent PAIR and were followed-up for 6 – 12 months. The mean age of patients that underwent PAIR was 24.5 years, S.D 15.5 years and ranged between 3 and 65 years. Other attributes recorded before surgery are summarised in **Table 3-14.**

Factor	Characteristic	n	Percentage
Gender	Female	204	78.2
	Male	57	21.8
Previous Surgery	No	255	97.7
	Yes	57	2.3
Cyst Nature	Multiple cysts	96	36.8
	Single cyst	165	63.2
Cyst Type	CE1	186	71.3
	CE2	16	6.1
	CE3	10	3.8
	CE4	49	18.8

Table 3-14: Attributes of patients treated with PAIR

Those that showed cysts disappearance or complete obliteration of the internal structures of the cyst mostly leaving just a thin echogenic linear scar were considered cured. Other cysts showed partial obliteration or calcification of the cyst contents and were considered inactive. Inactive was also recorded for cysts that showed a folding of the endocyst with significant size reduction and absence of daughter cysts. Other cysts appeared to be regaining their original size and morphology or progressing to active types from inactive and were recorded as regenerating cysts. Analysis of the outcome of PAIR sought to find out which cyst type had responded best to treatment and a summary is shown in **Figure 3-28**

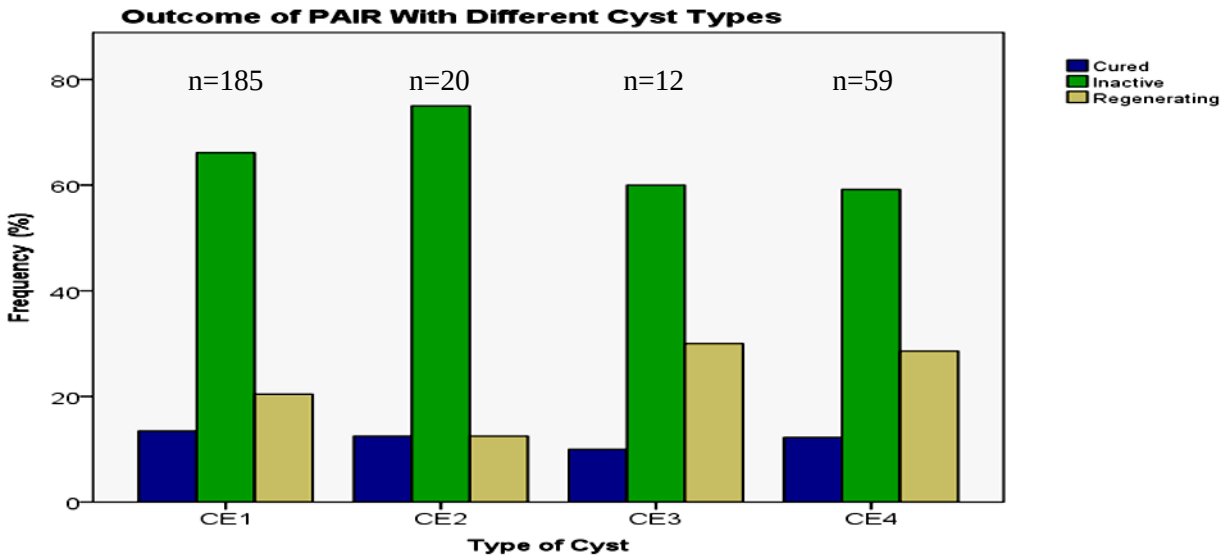


Figure 3-28: Proportions of Outcome of Pair in Different Cyst Types

The highest number of cysts that underwent PAIR was in the CE1 group with 186 cases which was 71.3 % of all cases. Of these, 25 (13.4%) were considered cured, 123 (66.1%) were inactive and 38 (20.4%) were regenerating cysts. CE1 cysts consisted of 73.5 % of all cured cysts, 72.4 % of all inactive cysts and 66.7 % of all regenerating cysts. There was no statistical significance between CE1 and outcome ($\chi^2(2) = 0.772$, $p = 0.680$). Compared to CE1 cysts, there were few cases of CE2 cysts that underwent PAIR (6.1%, n = 16). Two cysts (12.5%) from this group attained cure, 2 (12.5%) cysts regenerated by the end of follow-up and 12 cysts (75%) were considered inactive. Similar to CE1 cysts, there was no association between CE2 and outcome ($\chi^2(2) = 0.265$, $p = 0.876$). Only 10 CE3 cysts underwent PAIR. Of these, 1 (10%) cyst was considered cured, 6 (60%) were inactive and 3 (30%) regenerated. There was no association between cyst type and outcome in CE3 cysts (($\chi^2(2) = 0.275$, $p = 0.872$). In CE4 cases, 49 patients underwent PAIR. There was a 12.2 % cure rate in this group, 59.2% (n= 29) had inactive cysts while 28.6% (n = 14) of the cysts showed regeneration by the end of the study. Like all other cyst types, there was no association between the cyst type and outcome ($\chi^2(2) = 0.275$, $p = 0.872$).

3.8.2.4 Surgical Cases

Three hundred and seventy-eight cases harbouring 556 abdominal cysts underwent surgical endocystectomy. The mean age 27.5 years, SD 13.1, range 3 -60 years. Other patient factors at surgery are shown in **Table 3-15**.

Factor	Characteristic	n	Percentage
Gender	Female	438	78.8
	Male	118	21.2
Previous Surgery	No	528	95.0
	Yes	28	5.0
Cyst Nature	Multiple cysts	336	60.4
	Single cyst	220	39.6
Cyst Type	CE1	218	39.2
	CE2	85	15.3
	CE3	68	12.2
	CE4	185	33.3
Intervention	Surgery	475	85.4
	Surgery & Chemotherapy	81	14.6

Table 3-15: Attributes of patients who underwent endocystectomy

Post-surgical follow-up was done by ultrasonography to monitor the recovery of the residual cavity for between 8 months to 1 year. Recurrence was recorded where there was presence of a live cyst at the location of a previously treated one. Twenty-eight cases (5%) had had surgery before and were either recurrences or reinfections. Surgery without chemotherapy was performed on 457 cases while in 103 cases surgery was accompanied by chemotherapy prior and after. In all cases, accompanying surgery with chemotherapy reduced the rate of recurrence though this was not significant (χ^2 = 0.158, p=0.408). This reduction was greatest in cases under 18 years of age and in children between the ages of 5 and 12 accompanying surgery with chemotherapy reduced recurrence rate from 28.4% to 10%. Patients over 46 years old who received chemotherapy with surgery had a higher rate of recurrence than those who received surgery alone as well as those that remained untreated. Overall, cases who did not receive chemotherapy before and after surgery had a cure rate of 86.9% (397) and a recurrence rate of 13.1% (60) while those that did had a cure rate of 93.5% (n = 101/108) with a recurrence in 6.5 % (7)cases (**Figure 3-29**). The overall cure rate was 89.1 % recurrence rate was 11.9%.

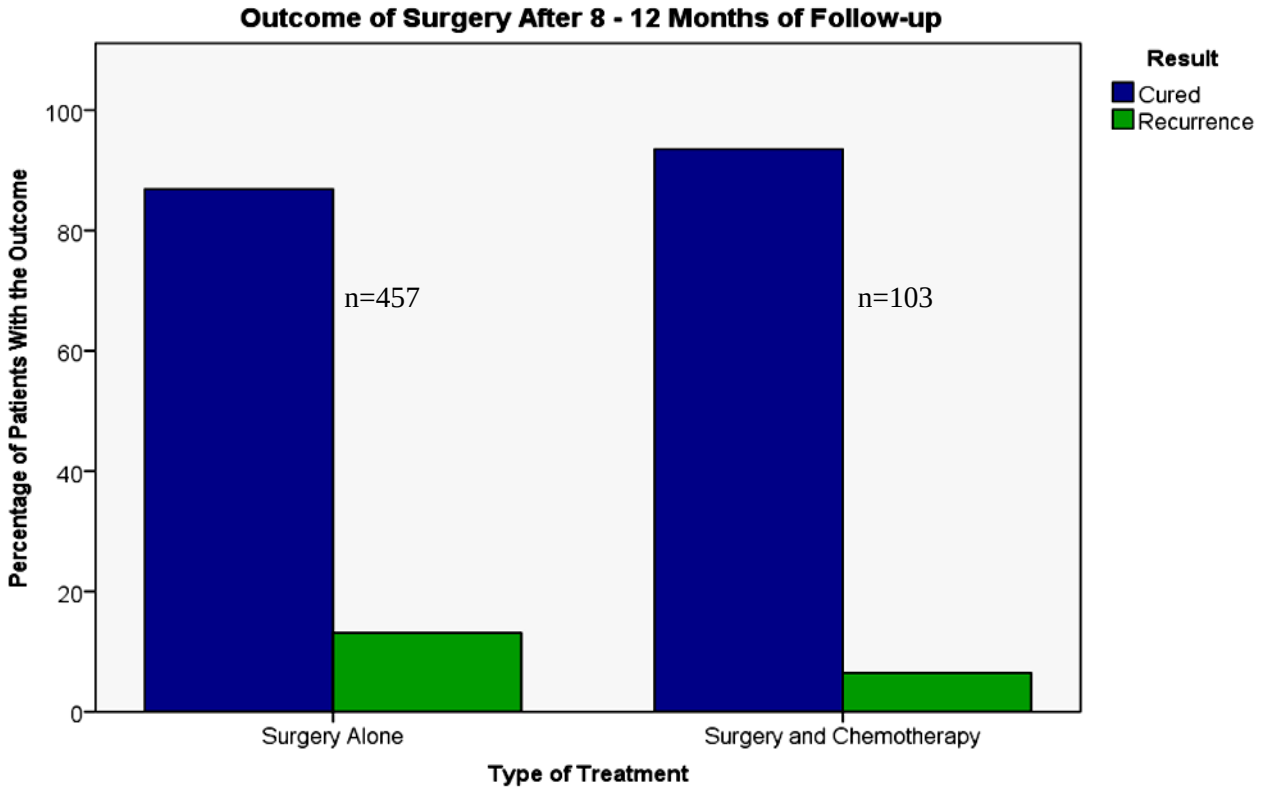


Figure 3-29: Outcome of surgical treatment with and without chemotherapy.

3.9 Discussion

Surveillance data, collected and analysed effectively, is a robust tool in defining the problem, providing a rationale for intervention strategies, evaluating the success of the control program and quantifying the infections in human (Craig et al., 2006). In CE, Infections are more of a measure of prevalence rather than incidence because cyst growth in humans can be slow and is variable (Seimenis, 2003). The clinical diagnosis of CE is usually only representative of a small proportion of the infection because most people are asymptomatic and can remain so for many years (Herrador et al., 2016)

From the start of the study, there were noticeable differences in the ratio of infection between the sexes with females being more infected than males. The ratio varied between age groups, but the youngest and the oldest age groups showed little or no difference. The greatest differences were seen in the post-pubertal and childbearing ages where the ratio was nearly 1:3. Some of the factors contributing to these differences may be immunological and/or cultural. The highest infections were found in women between 18 – 35 years old. Immunity may be reduced in childbearing and breastfeeding women who are also culturally unequally exposed to dogs. Egg viability after shedding from dogs is temperature dependent. In the sunlight, it is lost within 3 hours, in the huts after 48 hours and in water may remain viable for up to 300 hours (Wachira et al., 1991). In the heat of the day, family dogs lay inside the houses increasing proximity to women compared to men who spend their days out in the pastures with the animals. Studies have suggested a presence of sexual dimorphism that makes female cellular immune responses inferior to male ones(Giefing-Kröll et al., 2015; Klein et al., 2016) a fact that has been demonstrated in experimental murine infections (Bubb et al., 2012). This dimorphism was also recognised in medicine in that males and females often respond differently to drug therapy (Sherman et al., 1995)

At infection, cysts are small and unilocular. Some go on to develop in size as well as morphology while others have the development arrested at some stage and may remain in that state for long periods of time (Rogan et al., 2015). Variations of cyst sizes with cyst stages showed that most of the small to medium sized cysts were CE1 and CE2 types while CE3 and CE4 tended to be larger. The rate of growth has been estimated to be between 0 – 3cm per year (Eckert et al., 2004) although some cysts were observed to remain the same size after 14 years of follow-up (Acarli, 2004). In this study, the rates varied considerably between subjects. Differences were observed even with cysts in the same subject and same organ and the average increase was 24 mm in a year. Possible explanations for the variation maybe parasite related according to genotype as well as levels of virulence or host related with variations in immune responses (Rosenzvit et al., 1999). However the genotype of the infecting species have been shown to be mainly *E.granulosus* sensu stricto. Other infecting species are *E. Canadensis* and *E.orteppi.* The infecting species has similar patterns in Kenya (Mulinge et al, 2018), China (Shang et al 2019) and Iraq (Salam Jumaah Hammad et al, 2018).

It is proposed that cysts become symptomatic on reaching 10 cm diameter (Larrieu et al., 2001) but in Turkana, many larger cysts were observed with the subjects reporting no pain.

Following up on cyst structural changes is important in keeping track of morphological changes as some cysts may remain stable for many years and undergo a sudden change. It is also essential in monitoring therapeutic results. Changes in cyst type over time followed the natural progression which supported the WHO classification (Brunetti et al., 2010; WHO Working Group, 2003) in both chemotherapy-induced and natural progression. In the cases that were followed up without treatment and CE1 cysts were observed, they either progressed to CE2 or remained the same, CE2 cysts progressed to CE3 or did not change, CE3 cysts progressed to CE4 mostly although some transitioned back to CE2 cysts and most CE4 and CE5 cysts maintained the initially observed stages but a few retrogressed and went the other way.

Variations in cyst types with age were observed with CE4 cysts being seen more in older cases. The differences in cyst types at diagnosis with age are assumed to be indicative of length of time from infection. These variations are also useful in following the natural history of cyst development where internal modifications can be perceived as progressively or degenerative.

The increase in average age at diagnosis over the years may indicate fewer new infections and provide evidence that the control program was working

Cysts numbers also increased with age and most of the younger cases diagnosed had a single cyst. Multiple cysts were most likely to depict multiple exposures with a single cyst infection each time. This was evidenced by the observation that most paediatric cases only harbour 1 cyst. However, the possibility of multiple infections in one exposure was a possibility as it is difficult to establish when the infection occurred. On the other hand, many patients with multiple cysts had similar cyst types and sizes, even in different organs which may be indicative of infection at the same time or that cyst development stopped at a certain stage. Many untreated followed up cases showed no changes over long periods of time with cysts at every stage of development. This has been observed in other studies (Chihai et al., 2016; Piccoli et al., 2014)

Advancements in both serological and imaging techniques allow for screening of large numbers of populations at risk as the majority of infected cases are usually asymptomatic. Therapeutic strategies in diagnosed CE are determined by the clinicians depending on available options. The evidence base that would assist in such decisions is lacking, a problem further complicated by the setting where CE is endemic and limitations in the availability of resources. Guidelines from WHO exist (Group, 2003), but there is no standard medical management and clinicians determined treatment initiation, dose and duration. One of the dilemmas facing clinicians faced with CE cases especially in the endemic areas where cases are many is whether

to treat or not. Here we aimed at assessing the outcome of chemotherapy treatment and compare the results with those of untreated groups in different cyst types

Many of the cases diagnosed were not offered immediate therapy (watch and wait) especially if they were asymptomatic. One major reason for this was logistical as more were diagnosed than could be treated. Others did not seem to require it as their life was not impacted by the infection through compromised organ function or discomfort. Clinicians can decide not to treat as all treatment comes with risk (Ammann et al., 1996; El-Mufti et al., 1993). Regular follow-up was considered enough in asymptomatic cases and this proved a logical decision as with time, some cysts calcified and became inactive while others remained stable or improved with time. Other patients refused treatment even when it was offered, a decision that was respected by the clinicians and long-term follow-up with imaging was done instead.

At infection, cysts are small and unilocular. They may remain so for long periods of time or may change in morphology and increase in size (Pedrosa et al., 2000). In this study, the development in most cysts seen appeared to have been arrested at a different stage and remained stable for long periods. Factors influencing this are unknown and monitoring cysts is important as clinicians base their decisions and therapies on observed changes. Questions on efficacy of chemotherapy have been raised with some authors contend that with time, *E. granulosus* will self-resolve and disappear. Some have even suggested that changes seen after treatment are spontaneous and not therapy induced (John, 2003). In this study, the outcome in untreated cases seemed better than those that underwent chemotherapy. However, the number was too small for fair comparison and only the stable cysts were left untreated. The fact that over 10 times more patients were treated might indicate that although spontaneous cure does occur cysts are most likely to progress, and intervention becomes necessary at some point.

In this study as with other publications, responses of cysts to albendazole varied widely even in the same patient, further complicating evaluation. Symptomatic patients and those with cysts showing progression or size increase through time were offered chemotherapy as 1st choice of treatment. Use of chemotherapy for treatment of CE has gained traction since its efficacy was first published 40 years ago (Morris et al., 1983; Saimot et al., 1983; Vachon et al., 1985). Since then, many case studies have been published. There are better imaging techniques usable in the field allowing regular follow-up on both untreated and treated cases. In our study, chemotherapy seemed to cause stability to the disease more than cure it. After 1 cycle of chemotherapy, nearly 6 % were cured, 16 % improved, 72.8% did not change and 5.1 % deteriorated. After 2 cycles, 4.7 % were considered cured, 34.7 % improved, 51.8% remained the same and 8.8% of the cysts got worse. For the cases that received 3 courses of chemotherapy, 4.7 % were cured, 15.6 % improved, 75 % did not change and 4.7% got worse. Increasing the number of doses of chemotherapy, a patient had from 1 to 2 did not have the expected effect in increasing cure rate. Possible reasons may have been lack of compliance or reduced bioavailability since the recommendation is to have the medication with a fatty meal which is difficult in Turkana due to poverty. However, improved cases doubled after second dose compared to those who received one dose. The third dose did not seem to have a big impact on the cysts. Treatment was offered to the cases that had symptomatic and progressive cysts and chemotherapy appeared to stabilise them stopping further progression in morphology and size. The rate of cure after chemotherapy in cysts that were treated with chemotherapy compared to that of surgery was small. However, improvement of cysts to asymptomatic types and sizes allowed for continued follow-up without further treatment if that cyst stability was maintained. Other studies have found similar therapeutic results where after 12 months of evaluation. Cure was found in 7 – 30 % of the cysts, significant size reduction in 12 – 50 %

and no morphological changes no 20 – 50% (Horton, 1997; Nahmias et al., 1994). Results from WHO showed 39 % favourable response (Saida, 2011).

Intermittent treating was used in nearly all the cases in this study and 15 patients had their treatment terminated due to jaundice. Questions exist on whether continuous treatment is a better option. Although albendazole is well tolerated in most patients, increase in hepatic enzyme levels occur in 20% of the cases though few are severe (Todorov 1995) and they return to normal on cessation of treatment. The rationale for "dry out" period was to allow recovery before the next round of therapy and protect liver function ((Davis et al., 1989; Hao et al., 1994). However, a 20 year study in Rome using continuous treatment showed no increase in side effects (Franchi et al., 1999). The rises in hepatic enzymes during treatment are considered to be caused by drug toxicity. However, Rigano et al. (1995) found them to be useful immunological markers for albendazole efficacy and other studies concurred (Eckert et al., 1995; Mufit et al., 1998). It is now believed that the changes result from immune responses to the cyst after it has been damaged by chemotherapy. Liver function test should continue to be monitored for idiosyncratic drug reactions but with less concern over changes that may be indicative of efficacy (Morris, 1987). On the other hand, intermittent dosing has been argued to cause tolerance and reduce efficacy in subsequent doses and cysts have been known to increase in size during the rest period (Junghanss et al., 2008). This may explain some of the deterioration of cysts seen in this study. Overall there is a need to improve efficacy. In Turkana, the ease of administration of chemotherapy, coupled with the fact that the available hospital amenities are rudimentary, better efficacy would greatly improve outcome and reduce the need for surgery. Suggestions include increasing drug concentration and sustaining higher blood level (Morris et al., 1990; Naseri et al., 2016), albendazole administration with a fatty meal increased plasma levels 5 fold (Lange et al., 1988; Pawluk et al., 2015) and double dosing of albendazole with praziquantel (Homeida et al., 1994; Torabi et al., 2018). Praziquantel in E.

granulosus has been shown to be most effective on cysts that are not fully developed like those released from daughter cysts making it very useful after surgery (Al Karawi et al., 1992). Another advantage of this combination therapy is that the 2 drugs interact increasing plasma concentration for both but the safety of using praziquantel with multiple dosage lacks evidence (Hong, 2018). Other adverse effects of using albendazole include alopecia and bone marrow depression causing neutropoenias or aplastic anaemia although all are reversible (Bakhsh et al., 2017; Steiger et al., 1990). Albendazole is also teratogenic and its use in pregnancy is counter-indicated (Choi et al., 2017; De Silva et al., 1999). WHO recommendations are for a 3 – 6 cycles of 28 days and 14 days breaks, or a 3 to 6-month continuous cycle (Brunetti et al., 2010).

As a conservative surgical procedure, PAIR aims at evacuation and sterilisation of cyst contents. When first introduced in 1986, fear was that the cysts may puncture once pierced or that injecting a sclerosant into a cyst that had biliary communication may cause ascending cholangitis, (Filice et al., 1990; Yaghan et al., 2004). In practical use, with precautions in place, PAIR is safe and has been used as standard treatment for cysts between 5 – 15cm in diameter in many centres replacing open surgery in many cases (Wen et al., 2019). Sclerosants used include hypertonic saline, alcohol and cetrimides with suggestions of albendazole also included (de Reuver et al., 2018). Used alongside chemotherapy, PAIR has proved to have greater clinical efficacy, less recurrence rates and less complications compared to surgery (Khuroo et al., 1997; Silva et al., 2015). Recommendation for chemotherapy is to use for 1 month post-surgery to prevent reactivation or recurrence from accidental damage (Eckert et al., 2001). The average stay in hospital was 4 days (range 2 – 7 days) which shorter than surgery whose average hospital stay was 9 days. Recurrence is not uncommon over long periods of follow-up and this study was only done for 6 months. However, in studies where longer follow-up was possible, the results were still favourable after 5 years (Craig, 1994) but evaluation on allergic reactions

and secondary echinococcosis still require further evaluation allergic reaction was reported in 0.63% (Filice et al., 1997)

Surgical procedures for CE range from radical transplant to conservative partial pericystectomy (da Silva, 2015). Surgery has the capacity to eliminate the disease and treat complications associated with it. It is however associated with higher morbidity and mortality both during and post operation compared to other forms of treatment. (Manterola et al., 2016).

In this study, surgical treatment of CE was performed on very large cysts that caused significant morbidity. Conservative partial pericystectomy was performed to reduce complications related to more radical surgery and the number of days in hospital. Surgical cases considered cured at the end of follow-up were 88% (493/560) and 12% were considered as recurrences as the cysts appeared in the residual cavity. This is similar to other studies where conservative surgery was done (Chautems et al., 2003; Jerraya et al., 2015; Kapan et al., 2004; Sielaff et al., 2001). Other postoperative sequelae were not adequately followed up, but 1 patient died on the operating table and another one 3 days after surgery due to surgical complications.

There are frequent recurrences especially with conservative surgery as the residual cavity is a potent source of daughter cysts and has a likelihood of dispersion to multiple sites often with the disease being more extensive than before (Georgiou et al., 2015). Post-surgical recurrence may result from spillage of protoscoleces or inadequate treatment of the cyst during surgery. Radical surgical procedures show lower recurrence rates, but the rate of mortality increases(Pang et al., 2018). These drawbacks are exacerbated by poor facilities in a disease that generally infects the marginalised poor in developing countries.

Recurrence is not an early complication and its incidence is difficult to establish as it is dependent on quality and length of follow-up. Follow-up in this study was a maximum of 12 months and the rate of recurrence may have increased over the years. Recurrence may be local

to the organ, regional or distance. The question of whether a new cyst is a recurrence, or a new infection persists especially if the monitoring is inadequate. Recurrence occurs between 2 – 20% of the cases (Velasco-Tirado et al., 2017). The time it takes for the cyst to recur after endocystectomy varies from a few months to several years (Eckert et al., 1995; He et al., 2015). Differentiating between residual cavity and recurrence may not be clear during imaging (He et al., 2015) and follow-up should continue until the whole cavity has been reabsorbed and invisible. Other complications of surgery include exudates from the residual cavity, incisional fistula and excess bile secretion (Cirenei et al., 2001).

Limitations

The study as indeed the data was very fragmented due to difficulties associated with collecting longitudinal data from different sources. Measurement of the outcomes was limited to short periods due to inability to have a regular follow-up on the transhumant subjects who moved freely in and out of the control area. The difficulty of working with unregulated retrospective data collected by different people over long periods of time was also problematic to analyse. It was impossible to determine that the samples had been constantly stored at -20^0C as power outage is a common occurrence in Turkana as well as Nairobi where samples were stored for a long time. Even in the presence of a generator, it is possible that the samples thawed a few times.

Another problem encountered in follow-up was similarity in names where it was difficult to determine if it was a repeat patient or first diagnosis. This was reduced by use of patient numbers instead of names. However, this was only useful where the patient brought their records and its possible that there was duplication. A different method of patient identification like fingerprinting may be more useful in the future.

2.9 Conclusion

This study gives better under understanding of the effectiveness of existing approaches. This longitudinal study shows that there is considerable variation in the development of cysts. The spontaneous disappearance of 3 cysts and the collapse of 3 others have implications both for the selection of patients for surgery and the assessment of chemotherapy. Surgical removal of asymptomatic cysts should only proceed after a period of observation to establish cyst growth. This is especially pertinent in Turkana where the fertility of the cysts and the subsequent incidence of dissemination at surgery resulting in widespread inoperable disease are high (McPherson 1985). Although 66% of the cysts grew during the period of observation, 34% remained static, collapsed, or disappeared.

Controlled trials are needed to determine the efficacy of drugs such as albendazole in continuous treatment as opposed to intermittent.

4 RECOMBINANT ANTIGEN PRODUCTION AND STANDARDIZATION

Sensitive diagnostic tools are important in clinical diagnosis, epidemiological surveillance and infection control. We identified 5 antigens from literature which may have different properties and appeared to have differential immunoreactive properties towards serum antibodies in patients with CE (Carmena et al., 2006; Zhang et al., 2003). Elongation factor 1 (EF-1β/δ).Thioredoxin peroxidise (TPx), *E. granulosus* fatty acid binding protein 1 (EgFABP1), Heat shock protein 70 (HSP70) and EgAgB. After purification, the antigens showed a good immune response towards a pool of positive sera and poor responses towards negative sera. This was a good basis for the next two chapters where these antigens were used for diagnosis and follow-up of confirmed CE patients.

4.1.1 Serum samples and patient information

Serum samples were obtained from Mr Eberhard Zeyhle of the African Medical & Research Foundation (AMREF) which has run a hydatid control program in Northwest Turkana between 1983 and 2015. The study involved mass ultrasound screening of the nomadic Turkana people for Cystic Echinococcocus (CE) infection. Treatment was given when required and patients were then followed-up for as long as was possible and/or necessary. For each positive case, information was gathered on age, gender, medical history and cyst type. At each screening a serum sample for any positive case was taken, labelled, bagged together and stored in freezers at -20°C and a sample of these was transported to the UK after obtaining necessary permits.

4.1.2 Recombinant antigen purification

3.1.2.1 Protein Expression

Five recombinant antigens of *Echinococcus granulosus* origin were used in this study. Stocks of *Escherichia coli* BL21(DE3) cells harbouring the recombinant pET19b *Eg*FABP1,

*Eg*EF1β/δ, *Eg*HSP70, *Eg*TPx and *Eg*AgB plasmids had previously been synthesised by Dr. Tony Bodell (University of Salford) and stored as frozen glycerol stocks (10%). These antigens were cloned, purified and standardised for use in this study by the researcher.

Briefly, for each recombinant antigen, a frozen scraping from glycerol stocks (10%) was inoculated in 10 ml of Luria-Bertani (LB) (Thermo Fisher Scientific, Runcorn, UK) medium and supplemented with ampicillin (100mg/ml) to generate a starter culture. This was then grown overnight at 37°C in an incubator with a shaker (200rpm). The following day 500 ml conical flasks containing 200 ml LB-amp were seeded with the overnight cultures at a 1:50 dilution and grown to mid logarithmic phase. Cultures were then induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a concentration of 0.4mM (100mM stock) and incubated shaking at 37°C (200rpm) for 4 hours.

The 200ml cultures were processed using 50 ml falcon screw cap tubes to obtain pellets by centrifugation (5 min at 5000g), and pellets were inverted and left to air-dry. The pellets were re-suspended in BugBuster protein extraction reagent (His-Bind Purification Kit, Novagen, Watford, UK) using 5ml of 1X reagent per gram of wet cell paste. The cell suspensions were shaken vigorously on a rotating platform for 20 minutes at room temperature, and subsequently centrifuged (16,000g for 20min at 4°C). The supernatants were transferred to a new tube and a 1 ml aliquot was analysed by SDS-PAGE/Coomassie blue staining. The remaining soluble cell lysates were subsequently purified by Immobilised Metal Affinity Chromatography (IMAC) as per the manufacturer's instructions (Novagen, Watford, UK)

3.1.2.2 SDS PAGE analysis

Presence of purified target proteins was confirmed on 12.5% tris-glycine gels using the Laemmli (1970) system.(add ref) Stacking buffer (0.5M Tris.HCl, pH 6.8), separating buffer (1.5M Tris.HCl, pH 8.8), Sodium dodecyl sulphate (10% w/v), 40% Acrylamide solution

(acryl: bis, 37.5:1, pH 8.8), 10% Ammonium persulphate and 0.04% Temed. Sample buffer (100 mM Tris-Cl pH 6.8, 4% (w/v) sodium dodecyl sulphate, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT (dithiothreitol) was used at a 1:1 ratio (v/v) with purified recombinant proteins and loaded on gels at 10 µl per well.

The gels were stained in Coomassie blue stain (0.1% Coomassie Blue, 10% Glacial acetic acid [v/v], 50% Methanol [v/v], 40% H_2O [v/v]) and incubated covered on a rocking platform set at low speed at room temperature for 1 hour. Gels were de-stained twice (7% glacial acetic acid [v/v], 12% methanol [v/v], 81% H_2O [v/v]) for 1 hr and 3 hours respectively using fresh de-stain. Protein bands were visualised using a G-Box imaging system (Syngene, Cambridge UK) and molecular sizes estimated using *SeeBlue Plus2* pre-stained standard marker (Invitrogen, Paisley, UK). Protein sizes were calculated using the protein sequences inputted into the online Expasy available at: http://www.expasy.ch/tools/.

3.1.2.3 Western Blotting

Proteins separated under reducing conditions were transferred onto nitrocellulose membranes using an Iblot semi-dry system as per the manufacturer's instructions (Invitrogen, Paisley, UK).

The blots were subsequently blocked in phosphate buffered saline (PBS) (0.3%) $Tween_{20}$ and 5% dry milk powder (blocking buffer), covered and incubated at room temperature on a rocking platform set at low speed for 1 hour. The membranes were washed three times in washing buffer 0.1% PBS T_{20} for 3 minutes per wash.

Using an anti-histidine detection system, the nitrocellulose blots were incubated with polyclonal mouse anti-histidine primary antibody (Novagen Watford, UK) diluted in blocking buffer at 1:2000, covered and placed on a rocking platform set at low speed for 1 hour at room temperature. Blots were washed three times and incubated in anti-mouse secondary antibody

conjugated to alkaline phosphatase (Sigma, Dorset, UK) at a dilution of 1:16,000 in 0.3% PBS T_{20}.

Subsequently, the membranes were washed three times and then covered in 5-Bromo-4-chloro3-indolyl phosphate/Nitro blue tetrazolium substrate buffer (NBT/BCIP - BCIP 0.15 mg/ml, NBT 0.30 mg/ml, Tris buffer 100 mM and $MgCl_2$ 5mM, pH 9.25–9.75), until they had developed, and bands were visible. The reactions were stopped with deionised water and blots were viewed in a G-box imaging system (Syngene, Cambridge UK). Protein concentration determined using Bradford Assay

3.1.3 ELISA ASSAYS

All enzyme-linked immunosorbent assays (ELISA) were performed as described by Wen and Craig (1994). Optimum antigen concentrations for well coating and sera dilutions were predetermined by checkerboard titration (Voller et al., 1976). Total IgG was determined using direct ELISA and IgG subclasses were determined using indirect ELISA (**Figure 3-1**).

