EVALUATION OF THE USEFULNESS OF LAMINATED LAYER ANTIGENS IN THE SEROLOGICAL FOLLOW UP OF CYSTIC ECHINOCOCCOSIS IN HUMANS

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DEDICATION

This work is dedicated to my DAD and the ever beautiful and loving memory of my Mum and brother, may their souls continue to rest in perfect peace.

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List of Abbreviations

HCF	Hydatid Cyst Fluid
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
WHO	World Health Organisation
PNPP	p-nitrophenylphosphate
PBS	Phosphate buffered saline
PAIR	Puncture, Aspiration, Injection and Re-aspiration
ELISA	Enzyme-Linked Immunosorbent Assay.
LL	Laminated layer
GL	Germinal layer
CE	Cystic Echinococcosis
AE	Alveolar Echinococcosis
TEMED	Tetramethylethylenediamine
TRIS	Trishydroxymethylaminomethane
BCB	Bicarbonate carbonate buffer
BCIP	Bromochloroindolyl phosphate
KDa	Kilodalton
NCP	Nitrocellulose paper
AgB	Antigen B
Ag5	Antigen 5
BSA	Bovine serum albumin

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ABSTRACT

Cystic Echinococcosis is a zoonotic infection of humans caused by the metacestode (larval) stages of the cestode *Echinococcus granulosus* (family *Taeniidae*). Diagnosis of the infection often involves immunodiagnostic approaches using cyst fluid antigens and these have also been used in serological follow up of patients after surgical treatment or chemotherapy. However the usefulness of other metacestode antigenic extracts for these purposes has not been fully investigated.

The laminated layer is a polysaccharide/protein complex that surrounds the outside of the hydatid cyst and is a structure unique to the genus *Echinococcus*. In the current study a crude extract of this layer was prepared by sonication and tested for reactivity against sera from hydatid patients from Turkana, Kenya. This extract reacted both in ELISA and in Immunoblotting, primarily recognising antigenic bands around 55 kDa and 12-36 kDa. This latter region appeared to be more specific in terms of total IgG and IgG1 and IgG 4 subclass responses. The glycoproteins in this region also bound particular lectins such as soyabean Aglutinin and a lectin affinity purification column was produced to try and isolate the more specific glycoproteins. However this part of the project was not successful and no purified fraction was produced. The crude laminated layer extract was then compared with hydatid fluid to look for differences in antibody profile in treated and untreated Turkana patients over time in an attempt to identify possible markers of disease progression/regression. Sera were obtained from 10 albendazole treated patients over time courses ranging from 9 months to several years. Similar samples were also obtained from 4 patients who had refused treatment. Samples were analysed by ELISA against against Hydatid cyst fluid(HCF) and Laminated layer(LL) . Results of the time courses showed that antibody levels fluctuated in both treated and untreated patients and

that some of these changes were associated with changes in cyst morphology. In some cases the laminated layer showed similar recognition patterns to HCF but in others there were peaks of activity against one antigen which was not evident against the other.

CHAPTER 1

INTRODUCTION

1.1 Echinococcosis

The genus *Echinococcus* of cestode parasites belonging to the family *Taeniidae* includes also the well-known tapeworms such as *Taeniasaginata*. *Echinococcus* species have life cycles that always involve a definitive host harbouring an adult worm, and an intermediate host carrying the metacestode or the larva. The definitive host (mostly carnivore) become infected by the ingestion of protoscolices that are contained in the bladder-like metacestode lodged in intermediate host viscera (Diaz e*t.al*, 2011) Protoscolices later develop into 3mm long gut –dwelling adult tapeworms, which produce eggs. The intermediate host is infected by accidentally ingesting eggs passed out with faeces from definitive host. Hydatid disease is the term associated with infection by larval *Ehinococcus* (Diaz *et. al*, 2011). Traditionally, four species are recognised in the genus. They are *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus oligarthrus* and *Echinococcus vogeli*. Canids are definitive hosts for all species in the genus with the lion strain of *E. granulosus* being an exception (Diaz *et. al*, 2011).Domestic ungulates mainly act as intermediate hosts for *E. granulosus*, while wild rodents and lagomorphs carry out this role for the other three species.

Humans are accidental intermediate host for the genus. The major concern associated with *Echinococcus* is human hydatid disease and this is comprised mainly of cystic hydatid disease, which is caused by *E. granulosus* and alveolar

hydatid disease caused by *E. multilocularis* (Brunetti, *et. al*, 2010). Cystic hydatid disease can remain unobserved for many years, as the slow growing hydatid cyst by and large causes pathology only through the compression, contracting and putting pressure on the host organ, which is most commonly liver or lung, or any other organ. Consequently, symptoms are rather non-specific and are dependent on the precise location. Complications not associated with organ contraction can be experienced and are also important; they include bacterial superinfection and cyst rupture, which can bring about the risk of anaphylactic shock and or the initiation of a secondary infection. The disease has a worldwide distribution. It is a highly prevalent disease, especially where pastoralism is an important activity and regular dosage of domestic dogs with the drug praziquantel have not been allowed due to politics, institutional conditions. Hence, cystic hydatid disease is important in areas of Central Asia, South America, China, and Africa (Jenkins *et. al*, 2005).

Alveolar hydatid disease has the liver as the only primary site. After being asymptomatic for several years, the infection becomes apparent, initially with symptoms such as jaundice and abdominal pain. The host liver parenchyma and in some cases, other organs is aggressively invaded by the metacestode, the disease becomes grave, even lethal except treated appropriately (Diaz *et. al*, 2011). Restriction of this infection is to the cold and temperate climates in the northern hemisphere, significant especially is the Tibetan plateau and some other remote areas of China, Hokkaido in Japan and parts of Central Europe (Jenkins *et. al*, 2005).

1.2 Distribution/Epidemiology

The genus *Echinococcus* is an important one because it consists of a number of zoonotic species that can cause serious ill health in man. The genus is consisted of at least 4 species, but evidence gathered from recent molecular studies suggests there should be a taxonomic revision to at least 5 species or even possibly 6 (Le *et al*, 2002; McManus, 2002; Thompson & McManus, 2002). With the species *E. granulosus*, there is also a significant strain variation. The definitive host with each species is a carnivore, whilst the intermediate host could be any of a large number of mammalian species. The parasite is pathogenically and economically significant in intermediate and unusual intermediate hosts, where the larval parasite develops into a hydatid cyst. The genus is distributed worldwide, although geographical distribution of a number of species is limited (Torgerson and Budke, 2003).

E.granulosus is distributed globally, it is found on all continents, with highest prevalence in parts of Eurasia (especially the Mediterranean countries, the Russian Federation and adjacent independent states, and China), East and North Africa, Australia and South America (Eckert *et al*., 2001). According to Eckert *et al* (2001) and Ito *et al* (2003), there is also clear evidence for the emergence and re-emergence of human cystic echinococcosis in parts of China, central Asia, Eastern Europe, and Israel. Communities that are involved in sheep farming are known to harbour the highest rates of infection, emphasising the public health importance of the sheep-dog cycle and the sheep strain of *E. granulosus* in transmission to people. (Thompson and McManus, 2001; McManus, 2002).

Epidemiologically, human cystic echinococcosis occurs mainly in poor pastoral communities where sheep and other livestock are raised and dogs are kept for

guarding and or herding animals. Transmission of *E. granulosus* is predominantly in a cycle between dog definitive hosts harbouring the small intestinal tapeworm, and livestock (especially sheep). The distribution of this parasite in the United Kingdom is restricted, being found mainly in mid and southern Wales. The zoonotic strains of *E.granulosus* are present in every country in Europe, except Ireland, Iceland and Denmark (Torgerson and Budke, 2003). It is most extremely endemic in large parts of China and a significant re-emerging zoonosis in the former Soviet Republics in central Asia (Torgerson *et al*, 2002a,b).

The parasite is also present throughout the Indian subcontinent and the Middle East. *E.granulosus* is widely circulated or diffused in Africa, and of a major problem in Northern African countries such as Tunisia, Morrocco, Libya and Algeria. There are specific concerns for the parasite in the South of the Sahara in certain locations like the Turkana in Kenya. The parasite is present in Canada and Alaska of the North America region, and appears to put on mainly a sylvatic cycle. In the continental USA, the parasite occurs at irregular intervals with just a few communities, foci such as certain communities in Utah and California (Torgerson and Budke, 2003).

It is also widespread in South America, especially in Argentina, Uruguay and Peruvian Andes. In Australia a sylvatic cycle between dingoes and wallabies can also occur with over 25% of dingoes and up to 65% of macropod marsupials infected (Jenkins and Morris, 1995; Jenkins, 2002).

Echinococcus granulosus can have both sylvatic cycles, which often times involves wild carnivores and ungulates; and domestic cycles, with dogs and farm livestock

usually involved. The latter transmission cycle is the commonest and presents the greater risk, threat to human health. The highest incidence rates in man have often being from, or noticed in areas where there are close relationship with man and domestic livestock, with man often using dogs as working dogs. The common source of infection for dogs is by feeding on offal from infected sheep, which most times harbour G1 zoonotic strain that is in many cases responsible for human CE.

Consequently, the high infection levels in these dogs then present a risk to human contacts (Torgerson and Burdke, 2003). There is a very high potential for transmitting *E. granulosus* domestically, in poor countries, where level of education may be low, inadequate veterinary services and a widespread practice of home slaughtering (Torgerson and Burdke, 2003). The infection rates in dogs in such circumstances can reach between 20% and 50%, with possibly an excess of 50% of the sheep population being infected. The degenerating situation in Central Asia are an illustration of the risks associated with infection. CE in man was at relatively low levels, prior to the breakup of the Soviet Union. Nevertheless, following the Central Asian republics independence, there was diffused economic and structural reform. With this came about farm privatisations, centralised meat processing facilities were either withdrawn or abandoned, and there was a return to small subsistence-type agricultural practices (Torgerson and Burdke, 2003). Lack of government funding saw the collapse of veterinary services, resulting in an epidemic of human CE, with annual incidence reported surgical cases by hospitals in an excess of 4-5 times the number reported prior to 1991 (Torgerson *et al*, 2002a,b, 2003).

Emerging also in other former communist countries like Bulgaria is a similar pattern (Todorov and Boeva, 1999).However, providing resources were made available, a decrease, reduction in prevalence, even eradication would have been a possibility. This mainly is due to the factors that affect dynamics of transmission.

1.3 Public Health Implications

Worldwide currently, over three million people are afflicted with echinococcosis, and the extent of the morbidity associated with both AE and CE is estimated to result in more than 1.5million disability adjusted life years (DALY'S) lost (Budke *et. al*, 2006; Togerson *et al*, 2010). AE is of significant public health concern, especially in parts of Central and Eastern Europe and notably, Northwest China (Vuitton *et. al*, 2003). Even though, in many endemic areas, the annual incidence of AE may appear low (0.03-1.2 per 100 000 inhabitants) (Vuitton *et. al*, 2003), the estimation is that there are many cases remaining undiagnosed (Brunetti *et al*, 2010).

Endemicity of human CE is in many pastoral communities, especially in eastern Europe (Jimenez *et. al*, 2002) and north China (Whang *et. al*, 2001). According to Eckert *et. al*, 2000), in many endemic regions and/ or countries, cotransmission of *E. granulosus* and *E. multilocularis* have been recognised, including Northern Japan, China, North Africa, North America and several Eurasian countries.Nearly 95% of all AE cases according to Wen and Yang (1977) worldwide are restricted in five endemic provinces/ autonomous regions of Sichuan, Quinghai, Gansu, Xinjiang and Ningxia, in north and northwest China. AE and CE are co-endemic in all five, with the latter being endemic also to a further 16 of the 33 provinces in China.

1.4 Life Cycle of *Echinococcus granulosus*

Two hosts are required for the life cycle of the parasite, a definitive and an intermediate host. Dogs are the main definitive hosts for *E*. *granulosus* while foxes are for *E. multilocularis*. The major intermediate hosts for *E. granulosus* are ungulates and wild rodents for *E. multilocualris* (See *review by*, Ammann and Eckert, 1996). The intestine of the definitive host harbours the adult worm and eggs passed out in the faeces are ingested by the intermediate host. Gastroenteric enzymes digest the external coating of the eggs, following its oral ingestion and the oncosphere larva is freed. The embryos possess hooklets with which they attach to the mucosa of the intestine (Tüzün *et al*, 2002). They penetrate the wall and enter the portal venules and lymphatics, from where they are transported to the liver, lungs, organs and tissues of the systemic circulation. Ultimately, the oncosphere develops into a cyst within which the protoscoleces are produced.

The cycle is completed when viscera containing live larval form are eaten by the definitive host (Beggs, 1985; Ersahin *et al*, 1993; Ammann and Eckert,1996; Sinner, 1997; Gossios *et al*,1997). Humans may become accidentally infected as intermediate hosts after directly ingesting parasitic eggs from contact with a definitive host or indirectly from contaminated food or water (Ammann and Eckert, 1996 ; Gossios *et al*,1997 ; Tüzün *et al*, 1998 ; Haliloğlu *et al*, 1997). Humans are usually considered a dead end for the parasite since the life cycle relies on carnivores eating infected herbivores (*see review by*, Zhang *et al*, 2003).

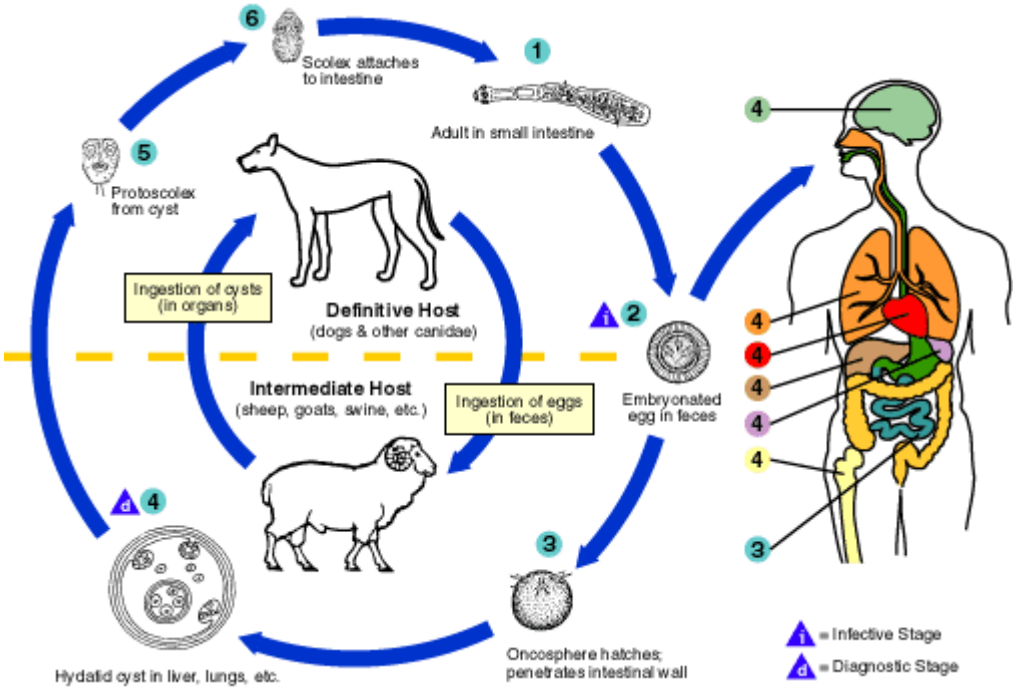


Figure 1.1: Life cycle of *Echinococcus granulosus*

(Source: http://images.google.co.uk/images?imgurl=http://www.biochemj.org/bj/362/0297/bj3620297f01.gif)

1.5 *Echinococcus* Metacestodes

After eggs have being ingested by the intermediate host, they go on to release embryos (oncospheres) that penetrate the gut wall, travel through blood or lymph and end up being trapped in internal organs where development into metacestodes takes place (Diaz *et. al*, 2011). These are surrounded by a thin cellular layer known as the germinal layer (GL) and fluid filled (hydatid cyst fluid, or vesicle fluid). The GL gives rise to brood capsules by budding towards the inside, generating protoscolices in return. There are variations to this basic structure amongst species. The *E. granulosus* is typical; it develops as a large unilocular, turgid cyst, which grows

through an increase in diameter. *E. oligarthrus* and *E. vogeli* develop in similar way but tend to form multi-chambered cysts. Growth by *E. multilocularis* is different and contrasting to what is obtained with the other species in that it grows by outward budding, thereby giving rise to a labyrinth of chambers and tubules (Diaz *et. al*, 2011).

The GL extends towards the outside, towards the apical plasma membrane of its syncytial tegument which carries truncated microtriches (Morseth D.J, 1967). Additionally, the GL possess non-syncytial cell types, including muscle, glycogen-storage and undifferentiated cells. There is neither a syncytial organization nor junction complexes between cells towards the cyst cavity, so that the intercellular fluid of the GL is apparently continuous with the cyst/ vesicle fluid (Lascano *et. al*, 1975) .The laminated layer (LL) is what separates the GL from host cells and / or host extracellular matrix. It is an acellular carbohydrate-rich sheath secreted by the GL. Approximately, the LL attains 10-12µm in thickness in *E. multilocularis*, up to 400µm in *E. vogeli* and up to 3mm in *E. granulosus* (Rausch, 1954; Bortoletti and Ferretti, 1978; Rausch, et al, 1981).The LL is related to cellular glycocalyses. Nonetheless, the inner most strata only of this huge structure are likely to be covalently anchored to the GL tegumental membrane. Therefore, the LL can appropriately be described as a specialised extracellular matrix, found only in the genus *Echinococcus*, designed evolutionarily for the maintenance of the physical integrity of metacestodes and for the protection of GL cells from host immunity. There is the probability that the first of these demands determines the impressive thickness of the *E. granulosus* LL: live hydatid cysts are turgid, and also pressure from the outside is a significant threat, as depicted by the occurrence of traumatic

cyst rupture (Brunetti *et. al*, 2010).

Budding off from the germinal membrane are the brood capsules and protoscolices (PSC) (*See review by*, Zhang *et al,* 2003). “A mature fertile cyst is frequently unilocular (having a single cavity) and visualized as a clear (anechoic), fluid-filled lesion, usually with a cystwall visible” (Rogan *et. al*, 2006). The cyst wall is made up of a syncitial germinal layer that gives rise to brood capsules and protoscolices, and a non- living laminated layer adjoining the host tissue. The living germinal layer is not distinct or differentiated within the cyst wall in ultrasound. However, cysts vary in both size (1-20cm or more) and internal structure (Rogan *et al*, 2006).

Host tissues usually enclose or surround the parasitic cyst or endocyst to form a pericyst. The endocyst is largely consisted of a thick (0.2-2mm) acellular laminated layer. A thin (10-20µm) germinal layer may be present in healthy cysts, which lines the inside of the laminated layer, which may give rise to brood capsules containing protoscolices. The central cavity of a healthy cyst is filled with clear fluid, and varying sizes of daughter cysts which are formed by internal growth; may also be present. In degenerating, degenerated or dead cysts, a viable germinal layer or protoscolices may no longer be contained in the endocyst, there is the possibility of infiltration, it may have occurred and pericyst often shows signs of partial or complete calcification. (Wang *et al,* 2003).

The fully developed metacestode is a unilocular cyst, and could reach large size. “The cyst cavity is filled with sterile hydatid fluid, which, is a complex mixture of parasite-derived molecules and host-derived serum components”(*see review by*,

Richard and Lightowlers, 1986; McManus and Bryant, 1995). The cyst wall is consisted of an inner thin multinucleated germinal layer and an outer thick acellualr laminated layer.

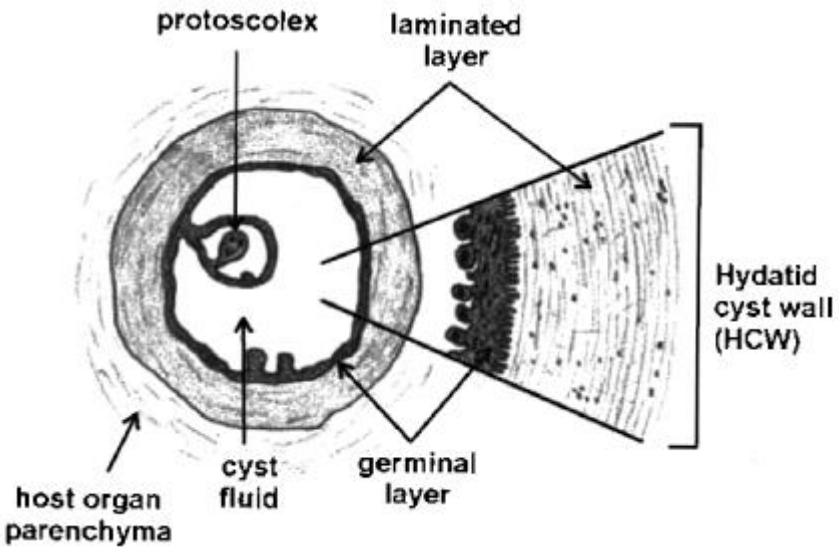


Figure 1.2: Structure of the Hydatid cyst.