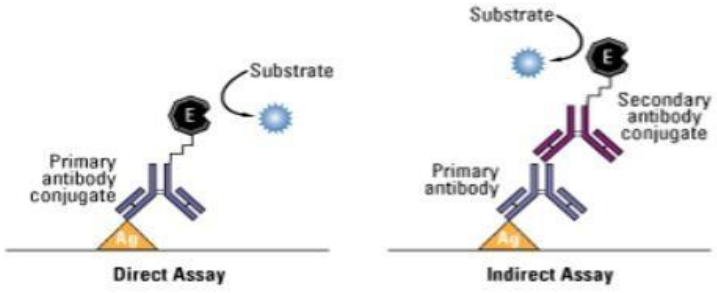


Figure 3-1: Direct and Indirect ELISA.

3.1.3.1 Standardization of Immunodiagnostic Reagents

To determine the optimum working concentrations for different antigens and antibodies, highly responding positive controls and low responding negative controls were screened to assess the level of total IgG antibody responses and determine a cut-off point for diagnosis.

3.1.3.1.1 Standardisation of recombinant antigens and crude HCF

To determine the optimal dilution for diagnostic use, checkerboard titrations were performed using sheep hydatid cyst fluid (HCF) and recombinant antigens against human serum antibodies.

A plate for crude sheep hydatid cyst fluid (HCF) and each pre-prepared recombinant antigen was set-up as follows: The first row of each immunolon microtiter plate (Thermo Fisher Scientific, Runcorn, UK) was coated with of HCF or recombinant antigen diluted in carbonate bicarbonate buffer (Sigma, Dorset, UK)) (0.05M) at pH 9.6. Each column was serially diluted across the plate from the neat concentrations determined by Bradford assay in 3.1.2.3 above.

The plate was incubated overnight at 4 ^{0}C and washed three times with phosphate buffered saline (PBS) (0.1%, pH7.2) Tween$_{20}$ (0.05%) (PBS) to remove any non-bound antigen. Nonspecific binding sites were blocked with PBS (0.3% pH 7.2) containing skimmed milk (5%) and Tween$_{20}$ (0.05%). Both positive and negative serum samples were diluted 1:25 with PBS (0.3% pH 7.2) containing skimmed milk (5%) in Tween$_{20}$ (0.05%) and pipetted in the first row followed by serial dilutions to 1/1600. A final row with no sera had PBS (0.3% pH 7.2) containing skimmed milk (5%) in Tween$_{20}$ (0.05%) only was used a control. The plates were incubated for 1 hour at room temperature, the liquid was discarded, and the wells washed (x3) with PBS to remove any non-bound serum. A secondary antibody, goat anti-human IgG whole molecule conjugated to alkaline phosphatase (Fc specific; Sigma, Dorset, UK)) was diluted to 1: 16,000 in PBS (0.3%)–T and added to each well (100 μl). The liquid was discarded, and the

wells washed (x3) with PBS. A chromogen *p*-Nitrophenylphosphatase (Sigma, Dorset, UK)) dissolved in (1M) diethanolamine (Sigma, Dorset, UK) and Magnesium Chloride (Sigma, Dorset, UK)) solution (0.5mM, pH 9.8) was added to each well and the plates were left to incubate at room temperature for a pre-determined time. The optical densities were measured at 405nm after 15 minutes, 20 minutes and 45 minutes, using an automatic microplate reader (Multiskan Ascent FC, Thermo Fisher Scientific, Runcorn, UK). Optimal dilution was determined at the point where the positive control began to titrate out ensuring maximum binding with lowest dilution of antigen possible.

3.1.3.1.2 Single antibody detection (Direct) ELISA assays

Optimum working concentrations were pre-determined from the above experiment on standardisation of recombinant antigens and crude HCF. They were ^{R}*Eg*AgB - 5.6µg/ml, ^{R}*Eg*EF-1β/δ- 6µg/ml, ^{R}*Eg*HSP70 - 87.5µg/ml, ^{R}*Eg*FABP1- 33.25µg/ml and ^{R}*Eg*TPx - 5.3µg/ml and HCF-1.5 µg/ml. Immunolon microtitre (immunolon B) plates were pre-sensitised with crude parasite antigen (HCF) and recombinant antigens with the above working concentrations. The plates were then washed three times with phosphate buffered saline (PBS) (0.1%, pH 7.2) $Tween_{20}$ (0.05%) (PBS), to remove non-bound antigen. Non-specific binding sites were blocked with PBS (0.3% pH 7.2) containing skimmed milk (5%) in $Tween_{20}$ (0.05%) (200ul) per well and the plates were covered and incubated at room temperature for 1 hr. The blocking buffer was discarded, and the plates were washed (x3) with PBS. Individual patient sera including negative and positive controls was diluted to 1:200 with PBS (0.3% pH 7.2) containing skimmed milk (5%) in $Tween_{20}$ (0.05%) and pipetted in duplicate to each well (100 µl). The plate was incubated for 1 hour at room temperature and washed (x3) with PBS. The antibody, goat anti-human IgG whole molecule conjugated to alkaline phosphatase (Fc specific; Sigma, Dorset, UK) was diluted to 1: 16,000 in PBS (0.3%) and added to the wells at 100 µl per well and the plates were covered and incubated for a further 1 hr at room

temperature. The liquid was discarded, and the wells washed (x3) with PBS. A chromogen, *p*Nitrophenylphosphate 5mg (Sigma, Dorset, UK)) dissolved in (1M) diethanolamine (Sigma, Dorset, UK)) and Magnesium Chloride (Sigma, Dorset, UK) solution (0.5mM, pH 9.8) was added to each well and the plates were left to incubate at room temperature for a pre-determined time. The optical densities were measured at 405nm after 15 minutes, 20 minutes and 45 minutes, using an automatic microplate reader (Multiskan Ascent FC, Thermo Fisher Scientific, Runcorn, UK).

3.1.3.1.3 Double antibody detection (Indirect) ELISA assays

Subclasses of IgG (IgG_1 and IgG_4) antibody detection ELISA assays were performed using previously standardise protocols (Lawn et al., 2004) and working concentrations of antigens calculated from the standardisation of recombinant antigens and crude HCF. These subclasses have been shown to be the predominant antigens in helminth infections e.g. filariasis, schistosomiasis, cysticercosis and echinococcosis (Restrepo et al., 1998; Siracusano et al., 2008).

Using 96-well immulon B4 micro-titre plates, crude HCF (1. 5 µg/ml) and recombinant antigens were coated at different optimum working concentrations pre-determined by checkerboard titration. These were (^{R}*Eg*AgB - 5.6µg/ml), (^{R}*Eg*EF-1β/δ- 6µg/ml), (^{R}*Eg*HSP70 - 87.5µg/ml), (^{R}*Eg*FABP1- 33.25µg/ml) and (^{R}*Eg*TPx - 5.3µg/ml) (**Table 3-1**) and were incubated overnight at 4°C. The plates were washed three times with phosphate buffered saline (PBS) (0.1%, pH 7.2)-$Tween_{20}$ (0.05%) (PBS). Non-binding antigens and non-specific binding sites were blocked with PBS (0.3% pH 7.2) containing skimmed milk (5%) in $Tween_{20}$ (0.05%) (200ul). The plates were covered and incubated at room temperature for one hour and washed three times with PBS. Individual patient sera samples, including negative and positive controls were diluted to 1:50 with PBS (0.3% pH 7.2) containing skimmed milk (5%) in $Tween_{20}$

(0.05%). Each sample (100ul) was pipetted in duplicate into mapped wells and incubated for one hour at room temperature. Monoclonal anti-human IgG1 or IgG4 antibodies raised in mice (Abcam, Cambridge UK) were diluted to 1:5000 in PBS (0.3%) and added to each well (100 µl). The plates were covered and incubated for one hour at room temperature. The antibody was removed from each well and the wells washed (x3) with PBS. Goat anti-human IgG whole molecule tertiary antibody conjugated to alkaline phosphatase (Sigma, Dorset, UK)) was diluted to 1: 16,000 in PBS and added to each well (100 μl). The plates were covered and incubated for one hour at room temperature, followed by a final wash (x3) with PBS. Chromogen *p*-Nitrophenylphosphate (Sigma, Dorset, UK)) dissolved in (1M) diethanolamine (Sigma, Dorset, UK)) and Magnesium Chloride (Sigma, Dorset, UK)) solution (0.5mM, pH 9.8) was added to each well and the plates were left to incubate at room temperature for a pre-determined time. The optical densities were measured at 405nm after 15 minutes, 20 minutes and 45 minutes, using an automatic microplate reader (Multiskan Ascent FC, Thermo Fisher Scientific, Runcorn, UK).

4.1.3 Results and Discussion

The preparation of the recombinant antigens *Echinococcus granulosus* antigen B (^{R}*Eg*AgB), *Echinococcus granulosus* elongation factor 1 alpha beta (^{R}*Eg*EF-1β/δ) *Echinococcus granulosus* heat shock protein 70 (*^{R}Eg*HSP70), *Echinococcus granulosus* fatty acid binding protein 1 (^{R}*Eg*FABP1), and *Echinococcus granulosus* thioredoxin peroxidase (^{R}*Eg*TPx) under investigation was achieved by cell culture of the recombinant antigen pET vectors previously prepared by Dr A. Bodell. The pET plasmid expression vector structures were based on the work carried out in the 1970's by Herbert Boyer and his post-doctoral scientists derived from their pBR322 plasmids (Greene et al., 1978).

Solubilisation of the crude proteins and purification was achieved using IMAC technology via affinity chromatography specific to histidine extensions of the protein sequences. The fine purification steps were followed using the standard procedures described by Merck4Biosciences with their HisBind Purification Kit. Imidazole elution afforded the proteins in high levels of purification and the protein concentrations were determination by the Bradford Assay (Bradford, 1976) method. **Table 4-1** shows the neat concentration levels achieved from the IMAC purification stage and the working concentrations that were then found to be optimum ELISA checker board experiments and later single and double antibody ELISA's.

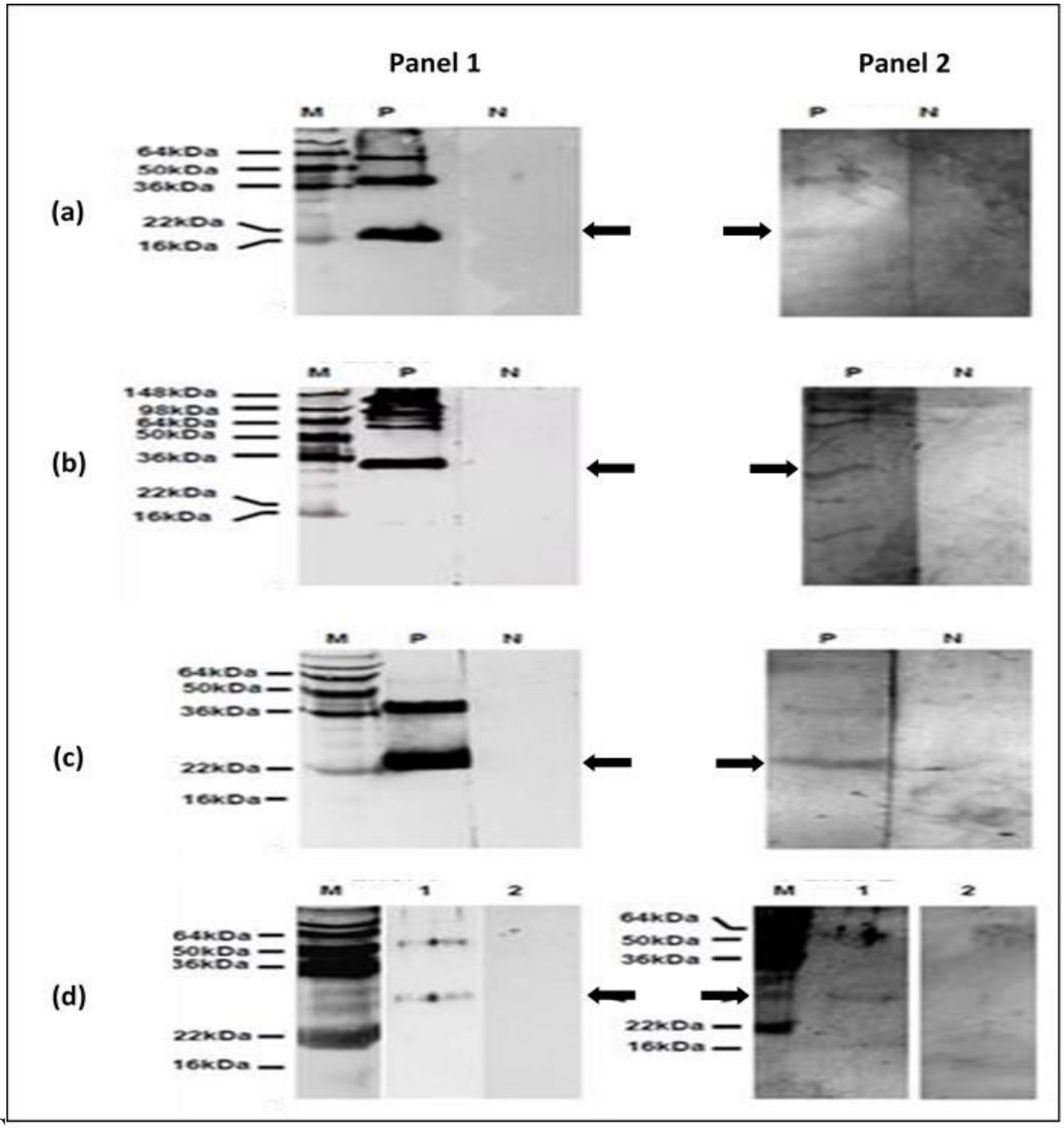
Antigen	Neat concentration	Working concentration
Hydatid cyst fluid	400 µg/ml	1.5 µg/ml
rEgAgB	90 µg/ml	5.6µg/ml
rEgEF-1β/δ	95µg/ml	6 µg/ml
rEgHSP70	1.4mg/ml	87.5 µg/ml
rEgFABP1	532µg/ml	33.25 µg/ml
rEgTPx	85µg/ml	5.3 µg /ml

Table 4-1: Antigen concentrations of the recombinant IMAC isolated proteins.

The recombinant fusion proteins *Eg*EF-1β/δ, *Eg*HSP70, *Eg*FABP1, *Eg*TPx and *Eg*AgB were partially characterised using SDS-PAGE and a 1mM Tris-glycine 12.5% gel. The molecular

weights were determined by comparing with the SeeBlue® plus2 pre-stained protein standard marker from Invitrogen, loaded in one lane of the gel.

The qualitative purification of each protein was observed by visualisation using a G-box imaging system. The number and density of the blue stained protein bands and the pseudo-quantitative evaluation of the recombinant protein sizes were calculated using the online Expasy Peptide mass calculator (Gasteiger et al., 2003), available from http://www.expasy.ch/tools/. shows the Coomassie stained gel of non-reduced blots for purified proteins in Panel 1 as well as their immune reactivity with pooled positive and negative sera (Figure 4-1).



C

Figure 4-1: Non-reduced blots for purified proteins

Key: Panel 1 Lane M, SeeBlue® Plus2 pre-stained protein standard 4–250 kDa; anti-histag assay; Lane 1, purified recombinant histag-fusion protein; lane 2, negative control. Panel 2: pooled sera assay: lane 1, pooled hydatid patient sera; lane 2, normal sera; a) EgFABP1; b) EgEF-1β/δ; c) EgHSP70; d) EgTPx; target proteins shown with arrow head.

4.1.4 Titration curves between human sera and crude HCF

The determination of the most effective working concentration of crude HCF was carried out using a checkerboard titration (Voller et al., 1976).

A pool of highly responding positive sera and another of low responding negative sera was used to work out optimal working concentrations. The optimum dilution was the point where there was maximum binding of HCF at the lowest dilution of antigen.

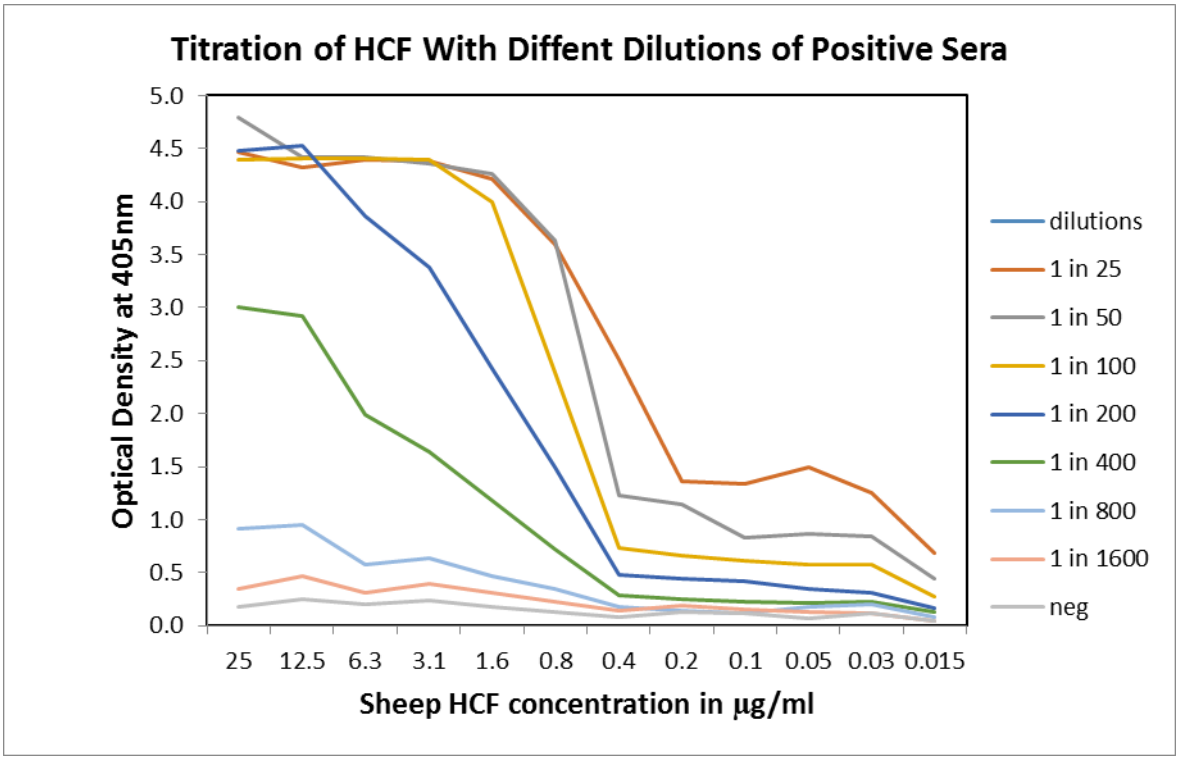


Figure 4-2: Titration of native HCF.

A titration of HCF against positive and negative human sera antibodies using antihuman total IgG conjugated antibodies was performed (**Figure 4-2**).

The results show that the human sera dilutions at 1:25, 1:50 and 1:100 all retain relative plateaus above the O.D. of 4.0 with similar HCF concentrations (25µg/ml to 1.5µg/ml). The HCF antigen concentration of 1.5µg/ml and the dilution 1:100 for sera were used for all proceeding work and identified as the optimal concentrations for sample experiments.

4.1.5 Titration Curves of Recombinant Antigens

A similar checkerboard titration method was used to assess the optimal concentration of recombinant antigens. This second checkerboard ELISA's were undertaken to assess the reactivity's of the IMAC purified positive and negative recombinant proteins derived from the clones: pET19b-EgTPx-2Cb; pET19b-EgFABP1-C10; pET19b-EgEF-1β/δ; pET19b-EgHSP70-C10; and pET19b-EgAgB and were compared to crude HCF.

Each recombinant protein was examined against the positive and negative antihuman total IgG conjugated human sera antibodies, shown separately in **Figure** 4-3 to **Figure 4-7**

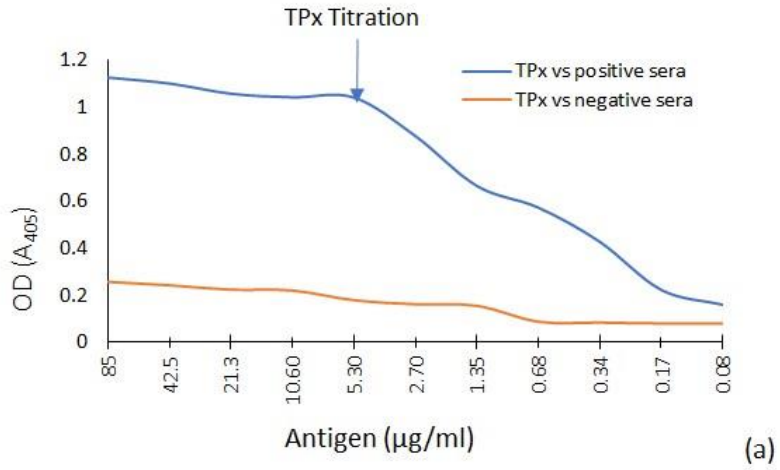


Figure 4-3: Titration curves for recombinant antigens TPx

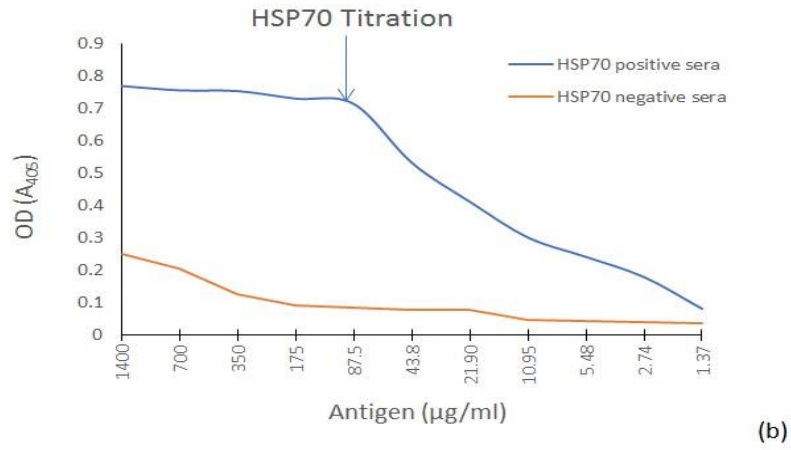


Figure 4-4: Titration curves for recombinant antigens HSP70

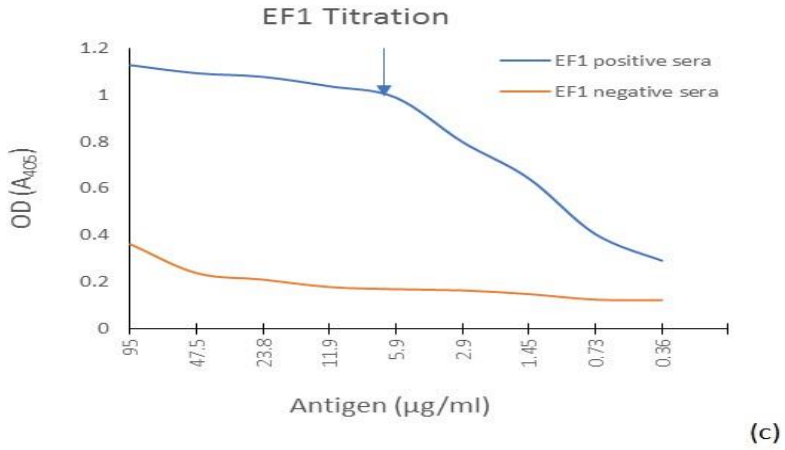


Figure 4-5: Titration curves for recombinant antigens EF-1β/δ

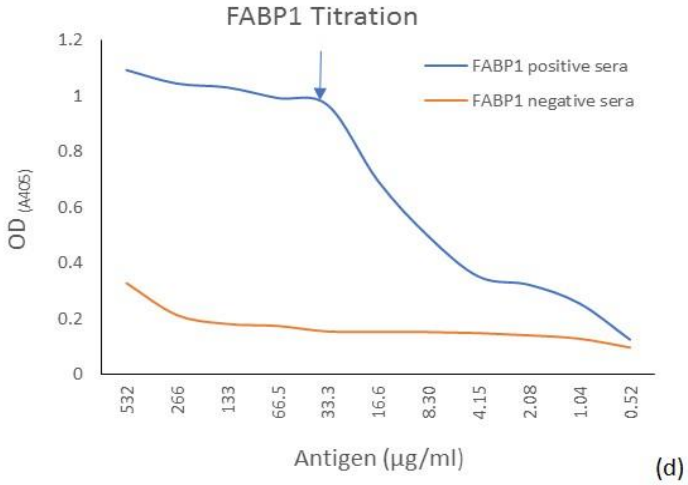


Figure 4-6: Titration curves for recombinant FABP1

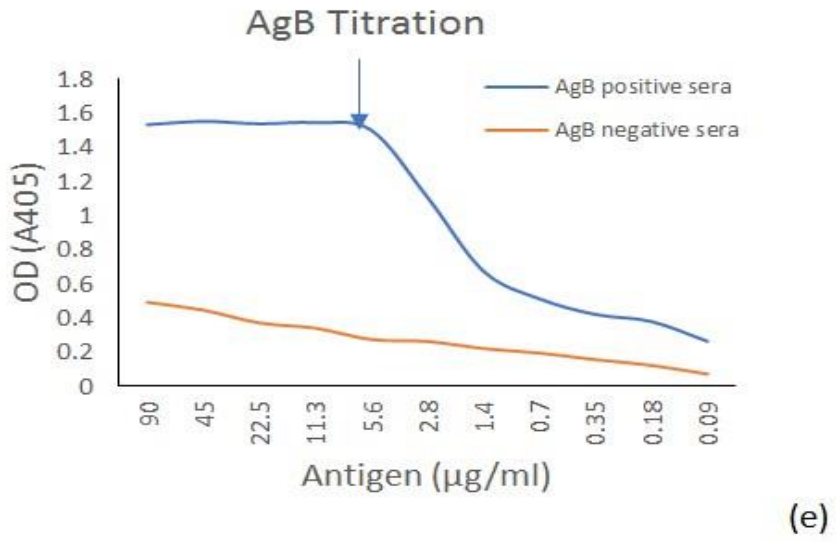


Figure 4-7: Titration curves for recombinant antigens AgB.

Antigens reactivity were tested against positive and negative human sera using antihuman total IgG conjugated antibodies. The titration curves for the positive sera responses were used to determine the working concentrations used in subsequent experiments. The reactivity of the recombinant proteins examined were lower compared to HCF. At the highest sera concentration, HSP70 starts with a response below an O.D. of 0.8. FABP1, TPx and EF-1β/δ all start with an O.D. of around 1.15, at the highest concentration.

Antigens reactivity were tested against positive and negative human sera using antihuman total IgG conjugated antibodies. The titration curves for the positive sera responses were used to determine the working concentrations used in subsequent experiments. The reactivity of the recombinant proteins examined were lower compared to HCF. At the highest sera concentration, HSP70 starts with a response below an O.D. of 0.8. FABP1, TPx and EF-1β/δ all start with an O.D. of around 1.15, at the highest concentration.

4.2 Discussion

Use of recombinant antigens in diagnosis of helminthic infections reduces cross reactivity and consequently the number of false negatives (Li et al., 2004; Ricciardi et al., 2015). In diagnosis of CE, no one antigen shows satisfactory results in either diagnosis or follow-up. In this study, we sought to explore the idea that perhaps the answer lies in using multiple antigens consecutively. Four potentially differentially immunoreactive antigens with towards serum antibodies in patients with CE (Manzano-Román et al., 2015; Ortona et al., 2004) were purified and optimised for immunodiagnostic testing along with the commonly used HCF and AgB.

Mouse monoclonal antibodies have exhibited high specificity for human IgG (Welling et al. 1985 and Kameny.,1987) demonstrated these characteristics. Working concentrations of antigens were determined using highly positive sera and low responding negative sera described in checkerboard ELISA experimental protocol 3.1.3.1. Optimal concentrations of antigens were taken when the lowest amount of antigen exhibited maximal binding. The lowest dilution of positive sera where the optimal concentration of antigen had the highest binding just before it began to titrate out was determined in the checkerboard ELISA with considerations to background noise and quantity of sera available. Working concentrations used in this study are summarised in **Table 4-1.**

Working dilutions of conjugates was based on manufacturers recommendations. These were verified and modified where necessary. Optimal concentrations were checked for each new set of sera, conjugates and reagents and new antigen preparations.

5 IMMUNO-SEROLOGICAL PROFILES IN HUMAN CYSTIC ECHINOCOCCOSIS

5.1 Introduction

Human cystic echinococcosis (CE) is a re-emerging zoonotic disease among the rural poor. It causes considerable morbidity and mortality in many parts of the world, particularly in underdeveloped regions. Pathology is typified by long term growth of unilocular fluid filled cysts which may contain thousands of protoscoleces (Macpherson et al., 2003; Thompson, 2015). *E.granulosus* is the only parasite known to produce lesions in so many anatomical sites. (Kiresi et al., 2003). WHO based classification of hydatid cysts on morphology and contents and placed them into five groups from CE1 to CE5. This formed the basis for clinicians to perform or recommend the appropriate interventions (Macpherson et al., 2003) and to define whether patients show active disease CE1 – CE2, transitional CE3 or stable/cured disease CE4 – CE5 (Brunetti et al., 2009; Lissandrin et al., 2016). These definitions are not clear cut as a dead cyst may harbour active CE2 cysts containing viable daughter cysts (Taherkhani et al., 2007).

Accurate early diagnosis of CE leads to cheaper management and better treatment outcomes (Velasco-Tirado et al., 2018) Today, diagnosis is based on clinical assessment, imaging and serology. Imaging techniques including ultrasonography (US), computed tomography and magnetic resonance imaging have been widely used in diagnosis of CE and the modality most helpful depends on size of cyst and affected organ (Wen et al., 2019) In areas where CE is endemic, the high cost of computed tomography and magnetic resonance imaging make them inaccessible and ultrasound is the main diagnostic method. Use of ultra sound revolutionized the diagnosis of intra-abdominal CE in individual cases of human infection, with sensitivity and specificity between 93–98% (Vicary et al., 1977; Wen et al., 2019), giving the clinicians information on the number, size, location and condition. The advantages include its portability,

lack of ionizing radiation, painless, harmless and non-invasive. Also, instant results which can be permanently recorded. Ultrasound is ideal for abdominal localizations where visualising the internal structures of the cysts provides conclusive diagnostic information in active cysts especially CE1 and CE2 (Macpherson et al., 2003).

Ultrasound is not always ideal for some cyst locations and may miss out on cysts in the lungs, bones or CNS (Shambesh et al., 1999). Other diagnostic techniques are therefore necessary for confirmatory testing. Serological studies are useful in community surveys and may provide partial diagnosis for some of those cases missed. They also allow a follow-up with better imaging techniques as well as confirming US imaging where cystic lesions presented are morphologically unclear (Brunetti et al., 2018; Rogan et al., 1997). In addition, when community studies are carried out, greater numbers of people are screened, and serological responses can be useful in identifying suspect cases not picked up by ultrasound screening (Shambesh et al., 1999). Torgerson et al (2009) suggested using a highly sensitive test to pick out all the initial cases, followed by a highly specific test to confirm the results. Immunodiagnostic tests for CE using *E.granulosus* hydatid cyst fluid antigen for primary screening has good sensitivity between 75 to 95 % and is ideal for primary testing but it has many inconsistencies due to cross reactivity with other helminths producing false positives (Craig, 1986). Other diagnostic variations arise due to patients showing low responses (Macpherson et al., 2003; Siracusano et al., 2008), variation in responses with cyst type, cyst size (Hernandez-Gonzalez et al., 2008; Piccoli et al., 2013) and location (Zhang et al., 2006).

To improve sensitivity and reduced the problem of cross reactivity with other helminths significantly, use of IgG subclass detection and recombinant antigens which can be mass produced and easily purified has been applied (Sarkari et al., 2015; Virginio et al., 2003). Sensitive assays, such as enzyme-linked immune-sorbent assay (ELISA), immunoblotting and immuno-fluorescence, have replaced the classical tests such as immuno-electrophoresis,

indirect hemagglutination. The efficiency of the assay is dependent on the quality of antigen used (e.g. crude, purified or recombinant,) as well as the characteristics of the sera (e.g. confirmed cases, suspected cases, low responders or high responders (Paul et al., 2001)

This study sought to compare the diagnostic value of HCF with 5 recombinant antigens and correlate the immune responses to defined CE pathology.

The aim of this chapter was to evaluate the relevance of cyst morphology to antibody profiles in relation to immunodiagnostic antigens.

The hypothesis was that different antigens are associated with different stages of cyst development.

5.2 Materials and methods

5.2.1 Serum samples

Samples from 100 ultrasound confirmed CE cases were selected for this study. All the patients were untreated and harboured single abdominal cysts. All sera samples originated from Turkana (Kenya) and had been collected during mass screening between 2002 and 2005 and had been stored in a freezer at 20^0C. The freezer was battery powered in the field and contents were transferred to an electric powered freezer as soon as that was possible. There is a possibility that the samples went through at least 3 freeze – thaws between the field and arrival in Salford. In all batches tested, there were high numbers of high reacting samples and therefore freeze thaw did not seem to be a problem. A patient was classed as untreated if they had not received chemotherapy treatment in the last 6 months or surgery in the last 18 months. Patients with multiple cysts were excluded from the study. The positive control was a pool of highly responding sera from individuals infected with CE1, CE2, CE3 and CE4 type cysts (4 samples per cyst type, n=16 individuals). Details of cyst were provided by US images and/or notes.

Patients with different cyst morphologies were selected for this study. A flow chart describing the inclusion criteria for sample selection is shown below **Figure 5-1**

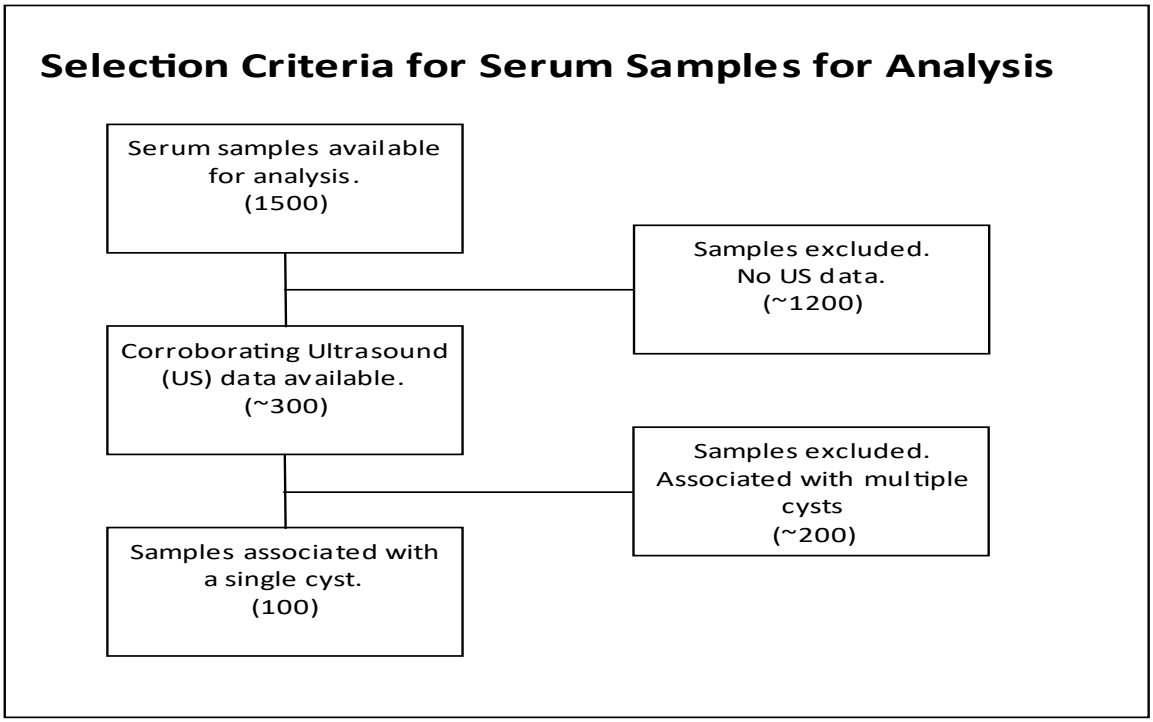


Figure 5-1: Selection criteria for serum samples

The classification of cyst types observed (CE1-CE4) was based on matching the ultra-sonographer's images and notes to the initial classification system of hydatid cysts (WHO-IWGE, 2003). It is acknowledged that this system has been updated by splitting CE3 cysts into CE3a and CE3b, but this was not established at the time of serum collection and therefore is not employed. Among those selected, 33 patients had CE1 cysts, 19 had CE2 cysts, 21 patients with CE3 cysts and 27 patients had CE4 cysts. Cyst locations are shown in **Table 5-1**.

Cyst	Numbers of	Liver	Lower	Omen	Kidne	Spleen
CE1	33	24	3	3	2	1
CE2	19	11		8		
CE3	21	15	1	5		
CE4	27	24	2	1		

Table 5-1: Cyst Morphology and Localization

5.2.2 ELISA analysis

ELISA analysis was done to determine total serum immunoglobulin (IgG(w)) and IgG1 and IgG4 subclass antibodies expression in response 5 recombinant and 1 native parasite antigens.

These had been previously purified and optimised in chapter 3. The optical density was calculated as the average of duplicate samples with the blank subtracted. Duplicate samples that had more than 10% difference were repeated.

5.2.3 Cut off results

Traditionally, ELISA cut-off values are calculated by averaging a panel of known negative controls and adding two or three standard deviations, the Gaussian approach (Allan et al., 1990). In this study, negative cut off points were calculated as the mean optical density plus 2 SD of control negative sera samples (Ito et al., 2002; Jiang et al., 2012). The sera were chosen from 50 patients who were CE-negative but had blood taken for other unrelated conditions e.g. malaria and typhoid; but the details of the conditions were not available. It is acknowledged that this may have influenced the immune responses. The patients all gave consent for their serum to be used for scientific research. The lowest reactive samples from 20 individuals in Turkana ultrasound results was used. The negative cut off point of expression of total IgG and IgG subclasses 1 and 4 for different recombinant antigens were calculated with the results shown in **Table 5-2**.

Antibody (Antigen)	MEAN O. D	Standard Deviation	CUTOFF
Total IgG (HSP70)	0.117	0.060	0.237
Total IgG (EF1)	0.090	0.075	0.241
Total IgG (FABP1)	0.108	0.052	0.212
Total IgG (TPx)	0.079	0.055	0.189
Total IgG (AgB)	0.065	0.031	0.127
Total IgG (HCF)	0.156	0.120	0.397
IgG1 (HSP70)	0.081	0.037	0.155
IgG1 (EF1)	0.097	0.059	0.216
IgG1 (FABP1)	0.082	0.022	0.125
IgG1 (TPx)	0.062	0.015	0.093
IgG1 (AgB)	0.098	0.034	0.167
IgG1 (HCF)	0.188	0.076	0.341
IgG4 (HSP70)	0.074	0.032	0.138
IgG4 (EF1)	0.079	0.035	0.149
IgG4 (FABP1)	0.076	0.033	0.143
IgG4 (TPx)	0.080	0.038	0.156
IgG4 (AgB)	0.074	0.030	0.134
IgG4 (HCF)	0.154	0.091	0.336

Table 5-2: ELISA negative cut off values

A pool of 10 highly responding positive sera were used a positive control. All sera were stored at -20°C prior to being assayed

5.2.4 Statistical analysis

Data were analysed using scatter computer graphics software (GraphPad Prism version 6, SanDiego - CA USA) and SPSS statistical package version 23 (SPSS Inc Chicago, IL). Differences between total IgG and IgG subclasses based on the same antigen were compared using chi-square test. Statistical significance was considered at P-value ≤ 0.05 unless otherwise stated.

5.3 Results

5.4 Specificity of total IgG and IgG subclasses.

Test for specificity was done using 21 sera samples from individuals found in the same community as test cases but known be to disease free. Specificity was calculated as sum of negative cases shown by the particular antigen, divided by total number of cases and multiplied by 100. **Table 5-3** shows the specificity of total IgG and IgG sub classes in response to different antigens. All antigens showed good specificity of between 90 and 100%.

Antibody	HSP70(%)	EF1(%)	FABP1(%)	TPx (%)	AgB (%)	HCF (%)
Total IgG	95	95	95	95	90	95
IgG1	95	90	100	100	95	95
IgG4	100	95	100	95	95	100

Table 5-3: Specificity of total IgG and IgG sub classes in response to different antigens

5.4.1 Differential antigen recognition with changes in cyst type

One hundred CE cases were evaluated for antibody responses towards 6 different antigens with total serum IgG and IgG subclasses IgG1 and IgG4. Of these 33 harboured CE1 cysts, 19 had CE2 cysts, 21 had CE3 cyst and 27 had CE4 cysts. All the cases had been previously confirmed and cyst type determined by ultra sound.

5.4.1.1 Antigen Recognition Towards CE1 Cyst Types.

Sera from 33 untreated individuals diagnosed with CE1 cysts were tested using ELISA against 5 recombinant antigens and 1 native antigen to detect levels of total IgG and IgG subclasses 1 and 4 immune responses. The diagnostic performance of different antigens was assessed and graphed in **Figure 5-2** to **Figure 5-5**

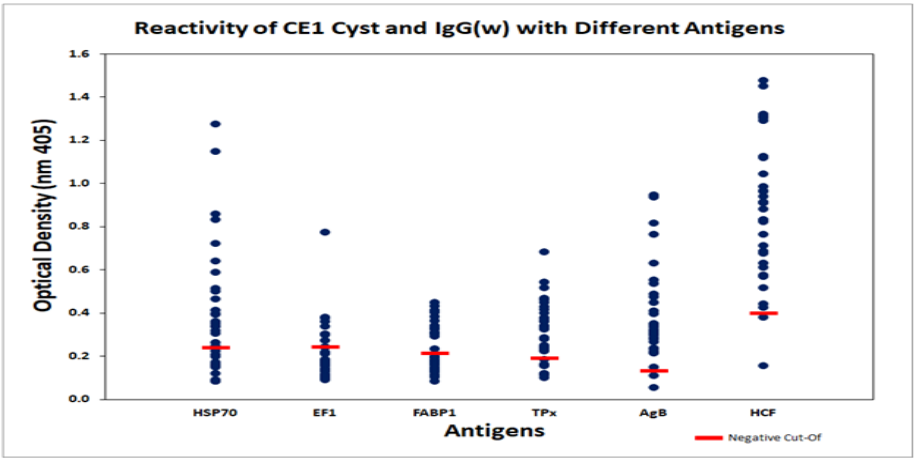


Figure 5-2: Expression of Total IgG in responses to echinococcal antigens in patients with CE1 cysts

Figure 5-3 shows the above data in a box and whisker plot as a comparative representation

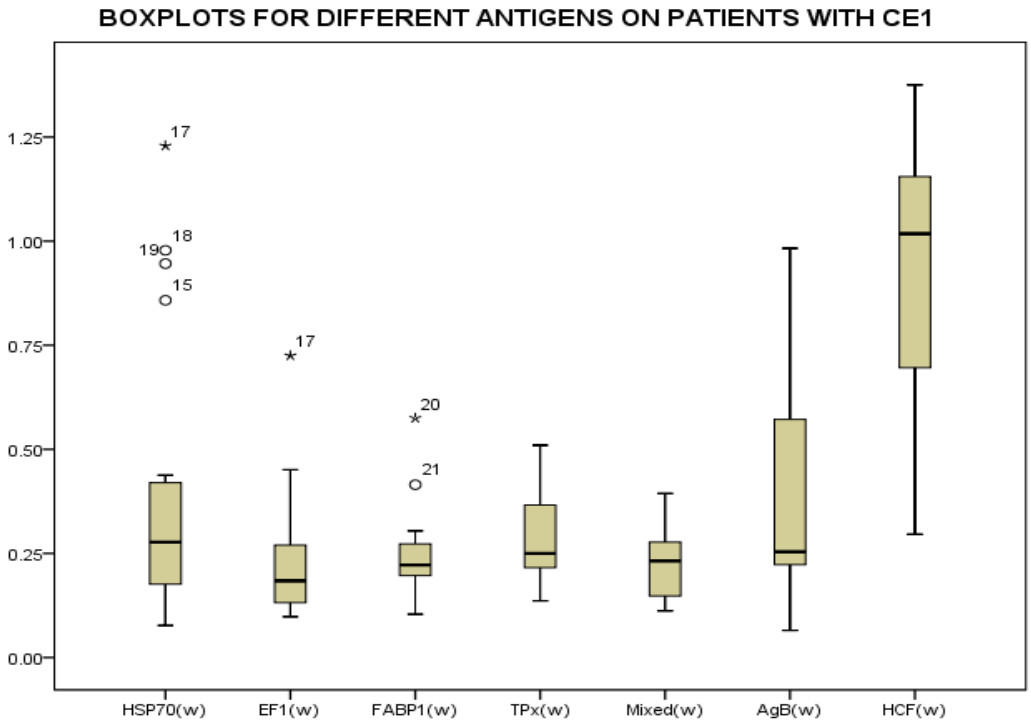
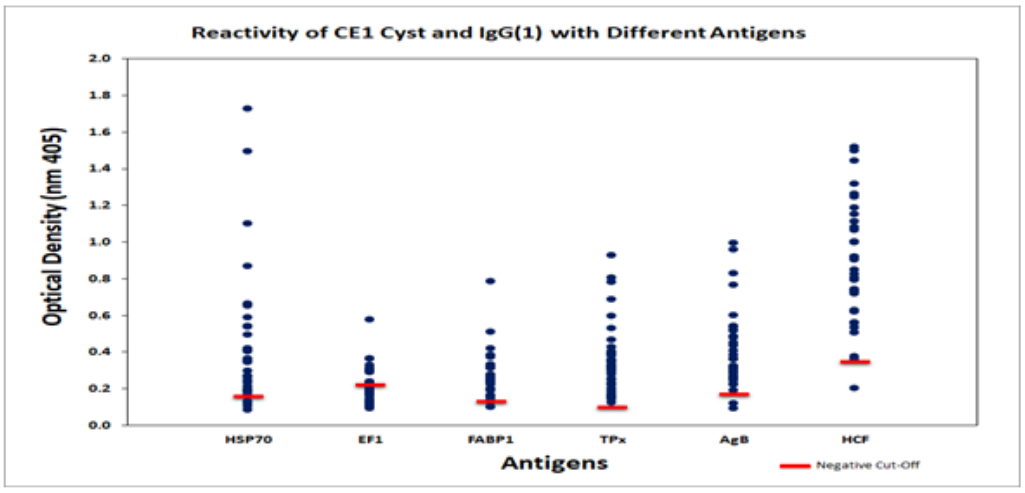


Figure 5-3: Total IgG Expression in responses to echinococcal antigens in patients with CE1 cysts



b)

Figure 5-4: Expression of IgG1 in responses to echinococcal antigens in patients with CE1 cysts

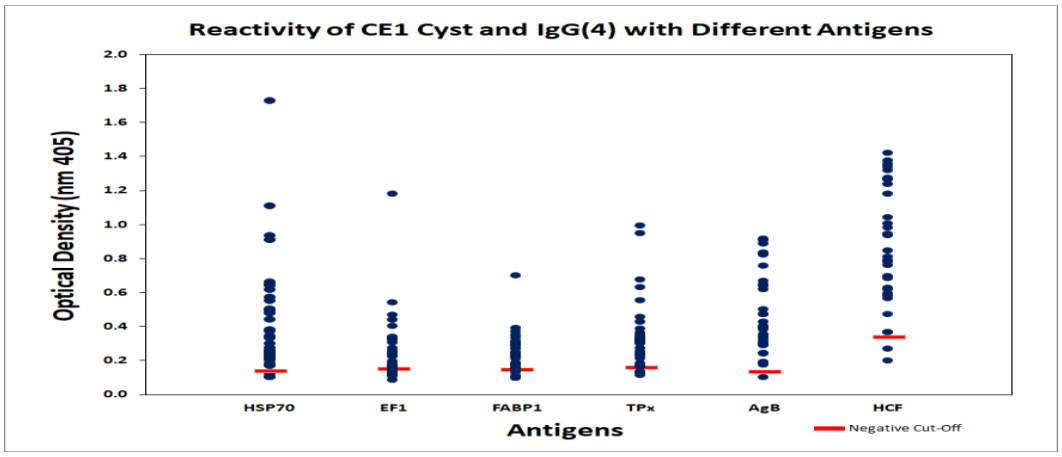


Figure 5-5: Expression of IgG4 in responses to echinococcal antigens in patients with CE1 cysts

ELISAs determining total IgG (IgGw) expression in response to HSP70 in CE1 cases showed a seropositive rate of 67% (22) with an average OD of 0.398. The diagnostic value was improved to 88 % with both subclasses IgG1 and IgG4 with a higher average O.D of 0.430 and 0.45 respectively. The differences between total IgG and the subclass seropositive rates were highly significant with IgG1 ($\chi^2 = 9.1$, $p = 0.008$) and IgG4 ($\chi^2 = 9.1$, $p = 0.008$). Also, the seropositive rates between IgG1 and IgG4 were significant ($\chi^2 = 16.8$, $p = 0.003$).

Total IgG expression in response to EF-1β/δ was poor with only 30 % (10) of the cases confirmed as positive. It also had low average OD at 0.220nm but the spread of immune

responses was minimal with one highly responding outlier. These responses were marginally improved with IgG1 which confirmed 42 % (14) were positive and markedly improved with IgG4 where 64% (21) of the cases were seropositive. The average optical density in IgG1 and IgG4 responses were 0.216 and 0.255 respectively. The difference between total IgG and subclass IgG1 seropositive rates were highly significant ($\chi^2 = 13.3$, $p < 0.001$) and IgG4 ($\chi^2 = 18.2$, $p = 0.005$) and IgG1 vs IgG4 was also highly significant ($\chi^2 = 13.9$, $p < 0.001$).

Total IgG expression towards FABP1 were also poor with only 45 % (15/33) seropositivity and average titre value of 0.241. Better diagnostic value was found with subclass responses where 91% (30) were seropositive with IgG1 and 85% positive with IgG4. The average 0D was also improved with 0.258nM with IgG1 and 0.238 with IgG4. The difference between total IgG and subclass IgG1 seropositive rates were not significant (IgG(w) vs 1gG1, $p = 0.23$, IgG(w) vs IgG4 ($p = 1$) and IgG1 vs IgG4 was also not significant ($p = 0.53$).

Better diagnostic values with total IgG were achieved with TPx with positive responses at 73% (24) with average OD of 0.304. Similarly, diagnostic value was improved by using subclasses and positive responses with IgG1 were 100% (33) with average OD of 0.366 and IgG4 88 % (29) with average OD of 0.336. The difference between total IgG and subclass IgG1 seropositive not significant ($\chi^2 = 3.2$, $p = 0.24$) but was significant with IgG4 ($\chi^2 = 10.86$, $p = 0.02$) and IgG1 vs IgG4 was not significant ($\chi^2 = 5.3$, $p = 0.16$).

Total IgG expression in response to AgB was 70% (23) with an average OD of 0.342. With subclasses, 94% (31) were seropositive with IgG1 with average OD of 0.411 and 97% (32) with IgG4 with an average OD of 0.472. The difference between total IgG and subclass IgG1 seropositive rates was highly significant ($\chi^2 = 33.01$, $p = 0.02$) but not significant with IgG4 ($\chi^2 = 15.9$, $p = 0.061$) and IgG1 vs IgG4 was also not significant ($\chi^2 = 15.9$, $p = 0.061$).

Responses of total IgG towards HCF showed a sero-positive rate of 88% (29) and average OD of 0.864. Reponses of IgG1 showed 91% (30) seropositivity and IgG4 94% (31) with an average OD of 0.882 and 0.869 respectively. The differences between total IgG and subclass IgG1 seropositive rates were not significant (χ^2 = 15.98, p =0.119) and IgG4 (χ^2 = 7.220, p = 0.061) and IgG1 vs IgG4 was significant (χ^2 = 15.98, p = 0.061).

Therefore, specific IgG1 subclass was predominant in responses AgB while specific and IgG4 responses were the predominant towards TPx. Responses towards HSP70 and EF1 were significantly different between all antibodies while those against FABP1 and HCF had no statistical significance differences across all antibodies tested.

5.4.1.2 Antigen recognition towards CE2 cyst types

Sera from 19 untreated CE2 cases was tested using ELISA against 5 recombinant antigens and 1 native antigen to detect levels of total IgG and IgG subclasses 1 and 4 immune responses. The diagnostic performance of different antigens was assessed and graphed in **Figure 5-6** to

Figure 5-8 Figure 5-8: Expression of IgG4 in responses to echinococcal antigens in patients with CE2 cysts

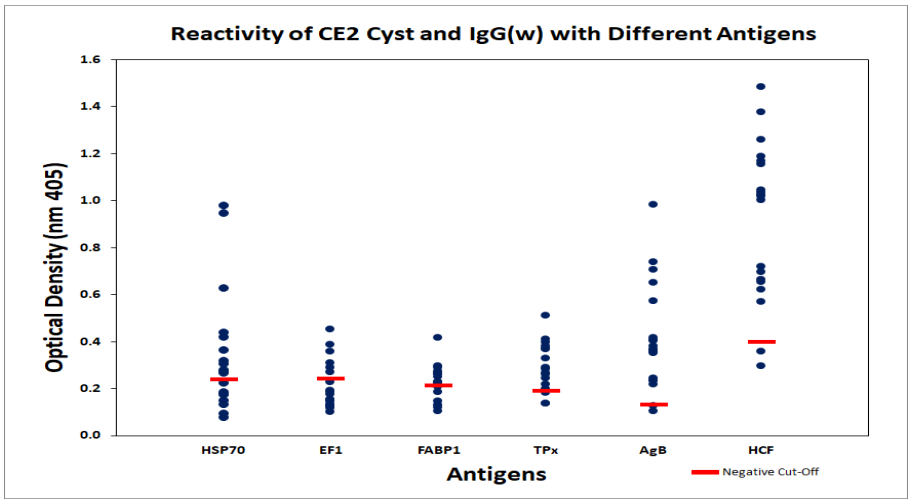


Figure 5-6: Expression of total IgG in responses to echinococcal antigens in patients with CE2 cysts

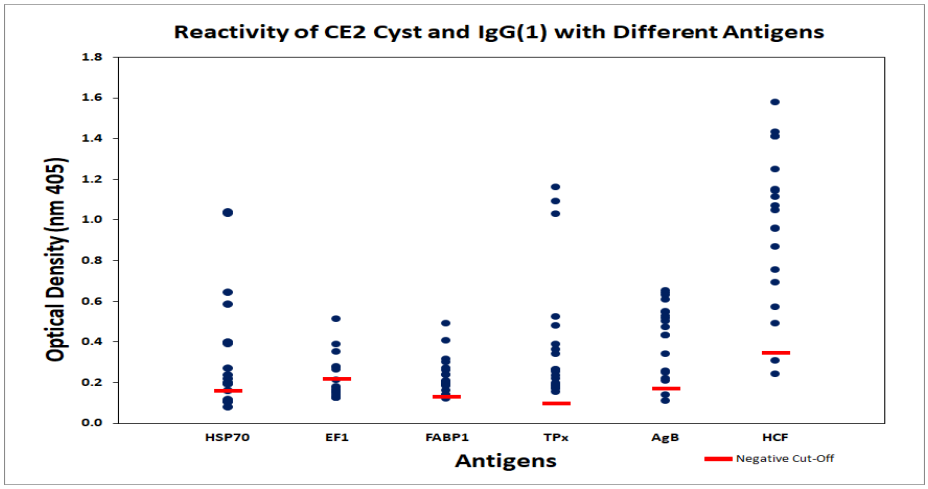


Figure 5-7: Expression of IgG1 in responses to echinococcal antigens in patients with CE2 cysts

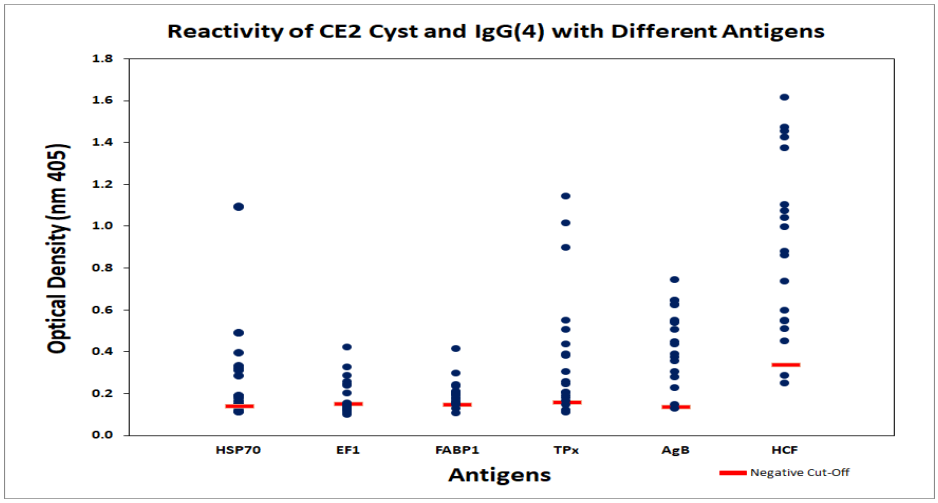


Figure 5-8: Expression of IgG4 in responses to echinococcal antigens in patients with CE2 cysts

Assays for total serum IgG antibody showed that 58% (11) of CE2 cases were sero-positive in response to HSP70 with an average O.D of 0.33. assays for subclass IgG1 showed a seropositivity of 79% (15) with a mean O.D of 0.36 and IgG4 79% (15) of the cases to be seropositive with an average O.D 0.331. The differences between total IgG and the subclass seropositive rates were significant with IgG1 ($\chi^2 = 16.9$, $p = 0.018$) and IgG4 ($\chi^2 = 6.9$, $p = 0.018$). Also, the seropositive rates between IgG1 and IgG4 were significant ($\chi^2 = 19$, $p < 0.001$).

In CE2 cases, similar to CE1 cases, the poorest performing antigen against total serum IgG was EF-1β/δ where six patients (32 %) showed positive response with a mean O.D of 0.212. subclass IgG1 had similar results at 32 % (6) mean O.D of 0.121 and unlike in CE1 cysts, subclass IgG4 did not improve the performance very much with a seropositivity 42 % (8) and a mean O.D of 0.183. The difference between total IgG and subclass IgG1 seropositive rates were highly significant ($\chi^2 = 19$, $p < 0.001$) and IgG4 ($\chi^2 = 12.2$, $p = 0.005$) and IgG1 vs IgG4 was also significant ($\chi^2 = 14$, $p = 0.01$). Total IgG responses in response to FABP1 showed a s 68 % (13) seropositivity with an average O.D 0.224. With the subclasses, IgG1 had a seropositivity of 95 % (18) and a mean O.D of 0.233 while IgG4 had a seropositivity rate of 89% (17) and an average OD of 0.199. The difference between total IgG and subclass IgG1 seropositive rates were not significant ($\chi^2 = 2.3$, $p = 0.32$) and IgG4 ($\chi^2 = 0.531$, $p = 0.105$) and IgG1 vs IgG4 was also significant ($\chi^2 = 8.9$, $p = 0.422$).

Antigen TPx showed a good diagnostic performance with responses to total IgG at 84 % (16) with average O.D of 0.281, responses with IgG1 100% (19) average O.D of 0.403 and responses of IgG4 at 84 % (16) with average O.D of 0.390. The difference between total IgG and subclass IgG4 seropositive not significant ($\chi^2 = 0.825$, $p = 0.422$)

Total IgG expression in response to AgB was 89% (17) with an average OD of 0.412. With subclasses, 89% (17) were seropositive with IgG1 with average O.D of 0.421 and 89% (17) with IgG4 with an average OD of 0.446. The difference between total IgG and subclass IgG1 seropositive rates was highly significant ($\chi^2 = 19$, $p < 0.006$) but not significant with IgG4 ($\chi^2 = 8.9$, $p = 0.105$) and IgG1 vs IgG4 was also not significant ($\chi^2 = 8.9$, $p = 0.105$).

Responses of total IgG towards HCF showed a sero-positive rate of 88% (29) and average OD of 0.864. Reponses of IgG1 showed 91% (30) seropositivity and IgG4 94% (31) with an average OD of 0.882 and 0.869 respectively. The differences between total IgG and subclass

IgG1 was highly significant ($\chi^2 = 19$, $p = 0.006$) and IgG4 ($\chi^2 = 19$, $p = 0.006$) and IgG1 vs IgG4 was also significant ($\chi^2 = 19$, $p = 0.006$).

5.4.1.3 Antigen recognition towards CE3 cyst types

Sera from 21 untreated CE3 cases was tested using ELISA against 5 recombinant antigens and 1 native antigen to detect levels of total IgG and IgG subclasses 1 and 4 immune responses. The diagnostic performance of different antigens was assessed and graphed in **Figure 5-9** to **Figure 5-11**.

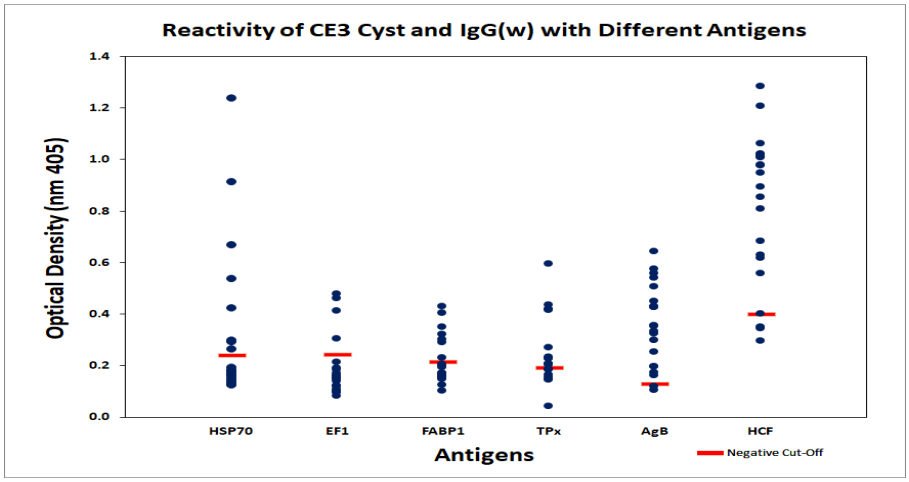


Figure 5-9: Expression of total IgG in responses to echinococcal antigens in patients with CE3 cysts.