(Source**:**http://images.google.co.uk/images?imgurl=http://pathmicro.med.sc.edu/parasitology/EchinococcusLifeCycle)

1.6 The Laminated Layer

The laminated layer (LL) is made up of a number of laminations, and in addition to giving support to the cyst, it is presumed to protect the parasite from the hosts

immune responses. (Coltorti and Varela-Diaz, 1974; Bortoletti and Ferreti, 1978; Richards *et al*, 1983 ; Harris *et al*, 1989 ; Rogan and Richards, 1989; Holcman *et al*, 1994; Gottstein and Felleisen, 1995). It consists of a protein-polysaccharide complex: the carbohydrate component appears to be built up of glucose, galactose, glucosamine and galactosamine (Kilejian and Schwabe, 1971; McManus and Bryant, 1986; Leducq and Gabrion, 1982). Protection of the parasite from the hosts immune response could stem from the carbohydrate (Coltorti and Varela-Diaz, 1974; Leducq and Gabrion, 1982; Rogan and Richards, 1986), presumably inhibiting complement activation (Smyth and Mcmanus, 1989). The laminated layer is parasitic in origin, secreted by the germinal layer (Bortoletti and Feretti, 1978; Harris *et al*, 1989; Holcman *et al*, 1994). Both layers, laminated and germinal layers are joined together by cystoplasmic connections.

The germinal layer is made of undifferentiated, proliferative totipotential cells, which produce brood capsules that project into the lumen of the mother cyst or daughter cysts. Formation of the protoscolex occur from budding of the germinal layer of the brood capsules which eventually may break away from their attachment to the germinal membrane and form ”hydatid sand” in the cyst fluid. When ingested by the definitive host, each protoscolex may develop into adult tapeworm. The cyst wall of metacestodes is consisted of inner, middle and external layers, with the middle (laminated) layer been unique to the genus *Echinococcus* when compared with other larval Cestodes. The presence of this layer is not noticed in very young cysts until it is about 14-18days when it appears first as a thin, clear layer on its outer margin. The laminated layer is an acellular, polysaccharide protein complex that is strongly

stained by periodic acid, Schiffs reagent (PAS) and in histological studies provides a useful marker (Kilejian *et al*, 1962 ; Craig *et al*, 1995). This layer is developed from or given rise to by the germinal (inner) layer (Bortoletti and Ferretti, 1978; Holcman *et al*, 1989), and its structure may also be contributed to by host material (Kilejian and Schwabe, 1971; Pezzella *et al*, 1984). It has been shown that more galactosamine than glucosamine is contained in the laminated layer (LL) of *E. granulosus*. Nevertheless, there are more glucosamine than galactosamine in protoscoleces (Px) and hydatid cyst fluid (HCF). Also presented in this layer is acid muco-polysaccharide (Richards, 1984).

1.7 Development In The Intermediate Host

Hatched parasitic embryos migrate through the intestinal mucosa and enter venules and lymphatics. Between 60-70% of the embryos are filtered by the liver, and 15-25% by the lungs, 10-15% reaches other organs via the systemic circulation (Sinner *et. al*, 1991). Undestroyed embryos are transformed into small cysts that will grow 2-3cm each year. The parasitic cyst wall is consisted of a germinal layer (endocyst) and a laminated proteinaceous membrane (ectocyst). The host forms a dense fibrous capsule (pericyst) which is in reaction against the cyst, and this contains blood vessels that provide nutrients to the parasite (Sinner *et. al*, 1991).

In order to comprehend the host-parasite relationship, the knowledge of post-oncospheral differential is vital since structural changes of the developing hydatid

cyst tegument may reflect the parasites immunoprotectivemechanism (Holcman *et. al*, 1994). Mature eggs of *E. granulosus* possess a thick embryophore and its ultrastructure shows it consists of thick elongated blocks that are united by electron-lucid cement (Holcman *et. al*, 1997), and surrounding the oncosphere is a thin cytoplasmic oncosphere membrane. The most eminent granules of penetration glands occupy the region of the nuclei in the hatched oncosphere. Various different functions are ascribed to the secretion of the penetration glands. They are involved in penetration and are totally pushed out during this process (Holcman *et. al*, 1997). The secretion causes lysis of host tissue in the surrounding of the invading oncosphere, enabling the oncosphere to resist the host cellular attack by maintaining a zone of necrosis of surrounding cells during the development of laminated layer (Heath, 1971).

The penetration glands support and assist in adhesion and protection against digestive enzymes or immune response of the host (Lethbridge, 1980; Fairweather and Threadgold, 1981), they may also contribute to the formation of microvilli (Harris *et. al*, 1989; Holcman *et al*, 1994). There are three recognised types of penetration glands (Swiderski, 1983), displaying a range of electron densities (Holcman *et. al*, 1994) different functions could be ascribed to different secretions. Harris *et. al*, (1989), suggested that much of the membrane needed for the extension of the microvilli from the epithelium could come from the fusion of penetration granule membranes with the outer plasma membrane. The three pairs of hooks located in the region opposite to the nuclei are equipped with a complex muscle system.

The oncosphere may be assisted in penetrating the host intestine possibly by secretions from the penetration glands. Hooks may be put to work as blender knives

to cut tissue for penetration, in contrast to the hooks found on protoscolex or scolex which are lacking in independent musculature and only function to provide anchorage (Antoniou and Tselentis, 1993). The hook region, seen as the smaller lobe at light microscopy is incorporated in the metacestodes eventually (Heath and Lawrence, 1981), by day 2-3 after activation. The post-oncospheral development is a complex process of acquisition of biochemical and morphological properties difficult to be observed in vivo. “There is a great number of studies on *in vitro* culture of protoscolex from fertile hydatid cysts to adult strobilate stage and a significant number on the ultrastructure of *E*. *granulosus* protoscolex tegument and of the germinal and laminated layers of the hydatid cyst” (Morseth, 1967; Bortoletti and Ferretti, 1973, 1978; Lascano *et al*, 1975; Conder *et al*, 1983, Rogan and Richards, 1986; Casado *et al*, 1992).

However, studies on in vitro culture of oncospheres are few, this, perhaps is due to the risk associated with handling of *E. granulosus* eggs (Heath and Smyth, 1970; Heath and Lawrence, 1976, 1981) and only two studies deal with ultrastructural development of the oncosphere to early metacestode (Harris *et. al*, 1989, Holcman *et*. *al*, 1994). In the obvious absence of cell multiplication, cellular reorganization of an early metacestode takes place. There is a rapid increase in number and size of the microvilli by day 1 and up to day 2. In the epithelium of the metacestodes, electron-lucid vesicles start to appear increasing in size and number continuously.

“In a 3days old metacestode, long microvilli are substituted by old short microtriches and the first laminated layer surrounding the metacestode appears as an electron-dense matrix composed of fine microfibrillated material and remnants of depressed

microvilli". (Holcman& Heath, 1997). This layer is the first of a series that stems from the germinal membrane, and eventually appears to be a series of adjacent laminations. The large microvilli are completely by day 5 substituted by short and microfilamentous microtriches that project into the laminated layer. The appearance of the second laminated layer is between day 6-8. Its more electron-dense than the first lamination and is represented on its outer and inner surfaces by particulate material.

"Some microtriches appear to open into or be covered by the particulate material of the second lamination" (Holcman and Heath, 1997,). The laminated layer that surrounds the metacestode of the *E. granulosus* is involved in protection of the parasite from the host immune response. Lamination first appears very early in post-oncospheral development. The organization of the laminated layer in the early metacestode of *E. granulosus* suggests that the outer sheet of the laminated layer is likely to be constantly replaced. The cyclical production possibly, is an intrinsic characteristic of the laminated cover essential in the creation of layers that could eventually be depressed as the cyst grows, and serve to divert host cellular response to the parasite. Before a naive host would be expected to mount an antibody-mediated immune response, the full development of the first lamination is completed (Holcman and Heath, 1997).

1.8 Human Hydatidosis

Human disease in cystic echinococcosis is as a result of the development and growth of fluid-filled cysts that are found mainly in the liver and the lungs, although

the abdominal cavity, heart, bone, muscle, nervous system, and or other locations can be affected (Khuroo, 2002; McManus *et al*, 2003). The cystic larvae grow slowly, and its growth is well tolerated by the host, leading occasionally to large parasitic masses (Moro, et al, 1999; McManus *et al*, 2003). Human echinococcosis occurs when eggs that have been shed in the faeces of definitive hosts are ingested by man.

Usually, the initial phase of CE is asymptomatic with small, well encapsulated cysts, which after an undefined period of several months to years; the infection may become symptomatic as a space-occupying lesion.

However, according to Pawlowski *et. al,* (2001), 60% of infections will remain asymptomatic. The commonest organ involved is the liver, with over two third of cysts usually. Infection in the lungs accounts for 20% of cases, with involvement of other organs accounting for less than 10% of cases (Torgerson and Burdke, 2003).

1.9 Pathology

Once the establishment of infection has taken place, the parasites are able to survive and grow throughout the lifetime of the host. Cysts growth can be unrestricted and CE cysts can sometimes reach sizes in excess of 13cm in diameter (Yang *et. al*, 2005). Nevertheless, because the growth of the parasite is very slow, often, symptoms arise years after infection when the disease is well advanced. In untreated or in inadequately treated AE patients, the mortality rate is more than 90% within 10-15 years of infection (Togerson *et. al*, 2008). The mortality rate due to CE is lower (about 2-4%) but significantly increases if medical treatment is inadequate or

unavailable (Brunetti *et. al*, 2010). CE cases remain asymptomatic most times until the cyst compresses or ruptures and there is spillage of its contents into neighbouring tissues and organs, by which time the disease is well advanced already (Brunetti *et. al*, 2010).

The pathological damage or dysfunction caused by cysts is mainly by the gradual process of space-occupying repression or the displacement of vital host tissue, vessels or organs. Clinical manifestations are consequently determined primarily by the site and number of cysts and these are quite variable. A massive release of cyst fluid and dissemination of protoscolices is what can follow and in most cases is what follows accidental rupture of cysts, resulting, occasionally in anaphylactic reactions and or multiple secondary cystic echinococcosis, since protoscolices have the potential of developing into cysts within the intermediate host (Schantz and Gottstein, 1986).

There have been reports of cystic echinococcosis presenting for medical attention in people that are aged from younger than 1year to older than 75years, with fairly similar rates in both sexes. Following surgery on primary cysts, recurrence may occur. About 60% of all cases of cystic echinococcosis may be asymptomatic, although an unknown proportion may become symptomatic. “About 0.2 per population of 100,000 has been estimated as the mortality rate, with a case fatality rate of 2.2%” (Menghebat *et al*, 1993)**.** A good percentage of cysts (90%) occur in the liver, lungs, or both. Cysts that are symptomatic have been occasionally reported in the spleen, kidney, peritoneal cavity, and the skin muscles (2-3% each); and

seldomly in the heart, brain, ovaries, vertebral column (1% or less each) (Menghebat *et al*., 1993). Symptoms presented by cystic echinococcosis can be highly variable, and can be dependent not only on the organ involved, but also on size of cysts and their position within the organ, the mass effect within the organ and upon surrounding structures, and related complications to cyst rupture and secondary infection. In response to cyst leakage or rupture, manifestations of systemic immunological responses may be evident. Common complications involving cystic echinococcosis include rupture into the biliary tree with secondary cholangitis, obstruction of the biliary by daughter cysts or extrinsic compression, rupture into the bronchial tree, intracystic or subphrenic abscess formation, development of a bronchobiliary fistula and intraperitoneal rupture (with or without anaphylaxis).

According to Ammann and Eckert (1996), 10% of all intraperitoneal ruptures had anaphylactic complications, with the remaining patients developing multiple intraperitoneal cysts, and anaphylaxis accounted for two of the 221 (0.9%) reported complications of cystic echinococcosis. As cysts grow and enlarge, however, they can put pressure on surrounding organs and can cause several pathological changes (Pawlowski *et al*, 2001). More common are fever, jaundice and abdominal pain, but more serious problems, such as hepatomegaly (enlargement of the liver), inflammation of the bile duct (cholangitis) and high blood pressure in the portal venous system (portal hypertension) also can occur (Pawlowski *et al*, 2001).Complications also arise due to or by the possibility of cyst rupture which can result in anaphylactic reactions due to the large amounts of hydatid fluid being released. A secondary hydatid infection can result from rupture, caused by the

release of many thousands of larvae (protoscolices), with each having the capability to differentiate into another hydatid cyst (Rogan *et al*, 2006).

1.10 Diagnosis

The detection of the space occupying cysts or lesions caused by metacestode(s) of *Echinococcus* species that are developing, dying, or dead is largely dependent on imaging techniques. The quality of the management and treatment of cystic echinococcosis can be improved essentially by early diagnosis. With the early stages of infection being asymptomatic in most cases, cheap methods and that are quite easy to use are needed for large-scale screening of populations that are at high risk. For most cases of cystic echinococcosis in man, the definitive diagnosis is usually by physical imaging methods, such as computed tomography (CT scanning), ultrasonography, radiology and magnetic resonance imaging (MRI), (Pawlowski *et al*,2001), although in isolated communities, such procedures are not readily available (Mcmanus *et al*, 2003; Raether and Hänel, 2003; Polat *et al*, 2003 ; WHO/IWGE,2003; Macpherson and Milner, 2003; Eckert and Deplazes, 2004; Kjossev and Losanoff, 2005). The overall method of choice for diagnosing cystic echinococcosis is the usage of ultrasound scanning. This choice of method is applicable to both community and routine hospital settings (Whang *et al,* 2003). Since the late 1970s, ultrasound has been used for the detection of pathological lesions due to CE (Vicary *et al*, 1977; Macpherson, 1992). The ultrasound scanning method has also been used for the examination of hydatid cyst development over time (Romig *et al*, 1986 ; Frider *et al*, 1999) and post-treatment (Caremani *et al*,

1997 ; Gharbi *et al*, 1997). Diagnosing CE early can bring about significant improvements in the quality of the management and treatment of the disease. Early stages of the infection are asymptomatic in most cases, so, cheap and relatively easy to use methods are required for large-scale screening of populations at high risk. Providing such an approach is immunodiagnosis, which can also confirm clinical findings (Zhang *et al*, 2003).

Immunodiagnosis is vital in that it plays an important as well as a complementary role. Its usefulness is not only for primary diagnosis but also for follow-up of patients after surgical or pharmacological treatment. Detection of antibody in sera is more sensitive than the detection of circulating antigen, and remains the method of choice (Zhang *et al*, 2003). Serological testing of cystic hydatid disease (CE) has a very long history, and almost all serological tests that have been developed have been used in the diagnosis of human cases. Among the various tests, there are considerable differences in sensitivity and specificity. Non-specific and insensitive tests, like the Cassoni Intradermal test, the complement fixation test, the latex agglutination test, the indirect haemagglutination test have been replaced by the enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence antibody test, immunoelectrophoresis (IEP), and immunoblotting (IB) in routine laboratory procedures, applications (Lightowlers and Gottstein, 1995).

“The lipoproteins antigen B (AgB) and antigen 5 (Ag5) (Oriol and Oriol, 1975), the major components of hydatid cyst fluid, have received the most attention with regard to diagnosis. Along with HCF, they are the most widely used antigens in current assays for immunodiagnosis of CE. Both antigens have been well characterized by

immunoblotting and or by immunoprecipitation of radiolabelled antigen and SDS-PAGE" (Shepherd and Mcmanus, 1987; *see review by*, al-Yaman and Knobloch, 1989; Lightowlers *et al*, 1989; Shapiro *et al*, 1992).

"Antigen B, with a molecular mass of 120KDa is a polymeric lipoprotein that can be measured as a circulating antigen in patients blood (Kamiya and Sato, 1990; Liu *et al*,1993) has been suggested to play an important role in the biology of the parasite-host relationship (Shepherd et al, 1991; *see review by*, Rigano *et al*,2001). Antigen B is a highly immunogenic molecule, appearing ladder like under reduced condition on SDS-PAGE, with three bands with molecular sizes of approximately 8 or 12, 16, and 24KDa (Chordi and Kagan, 1965 ; Oriol *et al*, 1971 ; Shepherd and Mcmanus, 1987 ; Lightowlers *et al*, 1989 ; Leggatt *et al*, 1992), suggesting that it comprises polymers of 8KDa subunits. The smallest subunit has proved the most useful target in diagnostic studies" (Ortona *et al*, 2000; Rott *et al*, 2000).

Ag5 is a lipoprotein with a very high molecular mass complex composed of 57 and 67KDa components that dissociates into 38 and 22 to 24KDa subunits under reducing conditions (Lightowlers *et al*, 1989). "According to history, the demonstration of serum antibodies precipitating antigen 5 (arc5) by immunoelectrophoresis or similar techniques has been one of the most widely used immunodiagnostic procedures for CE" (Shepherd and McManus, 1987).

The current standard of practice for serology for human cystic echinococcosis is based on the detection of IgG antibodies to hydatid cyst fluid-derived native or recombinant antigen B subunits, either in ELISA or in immunoblot formats (Wen and

Craig, 1994; Eckert and Deplazes, 2004). Studies on the hydatid cyst of *E. granulosus* have indicated the occurrence of high levels of host IgG heavy chain in the germinal layer of non-fertile cysts and suggests the host immune response could be destructive of protoscolex production by bringing about or causing apoptosis of the germinal membrane, possibly opening up an avenue for vaccination against established cyst (Blanton *et al*,1991 ; Lawn *et al*, 2004).

Following the knowledge and understanding of the smallest size of the lipoprotein of antigen B to be 8KDa and believed to be *Echinococcus* specific with diagnostic potentials, Barbieri et al (1993) went on to prepare a mixture of lipoproteins antigens that contained the relevant diagnostic AgB and Ag5 from bovine hydatid cyst fluid by heparin- affinity chromatography. A standardized antigen mixture of high sensitivity and specificity for human hydatid serology has been provided by this heparin-binding lipoprotein fraction (HBLF) (Barbieri, et al, 1993; Barbieri, et al,1994). Although, a constant supply of parasite material is demanded by its preparation and there is the observation of false positive results with purified antigen. Fernandez et al (1996) in theoretically analysing how a mixture of recombinant proteins can help in solving the drawbacks observed and or obtained with the use of native antigens: production of unlimited amounts can be achieved under controlled conditions and it may be a possibility to identify and remove the cross- reactive epitopes without the loss of diagnostic sensitivity. In search for recombinant proteins that would allow the preparation of antigenic mixture with such characteristics, Fernandez et al, (1996) screened an *E. granulosus* protoscolex cDNA library using a rabbit anti- HBLF serum and went further to describe the characterisation of a cDNA coding for an antigen similar to, but distinct from the already described 8-KDa subunit of AgB (Shepherd, et al, 1991; Frosch, et al, 1994).

The cDNA library prepared with *E. granulosus* protoscolices obtained from hydatid cysts of Uruguayan sheep were immunoscreened and allowed the isolation of a reactive phage clone (ʎ3C3) which was characterised further. Their results showed affinity- purified monospecific polyclonal antibodies against lambda 3C3 reacted by western blotting with HBLF bands of 8, 16, 24 and 32 KDa apparent molecular mass. Consequently, the pattern described as corresponding to AgB subunits (Lightowlers, et al, 1989) was reproduced with anti- ʎ3C3 antibodies.

Recent research has demonstrated that AgB, encoded by a gene family constituted of member genes, exhibits variation to a very high degree (Frosch, et al, 1994; Chemale, et al, 2001; Arend, et al, 2004; Muzulin, et al, 2008). Five 8KDa subunit genes from *E. granulosus* so far have already being identified. They are named as EgB8/1, EgB8/2, EgB8/3, EgB8/4 and EgB8/5 (Haag, et al, 2004). EmB8/1-EmB8/5 have also being identified in *E. multilocularis* (Mamuti, et al, 2006, 2007). AgB recombinant subunits were found to self assemble by Monteiro et al, (2007) into high molecular mass homo-oligomers with structural features that are similar to those of the parasite- produced AgB while they studied the recombinant subunits of AgB1, AgB2 and AgB3.

Gene polymorphism, strain variability, differential expression and source of hosts are a few of many problems associated with the usage of AgB antigen in diagnosis (Jiang, et al, 2012). Therefore, the characterisation of AgB subunits immunologically is vital to the assessment of their actual diagnostic value. Majorly, and to date, the immunological studies on AgB subunits have focused mainly on AgB1 and AgB2 (Rott et al, 2000; Virginio et al, 2003), the initially identified subunits. This study was carried out by Jiang Li et al, (2012) to clone and express all of the 5 identified subunit

genes of AgB antigen family, to investigate their serological reactivity and differences in the recognition of specific antibodies, to identify potential subunit antigens for immunodiagnostic tests and to proving a basis for standardization of AgB antigen.