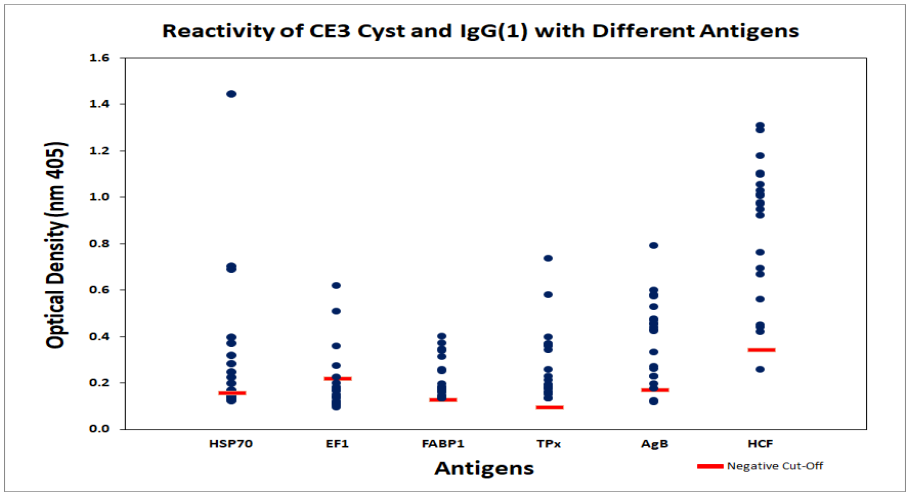


Figure 5-10: Expression of IgG1 in responses to echinococcal antigens in patients with CE3 cysts

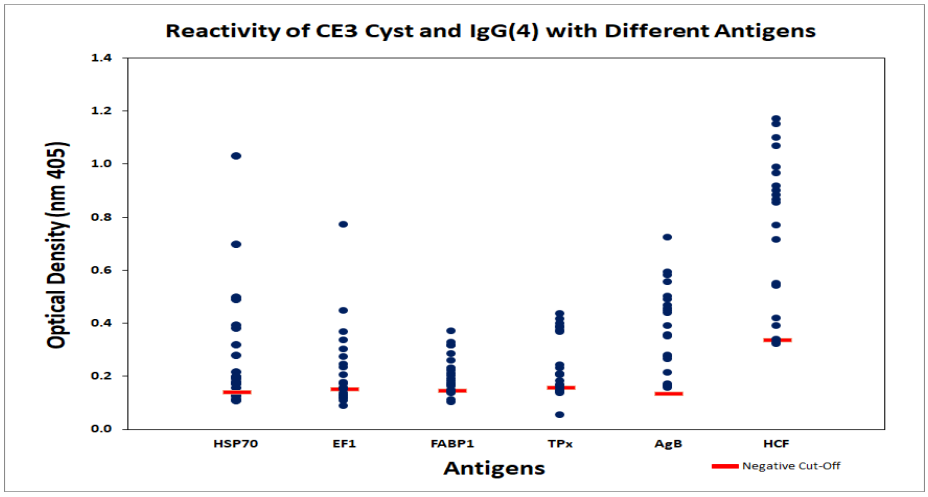


Figure 5-11: Expression of IgG4 in responses to echinococcal antigens in patients with CE3 cysts

In CE3 cases, expression of total serum IgG antibodies in response to HSP70 was lower than the active CE1 and CE2 cysts and only 38% (8) of the cases were sero-positive with an average O.D of 0.317. there was improvement in diagnostic value with the use of subclasses and IgG1 showed seropositivity of 62% (13) with a mean O.D of 0.31 and IgG4 a seropositivity of 80% (17) of the cases and average O.D 0.294. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 7.95$, $p = 0.006$) but not significant with IgG4 ($\chi^2 = 3.04$, $p = 0.12$) and IgG1 vs IgG4 was also not significant ($\chi^2 = 0.297$, $p = 0.618$).

As with the active cysts, the poorest performing antigen with total serum IgG remained EF-1β/δ where 4 patients (19 %) showed positive response with a mean O.D of 0.192. The response remained poor with subclass IgG1 at 23 % (5) with a mean O.D of 0.198 but improv ed greatly with IgG4 to 67 % (14) with mean O.D of 0.228. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 15.78$, $p < 0.001$) but not significant with IgG4 ($\chi^2 = 2.42$, $p = 0.255$) and IgG1 vs IgG4 was also not significant ($\chi^2 = 3.28$, $p = 0.123$).

Another poor performance with total IgG was found with FABP1 where 33% (7) seropositivity was found with FABP1 with an average O.D 0.218. With the subclasses, responses of IgG1 FABP1 picking up all the cases at 100 % (21) and a mean O.D of 0.213 and IgG4 76 % (16) with average O.D of 0.208. The difference between total IgG and subclass IgG1 seropositive rates were not significant.

Expression of total IgG in response TPx were 62 % (13) seropositive with average O.D of 0.250, 100 % (21) and had an average O.D of 0.278 in IgG1 responses and 76 % (16) with average O.D of 0.244 with IgG4 responses. The difference between total IgG and subclass IgG1 seropositive rates were not significant.

Responses of total IgG towards AgB were moderate at 67 % (14) average O.D of 0.353. while seropositivity with IgG1 was 81% (17) and IgG4 was 100% (21). The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 21$, $p = 0.005$).

The best performing antigen with total IgG was HCF recognising 81% (17) with average O.D of 0.806 which a similar result with an average and 0.862 IgG1 and 90 % (19) of the cases, having an average O.D 0.750 with IgG4. The difference between total IgG and subclass IgG1 seropositive rates were not significant.

The best performing antigen was TPx against IgG1 antibodies as it picked up all the cases. The poorest performing antigen with CE3 cases was EF1α/β though the performance was improved with IgG4 antibodies compared to total serum IgG and IgG1 similar to CE2 and CE1 cases.

5.4.1.4 Antigen recognition towards CE4 cyst types

Sera from 27 untreated CE4 cases was tested using ELISA against 5 recombinant antigens and 1 native antigen and the levels of total IgG and IgG subclasses 1 and 4 immune responses assessed. The diagnostic performance of different antigens was assessed and graphed in **Figure 5-12** to **Figure 5-14.**

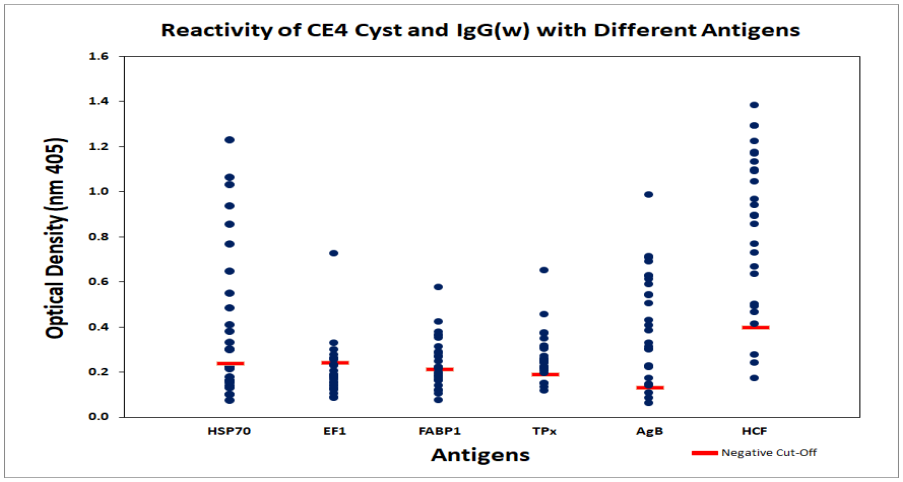


Figure 5-12: Expression of total IgG in responses to echinococcal antigens in patients with CE4 cysts

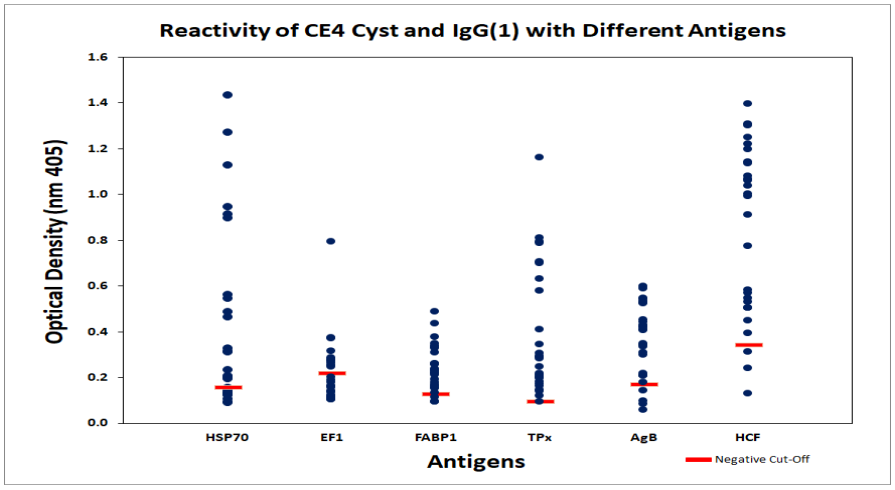


Figure 5-13: Expression of IgG1 in responses to echinococcal antigens in patients with CE4 cysts

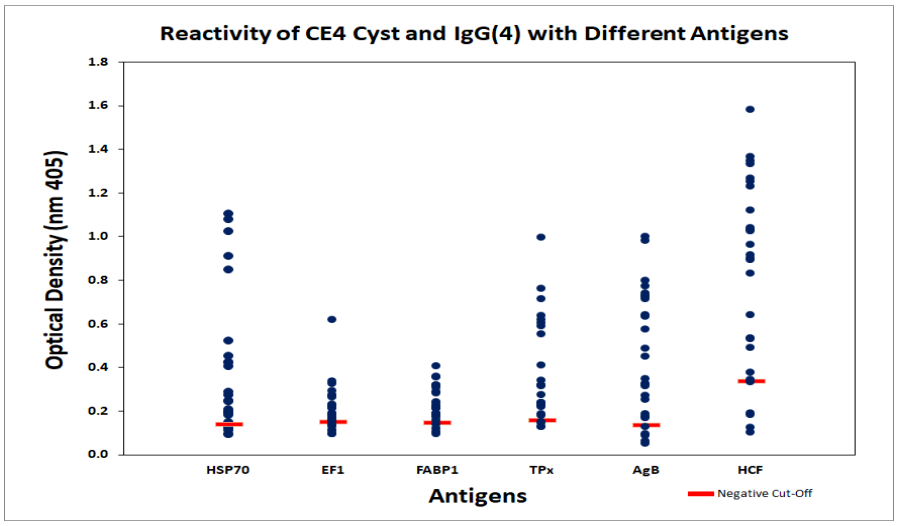


Figure 5-14: Expression of IgG4 in responses to echinococcal antigens in patients with CE4 cysts

The responses of total serum IgG antibody in CE4 cysts showed that 52% (14) were sero-positive in response to HSP70 with an average O.D of 0.421. Responses of IgG1 antibody with the 27 cases improved the diagnostic value, similar to all the other cyst types to 70% (19) with a mean O.D of 0.433. and with IgG4 antibody to 78% (21) with average O.D of 0.377. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 12.24$, $p = 0.001$) but IgG4 was not significant ($\chi^2 = 3.84$, $p = 0.067$) and IgG1 vs IgG4 was also significant ($\chi^2 = 10.67$, $p = 0.04$).

The poorest performing antigen with total serum IgG was EF1 where 7 patients (26 %) showed positive response with a mean O.D of 0.207. This was also found in response towards IgG1 at 41 % (11) with a mean O.D of 0.221 but the diagnostic performance of EF1 was the best with CE4 cysts at 74 % (20) with mean O.D of 0.209. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 13.74$, $p < 0.001$) but with IgG4 was not significant ($\chi^2 = 7$, $p = 0.137$) but IgG1 vs IgG4 was not significant ($\chi^2 = 0.22$, $p = 0.022$).

Seropositivity with FABP1 was 56 % (15) with an average O.D 0.243. Improved performance was observed in all other antigens with IgG1 being seropositive for 89 % (24) the cases and a mean O.D of 0.241. and with IgG4 seropositive for 85% (23) with average O.D of 0.231 The difference between total IgG and subclass IgG1 seropositive rates were not significant ($\chi^2 = 4.21$, $p = 0.075$) but was significant with IgG4 ($\chi^2 = 3.04$, $p = 0.028$) and IgG1 vs IgG4 was also significant ($\chi^2 = 19.4$, $p = 0.001$).

Responses of total IgG to TPx were 85 % (23) seropositive with average O.D of 0.266. The best performing antigen was TPx with 96 % (26) of seropositive cases and an average O.D of 0.383 Seropositive cases with TPx reduced to 85 % (23) with average O.D of 0.368. The difference between total IgG and subclass IgG1 seropositive rates were not significant ($\chi^2 =$

5.97, p = 0.148) but with and IgG4 was highly significant ($\chi^2 = 27$, $p < 0.001$) and IgG1 vs IgG4 was not significant ($\chi^2 = 5.97$, $p = 0.148$).

The best diagnostic antigen with total serum IgG was AgB with a seropositivity of 89% and IgG1 response rate was 85% (23), mean O.D 0.357 and IgG4 picked up 81% (22) with an average O.D of 0.444. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 19.4$, $p < 0.001$) and IgG4 ($\chi^2 = 14.85$, $p = 0.003$) and IgG1 vs IgG4 was also significant ($\chi^2 = 9.28$, $p = 0.013$).

HCF was seropositive for 74% (20) with an average O.D of 0.839 responses to HCF were similar to FABP at 89% (24) with mean O.D of 0.837 Second best performing antigen was AgB and HCF which picked up 81% (22) each with an average O.D of 0.444 and 0.811 respectively. There were no significant differences in antibody responses across all the antigens. The difference between total IgG and subclass IgG1 seropositive rates were significant ($\chi^2 = 10.547$, $p = 0.026$) and IgG4 ($\chi^2 = 14.8$, $p < 0.025$) and IgG1 vs IgG4 was also significant ($\chi^2 = 14.8$, $p = 003$).

Overall, a logistical linear regression model was fitted to analyse the performance of all the antigens with different cyst types. It was found that expression of IgG1 in response to HSP70 were significantly different in CE1 cyst compared to all other cyst types ($p = 0.041$). In CE2 cysts, expression of IgG4 in response EF1 were not significantly level ($p = 0.062$). expression of total IgG in response to HSP70 was also significantly different in CE3 cysts ($p=0.09$) while in CE4 cysts it was the expression of IgG4 in response to AgB ($p = 0.015$). A summary of differences in significance is shown below **Table 5-4**

CE1

Antigen	IgG (w) vs IgG1	IgG (w) vs IgG4	IgG1 vs IgG4
HSP70	0.008	0.008	0.003
EF-1β/δ	0.001	0.005	0.001
FABP1	0.23	1.0	0.53
TPx	0.24	0.02	0.16
AgB	0.002	0.61	0.061
HCF	0.119	0.61	0.061

CE2

Antigen	IgG (w) vs IgG1	IgG (w) vs IgG4	IgG1 vs IgG4
HSP70	0.018	0.018	0.01
EF-1β/δ	0.001	0.005	0.01
FABP1	0.32	0.105	0.422
TPx	0.422	0.422	0.422
AgB	0.006	0.105	0.105
HCF	0.006	0.006	0.006

CE3

Antigen	IgG (w) vs IgG1	IgG (w) vs IgG4	IgG1 vs IgG4
HSP70	0.006	0.12	0.618
EF-1β/δ	0.001	0.255	0.123
FABP1	-	-	-
TPx	-	-	-
AgB	0.005	-	-
HCF	-	-	-

CE4

Antigen	IgG (w) vs IgG1	IgG (w) vs IgG4	IgG1 vs IgG4
HSP70	0.001	0.067	0.04
EF-1β/δ	0.001	0.137	0.022
FABP1	0.075	0.028	0.001
TPx	0.148	0.001	0.148
AgB	0.001	0.003	0.013
HCF	0.026	0.025	0.003

Table 5-4: Differences between different antigens in diagnosis

Significant differences between the expression of IgG1 and IgG4 subclasses were seen in responses to HSP70 and EF1 in CE1, CE2 and CE4 cysts. Antigen B and TPx also showed significant differences in expression of the 2 subclasses of IgG in cases harbouring CE4 cyst.

As a diagnostic approach in CE, detection of IgG1 responses to TPx had the best value with seropositivity of between 96 and 100%.

5.4.2 Variation of Immune Reponses with Different Cyst Types

Performance of serological tests varied with pathological stage of CE and the antibody used.

The CE-specific IgG subclasses (IgG1-4) showed a better performance than total IgG (**Figure 5-15** to **Figure 5-17).**

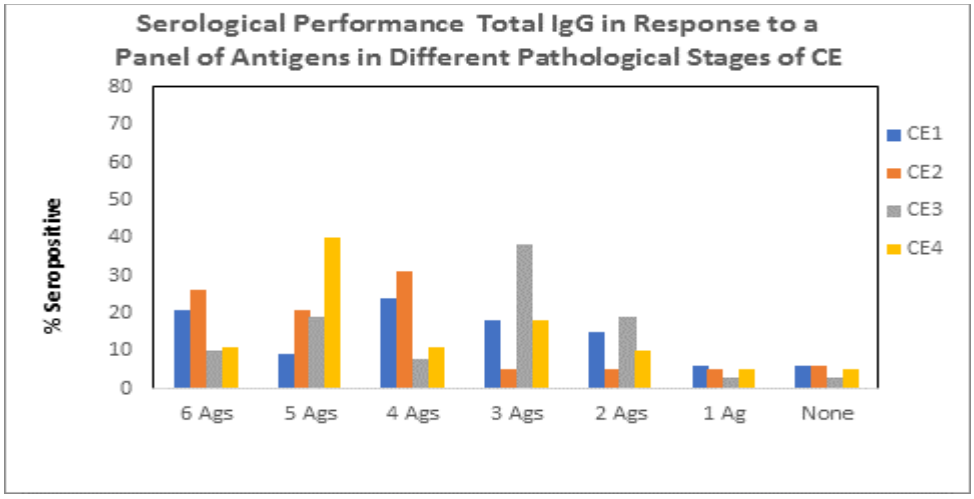


Figure 5-15: Level of total IgG responses in different cyst types

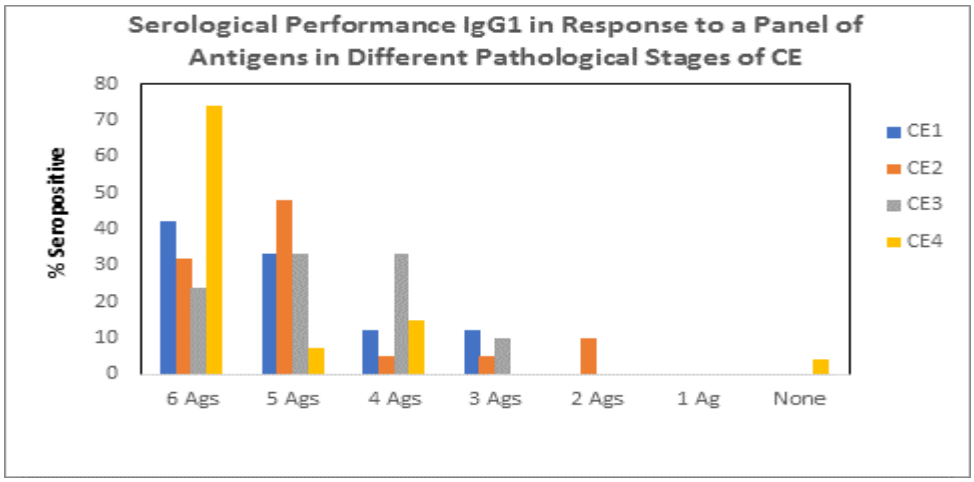
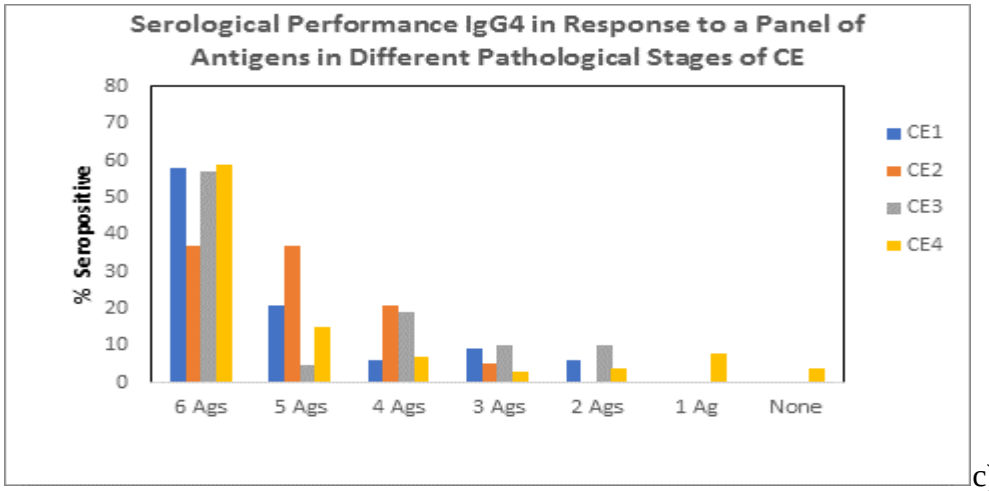


Figure 5-16: Level of IgG1 responses in different cyst types



c)

Figure 5-17: Level of IgG4 responses in different cyst types.

Expression of total IgG in response to a panel of 6 antigens. Active CE1 and CE2 cysts were more seropositive to all the antigens than inactive CE3 and CE4 cysts but CE1 and CE2 cyst had the largest number of patients that were seronegative. Every cyst type showed some seronegative cases. Over 70 % of all cases were seropositive towards 3 or more antigens but 20% of the cases were false negatives by all the antigens.

Expression of IgG1 subclass in in response to the same panel of antigens showed good diagnostic potential for CE4 cysts where 74% of the cases were seropositive to all 6 antigens compared to 42 % in CE1, 32% in CE2 and 24% in CE3 cases. Performance of IgG1 compared to total IgG was superior and over 90% of all the cases were seropositive for 3 or more antigens. About 99% of the cases were seropositive towards 4 or more antigens but 1 CE4 patient was seronegative in all the tests.

Responses of IgG4 showed the best diagnostic value for CE1 and CE3 cysts in response to all 6 antigens tested compared to the performance of IgG1 and total IgG. The same CE4 patient that was non-responsive with IgG1 was also seronegative with IgG4, but no other case was missed out by the subclasses. Compared to IgG1 responses where all but 1 case were seropositive with 4 or more antigens, there was a wide variation in responses with IgG4 and

only 80% of the cases were positive for 4 or more antigens. In diagnosis, IgG1 was found to be the best subclass antibody.

5.4.3 Variation of Immune Reponses with Different Patients

To assess the breath of the serological profile of patients harbouring different cyst types, expression of antibodies total IgG and subclasses IgG1 and IgG4 in response to a panel of 6 different antigens were assessed. There were 33 patients harbouring CE1cysts, 19 with CE2 cysts, 21 with CE3 cysts and 28 with CE4 cysts. For each patient, 18 tests were carried out for each serum sample (**Figure 5-18** to **Figure 5-21**). The highest responding patients showed a positive response in all 18 tests while some patients were seropositive for only 4 tests.

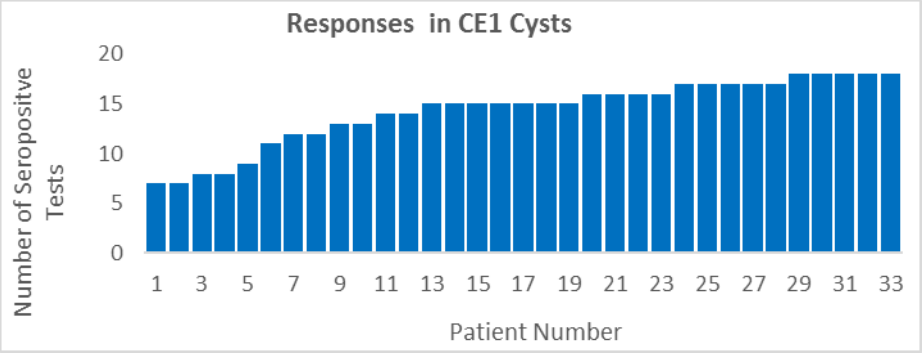


Figure 5-18: Number of seropositive tests, from a total of 18 in each patient among CE1 cases

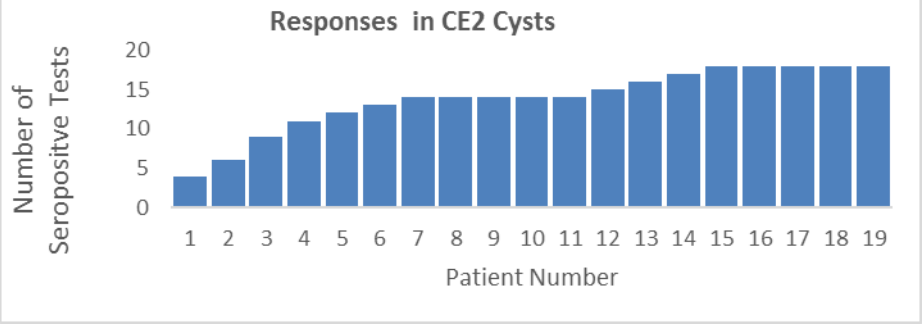


Figure 5-19: Number of seropositive tests, from a total of 18 in each patient among CE2 cases

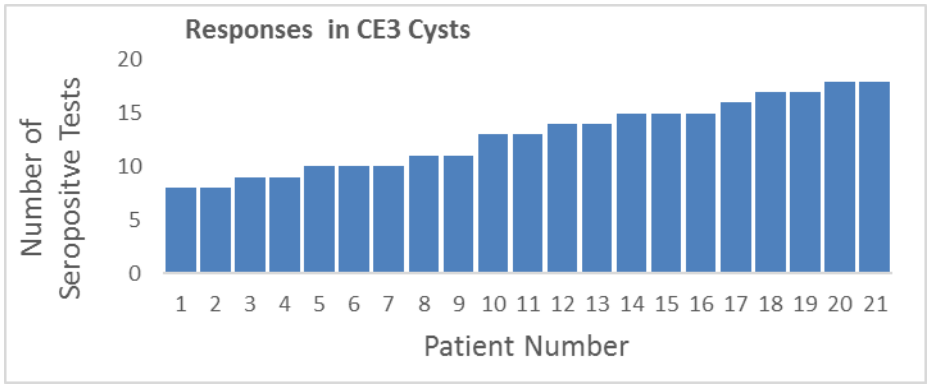


Figure 5-20: Number of seropositive tests, from a total of 18 in each patient among CE3 cases

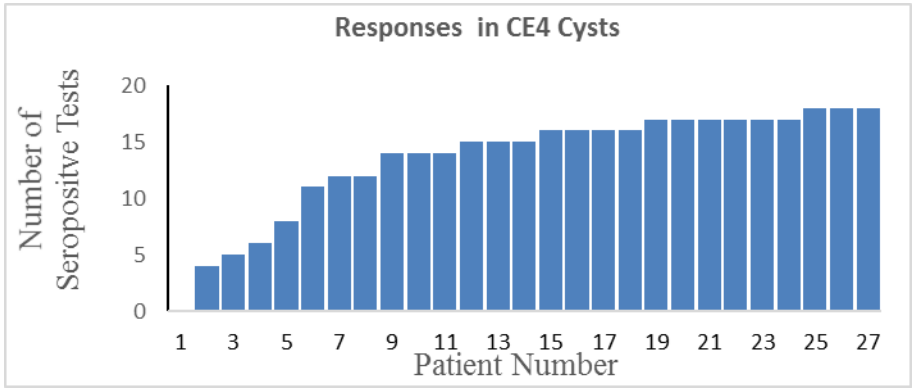


Figure 5-21: Number of seropositive tests, from a total of 18 in each patient among CE4 cases

Some patients showed positive responses to all 18 tests. These were 15% with CE1 cysts, 26% with CE2 cyst, 9% with CE3 cysts and 11% with CE4 cysts.

The group that had the highest number of highly responding patients was CE1 with 66% seropositive in 15 or more of the tests. In cases with in CE2 cysts there was 42% seropositive response for 15 or more tests, 38% in CE3 cyst and 55 % in CE4 cases.

All CE1 patients showed seropositive results to 7 or more tests and all CE3 patients showed positive results to 8 or more tests. On the other hand, one CE2 patient had positive results to only 4 tests while one CE4 case was seronegative to all tests.

5.5 Discussion

Serological diagnosis of CE depends on numerous factors, including cyst location, type, number and size. A great challenge in serological diagnosis is finding simple inexpensive methods for large scale sero-epidemiology. The main aim of this chapter was to generate a comparison *of E granulosus* antigens and to assess their diagnostic value as well their efficacy in defining cyst status.

Diagnostic tests are judged on their ability to recognise true disease. False positives are a problem with CE and studies have shown variations in diagnostic value with different populations. For example, total IgG-ELISA tests with HCF showed a low false positive results with specificity of between 87.5% in healthy Indian donors to 100% in Italian donors (Chirag et al., 2015; Tamarozzi et al., 2013). However, in Turkana this was shown to be up to 50 % (Craig, 1986). Cross-reactivity with other parasitic diseases such as alveolar echinococcosis (AE), cysticercosis, schistosomiasis and fascioliasis is quite high in patients (Moro et al., 2009; Zhang et al., 2011). Hydatid cyst fluid has also been shown not to be a good antigen for patients follow-up during the clinical management of CE (Moro et al., 2009), and anti-HCF IgG antibody reactivity may remain high many years after successful cyst removal (Galitza et al., 2006).

The antigens chosen for this work included *Eg*FABP1, *Eg*EF-1β/δ, *Eg*HSP70, *EgAgB* and *Eg*TPx that had been purified as described in chapter 3. We also included native HCF comparison purposes. Carmena et al. (2006) reviewed the diagnostic performance of different recombinant antigens and previous studies suggested they might be differentially expressed in different cyst types. They may therefore have potential use in diagnosis of fertile, deteriorating, unstable and viable parasite respectively.

Diagnosis was based on the detection of total serum IgG as well as specific IgG subclasses IgG1 and IgG4 antibodies. Detection of antibodies IgG isotopes rather than total IgG has also shown promise in follow-up in relation to cyst activity and relapses. Both IgG2 and IgG4 may be related disease evolution, and relapses (Benabid et al., 2013; Celik et al., 2009). Other studies have shown that IgG4 responses are mainly against CE1, CE2, and CE3 cyst types while IgG1, IgG2, and IgG3 were the predominant responses against CE4 and CE5 (Carmena et al., 2006; McManus et al., 2012). However, Sub class IgG are more under-detected in CE patients than total serum IgG (Cappello et al., 2013; Tawfeek et al., 2011). As a diagnostic tool, compared to total serum IgG, detecting IgG subclasses improved the diagnostic performance of our tests with nearly all the antigens. Significant differences in expression of IgG1 and IgG4 was only found in response to some antigens (**Figure 5-22** to **Figure 5-24**). In cases of CE1 cysts, IgG4 was the highly expressed in compared to IgG1 in response to HSP70 and EF1. All other antigens showed no significant differences. This was different from what was found in cases with CE4 cysts where IgG1 was the prominent antibody in response to HSP70, EF1 and HCF. This was also found by Tenguria et al. (2014) who stated that IgG1 is expressed highly in inactive cysts while IgG4 is more expressed in active cysts. However, this trend was not found with AgB which showed higher IgG4 antibodies in CE4 cysts. The diagnostic performance for HCF in detection of total IgG in ELISA is between 64.8% and 100% (Ahn et al., 2015; Akalin et al., 2014; Akisu et al., 2006). False negative results may be due to variability in antigenicity of HCF depending on source (Rahimi et al., 2011) or the infecting *E. granulosus* genotypes, e.g. G1 and G2 genotypes from Europe express higher quantities of antigen B2 in HCF compared to those from China (Jiang et al., 2012). In this study, HCF with total IgG had a sensitivity of 83% overall. Sensitivity to active CE1 and CE2 cysts was 88% and 89% respectively but this reduced to 81% and 74 % in CE3 and CE4 cysts.

The, diagnostic value of HCF was improved with IgG subclasses to 91% and 89% with IgG1 and IgG4.

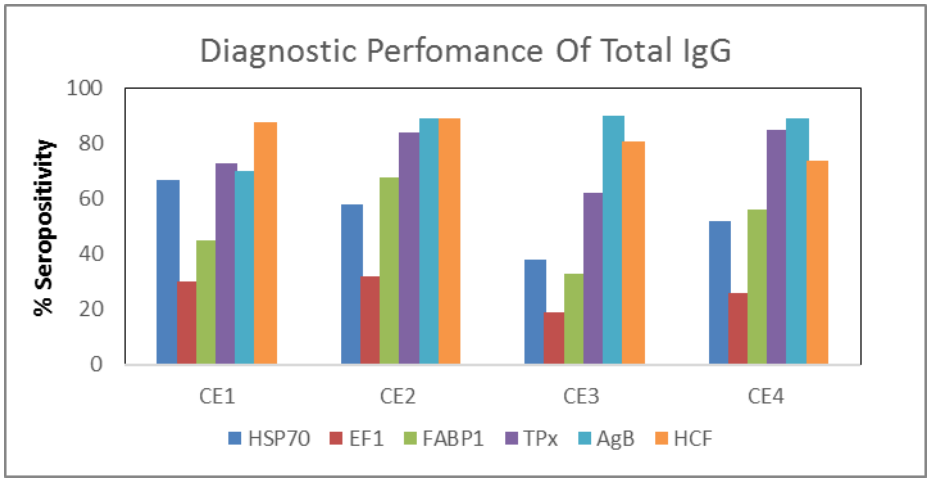


Figure 5-22: Diagnostic performance of total IgG

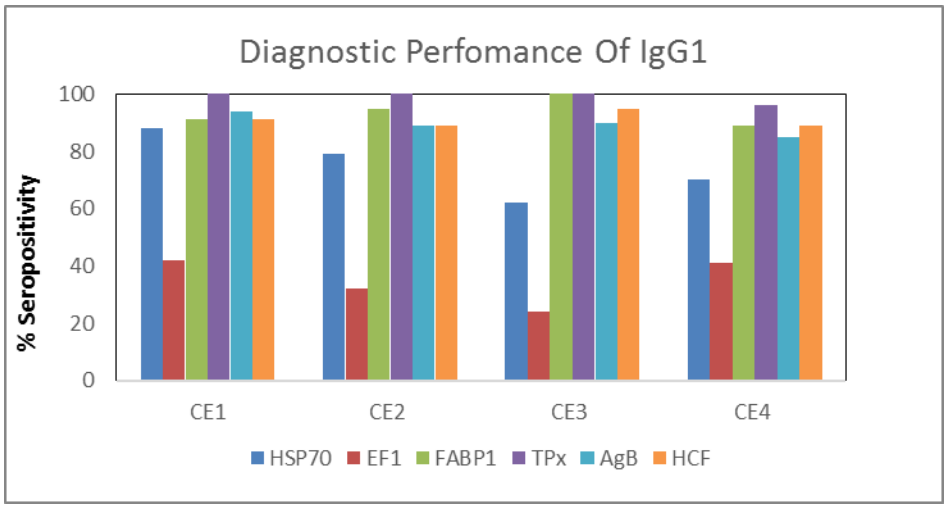


Figure 5-23: Diagnostic performance of IgG1

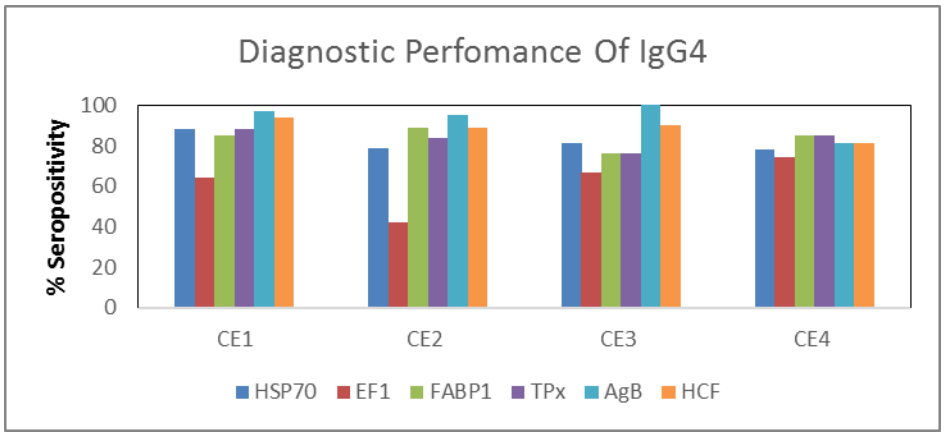


Figure 5-24: Diagnostic performance IgG4.

Antigen B (AgB) considered one of the most valuable *E. granulosus* antigens in diagnosis. The high immunogenicity of AgB presents an important diagnostic value for CE with sensitivity ranging from 60% to 85% in ELISA (Carmena et al., 2006; Virginio et al., 2003).in similarity to our study, Chen et al. (2014) found antigen B to be predominant with CE2 and CE3 cysts and also that expression of total serum IgG in response to AgB was lowest in CE1 cases with 70% seropositive rate. The diagnostic potential of AgB was improved with subclass IgG antibodies and it was the best diagnostic antigen overall for all cyst types. Responses of AgB IgG subclasses showed a positive diagnostic rate of over 85% in diagnosing all cyst types.

Immune responses towards *E. granulosus* EF-1β/δ antigen were most inferior in the panel with an overall sensitivity of 27%. Detection of EF-1 β/δ by total IgG was 27 % in our study while Ortona et al. (2001) found it to be 41%. On the other hand, the sensitivity to EF-1 β/δ when recognised by IgG4 in our study was 63 % compared to their 18.3 %. There were more seropositive cases in patients harbouring transitional CE3 and inactive CE4 cysts than active CE1 cyst. This was similar to (Ortona et al., 2003) who found that elevated levels were recorded in patients with inactive cysts while lower titres were found in patients with active cysts. A study by (Rigano et al., 1995) and another by Ortona et al. (2001) found that IgE was also significantly correlated to EF-1 β/δ. This and other literature point towards EF-1 β/δ release as being an indicator of cysts deterioration. We did not test for IgE responses but in our study, there were significant differences in recognition of EgEF-1 β/δ by different antibodies where IgG1 vs. IgG4 showed a $p < 0.000$, total IgG vs. IgG1 ($p = 0.12$) and total IgG vs. IgG4 ($p=0.12$). The best immune responses in recognition of EgEF-1 β/δ were IgG4. High expression of IgG4 is associated with active cysts and chronic disease (Boctor et al 1990). The antigen EgEF-1 β/δ may skew the Th1/Th2 cytokine balance towards a Th2 polarization CE patients (Ortona et al., 2001). This polarization has been associated with susceptibility to

CE (Rigano et al., 1995) suggesting that EgEF-1 β/δ has a role in inducing allergic reactions and susceptibility to human CE.

Heat shock protein has been identified as a dominant parasite immunogen and antibodies that recognize parasite HSP70 have been found in sera from patients with schistosomiasis, leishmaniasis and trypanosomiasis (Chen et al., 2017). Recognition of EgHSP70 by IgG and IgG subclasses has been associated with patients harbouring stable or younger or where the cyst had undergone trauma (Zhuo et al., 2017). In our study, the diagnostic value of total serum IgG in recognition to HSP70 was poor in cases with CE3 (38% seropositive rate) and CE4 cysts at 52% seropositivity and was marginally improved in CE2 cysts at 58% seropositivity and CE1 cysts at 67% seropositivity. A similar antibody reactivity to HSP70 was detected in the sera using immunoblotting technique where of 60% were detected for total IgG, 83% were positive with IgG1 and 31% were serodiagnosed using IgG4 (Ortona et al., 2003). In our study, both IgG1 and IgG4 showed an improvement in diagnosis compared to total serum IgG with 76% with IgG1 and 82% with IgG4.

A protein Thioredoxin Peroxidase TPx was previously described after isolation from *E. granulosus* cDNA library (Li et al., 2004). It was found to be an important factor for protoscoleces survival and defence against oxidative damage in the host during development (Wang et al., 2018). In our study, responses of total IgG to TPx had a sensitivity of 76 % overall. Detection of IgG1 in response to TPx improved the diagnosis to 99 - 100 % seropositive results for CE1, CE2 and CE3 cysts and 96% for CE4 cysts. Overall, this was the best diagnostically valuable antigen found in this study for both active and inactive cysts. Other ELISA findings in patients with both active and inactive disease suggest that the EgTPx protein is of potential interest in the host–parasite relationship. A study by Wang et al. (2018) showed

that silencing of EgTPx protein impaired the differentiation of protoscoleces into metacestodes both in vivo and in vitro through reducing parasite viability when subjected to oxidative stress. Some studies (Li et al., 2004; Margutti et al., 2008) dismiss it as a serologically unhelpful suggesting its role in immunomodulation of responses to *E. granulosus* or formation of antibody– antigen complexes. In contrast, we found EgTPx very effective in the immunodiagnosis of CE, a result that was also found by (Zhang et al., 2013). Responses of IgG4 subclass against TPx was 82% overall. There was little insignificant variation between cyst types.

Various classes of serum antibodies are found in patients with CE. Studies on different antibody responses show that IgG1 and IgG3 antibodies may correlate with inactive or stable disease and IgG4 and IgE antibodies correlate well with active or progressive disease (Manzano-Román et al., 2015). In our study, IgG subclasses recognized different hydatid cyst fluid antigens. Responses to HSP70 in inactive CE3 and CE4 cysts were greater with IgG4 than IgG1. Also, responses of IgG4 towards AgB in CE1, CE2, CE3 cysts were higher than IgG1 and EF1 was markedly increased with IgG4 in all cyst types. In contrast, responses to TPx and FABP1 were higher with IgG1 compared to IgG4. It is possible that there is a preferential recognition of different antibodies to different antigen molecule. This has also been reported in other studies where IgG1 preferentially recognized antigen 5 and IgG4 recognize antigen B or other immunomodulatory molecules (Rigano et al., 2004; Wen et al., 1994)3 Wen and Craig, 1994).

Differential expression of antibodies according to cyst type was only found in response to some antigens and cyst in our study. There were however differences in antigen recognition in different antibodies and TPx was better recognised by IgG1 while AgB was better recognised by IgG4. Like other studies total IgG showed the least diagnostic performance compared to subclass IgG1 and IgG4 (Lawn et al., 2004) and had false negatives in all cyst types. The best

performing antigen with CE1, CE2 and CE3 cysts was found to be TPx in determining expression of IgG1 antibodies as it resulted to no false negatives. The poorest performing antigen overall was EF-1β/δ though the performance was improved with IgG4 antibodies compared to total serum IgG and IgG1

Despite availability of well-defined purified and recombinant antigens, use of any single one in the field has proved suboptimal. One of the limitations is the complexity of immune responses in different individuals and populations. We demonstrated the differences in immune responses in different cases in the same community. This difference would be much wider in different populations. The solution of serodiagnosis may lie in using a combination of tests which proved more accurate in this study where despite variability in immune responses, there was only one false negative.

Although antibodies do not appear to be effective disease resolution, they may reflect functional purposes. Rigano et al (1995) found that patients who had high serum levels of specific IgG4 responses and low IgG1 responses towards HCF had poor response to chemotherapy while those who had low IgG1 responses compared to IgG4 responses had favourable responses to chemotherapy. In another study, high IgG4 responses were associated with chronic, symptomatic cases while high IgG1 responses were associated with acute and asymptomatic cases (Shambesh et al, 1997).

Serological tools to support imaging technique diagnosis and follow-up of CE patients would be desirable. It would be of even greater use if it contributes to the identification of cystic stages to assist in clinical management. This objective was not well demonstrated by the panel of antigens in our results. The reasons for this may be the small size of our sample, differences in immune responses between our subjects and those assessed in other studies or differences in quality of antigens produced

5.6 Conclusion

In general diagnosis, detection of IgG1 responses to TPx had the best value with seropositivity of between 96 and 100%.

In differential diagnosis, the difference between responses of subclasses IgG1 and IgG4 towards HSP70 was found to be a good indicator for active or inactive cysts.

6 IMMUNOLOGICAL FOLLOW – UP

6.1 Introduction

Antibody levels have been shown to fluctuate during the course of an infection depending on rate of development, antigenicity, transmission dynamics and sensitivity to chemotherapeutic agents (Daeki et al., 2000). Different antigens have been associated with those changes and studies on their use as markers for cyst fertility, viability or instability have been done (Carmena et al., 2004).

Tests based on antibodies against crude antigens have poor specificity and sensitivity in diagnosis and little or no use in follow-up of patients. Recombinant antigens have good potential for both diagnosis and follow-up, but lack of standardization is still a problem. The challenge persists on developing a reliable universal serology standard for the diagnosis and monitoring of CE patients. The answer may also lie in a panel of antigens as opposed to one.

A typical active cyst is filled with clear fluid containing viable protoscoleces. The cyst may remain in this state for many years with or without growth in size. Cysts may also unpredictably change in morphology by regressing and losing integrity, either naturally or after chemotherapy. This loss of integrity allows host cellular or bacterial infiltration and may lead to partial deterioration or death of the cyst. It is also possible that the cyst retains partial viability and may progress at a future date (Rogan et al., 2006). .

Ultrasound images in post-treatment follow-up may not give the whole picture and cysts undergoing micro-alterations in structure may not be detectable (Solomon et al., 2017). A means of predicting an imminent change in cyst morphology at a cellular/immunological level is needed. This would be particularly useful in monitoring treatment outcome during follow-up. Antibody levels fluctuate during the course of infection depending on structural changes to the cyst wall and the level of interaction between the cyst and the host immune system.

These variations may be different in response to drug treatment and may be indicative of outcome. In a study by Stojkovic et al. (2009) CE1 patients that showed a particular response to albendazole treatment had a 50-75% cyst inactive or disappeared within 2 years post treatment. One of the main drawbacks in serodiagnosis is that residual antibodies linger for months to years even after surgical cure giving rise to perpetual false positive results (Feng et al., 2010).

Ultrasound as a diagnostic tool would be complemented by a robust detection system of fluctuations in serum antibodies if they are useful in predicting the possible outcome of disease. Antibody fluctuations are a response to varied release of extraneous antigenic materials from the parasite (Mariconti et al., 2016). These fluctuations may be related to treatment, transition from one cyst morphology to another and changes in body immunity e.g. coinfection. During follow-up, discrete changes in the cyst may go undetected. Also, there may be no visible change in cyst morphology, but immunological indictors may be present. Cysts may transition from the inactive CE4/CE5 stages to active CE1 or CE2 cysts (Stojkovic et al., 2009) and immunological events may accompany these changes making serology especially important in follow-up after initial treatment (McVie et al., 1997).

The potential of finding antigens that are differentially expressed by defined cyst stages would give serology a potential usefulness in the description of cyst activity thus guiding clinical management of CE patients (Fotoohi et al., 2013). Effective serological tests for CE diagnosis would be more profitable if they could define and support cyst status and evolution (active: CE1, CE2, and CE3b, transitional: CE3a, or inactive: CE4 and CE5). The rationale is that in the host, intact healthy viable cysts should only elicit antibody responses to antigens that are native to cyst fluid (Nunnari et al., 2012). Cysts that have become damaged by natural causes or as a result of chemotherapeutical intervention are expected to trigger a more varied antibody response.

Specific antibody responses against many helminth infections have been reported including filariasis, cysticercosis and schistosomiasis with evidence of differential responses towards chronic infection where IgG4 responses are raised and acute infections where IgG1 responses are elevated (Atmadja et al., 1995; Mutapi et al., 2011). In CE, clinically expressed cases indicate a significant raise in IgG1 and IgG4 antibodies (Mourglia-Ettlin et al., 2016) and albendazole treated patients with good therapeutic and clinical outcome showed significantly lower serum specific IgG4 and higher IgG1 compared to no responders with poor outcome while untreated asymptomatic cases also showed lower IgG4 compared to clinically expressed cases (Rigano et al., 2004).

In chapter 4, five recombinant *Echinococcus granulosus* antigens, *Eg*FABP1, *Eg*EF1α/β, *Eg*HSP70, *Eg*TPx and *Eg*AgB and one native antigen HCF, were purified and assessed for performance as a tool of diagnosis for different cyst types. All these antigens showed variation in immune responses in different cyst types as well as IgG subclasses. The study now focusses on the use of these antigens in following up patients to assess their immunological potential as markers for disease progression or regression. Their reactivity towards total serum immunoglobulin IgG and its subclass antibodies IgG1 and IgG4 with sera obtained sequentially from patients harbouring cysts that either progressed or regressed over time was assessed.

The aim of this chapter was to link morphological changes in cysts to antibody profiles during long-term follow-up of individual patients with albendazole therapy.

Antibody levels fluctuate during the course of infection depending on structural changes to the cyst wall and the level of interaction between the cyst and the host immune system. These variations may be different in response to drug treatment and may be indicative of outcome. We hypothesised that this variations in antibody profiles may be useful in prediction structural changes in cysts.

6.2 Materials and methods

6.2.1 Sequential sera analysis

Serum samples collected in the endemic Turkana area by AMREF personnel during the years of surveillance were assessed for suitability of use in sequential sera analysis. A total of 9 cases that had at least five samples in enough quantities were selected for this study. Of these, 2 had cysts whose morphology did not change during follow-up, 4 cases changed from active to inactive while 2 patients harboured cysts that changed from inactive to active cysts with time. The sequential sera samples were used to test reactivity of previously purified echinococcal antigens. ^{R}*Eg*HSP70, ^{R}*Eg*FABP1, ^{R}*Eg*TPx, ^{R}*Eg*EF1-β/δ, ^{R}*Eg*AgB and native HCF antigens towards total serum immunoglobulin IgG and sub-classes IgG1/IgG4 antibodies in patients harbouring cysts which underwent morphological changes and others that did not.

6.2.2 Experimental procedure

All ELISA assays were carried out as described in chapter 3. Antigen concentrations were determined using Bradford assay and optimal working concentrations by checkerboard assays

Patients were classified according to the morphological changes that occurred during the months of follow-up. Nine patients were followed up for between 2 and 45 months. Variations in total IgG and sub classes IgG1 and IgG4 antibody levels in sera were monitored through their recognition of 6 different antigens described above. Patients were classified in to 3 groups depending on changes in cysts morphology during follow-up period. Three groups were described. Group 1 (P1 – P3) had cysts that did not change throughout follow-up, group 2 (P4 – P7) had cysts that transitioned from active to inactive and group 3 (P8 – P9) had cysts that changed from inactive forms back to active by the end of follow-up (**Table 6-1**)

Cyst Morphology	Change	Patient Number	Changes that took place during follow-up			
Unchanging Cysts		P1	CE1			
		P2	CE3			
		P3	CE1			
Changing Cysts	Active to Inactive	P4	CE2	CE4		
		P5	CE2	CE3		
		P6	CE2	CE4		
		P7	CE4	CE2	CE3	
	Inactive to Active	P8	CE3	CE1	CE4	CE1
		P9	CE2	CE3	CE4	CE3

**P1, P2 and P3 did not change, P4, P5, P6 and P7 changed from active to inactive, P8 and P9 changed from active to inactive*

Table 6-1: Characteristics of the cyst changes in followed up cases

6.2.3 Results

6.2.3.1 Changes in serum antibody levels in cases with unchanging cysts

Sequentially collected sera from 3 patients harbouring cyst type that did not change during follow-up were assessed for variations in immune responses. One case received albendazole treatment at the start of follow-up and the other 2 at different times in the course of follow-up. ELISAs determining total IgG and IgG subclasses 1 and 4 expression in response to different echinococcal antigens are described in **Figure 6-1** through- **Figure 6-3**.

The most responsive antibody was total IgG which showed optical density twice as high as that of the subclasses 1 and 4. With patient P1, the trends were similar in all antibody types. Follow – up started at treatment. Immune responses to all antigens showed an increase during the first week of treatment and continued to rise in the second week of treatment. Halfway into treatment at 2 weeks, all responses started to decrease at different rates apart from those towards FABP1 and TPx which only start declining at the end of treatment. Responses towards HSP70 show an increase at the end of treatment while those towards HCF remain largely the same a month post treatment.

Patient P2, harbouring a CE3 cyst that did not change, was followed up for 87 months. As in patient P1, all antibodies showed similar trends in response to different antigens. There were little fluctuations before chemotherapy. Post chemotherapy, the greatest variation in response was seen in to FABP1 where the antibody titres of both IgG1 and IgG4 doubled. There was marginal increase in responses to other antigens

Unlike the untreated CE3 cases where the antibody titres did not change much prior to treatment, the CE1 case P3 showed great variability of responses towards HSP70, HCF, FABP1 and AgB. There were also differences between antibodies. Responses of total IgG towards HSP70 showed an increase before chemotherapy. They had declined one week into

chemotherapy and increased again after the end of treatment to levels lower than prior to treatment. This was not the case with IgG1 and IgG4 where levels showed little fluctuations. Responses of IgG1 towards FABP1 showed a marked increase from start of follow up to 2 months post chemotherapy and thereafter declined sharply. This was not seen with the other antibodies. Responses towards AgB and HCF showed similar trends and with IgG1, the trends were opposite that of FABP1 where the antigen titres of AgB and HCF increase while that of FABP1 declined

Overall, AgB, EF1-β/δ and TPx antigens showed low responses towards total IgG antibodies in all 3 patients while HSP70 showed high responses in one CE1 patient and average responses with other CE1 and CE3 case. Post chemotherapy, levels of FABP1 were elevated significantly in 2 patients

ELISAs determining total IgG showed a significant variation in response to FABP1 ($p = 0.028$). Responses of IgG1 and IgG4 were significant towards AgB ($p = 0.044$). With total IgG, recognition of TPx and EF1-β/δ remained seronegative but were marginally positive with IgG1 and IgG4.

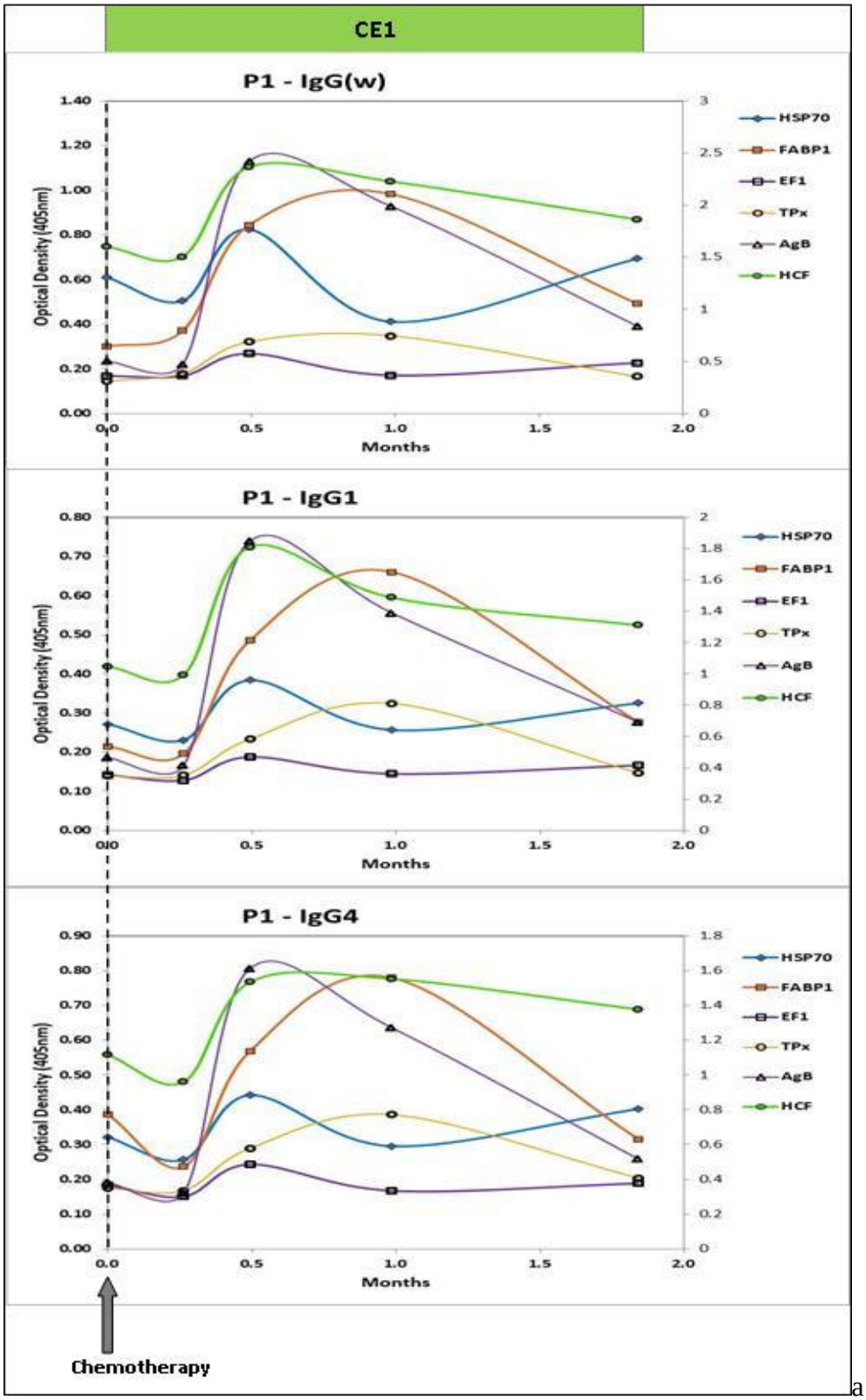


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Figure 6-1: Changes in antibody responses in unchanging CE1 cyst followed for 2 months.

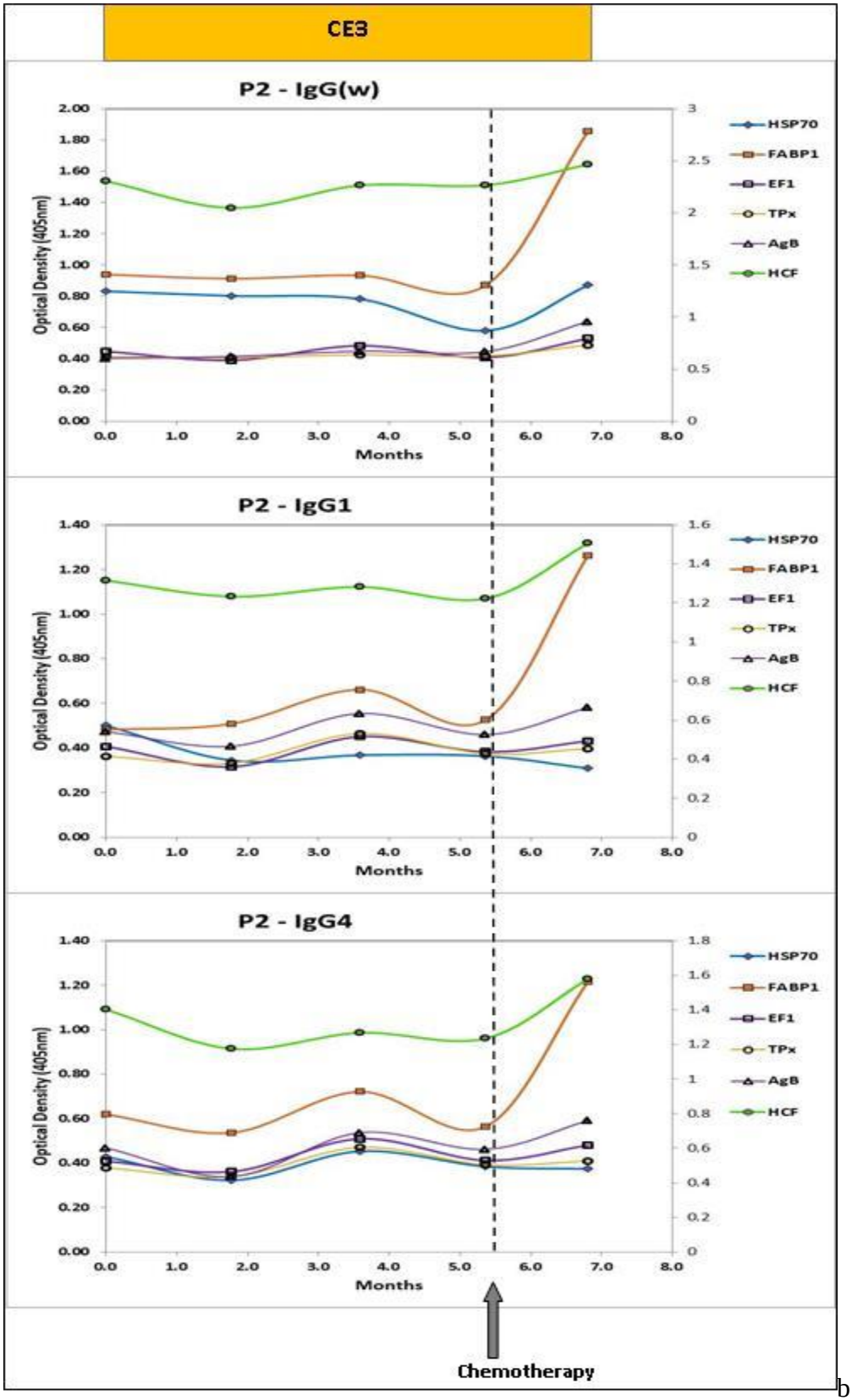

Figure 6-2: Changes in antibody responses in unchanging CE3 cyst followed for 8 months

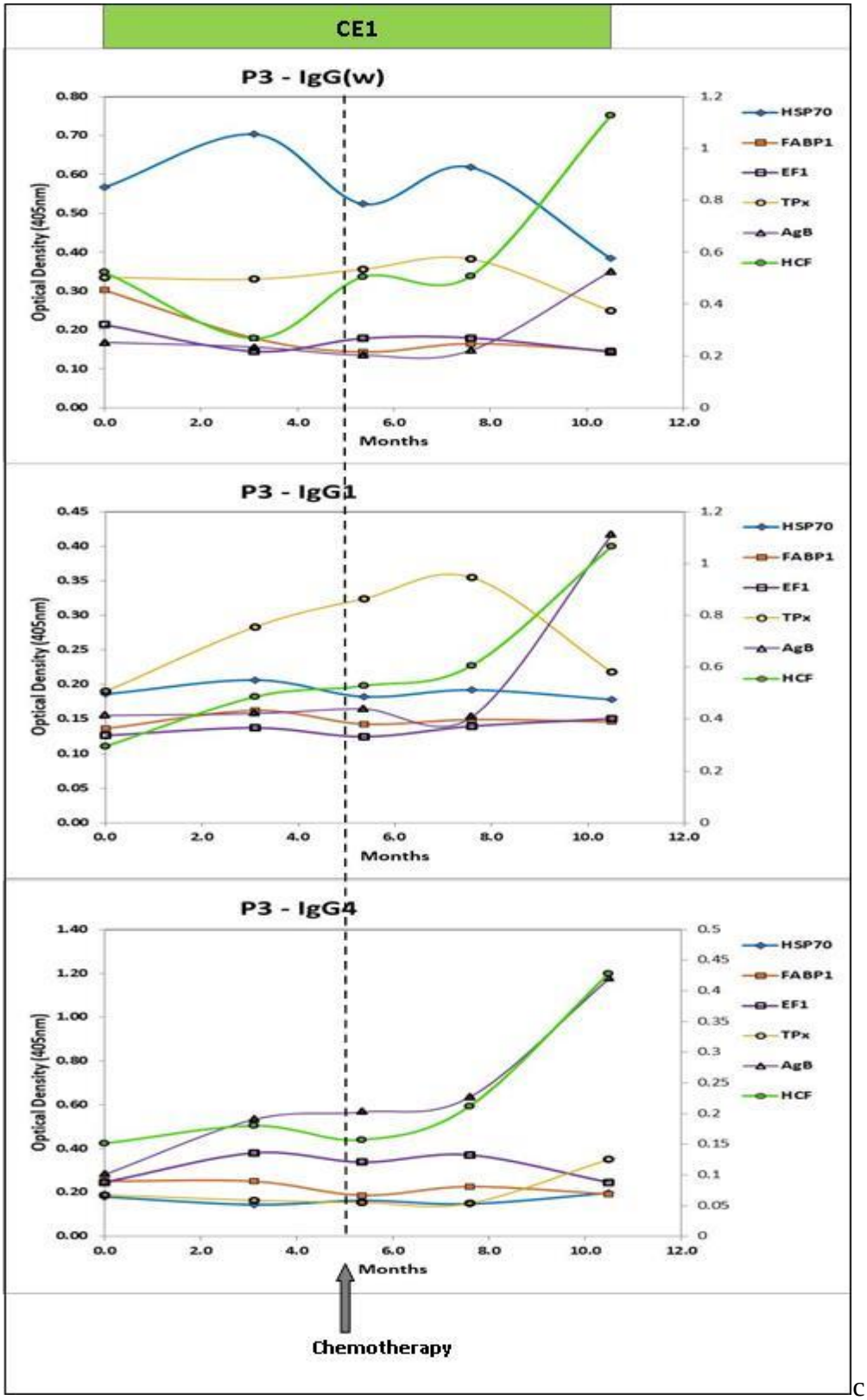

Figure 6-3: Changes in antibody responses in unchanging CE1 cyst followed for 12 months

6.2.3.2 Active to inactive

Four patients (P4 - P7, **Figure 6-4** to **Figure 6-7**). who had cysts that changed from active to inactive during were followed – up for between 7 and 16 months. Changes in expression of total IgG, IgG1 and IgG4 over time towards a panel of antigens was assessed. All the cases received chemotherapy during follow-up.