They (Jiang Li et al, 2012) analysed the reactivity of a panel of 243 serum samples from CE, AE, CC patients and NH with 8 recombinant subunit antigens by ELISA. They also made comparison of three paralogous subunits from *E. granulosus* (EgAgB1- EgAgB3) and *E. multilocularis* (EmAgB1-EmAgB3), respectively for their reactivities in CE and AE sera detection. Their results showed that all of the three orthologous subunits (EgAgB1 vs EmAgB1, EgAgB2 vs EmAgB2 and EgAgB3 vs EmAgB3) were not different statistically when detecting CE or AE sera and therefore suggested that there may be a similarity in their epitopes

The diagnosis of lung hydatid disease is based on chest imaging using X-rays or computed tomography (CT). Serological tools are used only to confirm the diagnosis because of low sensitivity and incomplete specificity (Santivanez and Garcia, 2010). The assay performance is dependent mainly on the format of the test and nature of antigen used but can also vary according to the characteristics of the disease such as organs involved, number of cysts and presence of any cyst complications (Zhang et al, 2003; Zhang and McManus, 2006). Previously, the use of synthetic peptides or recombinant antigens derived from sequences of the two major components of cystic fluid antigen B (AgB) and Ag5 have been proposed for use as reproducible antigens to improve test reliability and allow better standardization (Ortona et al, 2000; Virginio et al, 2003; Carmena et al, 2006).

The p176 antigen which is derived from AgB, is a 38-mer corresponding to the N-terminal extension of the subunit AgB8/1 (Gonzalez et al, 2000). Due to scarcity of

data on serological diagnosis of lung CHD and how published p176 studies do not allow estimations of its sensitivity or provide further details for pulmonary cases. Santivanez et al (2012) did a study and applied p176 ELISA in a series of known cases and responses of those patients were compared to the responses of noninfected controls to provide further information on the test performance of the assay for the diagnosis of lung CHD as well as its performance in relation to disease characteristics. The use of p176 was to counter the variability, high variability in results obtained with the use of cyst fluid as the antigenic source/ material.

Results obtained for the sensitivity of the p176 ELISA for the diagnosis of lung CHD cases was almost 80%, despite the fact that the restricted numbers of samples with isolated pulmonary CHD prevented a more precise assessment of sensitivity. In the long run, the simpler, cheaper, semiquantitative ELISA format and the potential for better reproducibility make this ELISA a good alternative for the diagnosis and posttreatment follow-up of lung CHD. Diagnosis of infection in human is based on the identification of infiltrative or cystic lesions by imaging techniques such as ultrasonography or computed tomography (Brunetti et al, 2010).

The diagnosis of AE is strengthened by immunodiagnostic tests such as ELISAs especially using native protoscolex or metacestode antigens, purified fractions (Em2 antigen), or recombinant antigens (II/3-10¯, Em10- or Em18- antigen) with variable sensitivities and specificities (Gottstein, et al, 1993; Brunetti, et al, 2010; Schweiger, et al, 2011). The study undertaken by Barth et al (2012) was to validate the immunohistochemical diagnosis of AE using the monoclonal antibody mAb Em2G11 on a large number of paraffin embedded samples from resection specimens and from cutting needle biopsies and fine needle aspirates of patients with AE or CE that have been confirmed histologically or with putative diagnosis. An epitope of a mucin-

type carbohydrate antigen called Em2 (Hulsmeier et al, 2002) which is a major antigen of the laminated layer of the *E. multilocularis* metacestode that is also present in the cyst fluid (Deplazes and Gottstein, 1991; Gottstein et al, 1992) is recognised by the monoclonal antibody mAb Em2 G11.

Barth et al (2012) were able to show that the mAb Em2 G11 is strongly positive in the laminated layer of *E. multilocularis* lesions in various human tissues in all samples studied. According to them, no protoscolices were found in all investigated material of 49 AE patients which confirmed protoscolices are a very inconstant diagnostic feature (Marty et al, 2000), and therefore submitted that the mAb Em2 G11- positive laminated layer is the crucial immunohistological hallmark for diagnosis of AE. The mAb Em2 G11 is also said to be species specific as no positive results were recorded at all for CE neither in the laminated layer, germinal layer, calcareous corpuscles nor in the protoscolices when stained with mAb Em2 G11 (Barth et al, 2012).

1.11 Treatment

In endemic regions, asymptomatic hepatic cystic echinococcosis are common and up to 75% of infected people may remain free of symptoms for more than 10years (Frider *et al*,1999). “Cysts may be seen to expand, become septate, or calcify when patients are monitored with serial ultrasound”. A greater occurrence of this condition have been identified in community studies with screening ultrasound compared to similar studies of patients presenting for medical attention (Larrieu and Frider, 2001). In spite of advances in chemotherapy, surgery still remains the main choice of treatment for hepatic CE (WHO-IWGE, 1996), and the increasing use of

percutaneous aspiration (Filice *et al*, 1990; Wang *et al*, 1994 ; Akhan, *et al*, 1996 ; WHO/OIE, 2001).

The possibility of cyst recurrence however remains the main problem with this form of treatment. Recurrence rates of cyst after surgery have been reported as being between 2% and 20% (WHO/OIE, 2001). However, these rates may be subjected to some level of inaccuracy since occurrence of cysts in patients after surgery could also be because it had been missed in the initial examination or because of subsequent reinfection after exposure to eggs. In establishing and validating whether cysts are truly recurrent after surgery or have by other means arisen, the use of ultrasound based cyst morphology could also be of benefit (Wang *et al*, 2003).

Surgical removal of the lesions is included in the treatment options for CE, and CE in most parts of the world is the most common reason for abdominal surgery. Ninety percent success rate has been attributed to surgery (Pawlowski *et al*, 2001). The PAIR (Puncture-Aspiration-Injection- Reaspiration) technique is an alternative to surgery (WHO/OIE, 1996). Chemotherapy, with drugs such as benzimidazoles, have also been used with some success. An indication for a wait and see approach to treatment is employed in calcified cysts (Torgerson and Budke, 2003).

1.11.1 Surgery

The principal and mainstay therapy for large cysts, infected cysts, those that are superficial and likely to rupture, and those in vital and anatomical sites or exerting considerable and substantial mass effect has always been surgery. “Surgical options include: pericystectomy, partial hepatectomy or lobectomy, open cystectomy (with or

without omentoplasty), or (palliative) tube drainage of infected cysts. Cyst extrusion (Barrett's technique) is also a surgical option for pulmonary disease". More radical surgery is associated with a higher complication rate but also a lower relapse rate. Recurrence usually is due to either insufficient cyst removal or previously undetected cysts. Percentage of reported recurrence rates range from 2-25% (Ammann and Eckert, 1996).

1.11.2 Pair

The puncture, aspiration, injection, reaspiration (PAIR) technique was introduced in the mid-190's (Gargouri *et al*,1990; Filice and Brunetti, 1997). Under ultrasound guidance, the cyst is punctured, as much cyst fluid is aspirated as possible, followed by the injection of a protoscolicide (e.g, 95% ethanol), and cyst contents reaspirated between 15-20mins later. This technique should only be undertaken by skilled practitioners, with intensive- care support on ground in the event of anaphylaxis. Assessment should be made of cyst aspirates for the presence of protoscolices or bilirubin. The use of PAIR should only be in, or, with patients with chemotherapeutic cover so as to minimise the risk of secondary cystic echinococcosis. This technique has not been performed or experienced with children and or pregnant women.

The use of PAIR is best for liver cysts that are 5cm or of greater diameter that anechoic (echoless), multiple or multiseptate. The PAIR technique has also been used in patients who have relapsed after surgery. For cysts that are superficial or not accessible, and for cysts that are solid, calcified or communicate with the bile ducts,

PAIR is inadvisable (Anonymous, 1996). Percentage rates of complication ranges from 28% in the absence of albendazole (Men *et al*, 1999), to 5-10% with concomitant chemotherapy (Pelaez *et al*, 2000 ; Aygun *et al*,2001).

“A multicentre survey on PAIR carried out by the WHO informal working group on echinococcosis (Filice *et al*, 2000), reported a 1% major complication (anaphylaxis or spillage) rate and a 13.7% minor (fever, rash, cyst infection, or haemorrhage)”.
“The usage of PAIR with albendazole chemotherapy has been shown to be as effective as pericystectomy for hepatic cystic echinococcosis in one randomised propective trial (Khuroo *et al*., 1997) with lower post-procedure morbidity and shorter hospital stay.

1.11.3 Chemotherapy

Albendazole and mebendazole, the benzimidazole compounds have been the bedrock, the fundamental chemotherapy for cystic echinococcosis. “Treatment with albendazole (10mg/kg in divided doses – usually 400mg – twice daily) results in the disappearance of up to 48% of cysts and a substantial reduction in size of a further 24%” (Horton, 1997). Mebendazole (40-50mg/kg per day in three divided doses) is less capable of producing desired effect than albendazole. (Horton, 1997).

Due to the limitation on toxicological data, the administration of albendazole was originally done in three to six 4-week cycles with intervals of 14days. However, more recent data have suggested that equivalent or improved efficacy with no increased adverse effect is achieved by continuous treatment (Liu, 1997; Franchi *et al*, 1999).

“Non-viability of cyst increases with duration of treatment – from 72% of cysts non-viable after 1 month to 94% of cysts non-viable after 3 months of treatment” (Gil-Grande *et al*, 1993), with the usual adverse effects including nausea, hepatotoxicity, neutropenia (which may not be reversible), and alopecia (occasionally).

All patients are advised to have regular monitoring of leucocyte counts and liver function tests. The protoscolicidal metabolite of albendazole is albendazole sulphide. Praziquantel (25mg/kg per day) has been used concurrently with albendazole for concomitant treatment of cystic echinococcosis, and early trial in man has shown improved efficacy over albendazole alone (Mohamed *et al*,1998). “Albendazole and mebendazole are listed as category C drugs in pregnancy in the USA (Gilbert *et al*, 2001), and category D and B£ respectively in Australia (Anonymous, 2000). Neither drug is definitely inadvised in pregnancy. Specialist advice should be sought if treatment during pregnancy is likely”.

1.12 Changes in Cyst Morphology and Follow Up After Treatment.

The potential success of treatment can be followed up using ultrasound or CT scanning. Morphological changes are shown by a significant number of cysts with chemotherapy and PAIR (Morris *et al*, 1984; Filice *et al*, 1992; Nahmias *et al*, 1994; Filice and Brunetti, 1997), but also, there is evidence that, cyst structure even in untreated people can change and cysts disappearance can happen in time without any intervention (Romig *et al*, 1986; Morris, 1986; Pawlowski 1997; Wang *et al*, 2003; Wang *et al*, 2006).

Every cyst begins as a typical small unilocular cyst with clear cyst fluid. In some cases, the cyst is termed to be sterile as no further development takes place. While in most other cases, there is subsequent development of protoscolices within the brood capsules attached to the germinal layer. “For many cysts, change from this form is not experienced, and ultrasound examination can show growth of the cyst but no change in internal structure over several years” (Rogan *et al*, 2006). Other cysts show a significant level of variation in internal structure, such as calcification, collapsed cyst walls and the presence of additional daughter cysts internally. Exhibition of such heterogeneity in cyst structure has for clinicians been an issue in terms of how to treat CE. The presence of daughter cysts, could be an indication that PAIR is a less favourable option; a distinct, small, unilocular cyst might give a good response to chemotherapy, while a small, calcified cyst might signify poor parasite viability and a good prognosis and would thus be a candidate for long term observation only (Pawlowski, 1997).

1.13 Cyst classification

A number of CE classifications have been proposed in the last 20years based on the appearance of the ultrasound images, with the most recent by WHO (Gharbi *et al*, 1981; Lewall and McCorkell, 1985 ; Caremani *et al*, 1997 ; WHO/OIE, 2001). The first, most enduring and lasting attempt to characterize human CE by ultrasound was by Gharbi (Gharbi *et al*, 1981) Gharbi based his classification largely on the sonographical analysis of the morphology and structure of hepatic hydatid cysts in 121 CE cases that were confirmed by surgery. Five categories to indicate CE pathological cyst types were proposed by Gharbi: “ Type 1, pure fluid collection,

Type 2, fluid collection with a split wall, Type 3, fluid collection with septa, Type 4, heterogenous echo patterns, and Type 5, presence of reflecting thick walls indicative of calcification" (Gharbi *et al*, 1981 ; Wang *et al*, 2003).

Gharbi's classification was the basis, foundation for all subsequent ultrasound classifications, which included largely minor modifications and or additional categories. Fifty nine clinical CE cases formed the basis of Lewall and McCorkell's CE classification and 3 categories were proposed by them. Type 1, simple fluid-filled cysts; Type IR, lesions containing wavy membranes, representing detached endocyst secondary to rupture; Type 2, lesions contained of daughter cysts and/ or a formed echogenic material; and Type 3, dead, densely calcified lesions (Lewall and McCorkell, 1985).

The 7 categories of CE classification proposed by Caremani were based on 113 CE cases which included many asymptomatics detected in Italy. "Type 1, simple CE, which was further divided into 1a and 1b, with (1a), being echo-free and or (1b), with fine echoes; Type 2, multiple CE, also sub-divided into 2a and 2b, (2a) being a multiple contiguous and or (2b) being multiseptated with rosette, honeycomb and wheel-like pattern; Type 3, with detachment of endocyst CE, is also sub-divided into (3a), with double layer image or, (3b), with water-lily sign; Type 4, mixed type CE, with fluid and solid aspect; Type 5, heterogenous CE, sub-divided into (5a), with ball of wool pattern or, (5b), with hypoechogenic image; Type 6, hyperechoic CE, (6a), being with snow-storm pattern or, (6b), with dyshomogenous aspect; and Type 7, calcified CE, (7a), with advanced calcification of the layer only or, (7b), with calcification of overall cyst (Caremani, *et al*., 1997).

With the use of ultrasound morphology to monitor cyst development or regression on the increase, a more universal classification was considered necessary. With this aim, the WHO Informal Working Group on Echinococcosis (WHO-IWGE) proposed recently a standardized classification to bring about the unification and simplification of the various ultrasound CE classifications (WHO/OIE, 2001).Six categories were proposed by WHO-IWGE: “Type CL, unilocular cystic lesion(s) with uniform anechoic content, with pathognomic signs that is inclusive of visible cyst wall and “snowflake” signs; Type CE2, is a multivesicular, and multiseptated cysts; Type CE3, anechoic content with a detached laminated membrane from the cyst wall visible as a floating membrane or as a “water lily”; Type CE4, hyperechoicheterogenous, or hyperechoic degenerative contents, daughter cysts not present; and Type CE5, are cysts that are distinctive by thick calcified wall that is arch-shaped, giving a cone-shaped shadow, variation in the degree of calcification may be from partial to complete. Also included in the WHO-IWGE standardized ultrasound classification of CE are the size and biological status of the hydatid cyst(s). In summary, cyst size ‹5cm is classified as small (s), medium/middle (m) size is from 5-10cm, and ›10cm is large (L). Biological status also should be classified as “active” (which comprises of group 1: Types CE1 and CE2), “transitional” (these are group 2: Type CE3), or “inactive” (comprising of group 3: Types CE4 and CE5) (WHO/OIE, 2001). This classification for CE by WHO is yet to be applied in many clinical situations or mass screening programmes (Wang *et al*,2003). Although being able to classify cysts on the basis of their morphology in order to perform surgery is important, it is equally important that the classification gives some indication of developmental or degenerative changes in the cyst that has either occurred naturally or after therapy. Detachment of the laminated layer from the

ectocyst, presence of daughter cysts and cyst calcification have all been highlighted by all of the proposed classifications, these may be considered as regressive signs though not consequently indicating total cyst non-viablity.

However, there is significant discussion on the possible sequential nature of these morphological changes. Largely, this is associated to the sequence of events surrounding daughter cyst formation. Cysts with a detached laminated layer have been classified by Gharbi as Type 2, and those with daughter cysts as Type 3. This order was reversed by the Caremani system and the WHO classification classifying cysts with daughter cysts as Type 2 and those with detached laminated layer as Type 3 (Wang *et al*, 2003). Pawlowski, 1997 has suggested that in some cases changes in cyst morphology may represent a natural progression or a natural history in addition to the morphology of cyst being useful, important, vital, and significant in planning surgical intervention or chemotherapy. There have also been recorded cases where hydatid cysts naturally disappear over a long period of time (Romig *et al*, 1986). It is therefore vital that developmental succession of cyst structure is reflected in the ultrasound classification (Wang *et al*, 2003).An international classification of ultrasound images of cystic echinococcosis which should in principle is used whenever ultrasound diagnosis is done has been produced by the WHO expert group on echinococcosis (WHO/IWGE, 2003). In addition, laboratory-based diagnosis can provide a useful confirmation of clinical infection and can also be applied to aid epidemiological surveys of cystic and alveolar echinococcosis in endemic regions.

The Current Classification System of Cystic Echinococcosis Cysts (WHO/IWGE,2003)

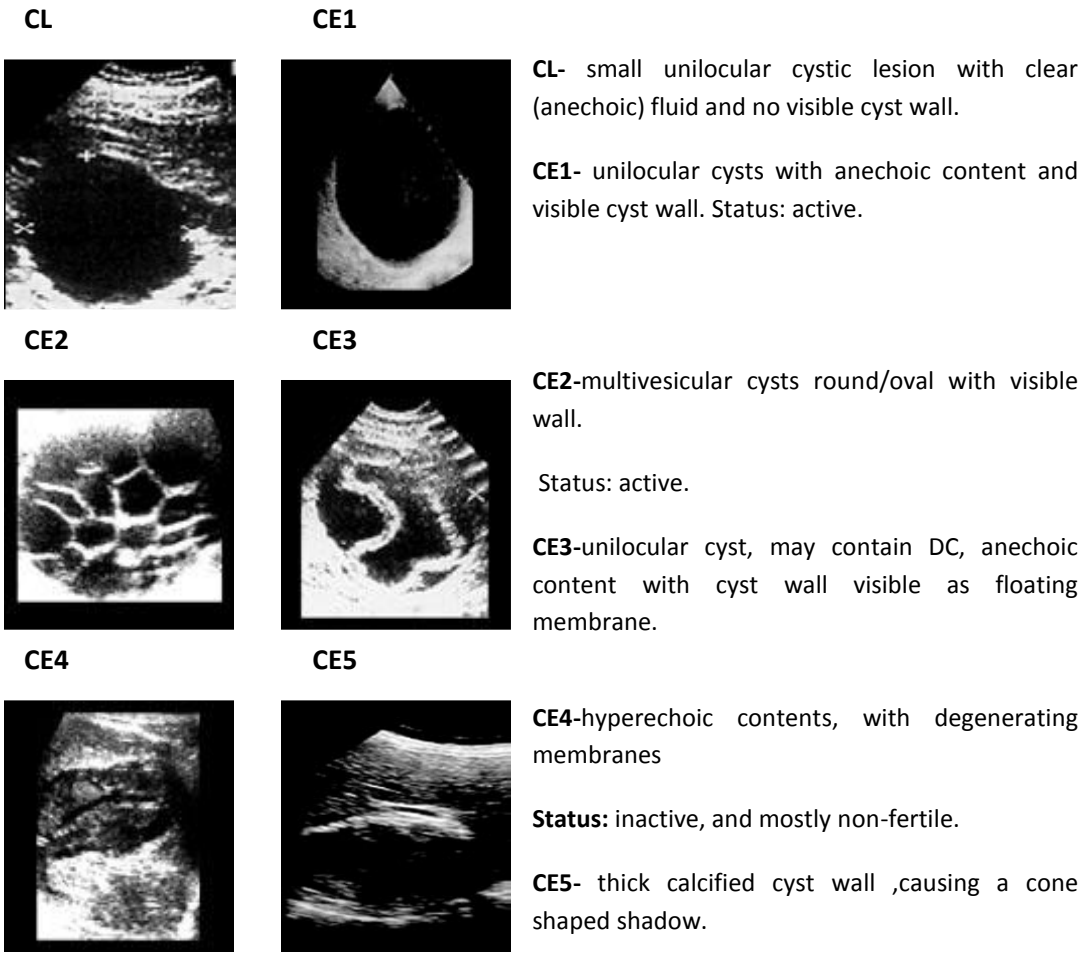


Figure 1.3: Classification of ultrasound images.
CE1-CE2 cyst types are considered fully viable, CE3 cyst type is considered and classified a transitional stage, and may remain viable or regress to a non-viable stage; CE4-CE5 are degenerative , non-viable and usually inactive stages.