In contrast to cysts that did not change, there was a marked difference in total IgG levels in response to HSP70 increasing by an average of 50% in two patients and decreasing by a similar margin in the other 2. IgG1 and IgG4 responses all showed an increase though one patient was seronegative with IgG1 and showed reduction in IgG4 responses throughout follow-up.

Responses of all antibodies tested to EF1 showed seronegative at the start but sero converted to positive with IgG4 in 2 cases. Total IgG expression in responses to TPx was also seronegative

Two patients, P4 and P5 were followed up before and after chemotherapy and for the other 2 follow-ups started from the date of first chemotherapy. Before treatment, the trends of the 2 untreated patients differed in that P4 had stable or increasing antibody titres whereas P5 had stable or decreasing titres. In P4 the increasing antibodies were in recognition of HCF and AgB and for P5 the decreasing ones were in recognition to FABP1 and HSP70. In both patients there were slight fluctuations in levels of antibodies against TPx, EF1 and FABP1.

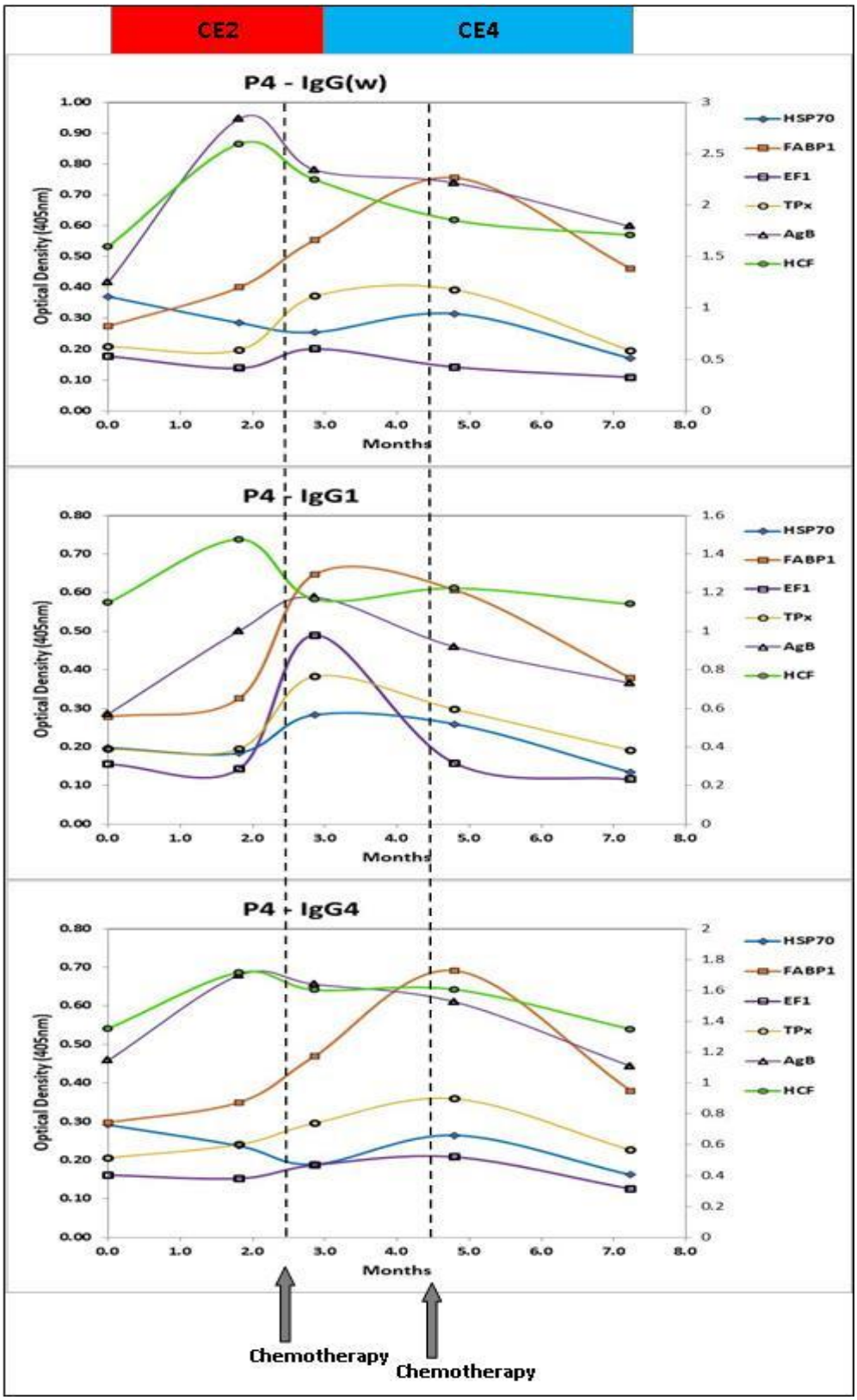

Figure 6-4: Changes in antibody responses in cysts changing CE2 – CE4

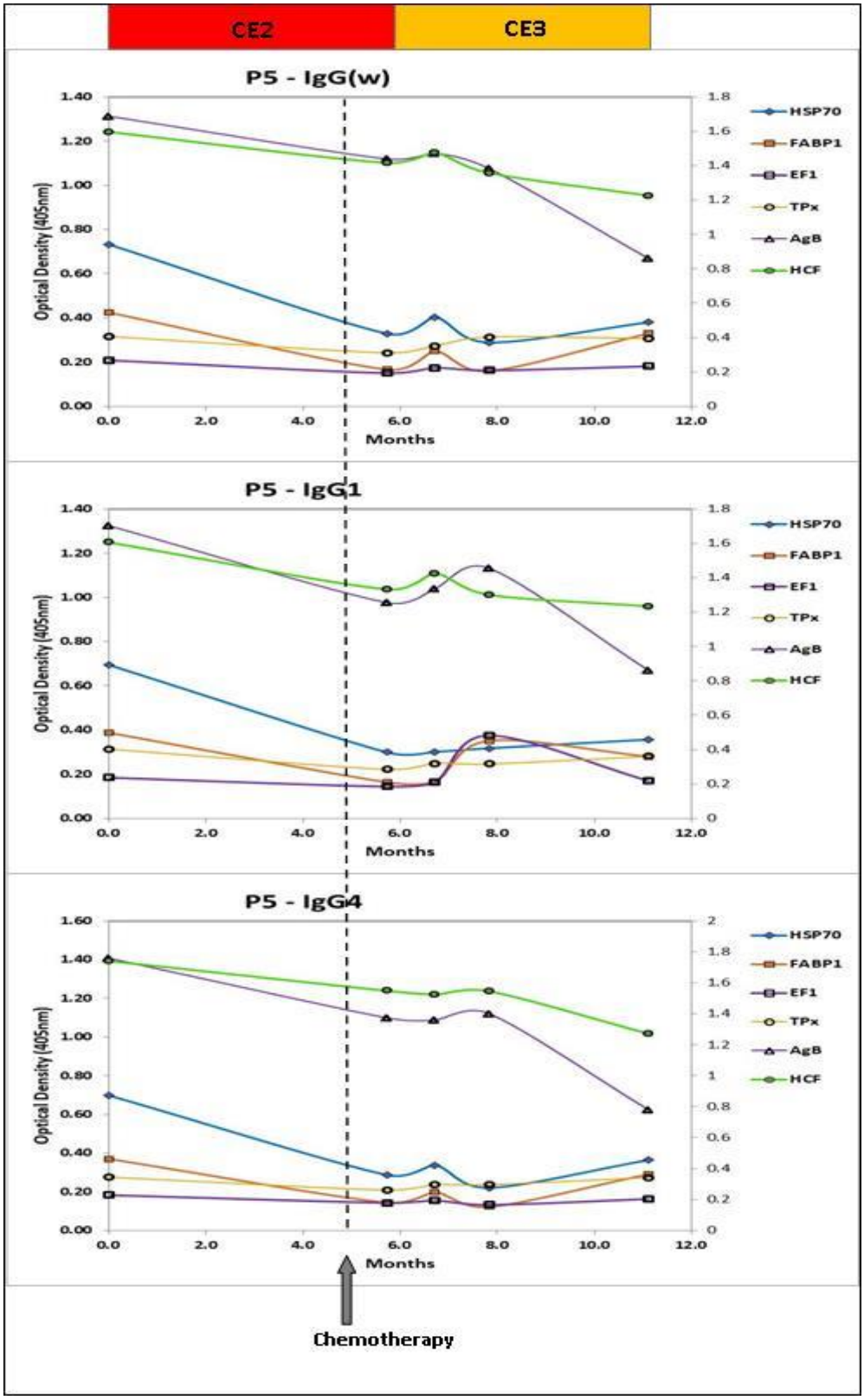

Figure 6-5: Changes in antibody responses in cysts changing from CE2 – CE3.

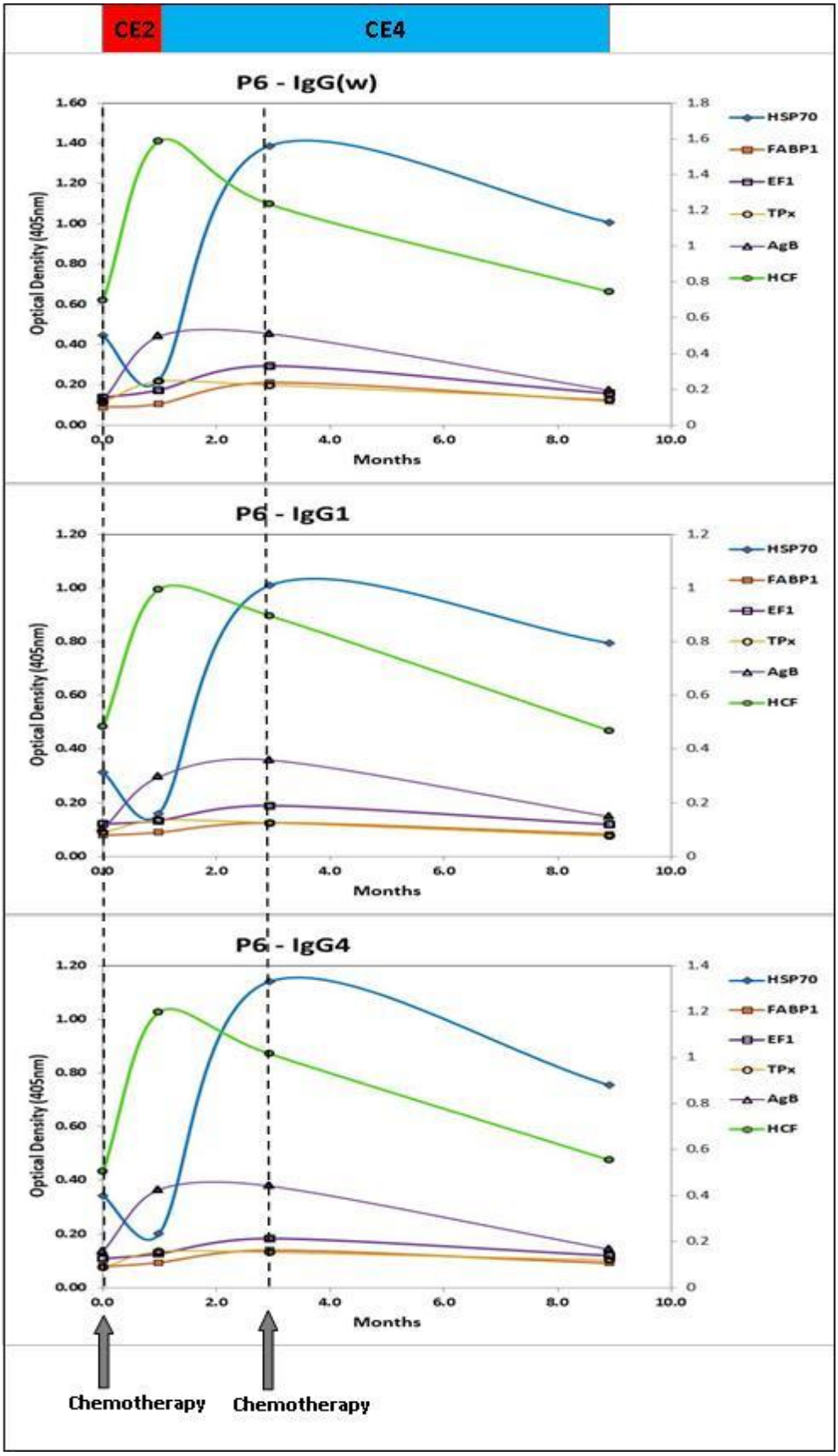

Figure 6-6: Changes in antibody responses in cysts changing from CE2 – CE4.

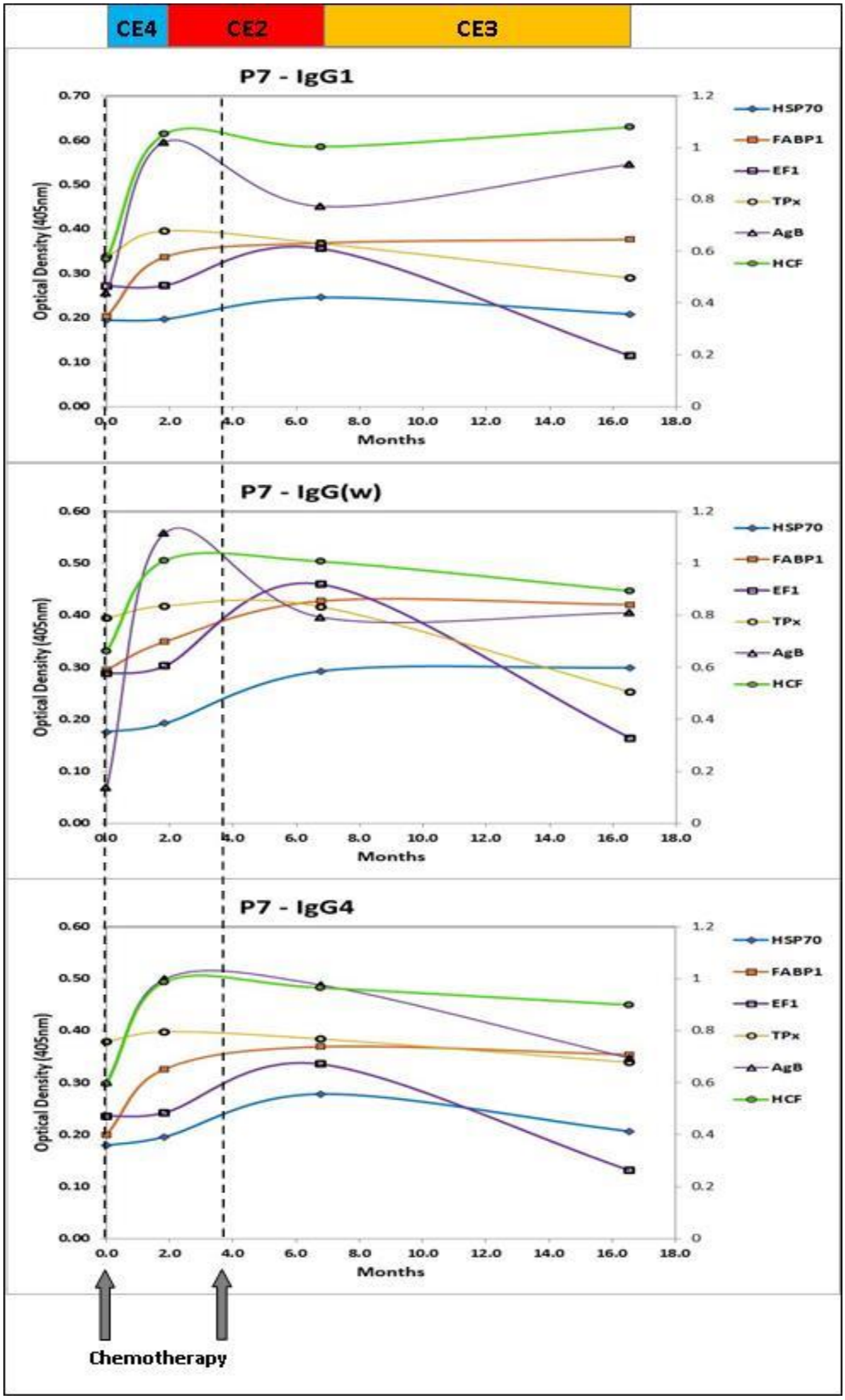

Figure 6-7: Changes in antibody responses in cysts changing from CE4 – CE1 – CE3.

Following chemotherapy, titres for all antibodies increased before returning to levels seen before treatment, an observation similar to responses seen in unchanging cysts with exception to AgB and HSP70. One patient, P5, antibody levels continued to decrease to levels below those found before treatment. For patients who received 2 doses of chemotherapy, (P4, P6 & P7), 2 changed from CE2 to CE4 (P4 and P6). There was an interesting outcome for the P7 where the inactive cyst CE4 became active CE2 after 1 dose of chemotherapy.

The second dose of chemotherapy did not produce an increase in the levels of antibody titres compared to the first dose. The cysts that had transitioned from active to inactive (P4, P6 &P7) after the first dose remained in the same state. However, P7 which had transitioned from inactive CE4 to active CE2 responded to the second dose of chemotherapy by transitioning back to inactive CE3.

Overall, cysts that changed to CE4 cysts showed a decline in antibody titres towards all antigens while those that changed to CE3 cysts showed less decline in titres levels. There was less than 20 % change in antigen titres from start to end of follow-up though there were many fluctuations between the two dates especially post chemotherapy. Compared to cysts that did not change, responses to AgB and HSP70 were higher in inactive cysts compared to active ones.

6.2.3.3 Inactive to active

Two patients (P8 & P9, **Figure 6-8** and **Figure 6-9**) transitioned from inactive to active and were followed up for 6 and 45 months respectively. In the 2 cases, the cysts transitioned between active and inactive but were active at the end of follow-up. The plots below show the variations over the follow up period. Patient P8 received 1 dose of chemotherapy and patient P9 received 2.

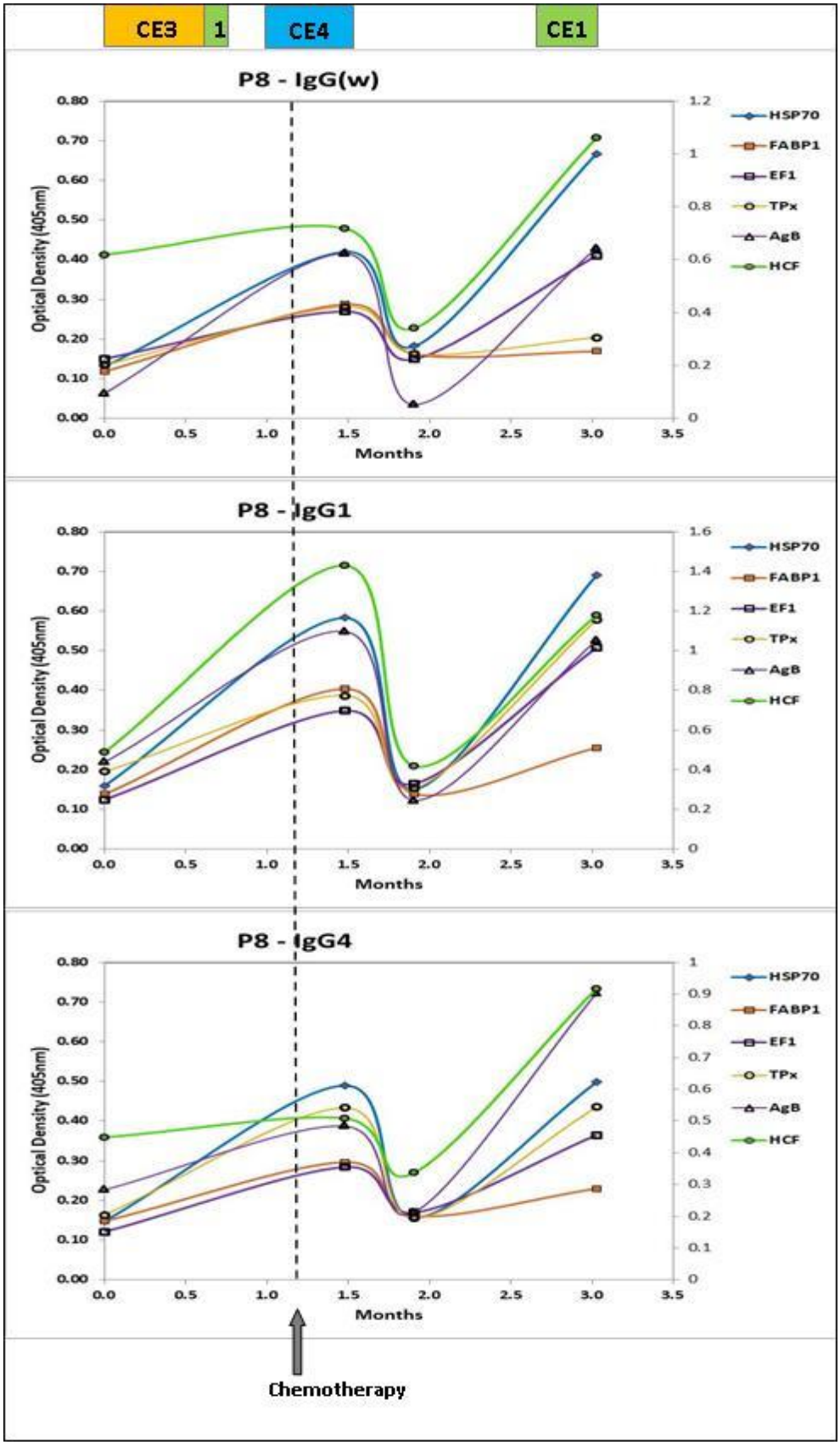

Figure 6-8: Changes in antibody responses in cysts changing from CE4 – CE1 – CE3.

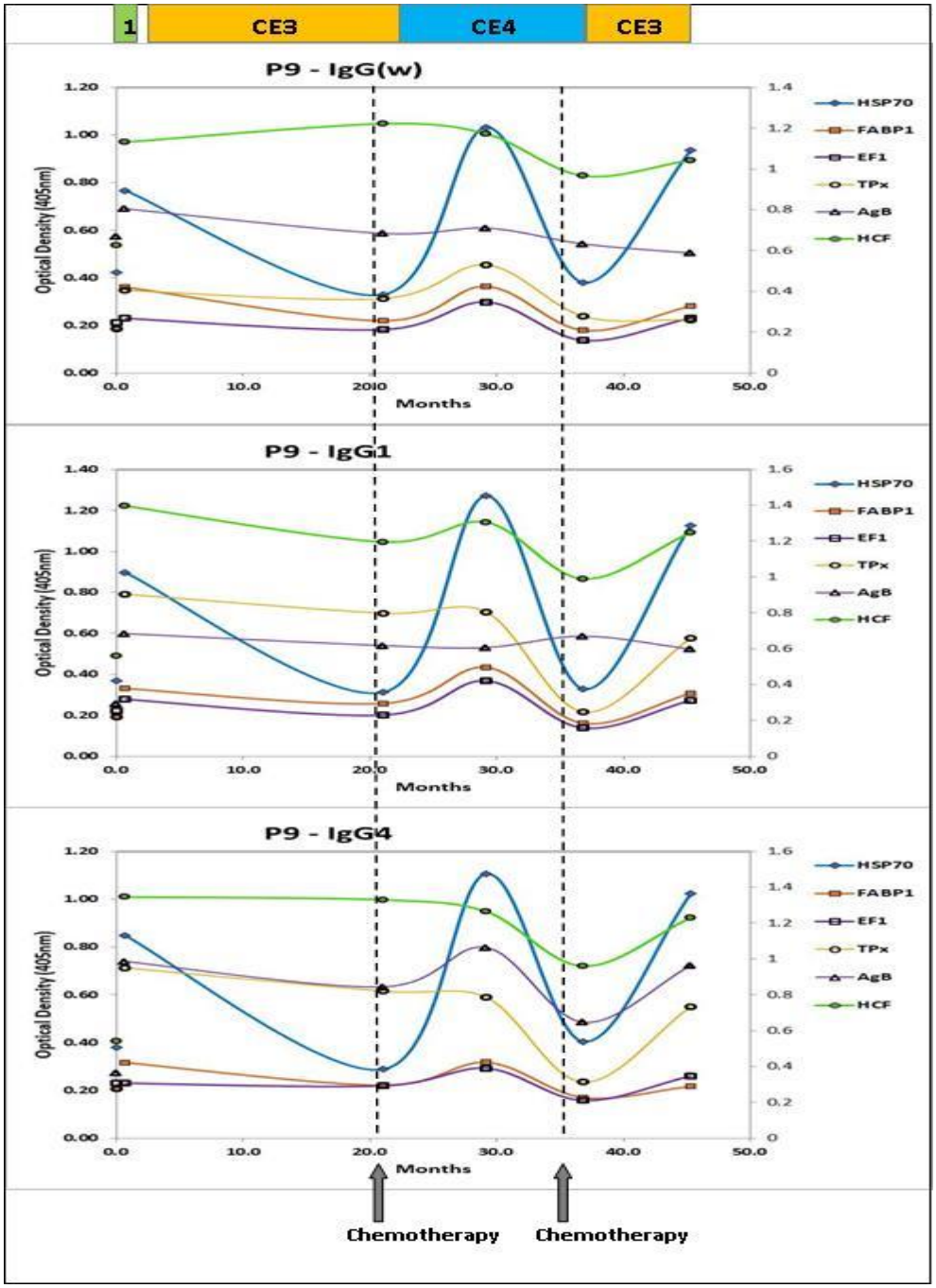

Figure 6-9: Changes in antibody responses in cysts changing from CE1 ~ CE3

Patient P8 had a CE3 cyst which transitioned in to a CE4 after treatment and returned to a CE1 cyst 3 months after treatment. The greatest response was towards HCF followed by AgB. The titre levels before treatment showed a remarkable rise up to 2 weeks during treatment and then fell sharply in the last 2 weeks of treatment. All the antigen titre levels increased again and

showed levels higher than or equal to the pre- treatment levels. They were still rising at the end of the follow-up period and the cyst returned to a CE1 cyst.

In Patient P9 Pre-treatment levels were stable except for HSP70 which gradually decreased until chemotherapy and HCF with IgG1 came down slightly. During chemo, all antigen responses generally went up but of significant increase was HSP70 and continued to increase with no other intervention over the next 10 months. This trend was reversed over the next 10 months down to pre-treatment levels. Over the next 15 months, all antigen titres started to increase again with HSP70 being of greater significance. This increase accompanied the change of the cyst from an inactive CE4 to a transitional CE3.

6.2.3.4 Summary of immunological changes during follow-up

As an overall assessment, and the first and last titres were used to calculate the differences in antibody expression towards different antigens. All cysts cases were assessed together, and amber colour indicates the cysts that did not change, green the cysts that changed from active to inactive forms and red the ones that regressed from inactive forms to active forms. Different facets in each box indicates the different antibody expression.

In response to HSP70, the greatest differences in responses were seen within 3 patients **(Figure 6-10)**. One case of a deteriorating cyst had titre levels increase 9-fold with total IgG, 6-fold with IgG1 and 3-fold with IgG4. Two other cases with improving cysts also had between 2- and 3-fold increases in expression of both total IgG and the subclasses IgG1 and IgG4. There was a decline or no change in responses towards all the cases that had unchanging cysts and one case of deteriorating cyst

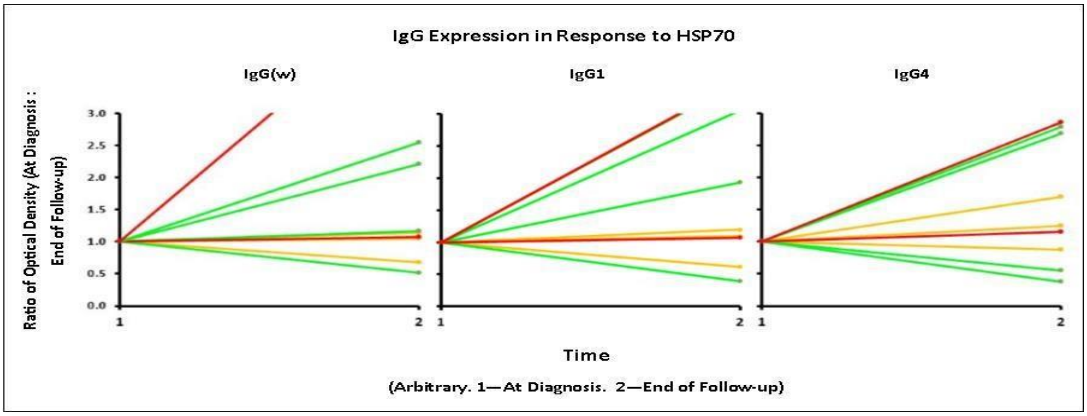

Figure 6-10: Differences in antibody expression towards HSP70 in 9 individual patients.

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

Similar towards responses with FABP1, the greatest variation between initial and final titres was in responses of IgG1 (**Figure 6-11**). Among those with unchanging cysts, there was a big variation in responses but those with deteriorating cysts showed a general increase while those with improving cysts showed either reduction or no change in titre level apart from one case who had increasing levels.

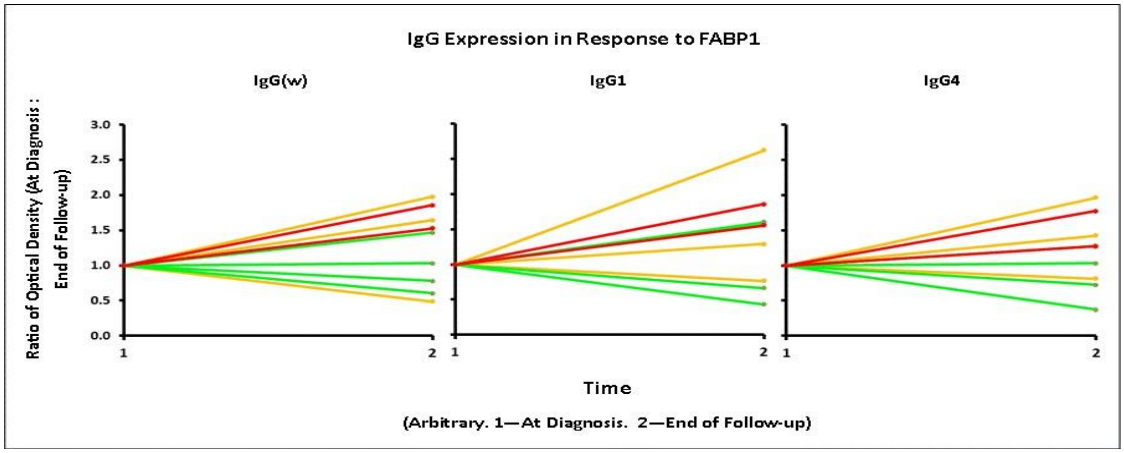

Figure 6-11: Differences in antibody expression towards FABP1

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

Responses towards EF1 were similar in all cases showing differences of ±25% overall apart from those who had deteriorating cysts where one case increase and one decreased (**Figure 6-12**). The increase was more than double with total IgG and more than 3 times with IgG1 and IgG4 while the responses of the other patient declined by half or less.