1.14 Natural History of Hydatid Cyst

A change in morphology might or might not be noticed in cysts over a relatively long period of time in treated or untreated individuals. Distinctly, slow degenerative changes are shown by some hydatid cyst and have a natural history or evolution. The details of changes are less well defined and have nonetheless been addressed by several authors (Pawlowski, 1997; Daeki *et al*, 2000; Teggi and Di Vico, 2001).

“The most recent interpretation is based on the current WHO classification as a progressive natural history of cyst development from CE1 to CE5” (Macpherson *et al*,2004). It is enormously important to understand the possible developmental fate of a cyst, to make possible the monitoring and prediction of disease progression, regression and recurrence (Rogan *et al*, 2006).

However, there is some debate and controversy as to whether the WHO progression is too simplified, and alternative classifications have been suggested by some authors that would also relate to size of cysts and indicate a different order of events (Wang *et al,* 2003).Follow up of cases can be for as long as 8-10years after treatment, but there is huge variation between studies in respect to monitoring points, making comparisons difficult. Therefore, it might not be completely as to what stages a cyst has passed through between scans (Rogan *et al*, 2006). Based on several literature descriptions, it appears that several developmental pathways can come from a typical unilocular CE1 cyst (Wang *et al*, 2003; Teggi and Di Vico, 2001). The viability of the parasite tissues within the cyst is the important feature; the cyst has the ability to regenerate in some form if viable portions of germinal layer, brood capsule wall or protoscolices are present. Usually, CE1 cysts are viable and fertile (possessing protoscolices), while CE5 cysts are dead and calcified (Rogan *et al*, 2006). The remaining types and their viability are more questionable.

Discussions mostly have revolved around the Type CE2 cysts (with daughter cysts) and the Type CE3 cysts (with collapsing cyst walls). The CE3 type of cysts are being accepted as transitional, showing degeneration as a result of the observed collapse of the cyst wall, although some parasite tissues that are viable could still be

contained, as demonstrated by some cases that had reverted to type CE1 (Larrieu *et al*,2004).CE2 cyst types are classified as active and are not accepted generally as showing degeneration. Viability of the parasitic material of the daughter cyst is not questionable, but the viability of the primary cyst is, and this is important when the natural history is being considered (Rogan *et al*, 2006).

The lack of understanding of the origin of daughter cysts is a main problem, as there is no histological support for the assumption that these arise from the germinal layer. This interpretation has come under arguments based on ultrasound images that seem to show a series of progressively larger daughter cysts forming around the periphery of the primary cyst. It is however impossible to say that the cysts have arisen from the intact germinal layer from these images given that the germinal layer is less than 1mm thick (Rogan *et al*, 2006). In addition, the internal production of a laminated layer would not be allowed due to the polarity and orientation of the tissues within the germinal layer (Bortoletti and Ferretti, 1978). The relevance of this is particularly with those ultrasound studies that conspicuously show formation of daughter cysts by internal growth, endogenous proliferation of the germinal layer (Czermark *et al*., 2001). A vesicle, in these cases seen forming from the mother cyst wall would have the laminated layer on the inside and the germinal layer on the outside, which according to Rogan *et al*., (2006), is incorrect.

“The contrasting argument is that daughter cysts arise from fragments of “disrupted” germinal layer or other parasite tissues” (Rogan *et al*, 2006). The capability and ability to form miniature hydatid cysts in vitro have been shown by the brood capsule, wall, the protoscolex attachment stalk and or the protoscolex itself (Smyth and Barrett, 1980; Rogan and Richards, 1986). The greatest ability to differentiate into

hydatid cysts is possessed by protoscolices and is directly involved with causing secondary hydatidosis, and altered physiological conditions can trigger cystic differentiation of protoscolices in vitro (Smyth and Barrett, 1980). Studies have also shown direct evidence from cysts in livestock that protoscolices show cystic development within degenerate primary cysts (Rogan, 1988). Although, the appearance of the fluid within daughter cysts is usually anechoic (clear), the fluid within the primary cyst is often more hyperechoic (dense), which signifies the presence of some sort of debris or infiltrate (Rogan *et al*, 2006). This material was referred to as “matrix” by Lewall and McCorkell (Lewall and McCorkell, 1985) and cysts such as this are often full of pus or leucocyte infiltrate at surgery and debris from a degenerate primary cyst (Abu-Eshy, 1998; Teggi and Di Vico, 2001). Bacterial infection, although not always, is sometimes present (Schipper *et al*, 2002). It is clear that the germinal layer and the cyst wall cannot be intact if cellular infiltrate or bacterial cells are present in the cyst cavity, therefore, the formation of daughter cysts is involved with damage or primary cyst degeneration (Rogan, 1988; Teggi and Di Vico, 2001; Wang *et al*, 2003).

1.15 The Role of Cytokines in Echinococcosis

In regards to the investigation of cytokine production in AE and CE patients, several studies have been undertaken to determine the underlying immunological responses to infection and disease. Much of the current understanding of *Echinococcus* infections have resulted from murine studies because of the difficulty in studying early-stage infection in humans. Nonetheless, it is generally obvious from these investigations that the onset of infection is biphasic, with an early predominant

induction of a Th1 response, recruiting Th1 cytokines such as IFN-γ (Mourglia-Ettlin *et. al*, 2011), which then switches to a Th2 response, predominantly inducing IL-4, IL-5, IL-10, and IL-13, in chronic and progressive disease stages, bringing about the hallmark response characteristics of most helminthic infections (Rogan, 1998; Mourglia-Ettlin *et. al*, 2011). Degenerating cysts in murine models are by contrast associated with Th1 cell activity and the production of IFN-γ (Rogan, 1998) which shows the protective effect of Th1 cytokines during infection and disease. Even though immunological studies of early infections are more difficult in human populations, according to data available from epidemiology studies by Yang *et. al*, (2009); Vuitton, (2003) have shown that different cytokine profiles are displayed by natural courses of human AE and CE at different stages of disease progression.

Hypotheses have been made that Th1 type response is associated with the very early stages of infection, while Th2 cell activity is more associated with active disease and a poor response to chemotherapy, thereby giving support to murine study findings that class switching occurs between Th profiles (Mourglia-Ettlin *et. al*, 2011). The ability of the *Echinococcus* parasites to stimulate Th2 cytokines have been shown by several studies to be antigen-specific, thereby indicating that the parasite stage plays a vital role in the Th1/Th2 paradigm. Particularly, antigen B (AgB) has been shown to hugely contribute to this Th2 polarization (Rigano *et. al*., 2001). In addition, the immune response is dose-dependent, so that the higher the antigen dose, the greater the Th2 response. This is in consistency with disease progression in humans-wherewith increased antigen levels are produced by metacestode development, which in turn brings about a greater shift in the Th2 response, furthermore protecting the parasite.

However, following successful drug treatment with albendazole, the polarized Th2 response in advanced disease tend to revert back to Th1 (Rigano *et. al*, 1995; Rigano *et. al*, 1999). It is important to note that both Th1 and Th2 cytokines are produced in patients with active and/ or progressive disease; nonetheless, it is only those patients who are able to elicit a Th1 response that have been shown to respond well to chemotherapy, where patients maintaining higher levels of IL-4 and IL-10 do not (Rigano *et. al*, 1995; Rigano *et. al*, 1995; Rigano *et. al*, 1999; Rigano *et. al*, 1999).A Th1 profile also correlates well with good prognosis in patients following the removal of cysts surgically and in those with inactive, late-stage of CE5 cysts (Rigano *et. al*, 2004; Rigano *et. al*, 2004). An Algerian study of 177 patients with CE showed that the Th1/Th2 skew is correlated and related to clinical stage, disease progression and prognosis, with Th1 cytokine being associated with protection and susceptibility to disease associated with Th2 (Mezioug and Touil-Boukoffa, 2009). The ability of the patient to maintain a Th1 response or yield to a Th2 response eventually decides whether he/she is vulnerable or resistant to disease and respond successfully to treatment (YuRong, *et. al*, 2012).

Early antibody production is thought to be most essential for the development of resistance to infection (Dempster, *et. al*, 1992). During early infection, an increase in antigen- specific IgG production is indeed observed, which is thought to trigger down-stream responses including the production of cytokine. Even though there is little understanding about early infection, given the expressed difficulty in early diagnosis of the disease, IgG1, IgG4, IgE and IgM are dominant in patients with chronic disease, but relatively low levels are attained by these isotypes in patients

with inactive or regressive disease (Craig, 1986; Daeki *et. al*, 2000; Khabiri *et. al*, 2006).Contrastingly, levels of IgG2 and IgG3 become elevated when cysts become infiltrated and/ or destroyed by the host (Daeki *et. al*, 2006), during which time clearly noticeable decreased levels of IgG1 and IgG4 are observed (Bayraktar, *et. al*, 2005). In murine studies of both AE and CE, antibody titers were found to be comparatively consistent in affected mice, notwithstanding the susceptibility of the host strains (Vuitton *et. al*, 2006), but changes according to the severity of the infection were observed (Vuitton *et. al*, 2006). This is in line, in consistency with the natural growth of cysts of both *E. multilocularis* and *E. granulosus* where protection against the immune response is provided by the intact cyst wall.

Nevertheless, for *E. granulosus*, where growth is rapid or where the cyst becomes excessively large, rupture may occur bringing about the rapid recruitment of host antibodies in response to antigen B, particularly isotypes of IgG2 and IgG3, which are capable of damaging cystic germinal membranes, giving rise to the killing of protoscolices and cyst degeneration (Siracusano *et. al*, 2008). Subsequent to the establishment of an AE infection, the acellular laminated layer (LL) of the parasite lesion, which is characterized by its rich high molecular-weight polysaccharide composition- with the mucin-type glycosylated Em2 protein antigen being a major component (Dai *et. al*, 2001), is capable of restricting the physical exposure of the germinal layer to the host immune system and bring about the production of low-avidity IgG isotypes. It is known that parasite antigens trigger antibody production, particularly antigen B, which excite the production of IL-4 and IL-13 and suppress the Th1 response through polyclonal antibody incitement (Rigano *et. al*, 2001; Mourglia-Ettlin *et. al*, 2011). Several studies, both in mice and humans have shown that Th1

cytokines, mostly IFN-γ, are well correlated with IgG2 levels and disease progression. Consequently, useful markers or indicators of disease activity and of the natural course of disease/cyst development can be provided by measuring cytokine and antibody profiles, particularly the IgG subclasses (Rigano *et. al*, 1995; Vuitton, 1997; Daeki *et. al*, 2000).