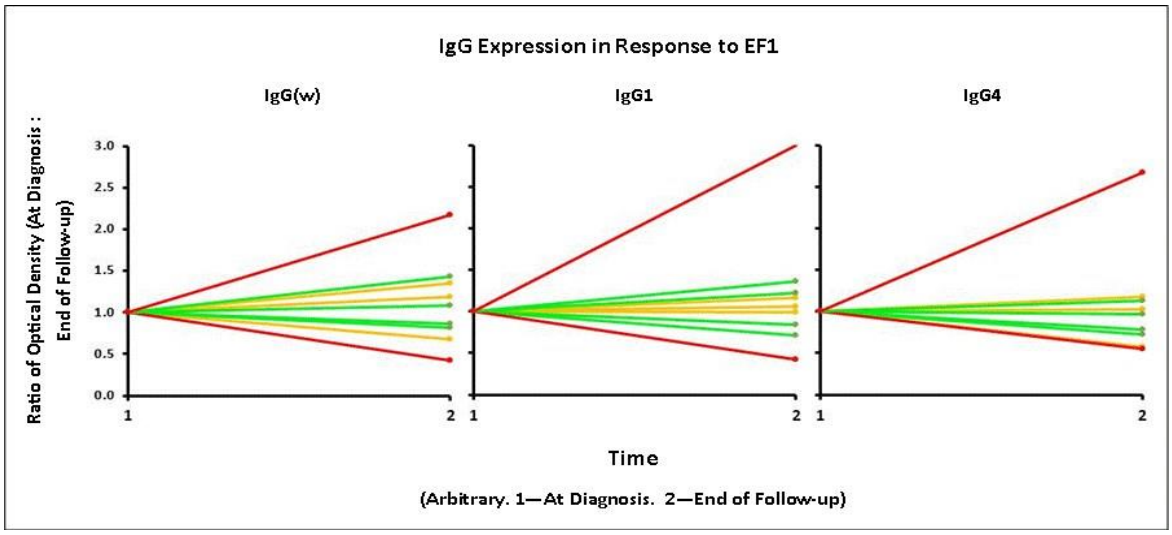

Figure 6-12: Differences in antibody expression towards EF1

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

In response to TPx, differences in expression total IgG overall were minimal apart from one deteriorating cyst that showed an increase of 35% (**Figure 6-13**). There were no noticeable differences according to changes in cyst morphology. Variations seen with IgG1 and IgG4 did not have value in follow-up as there were no obvious trends.

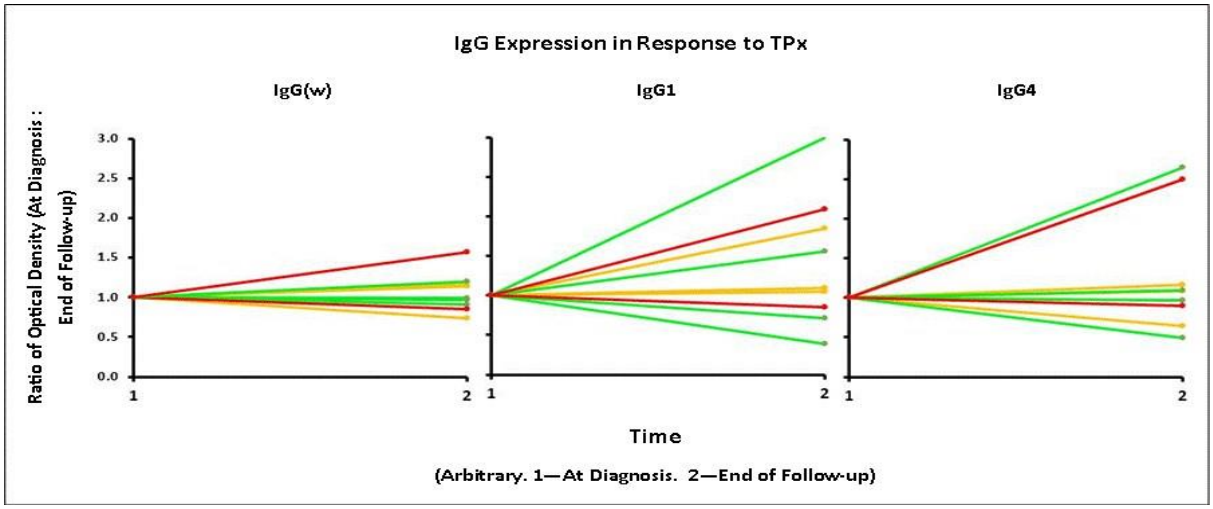

Figure 6-13: Differences in antibody expression towards TPx

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

Antibody responses to AgB showed the best promise in follow-up (**Figure 6-14**). The cases of deteriorating cysts both showed increase in antibody responses especially total IgG and IgG1 and the cases of improving cysts showed reduction or similar levels of IgG except 1. The unchanging cysts were more variable in responses. Overall, total IgG responses showed good qualities for follow-up.

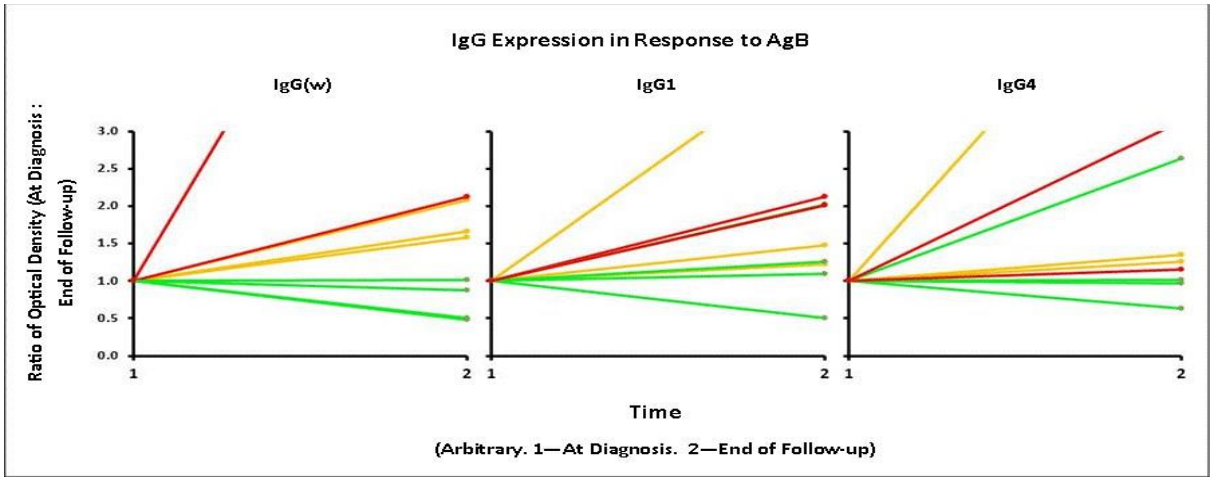

Figure 6-14: Differences in antibody expression towards AgB

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

In response to HCF, the greatest differences in responses were seen with IgG1 where 1 patient had 3 times the original titre value and another 2 patients more than doubled them (**Figure 6-15**). There was reduction in expression of total IgG in all the cases who had cysts that became inactive apart from 1. All cases with deteriorating cysts showed increase in titres but some cases with unchanging and improving cysts had increases in titres too.

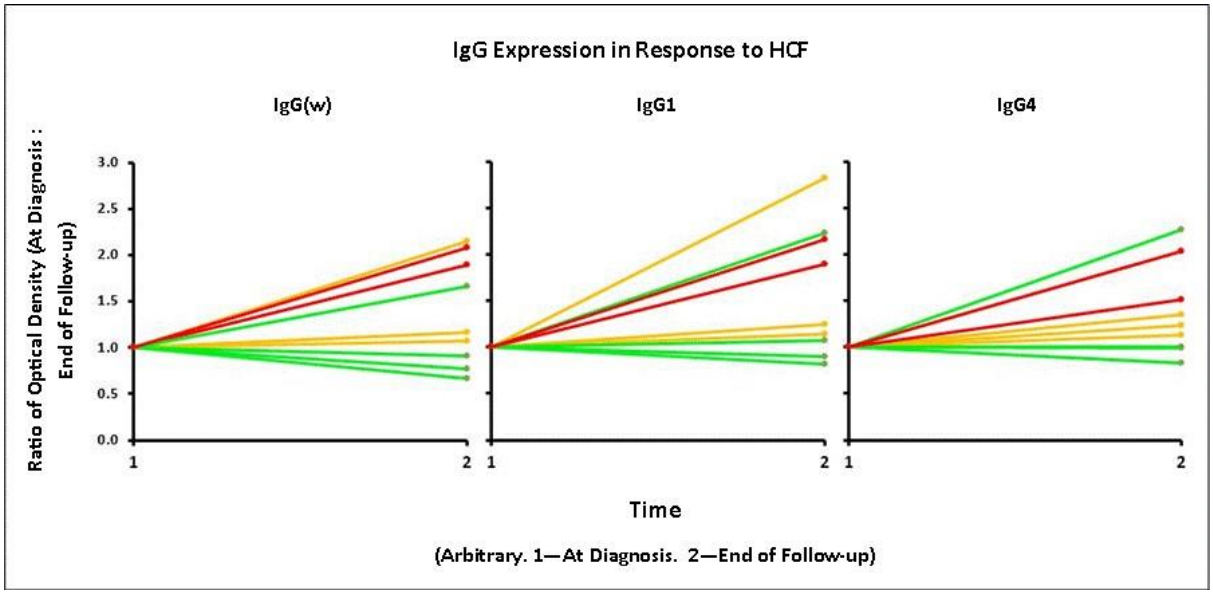

Figure 6-15: Differences in antibody expression towards HCF

Key: Amber colour indicates the cysts that did not change, Green the cysts that changed from active to inactive forms and Red the ones that regressed from inactive forms to active forms.

Antibody titres fluctuated throughout follow-up with levels significantly increasing after treatment and consequently decreasing. Some patients harbouring unchanging cysts as well as cysts considered to be improving showed increases in antibody titres in some patients.

6.3 Discussion

This chapter evaluate whether there was a longitudinal link between antibody profiles of individual patients and morphological changes in cysts. We sought to associate antibody responses of total serum IgG and IgG subclasses 1 and 4 to outcome of disease in follow-up. The usefulness of immune responses in monitoring cyst progression is not unequivocal but

some patients showed promise. Usefulness of native antigens is hampered by the long persistence of antibodies in patients after surgical removal or those with non-active cysts (Li et al., 2004). Some recombinant antigens have shown better correlation with specific antibodies against defined active cyst types after surgical treatment. Examples include EgAgP29 (Nouir et al., 2009) and the heat-shock protein 20 (Vacirca et al., 2011) which showed a low level of specific antibodies against EgB2 and EgHSP20 with EgB1 in the presence of inactive cysts. However, some CE patients did not recognize those antigens (Piccoli et al., 2014). Successful immunological monitoring of long-term CE cases would serve as an alert to a clinician before radiological and clinical manifestation reducing morbidity

Nine patients were followed up in this study. Of these, 3 were classified as having unchanging cysts whose morphology remained the same throughout follow-up even after chemotherapy. Four cases were classed as having active disease at the start of follow-up that transitioned to an inactive disease by the end. A third group of 2 cases had an inactive disease at the start of follow- up which then relapsed back to active disease. All patients received chemotherapy and there were marked immune responses post treatment without exception. Post treatment, there were differences in outcome where some cysts regressed, others progressed while some did not change at all. Reasons for this remain unknown but immunological factors, compliance to chemotherapy and age may play a role. We sought to provide immunological data which can be related to post treatment course of CE and provide a pointer to prognosis. Serological tests should show increase in relapsing cysts, no variation in unchanging cysts and show significant or gradual decline in successful treatment (Horton, 2018).

In unchanging cysts, ELISAs determining the expression of total IgG and IgG subclasses 1 and 4 in response to HSP70 showed an increase. Total IgG responses towards FABPI, HCF, EF1-β/δ, TPx and AgB remained the same or showed marginal decline while IgG1 and IgG4

responses showed variable results. The fact that cysts did not change during follow-up even after chemotherapy evidences the fact that chemotherapy does not always lead to improvement. These results support the findings in chapter 2 where questions on efficacy of chemotherapy were raised (Page 98). Continued rise in responses towards FABP1 2 months after chemotherapy in a CE3 cyst might indicate transition back to an active cyst since other studies have associated FABP1 with active cysts.

Four cases had cysts that transitioned from active to inactive forms. Responses of total IgG towards AgB showed a decline while all the other responses were variable. In 3 cases, responses of total IgG towards HSP70 increased in 2 cases but remained the same or declined in the other 2 cases.

In clinical practice, relapse of CE is difficult to detect early. Two patients relapsed in this study from inactive to active cysts. In one of these patients, there was marked increase in responses to HSP70, EF1-β/δ, TPx, AgB and HCF while the second patients showed increase in responses towards HCF and AgB. A correlation between HCF and AgB with relapsing disease seemed likely. Intermittent dosing was used as a treatment regime. It has been argued to cause tolerance and reduce efficacy in subsequent doses and may be accompanied by parasitic growth and deterioration during treatment interruptions (Horton, 2018). This may explain the scenario in patients P8 and P9 where retrogressive cyst changes were seen after treatment and previously inactive cysts became active again after chemotherapy. A similar trend was found in P7 although the cyst was inactive again after a second dose of chemotherapy.

Due to variations in titre levels during follow-up, the initial and the final levels were used to define a more precise measure of immunological follow-up criteria. Ratios of initial and final titres during the follow-up period were used and the trend assessed. In the cases of unchanging cysts as well as cysts considered to be improving, increases in antibody titres in contrast to

expectation were seen in some patients. These cases may have been recurrences that had not become clinically or morphologically detectable or may have been cases of multiple undiagnosed cysts. It is also possible that though these antigens had been found useful in diagnosis, they were unhelpful in follow-up in a significant number of cases. The follow-up period was short, and the sample was small but there were variations in antibody responses to different antigens. This maybe an indication that there is a differential expression of antigens and a longer follow-up period with more samples may define these expressions better.

Overall, only responses of total IgG towards AgB showed distinct differences according to changing cyst morphology and seemed applicable in follow-up. They increased in all the cysts that became relapsed, declined with all the cysts that became inactive and remained stable in all the unchanging cysts. The trends of other responses of different antibodies to different antigens were not as obvious but may have become more pronounced given longer follow-up and larger sample sizes. Other studies have shown that immune responses may linger even after surgical cure (Manzano-Román et al., 2015) but in other studies, they do decline with inactive disease and sometimes disappear though this have been shown to take years(Naik et al., 2016)

From chapter 4, many patients showed false negative results, and, in this chapter, this was followed further in followed- up patients. Total IgG responses towards EF1-β/δ and TPx remained negative throughout follow-up but were positive with IgG4. In a patient with an unchanging cyst, total IgG responses towards FABP1 declined to seronegative as did those of IgG4 towards HSP70. A third patient with CE3 unchanging cyst was highly responsive with all antibodies showing positive responses.

In the group where cysts changed from active to inactive, all immune responses towards EF1-β/δ were sero-negative

The results in this study showed that antibody levels persist post chemotherapy and this may be because cure was not achieved. In other studies where serology was used in follow-up, surgically cured patients have shown persisting IgG antibodies in 5 years of follow-up sometimes even showing increases (Manzano-Román et al., 2015)

7 ANTIGEN DETECTION

7.1 Introduction

The diagnosis of CE is generally based on radiological and ultrasound examinations along with immunodiagnostic methods (Parija, 1998; Sadjjadi, 2006). Many immunological assays have been developed to detect anti-hydatid cyst antibodies in the serum with variable degrees of success. Drawbacks associated with antibody detection in diagnosis of CE include sub-optimal performances of the available tests, difficulties with the standardization of antigen, inability to readily distinguish between past and present infections, significant numbers of false negatives and cross reactions with other cestodes (Doiz et al., 2001; McManus et al., 2012). Moreover, in villages where Echinococcus is endemic, up to 26% of the general population was seropositive for HCF antigens with only about 6% infected (Craig et al., 1986). Use of circulating antigens rather than antibodies as a diagnostic tool has been demonstrated in helminth infections (McManus et al., 2012), and it circumvents many of the problems above. Evidence points that circulating hydatid antigens are found in the serum only when there is an active infection with levels continually decreasing after a successful surgical removal or chemotherapy (Devi et al., 2003; Sarkari et al., 2015).

As a diagnostic tool, antigen detection requires antibodies that can be either commercially obtained or produced in house. Commercial antibodies can be used against a broad range of antigens including proteins, carbohydrates, short peptides, hormones and nucleic acids (Delves et al., 1997). Antibodies can be monoclonal i.e. those secreted by a single clone of B lymphocytes usually from immunized mice, or polyclonal i.e. those produced by a mixture of B lymphocytes, usually from immunised rabbits (Leenaars et al., 2005). Choice between polyclonal or monoclonal antibodies is dependent on time and money. Polyclonal antiserum

can be obtained within 4-8 weeks while monoclonal antiserum takes 3-6 months to produce (Leenaars et al., 2005). Another advantage of using polyclonal antibodies is the ability to recognise multiple epitopes on the antigen leading to better detection. Multiple antibodies also bind to the antigen at different epitopes, amplifying the signal and many research questions, polyclonal antibodies suffice.

To measure the amount of antigen in the sera, Sandwich ELISA, which measures the quantity of antigen between 2 layers of antibodies was used. The first antibody, capture antibody, is bound to a solid phase on the microtiter plate and then incubated with the solution containing the antigen. A second antibody conjugated to enzyme, conjugate antibody, is then incubated and a substrate is added. The amount of substrate hydrolysed is proportional to the amount of antigen in the test solution(Gottstein, 1984).

Commonly used enzymes for conjugate antibodies are horseradish peroxidise, alkaline phosphatise and β-galactosidase. Horseradish peroxidise (HRP) has the advantage of being cheaper, readily available and has a high turnover rate. Substrates used with HRP include 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) which has the advantage of detecting low levels of enzyme, is non-mutagenic and non-carcinogenic ((Mesulam, 1978)).

The aim of this chapter was to evaluate the relevance of antigen detection in immunodiagnosis of CE.

Use of circulating antigens rather than antibodies may show better result as a diagnostic tool as evidence shows that circulating hydatid antigens are found in the serum only when there is an active infection. The hypothesis was that variations in antigen profiles can predict structural changes in cysts.

7.2 Materials and methods

7.2.1 Antibodies

Several capture and conjugate antibodies previously purified and stored in a -80°C freezer at Salford University, UK were tested for an antigen recognition ELISA. These antibodies had been purified from sera obtained from rabbits immunized in Libya or China between 2 October 2000 and 23 October 2003. The rabbits had been immunized with either *Echinococcus granulosus* crude whole worm extract, excretory-secretory (ES) preparations or with a saline wash of intact *E. granulosus* worms (van Kesteren et al., 2015),).

7.2.2 Testing immunoreactivity of rabbit sera in ELISA

A total of 8 rabbit sera was tested for use a capture and conjugate antibody for this experiment. Different combinations were tested for optimal responses. To determine working concentrations, a checkerboard ELISA was prepared as described below.

A 4HBX plate (Fisher Scientific, Loughborough, UK) was coated with 1:400 hydatid cyst fluid (HCF) diluted in BCB (carbonate bicarbonate) buffer (Sigma-Aldrich, Dorset, UK) leaving 2 wells as blanks. The plate was incubated overnight at 4°C then washed three times with 0.1% PBS

buffer (1 Phosphate Buffered Saline tablet (Fisher Scientific, Loughborough, UK) per 100ml distilled water, 0.1ml Tween. All the wells, except blanks, were then blocked with 0.3% PBS (1 Phosphate Buffered Saline (Fisher Scientific, Loughborough, UK) tablet per 100ml distilled water, 0.3ml Tween with 5% milk powder and incubated for one hour at room temperature. The contents of the plate were discarded, and the plate washed three times with 0.1% PBS. A volume of 200µl of purified IgG from the sera of selected hyperimmune rabbits and normal rabbit rerum (the control) in 0.3% PBS and milk solution (1:25 dilution) were pipetted into the first column of wells, and subsequently serially diluted, to give concentrations ranging from

1:25 to 1:25600. After a one-hour incubation at room temperature, the contents of the plate were discarded, and the plate was again washed in 0.1% PBS. Apart from the blanks, 100µl of alkaline phosphatase labelled conjugate antibody (anti-rabbit IgG alkaline phosphatase, 1:2000 dilution, from Sigma-Aldrich, Dorset, UK) was added to each well, and incubated for one hour at room temperature. After this incubation the plate was again washed in 0.1% PBS and 100µl of PNPP (p- nitrophenylphosphatase, Fisher Scientific, Loughborough, UK) was added to each well (including blanks) and the plate was allowed to develop for 20 minutes. The plate was then read using a Tecan® Sunrise plate reader at 405nm. Sera R5 showed the best results for capture antibody while R91 was best for conjugate antibody.

7.2.3 Sandwich ELISA

Sandwich ELISA was performed to detect the amount of circulating antigen in human sera. Optimal concentration of rabbit anti-HCF IgG (5 µg/ml) was diluted in coating buffer and 100 µl of solution was placed into a 96-well ELISA plate followed by incubation at 4℃ overnight. The excess antibody was removed by washing the plate 5 times in PBS-Tween 20, pH 7.4 containing 0.05% Tween 20). After washing, 100 µl of blocking buffer (3% skimmed milk) was added and the plate was incubated for 1.5 hr at RT. Following the washing steps, sera from surgically confirmed CE patients along with samples from healthy people as negative controls was added into the wells and the plate was incubated for another 1.5 hr. After a washing step, 100 µl of horseradish peroxidase-conjugated anti-HCF IgG was added and the plate was incubated for 1 hr at RT. After being washed as before, the plate was incubated with chromogen/substrate (100 µl/well of 4 mg/ ml OPD, 0.025% H_2O_2 in 0.1 M citrate buffer, pH 5.0), The absorbance at 490 nm was monitored with a microplate reader (Bio-TEK- ELX-800). The cut-off point was set as 2SD above the mean of control samples.

7.3 Results

7.3.1 Usefulness of circulating antigens in diagnosis

A total 124 serum samples from proven hydatid patients from Turkana were tested for circulating antigens (cAg). Of these 34 harboured CE1 cysts, 42 had CE2 cysts, 21 had CE3 cyst and 27 had CE4 cysts. All the cases had been previously confirmed and cyst type determined by ultra sound. The diagnostic performance antigen detection was assessed (**Figure 7-1).**

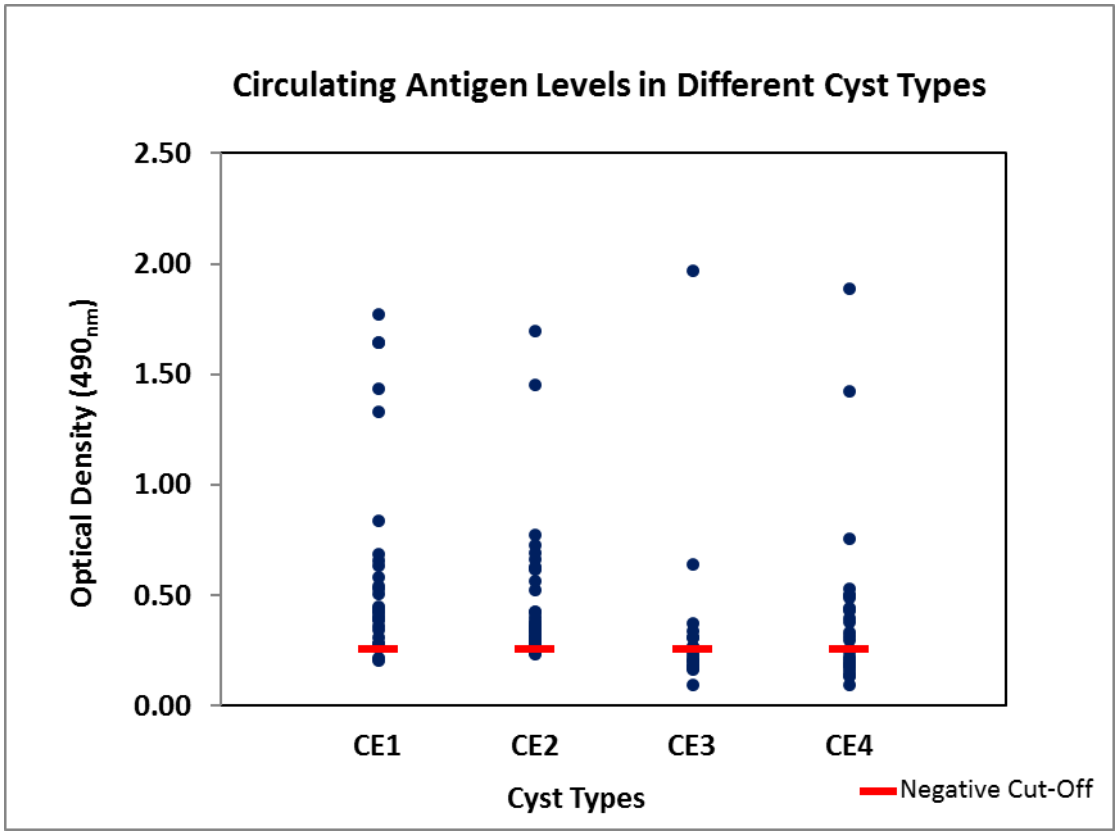

Figure 7-1. Circulating Antigens seropositivity in different cyst types

The results showed a significant variation in the level of cAg according to cyst type. The active cysts showed much higher titres of cAg than the inactive cysts. Overall hydatid cyst antigens were detected in 72.6 % (90) of the cases. In patients with CE1 cysts, antigen levels were

seropositive for 91.1 % (31/34) while in CE2 cases there were detectable cAg in 81% (34/42) of the patients. The levels were lowest in CE3 cases where only 42% (9/21) of the cases were seropositive for circulating antigens but number increased in CE4 cysts and 59 % (16/27) were seropositive

7.3.2 Assessment of circulating antigens for clinical follow-up

Sequential sera taken from untreated CE patients were assayed using ELISA for circulating antigens. The groups were divided in to 3, according to the cyst morphological changes during follow-up.

Eight patients with unchanging cysts were followed up for between 11 and 12 months. There was fluctuation in antigen levels over the months (**Figure 7-3**) but the first and last readings showed little difference (**Figure 7-2**). One case of improving cyst declined slightly.

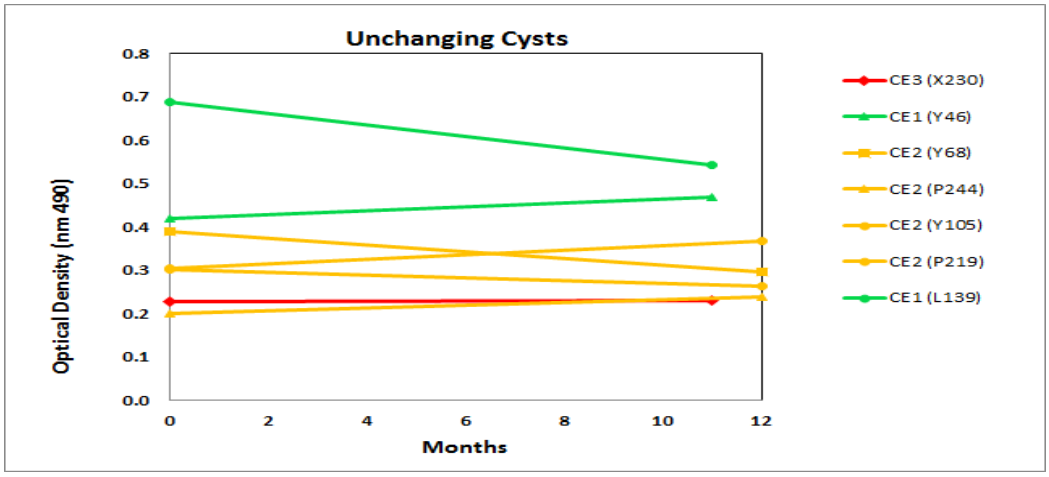

Figure 7-2 : Changes in levels of circulating antigens in unchanging cysts.

Green represents CE1 cysts, amber CE2 cysts and red CE3 cysts.

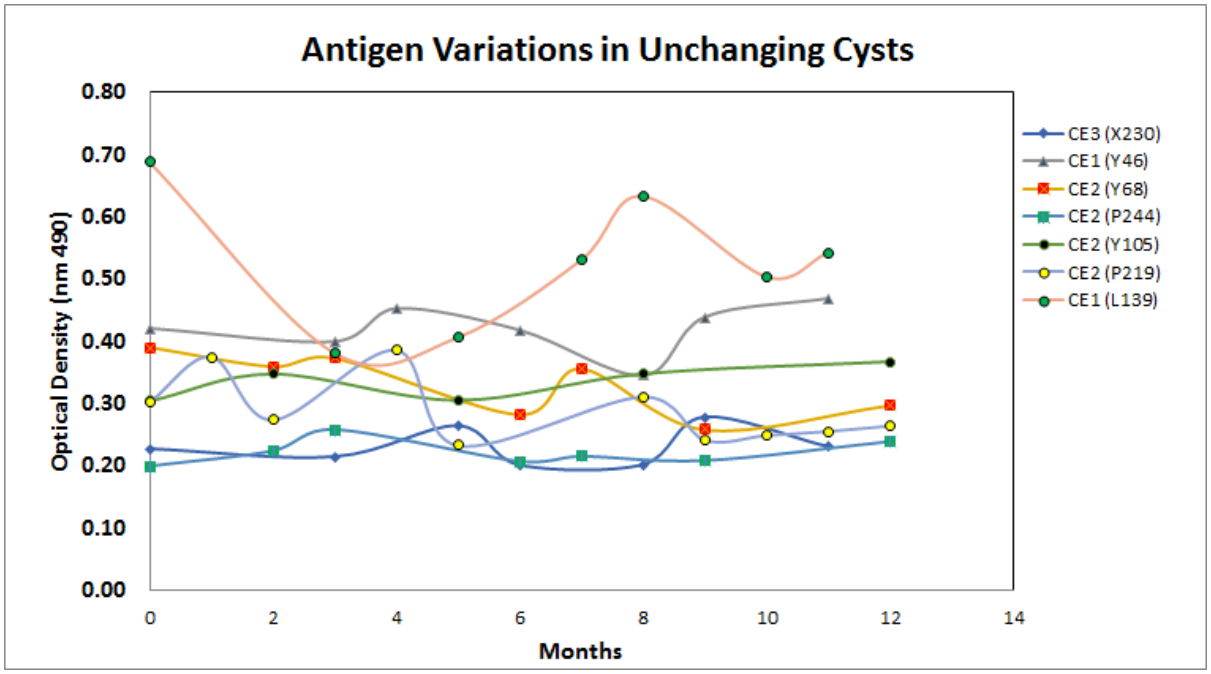

Figure 7-3. Fluctuations in levels of circulating antigens in Unchanging Cysts.

The above shows little variation in antigen concentration (OD range between 0.1 and 0.32) There were no obvious trends according to cyst type. Titres with the CE3 cyst fluctuated the least and had the same levels at the end of follow-up as at the start. The cases with CE2 cysts showed very little difference in titre levels with less than 0.1 nM change between the first and last titre. There were great variations during follow-up in all cases.

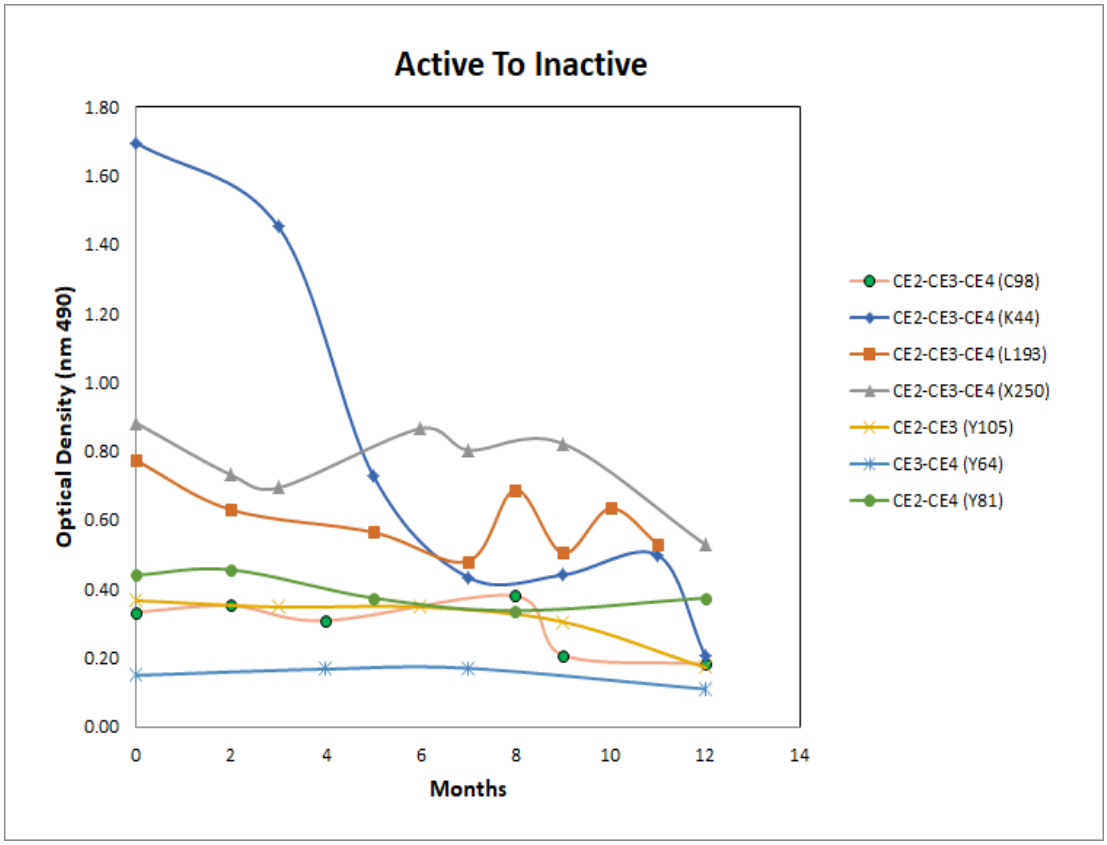

Figure 7-4. Changes in serum antigen concentration over time in improving cyst.

All the cases of improving cysts showed reduction in antigen titres. One case was seronegative all through follow-up while 2 cases became seronegative during follow-up. One case deteriorated rapidly while all the others had gradual decline (**Figure 7-4**).

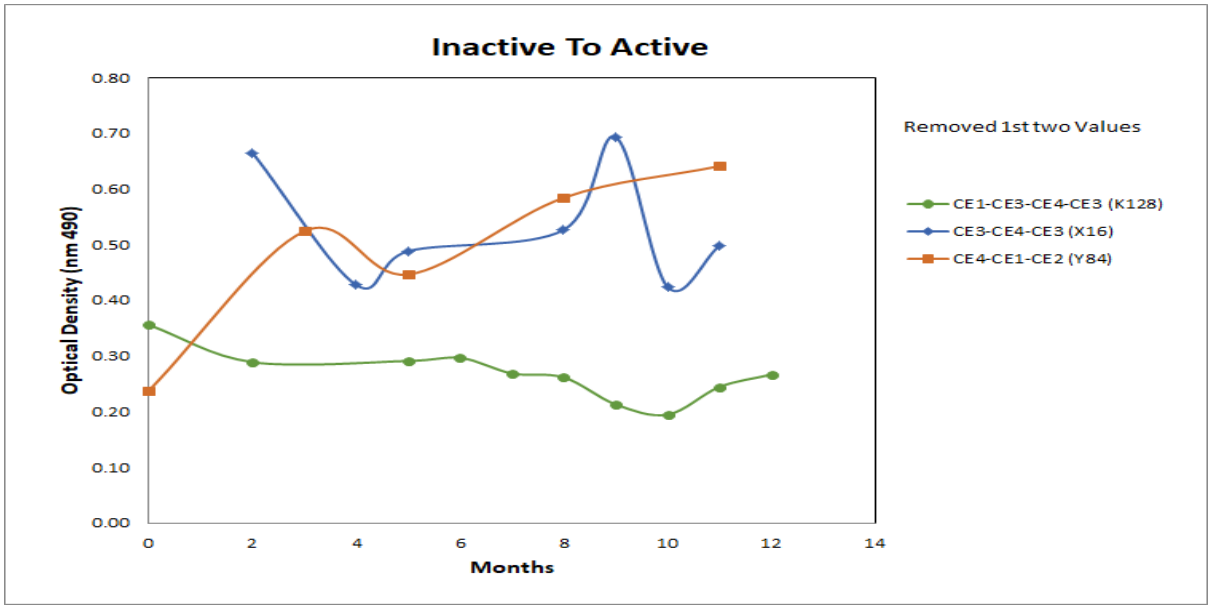

Figure 7-5. Changes in serum antigen concentration over time in deteriorating cyst.

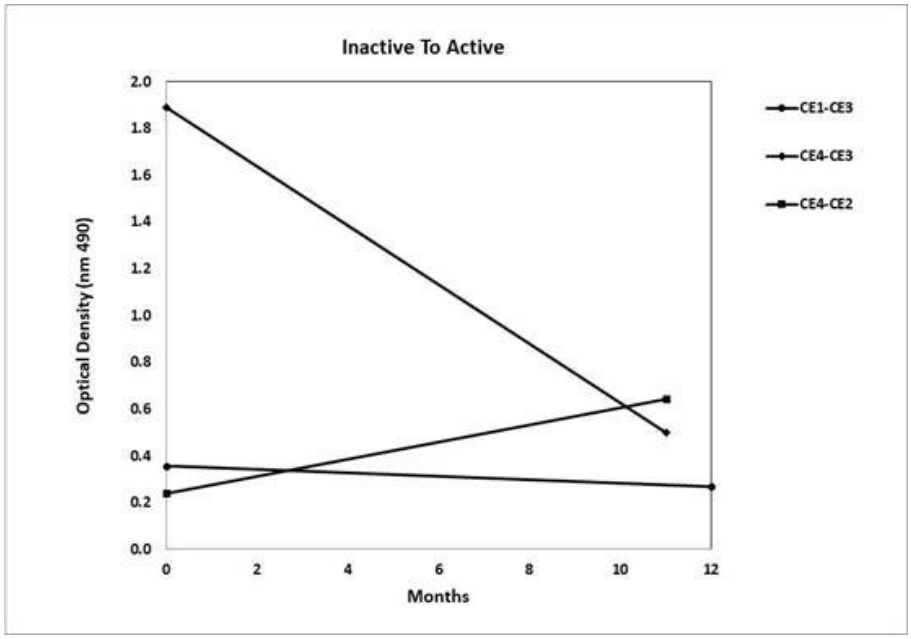

Figure 7-6. Changes in antigen concentrations over time in deteriorating cyst.

Serum antigen concentration in deteriorating cysts was followed up on 3 cases. One case decreased drastically, one case increased slightly, and the other patient did not show much

variation. The trends with deteriorating cysts did not show any value in follow-up (**Figure 7-5** and **Figure 7-6**).

Unlike antibody titres, chemotherapy did result in greatly changes in circulating antigens titres. Though most patients did not have detectable circulating antigen in their sera, those that did showed little variation during follow-up. There was no correlation between circulating antigens and antibody levels in this study though in one patient P4 there were opposite trends between circulating antigen and antibody responses towards **FABP1 (Appendix 2, Figure 10-1 and Figure 10-2).**

Discussion

Sero-diagnosis as a tool in clinical and epidemiological diagnosis and follow-up is especially important in Turkana due to lacks a macro diagnostic hospital and has nomadic population. A combination of ultra-sound imaging and serology gives the best diagnostic efficacy. Use of specific antibody detection results in sub-optimal sensitivity and specificity with unacceptably high rates of false negatives (Sunita et al., 2007). The level of antibody titres varies according to cyst properties such as size, location and type as well as patient properties such as age and immuno-competence. Antibodies against anti- E. granulosus may not be adequately raised for immuno-diagnosis but may persist long after cure as seen in surgically removed cysts leading to wrong diagnosis (Todorov et al., 1979).

Circulating *E. granulosus* antigens are only detectable in the serum during active infection with levels decreasing gradually after successful chemotherapy or surgical removal of the cyst (Devi et al., 2003). The passage of antigens from the cysts into peripheral circulation, formation of immune complexes and the subsequent clearance of both from circulation is ill understood. In this study the levels of circulating antigens in the sera of CE patients harbouring different cyst types was assessed. Positive responses in 72.5% had a better diagnostic accuracy than antibody detection in CE1 and CE2 cysts. Other studies have shown diagnostic accuracy of between 33-85%, with ability to detect anti- E. granulosus antibody (Gottstein, 1984; Ravinder et al., 1997). Low sensitivity was found in CE3 and CE4 cysts of the antigen detection assay. This may be due to inactive cysts releasing fewer antigens in the serum or formation of immune complexes which cannot be easily detected by assays. Some active cysts were also missed out in diagnosis and a reason for these maybe small intact cysts not releasing sufficient antigens detectable in the serum.

The findings of this study showed that antigen detection may be a useful diagnostic method for CE patients. Although the antibody detection had better sensitivity, antigen detection showed

good diagnostic value for active cysts CE1 and CE2. It may also be a useful tool in assessment of the efficacy post treatment.

Levels of circulating antigen were assessed during follow-up for up to 12 months in both treated and untreated cases. Levels of antigen titres were commensurate with variations in cyst type though in some cysts that did not change, antigen titres fluctuated during follow-up. In cases where the cysts progressed for inactive types, a fall in the level of circulating antigens declined slowly in response. The same was found in patients that underwent successful chemotherapy.

Serological assessment of chemotherapeutic responses is difficult due to variabilities in tests, patient and parasite. Indirect tests including cAg and antibody levels have shown suboptimal results in determining viability. The level of immune responses mounted is dependent on the patient's immune competence, cyst location, number, type and size. Additionally, live and dead cysts maybe present in one patient at the same time. Parasite factors may include differences in species, virulence and geographical origin.

Antibody responses may be affected by circulating antigen (CAg) or circulating immune complexes (CIC) taking up the some or all of circulating antibody and making it undetectable.

conventional analysis (Craig et al., 1984), (Craig et al., 1986).

7.4 Conclusions

Antigen ELISA showed value as a diagnostic tool in hydatid cysts has better sensitivity in active cysts. Serology adds accuracy to diagnosis as an alternative technique in the field

Circulating antigens were found to be unhelpful in follow-up

8 GENERAL DISCUSSION

Human cystic echinococcosis is a serious cyclo-zoonotic disease caused by the metacestode of *Echinococcus granulosus*. The parasite affects any body organ and pathology is typified by long term growth of unilocular cysts which may contain thousands of protoscoleces, each with the potential of generating another cyst upon rupture. Echinococcosis causes a substantial disease burden and an excess of 1 million people live with the diseases at any one time and the cost is estimated to be US$ 3 billion annually in for treating cases and losses to the livestock industry (Battelli, 2009).

With very some exceptions, the direction of disease progression was as predicted by the WHO CE ultrasound classification. CE1 cysts were significantly associated with younger people and therefore early infection and the progressive cyst changes were age increased. These observations are consistent with the findings of Brunetti et al. (2010), suggesting that the classification is correct in that early class cysts (CL, CE1, CE2) are fertile and progressive while late class cysts (CE4, CE5) are degenerative though they can revert to an earlier active state. If it is assumed that CE4 and CE5 cysts are older than CE1 cysts, then in communities where CE1 cysts dominate transmission from dogs is likely to be more recent that in communities where CE4 and CE5 cysts predominate (Rogan et al 2006). The progressive decline of CE1 cysts and the increase in average age at first infection was an indication that the control program was effective.

Treatment of human hydatid cases involves chemotherapy with albendazole or surgery, either radical endocystectomy or a more conservative procedure PAIR. In our study, many cases were left untreated and observed. Chemotherapy was offered to the cases that had symptomatic and progressive cysts as a first line of treatment.

The course of CE is indolent, and cysts may have incubation periods of months to years. The growth rate is generally estimated to be between 1 – 5 cm per year (Craig et al., 1986). Our

study had considerable variation in the development of cysts ranging from spontaneous disappearance to increases of 13 cm in one year. Evidence of spontaneous cure was observed in 7.9% of the cases that did not receive treatment. Previous studies in Tibet, Kenya and Argentina reported natural degradation in 18.9 %, 13.6% and 21% (Craig et al., 1986; Larrieu et al., 2004; Li et al., 2010). The number of cases followed up without intervention was small, mostly due to ethical issues as well as requirement for treatment when the cysts showed progressive changes. Besides self-cure, 32% of untreated cysts also showed improvement without intervention. These observations have great implications on assessment of patients and choice of therapy. This is especially pertinent in Turkana where the facilities are rudimentary and incidents of protoscoleces dissemination during surgery resulting to multiple cysts is high ((McPherson, 1986), Cooney et al., 2004)

Albendazole treatment stabilised cysts and arrested further progression in morphology and size rather than cure them. In our study, intermittent dosing was done, and number of courses limited to 3 in most patients though some received up to 8. In studies where continuous dosing was done, better outcomes were achieved (Barnouti, 2015). It is also possible that intermittent dosing in our study caused disease progression during rest period, a concept introduced by Junghanss et al. (2008) as some cases showed deterioration after treatment. Chemotherapy was used more frequently than any other form of intervention mostly due to lower logistical and technological costs as well as the possibility of improvement or cure without more invasive procedures. Three or more doses were found to be more effective on CE1 and CE2 cysts but not so much with CE3 and CE4 cyst. It would be recommended that a symptomatic CE3 or CE4 cyst gets a different kind of intervention if they have not shown enough improvement after the second dose.

Percutaneous treatment under ultrasound guidance provides an alternative to radical surgery or long-term chemotherapy treatment. The results of PAIR during the 12-month follow-up were favourable with 13% cured, 65% inactive and 21.2% showing regenerating cysts with an average of 4 days hospital stay similar to a study by Rajesh et al. (2013). Compared to surgery, there was poorer outcome with PAIR but with a reduced hospital stay and less post-operative complications. Better outcome may have been achieved in combination with chemotherapy.

Radical surgery is recommended for more complex cyst stages containing daughter cysts those features and similar to the other therapies, long term treatment is necessary (Thota et al., 2018). In our study, endocystectomy had the best overall outcome with 89.1% considered cured and 11.9% considered recurrences after 12 months of follow-up. Complications after treatment were more in the surgical group with the most common being bacterial infection in the residual cavity, continued morbidity due post-operative sequalae and 3 mortalities. Use of chemotherapy alongside surgery improved the cure rate from 86.9% to 93.5%.

Evaluation of recurrence is also difficult because surgical spillage may not become apparent until years later. It is also not possible to distinguish between recurrence and new infection and we assumed recurrence when a new cyst appeared in the residual cavity. Some patients already being followed up had cysts elsewhere in the body. In our study, we treated these as reinfections, but they could have been recurrences and our data maybe rather optimistic. Our follow-up period was also limited. The recommendation is to follow-up until the whole residual cavity is reabsorbed and in many of our cases with PAIR that was not achieved, and the cysts were considered inactive.

Diagnosis of CE is mainly confirmed through ultra sound sometimes in combination with anamnesis, clinical history and serological testing. Immunodiagnosis of CE is still problematic and commercially available tests show suboptimal performance. Factors that complicate

serodiagnosis include lack of standardized immunodiagnostic assays, discrepancy in antigen preparation and performance in different laboratories, cyst characteristics like genotype, size, stage and location and patient characteristics like age and immuno-competency. Serological test for CE have been negative in human immunodeficiency virus (HIV)-positive cases (Coupland et al., 2012). Serological assays still have a complementary role and research may improve diagnostic performance soon. False positives are one of the biggest problems in serodiagnosis. Available tests do not differentiate between past (cured or calcified cyst) from present (active or progressive) infection because antibodies have been shown to linger many years after surgical cure (Nouir et al., 2008; Piccoli et al., 2014). Performance of serological tests may also be dependent on the pathological stage of CE (Mariconti et al., 2014) and a single defined molecule may not be sufficient for diagnosis of all stages of CE.

We set out to explore the value of a panel of recombinant antigens for immuno-diagnosis and assess variations in antibody responses to cyst morphological changes. Purified parasite Antigens *Eg*FABP1, *Eg*HSP70, *Eg*TPx, *Eg*EF1-β/δ and *Eg*AgB were assessed diagnostic value using 100 sera samples from Turkana Kenya. Results showed difference in antigen reactivity towards total IgG, IgG1 and IgG4 isotype subclass antibodies. Although antibodies do not appear to be effective disease resolution, they may reflect functional purposes. Rigano et al (1995) found that patients who had high serum levels of specific IgG4 responses and low IgG1 responses towards HCF had poor response to chemotherapy while those who had low IgG1 responses compared to IgG4 responses had favourable responses to chemotherapy. In another study, high IgG4 responses were associated with chronic, symptomatic cases while high IgG1 responses were associated with acute and asymptomatic cases (Shambesh et al, 1997).

Higher IgG4 levels were found in active cysts CE1 and CE2 in response to HSP70 and EF1-β/δ compared to levels of IgG1, while higher IgG1 titres were found in inactive CE4 cysts

compared to IgG4. This was also found by Tenguria et al. (2014) who stated that IgG1 is expressed highly in inactive cysts while IgG4 is more expressed in active cysts. Daeki et al (2000) also found higher IgG4 levels to be associated with active cysts. In contrast, responses of IgG4 towards AgB was significantly higher in CE4 cases. Potential to link of the US images to immunological markers is needed especially during the cyst stages that are "silent" post-treatment when the cyst changes may go undetected (von Sinner et al., 1991). To the clinician, information on developmental pathway of disease with a means of predicting, instability or transition from degeneration to regeneration would be helpful in choice of intervention. The levels of subclass IgG activity with different cyst types showed discriminatory recognition in HSP70, EF1 and AgB between active and inactive cyst types. These immunoserological differences are especially helpful in characterisation of CE during field study

The purified parasite antigens were assessed for their usefulness as immunological markers for disease characterisation in long term follow-up. Antibodies fluctuate throughout the course of infection in response to varied release of extraneous antigenic material. Some cysts may have arrested development and not change for long periods while others may progress unpredictably and show much disparity in structure (Rogan, *et al.,* 2006). In the current study, levels of immunoglobulin in a defined panel of CE patients' sera showed discriminatory antibody responses in recognition to some recombinant antigens according to cyst changes. The study substantiates the premise that chemotherapy always elicits a wide range of responses towards different antigens. The responses peaked one to two weeks from start of treatment and generally declined gradually. This maybe an indication that at 2 weeks, chemotherapy treatment begins affecting cyst integrity causing release of extraneous antigenic material and leading to increased immune responses. From 2 weeks post treatment, it may be possible that the cyst losses viability or immune suppression occurs, and this is indicated by decrease in the immune responses in most cases to pre-treatment levels. The prognosis is considered

favourable if the decline continuous past pre-treatment levels and in cured cases become seronegative. The antibody levels persisted in all the cases followed up. This was post-chemotherapy, and this may be because cure was not achieved. In other studies where serology was used in follow-up, surgically cured patients have shown persisting IgG antibodies in 5 years of follow-up sometimes even showing increases (Manzano-Román et al., 2015)

Use of circulating antigens rather than antibodies as a diagnostic tool reduces false negatives and cross reactions with other cestodes (Doiz et al., 2001; McManus et al., 2012). Circulating *E. granulosus* antigens are only detectable in the serum during active infection with levels decreasing gradually after successful chemotherapy or surgical removal of the cyst (Devi et al., 2003). Antigen detection was found to be very useful in diagnosis of active cysts where 86% of CE1 and CE2 cases were seropositive and only 50.5% of inactive CE3 and CE4 were seropositive.

Taken together, findings of this study indicated that the antigen detection assay might be a useful method for diagnosis of patients with active hydatidosis. Also, the antigen detection assay might be a useful approach for assessment of the efficacy of treatment especially after removal of the cyst.

8.1 Conclusions

Evidence of spontaneous cure was observed in 7.9% of the cases that did not receive treatment. Besides self-cure, 32% of untreated cysts also showed improvement without intervention. Treatment should only proceed on deteriorating cysts after a period of observation.

Second and third dose of chemotherapy is unhelpful in treatment of CE3 and CE4 cysts and a different intervention should be considered if the first dose does not give favourable outcome.

The best tool in general diagnosis of CE is IgG1 responses to TPx with a sensitivity of 98% overall

Serological responses to each antigen may differ in individual patients and that reactivity may be useful in predicting disease progression or regression.

Antigen diagnosis is useful in diagnosis of active cysts but found no use in follow-up

8.2 Future work

This study showed some immunological complexity associated with hydatid cysts in diagnosis and their variations during transformation from one cyst-type to another during follow-up. However, the sample size was small and follow-up period was short. More intensive studies proving repeatability and consistency of our findings are essential. Other Limitations included insufficient patient data and missing US images. Further work would include a more comprehensive set of sequential patient sera and partnerships with other countries where human hydatidosis endemic. Test for cross reactivity of the antigens used with other helminths would also be helpful to determine specificity.

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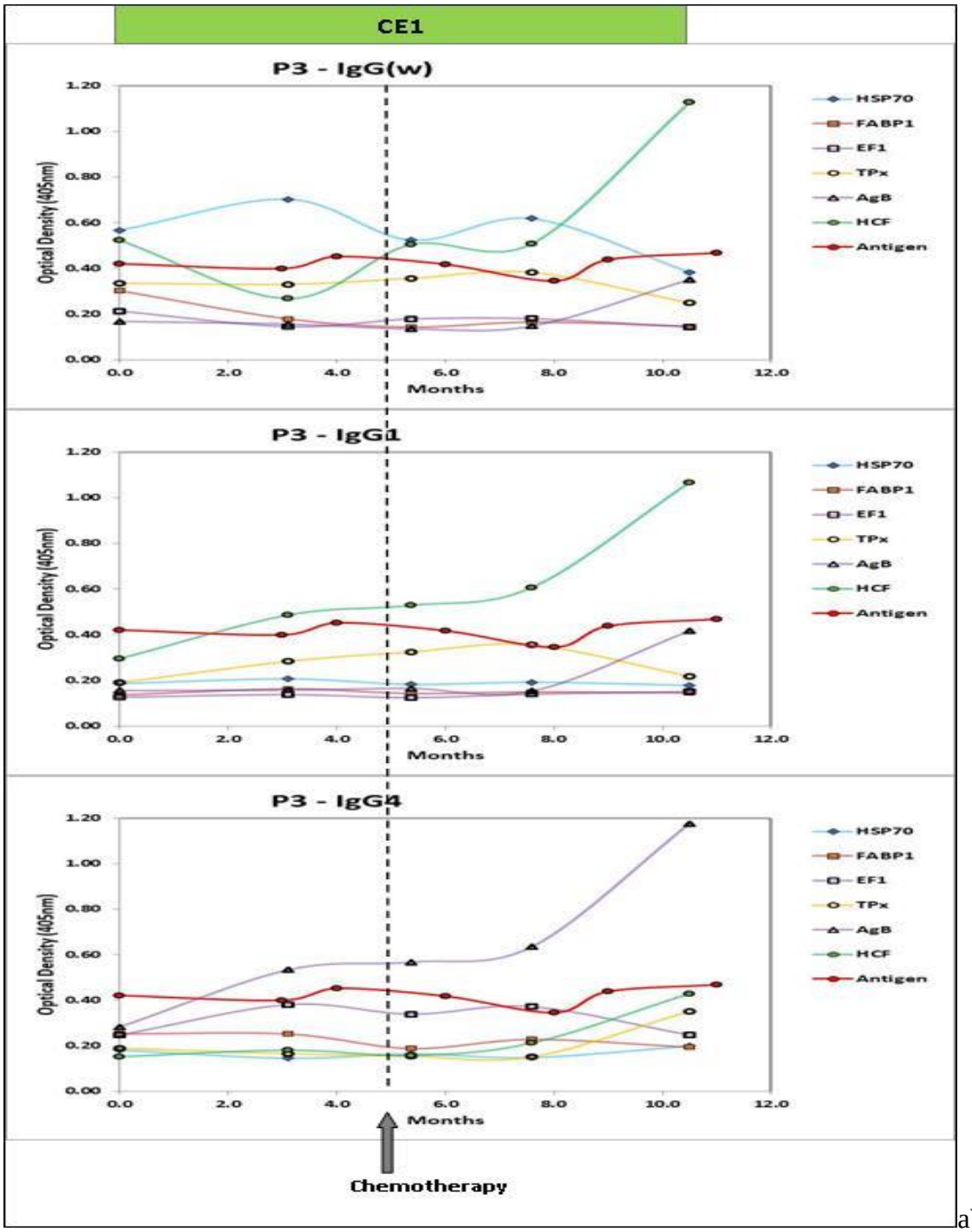
10 APPENDIX

10.1 Patient data collection sheet

Id number	15	30	12	8
location	Lowarengyah	Lokichoggio	Lokichoggio	Lokichoggio
age	15	30	52	21
sex	Male	Female	Female	Female
tribe	Toposa	Turkana	Turkana	Turkana
Previous surgery	Yes	No	No	No
Location of Surgery	Kakuma			
Sample date	02/05/1991	24/01/2003	05/04/1989	10/09/1985
visit	3	2	7	1
Follow-Up period (months)	55	0	65	0
US Result	Hydatid	Hydatid	Hydatid	Hydatid
Cyst Nature	Multiple cysts	Single cyst	Multiple cysts	Single cyst
Multiple cyst number			2 cysts	
Cyst in other location				
WHO classification R liver	CE2	CE3	CE3	
WHO Classification L liver				
WHO Classification R Kidney				
WHO Classification L. Kidney				
WHO Classification spleen				CE1
WHO Classification mesentery	CE1			
WHO Classification L. abdomen			CE4	
WHO Classification eye				
WHO Classification muscle				
WHO Classification Lung				
WHO Classification other		CE1		
Size Cyst in R Liver	large		Large	
Size Cyst in L liver				
Size Cyst in R kidney				
Size Cyst in L Kidney				
Size Cyst in Spleen				medium
Size Cyst in Omentum mesentery	large			
Size Cyst in lower abdomen			medium	
Size Cyst in abdominal wall				
Size Cyst in eye				
Size Cyst in muscle				
Size Cyst in lung			large	
Size Cyst in other location				
Treatment	No	Yes	no	No
Status of cyst			No	
Treatment PAIR				
Result of PAIR				
Treatment Surgery		endocystectomy		
Type of surgery				
Findings at surgery				
Result after surgery				
Albendazole after surgery				
Type of treatment Albendazole		Yes		
Dosage		1 dose		
Number courses				
Result after treatment Cyst in R Liver		No cyst visible		
Result after treatment Cyst in L liver				
Result after treatment Cyst in R kidney				
Result after treatment Cyst in L Kidney				
Result after treatment Cyst in Spleen				
Result after treatment Cyst in Omentum Mesentery				
Result after treatment Cyst in lower abdomen				
Result after treatment Cyst in abdominal wall				
Result after treatment Cyst in eye				
Result after treatment Cyst in muscle				
Result after treatment Cyst in lung				
Result after treatment Cyst in other location		recurrence		
Further Information	Surgery recommended		Self-cure	No change

10.2 Variations in circulating antibodies and circulating antigens during follow-up



a)

Figure 10-1: Comparison between circulating antigen and circulating antibody titres in Unchanging cysts

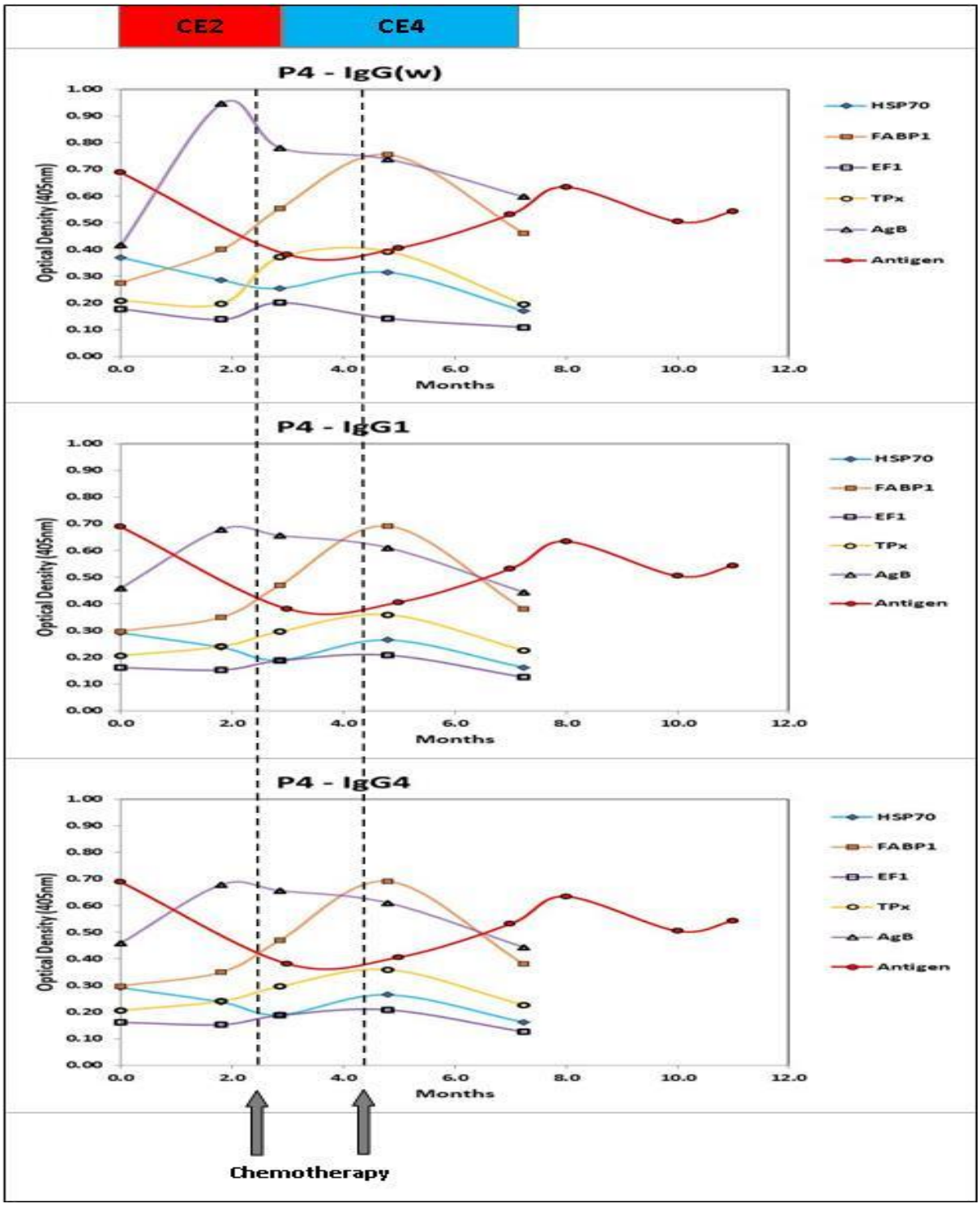


Figure 10-2: Comparison between circulating antigen and circulating antibody titres in cysts hanging from active to inactive.

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Date 30th March 2015

AMREF Nomadic Health Unit
Hydatid Control
Wilson Airport
Nairobi
Kenya

Re: PhD Student Ms Judy Mwangi, University of Salford

Dear Sir/Madam

I am writing in relation to some collaborative work which we have been doing with Dr Eberhard Zeyhle relating to antibody responses in hydatid patients under AMREFs care. My PhD student Judy Mwangi is now using serum samples taken from patients during treatment and relating this to a database of clinical information about each patient. As part of her PhD she must complete an Ethics Approval form for our University. At present all that is required is a letter of consent from AMREF stating that AMREF is aware of the nature of the work being done and has given permission for Judy to work on the sera. Could you please either write a separate letter of consent or complete the section below and return it to me. (Scanned document sent by email is acceptable).

Thank you very much

Professor Michael Rogan

(Project supervisor)

PhD Student Ms Judy Mwangi, University of Salford

We at AMREF are aware that Ms Judy Mwangi (University of Salford) has access to serum samples and clinical data from hydatid patients which are part of the Nomadic Health Care programme in Turkana, in order to carry out primary research on immunodiagnosis and immunological follow up of patients after treatment. We can confirm that the Nomadic Health programme has had full ethical approval in relation to diagnosis and treatment of patients and that Ms Mwangi has permission to work on the samples collected as part of this programme. In relation to her results, it is accepted that all patient identification information will remain confidential and that AMREF will be acknowledged in any publications arising from this collaborative work.

Signed .....Eberhard Zeyhle..... Date .....6th April 2015.....