An indication to whether a patient would respond to treatment or not may also is offered by these immune profiles. Regardless of this, there remains a substantial variance in the immunological response between patients that may be affected by the parasite strain and/ or antigen type produced, which influences the development of T helper subsets. Antigen dose and the genetic background of the host are other factors that may also contribute (Emery *et al*, 1997; Eiermann *et. al*, 1998). Contributing significantly also is the general well- being and health of an individual to disease susceptibility and this is especially influenced by conditions underlying such as malnutrition and/ or coinfections with tuberculosis (Vuitton, 2003) or HIV (Sailer *et al*, 1997; Wellinghausen *et. al,* 1999; Zingg *et. al*, 2004).

1.16 POST-TREATMENT FOLLOW-UP

The pre-requisite for the evaluation of failure or success of curing disease is the long-term post-operative treatment and serological surveillance**.** Surgery still is the main treatment of hydatid cyst, even though chemotherapy may be used in some cases (El-On, 2003; Kern, 2003). There has been more than 30% reported cases of local recurrence or secondary infection during surgery (Rafiei *et. al*,2008). Also, during chemotherapy, progress of treatment is difficult to ascertain. Consequently,

monitoring of CE patients after surgery and during chemotherapy has been emphasized. Despite limitations encountered with serological tests, due to their cost effectiveness and improvement facilities, they are probably best choice for follow-up assessment of CE after either surgery and / or chemotherapy (Rafiei *et. al*, 2008).

Serological diagnosis in a routine laboratory depends mainly on the detection of immunoglobulin class G (IgG) antibodies directed against different antigens of *E. granulosus* or *E. multilocularis* (Grimm *et. al*,1998). Sensitivity and specificity of the serological tests depend on the stage of the disease, the localization of the parasites, the antigens, and the techniques used (Gottstein, 1992; Craig, 1993). One of the most widely used antigens is the cyst fluid (CF) of *E*. *granulosus* cysts of sheep or cattle origin, and the enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used techniques in serodiagnostic laboratories.In cases of CE of the liver, antibodies against CF antigens can be detected with a high diagnostic sensitivity by this method (Grimm, *et. al*., 1998).

Rationale of study

To date, most of the work carried out on CE has been in serological diagnostic testing involving antigens derived from HCF and more recently, molecular techniques such as the PCR. Also, imaging techniques have been used to date in the classification of cyst types. There has not been so much done with regards to serological classification of cysts before treatment and in the area of follow-up after treatment using antigens other than the native hydatid cyst fluid (HCF) and purified

antigen B (AgB). To bring about the development of a means of measuring the immunogenic activity in sera of CE patients using antigenic markers, which, may potentially create antigen/ antibody profiles signalling the progression or regression of disease in relation to/ with particular categories of cyst.

The use of these markers in association with IgG subclasses may bring out and reveal information distinctively, with emphasis particularly on the development of a more specific means of monitoring success or failure of therapy during post-treatment, follow-up and surveillance. Studies by Doiz *et. al*, (2001) showed that antibodies from CE patients with specific proteins of molecular weights 39KDa and 42KDa from the antigen B/5 rich fraction of HCF by western blot analysis may be useful in the status of the disease, as these bands were present in patients with progressive disease, but absent in cured patients.

The use of western blot with a purified antigen was analyzed by Doiz *et al*,(2001**)**, so as to evaluate and determine its possible application in post-treatment monitoring. Following their purification procedure, they were provided with proteins of the following molecular weights 12-14, 16, 20, 24-26, 34, 39 and 42KDa and went on to prove that the western blot technique shows a disappearance of the bands in the case of cures, as well as persistence and appearance of new bands in the opposite case. In the usage of antigens other than HCF and antigen B, little work has been done. However, a recent work by Taherkhani *et al*,(2007) has shown that a significant proportion of hydatid patients recognised extracts of sheep hydatid cyst laminated layer containing proteins of low molecular weights.

The aim of this study is to investigate the recognition of crude antigens by sera of patients of confirmed hydatid disease of different cyst types and stages in relation to their reactivity with whole immunoglobulin (IgG) and IgG subclasses 1 and 4 in an attempt to identify, classify and determine disease categorisation using immunological markers.

The objective of the current study is to classify the laminated layer of *Echinococcus granulosus* using different immunological methods with a view to using it as an antigenic material in studies involving the diagnosis of hydatid disease and follow-up after treatment.

CHAPTER 2

MATERIALS and METHODS

2.1 Antigenic Materials

2.1.1 Hydatid Cyst Fluid (HCF)

Liver and lung of infected sheep were collected from a local UK abattoir brought back to the laboratory at University of Salford. Carefully, cysts were removed, aspirated and antigen or hydatid cyst fluids were collected according to the method described by Rogan *et. al*, (1991). A brief description, hydatid cysts were aseptically aspirated using sterile 5ml syringe and needle, the fluid was centrifuged and the supernatant obtained was used as the crude hydatid cyst fluid and this was put in sterile 50ml bottle and stored at -20℃ until use.

2.1.2 Laminated Layer (LL)

The laminated layer was prepared as described by Taherkhani *et. al*, (2007). The laminated layer was carefully removed from the whole cyst under magnifying microscope using forceps and scapels. After which the parasite layer was kept frozen, and then thawed and cut into 1cm strips, put in 1ml PBS and freeze-thawed twice. Upon thawing, the laminated layer was weighed, and for the purpose of this study 17g was used. The strips were cut into smaller pieces and ground to a pulp using mortar and pestle. The mixture, i.e.17g of laminated layer and 17mls of 10% PBS was transferred into a plastic beaker, put on ice, and sonicated in a 150W sonicator for 2minutes at 10secs on and 10secs off cycle. The resultant milky-like liquid was transferred to sterile 1.5ml eppendof tubes and centrifuged at 10,000 rpm for 15mins. The supernatant was transferred to new sterile tubes and stored at -20℃ until use.

2.2 Sera.

Serum samples used for the purpose of this current study were collected as part of the African Medical and Research Foundation (AMREF) Hydatid Control Programme, based in Lokichoggio, Kenya under the management of Dr Eberhard Zeyhle. Collection of many of the samples was made over a long period of time, but all samples were stored at -20°C without repetitive freeze thaw. Each patients diagnosis was confirmed at AMREF by the using ultrasound examination. Each patient was monitored at regular intervals by ultrasound and details of cyst size and morphology recorded by photographs and notes. The classification of cyst types observed was based on matching the ultrasonographers' notes to the WHO 2003 classification system under the guidance of Dr Zeyhle. Serum samples were taken at each observation point. Most patients were put on a schedule to take one or more courses of albendazole at 20mg/kg/day (see Table 2.1). Some patients experienced a delay before their first course of albendazole was taken and these could be regarded as "untreated" for the preliminary part of their observation. Treatment was refused by some other patients and these also were regarded as untreated.

Several hundred serum samples from 45 confirmed hydatid patients in total were supplied by AMREF. These samples had not been sorted into any order at the beginning of the current study. In order to select panels of sera that are usable, a database of patient details and clinical (ultrasound) information was established using data collected at AMREF. Serum samples matching the clinical data were then identified comprising 22 patients who had undergone treatment and who had continuous sampling times, and 9 untreated patients.

For optimisation pourposes of the crude antigen ELISA assays and analyses of CE patients sera, negative controls comprised a pool of normal sera from a CE endemic area (Turkana, Kenya). For Western blot analysis and ELISA detection of total serum IgG and IgG subclass antibodies from the sera of infected individuals probed, normal human sera was used for negative controls (Sigma-Aldrich, UK).

Table 2.1 Summary of Patient Information

Patient ID	**Gender/ Age**	**Cyst series**	**Organ Involvement**	**ALB Chemotherapy (mg/kg body weight)/ surgery**	**Sera Timeline**
B142	Female 6	CE1, CE4	R/ liver	Untreated	0-42.7months
P160	Female 16	CE2, CE3	R/ liver	Untreated	0-33.9months
X345	Female 20	CE2, CE3	Omentum/ mesentery	Untreated	0-3.6months
X161	Female 17	CE3, CE4	R/ liver	27.6mths ALB (20mg).	0-31.6months
Y88	Female 5	CE1	R/ liver	4.8mths 2 courses ALB (20mg); 17.8mths ALB (20mg).	0-8.4months
Y13	Female 20	CE1, CE3, CE4, CE5	R/ liver, lower abdomen	3.8mths 3 courses ALB (20mg); 22.2mths ALB (20mg); 58.7mths ALB (20mg); 62.3mths endocystectomy.	0-209.8months
Y28	Female	CE1,	R/ liver	5.5mths 3	0-

	20	CE3		courses ALB (20mg); 41.6mths cystectomy and 2 courses ALB (20mg).	46.9months
Y51	Female 10	CE1, CE3, CE4	R and L/liver, Omentum/mesentery	1.6mths 1 course ALB (20mg).	0-10.5months
X269	Female	Data not available	Kidney	Untreated	Data not available
Y63	Female 25	CE1, CE3	R/ liver	At point "0" 1 course ALB (20mg).	0-47.3months
Y179	Female 22	CE2, CE£	R/ liver	0.1mths 3 courses ALB (20mg); 15.1mths ALB (20mg); 23.9mths ALB (20mg).	0-42.7months
Y235	Female 46	CE2, CE3	R/ liver	2.2mths ALB (20mg).	0-6.2months
Y111	Female 30	CE1, CE2, CE4	R and L/ liver	0.1mths 3 courses ALB (20mg); 15.1mths ALB (20mg); 23.9mths ALB (20mg).	0-46.2months
156	Female 33	CE2	R and L/ liver, Omentum/ mesentery	1.8mths 2 courses ALB (20mg); 4.2mths had endocystectomy.	0-4.1months

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) was performed following previously standardized ELISA protocols by Rogan, (1997). The pooled positive and negative human serum was tested for *Echinococcusgranulosus*antigens. Optimum antigen concentration and dilutions were pre-determined by checkerboard titration, and an antigen concentration and dilution of 1:100 (1ml of antigen and 9ml of Bicarbonate carbonate buffer) was used for the purpose of this study.

Immunolon B1 microtiter plates were coated with HCF, LL at 100µl per well, and left to incubate overnight at 4℃. The next day, plates were brought out and washed three times with washing buffer, 0.1%PBS Tween 20, as was at every stage of the assay. Subsequently, plates were blocked with 0.3%PBS Tween 20 and 5% skimmed milk powder and left to incubate for 1hr at room temperature. All human sera samples were diluted to 1:100 in 0.3%PBS T20 and 5% skimmed milk powder and incubated for 1hr at room temperature, which was followed by incubation at 1:10,000 (for HCF) dilution of anti-human IgG (whole molecule) conjugated to alkaline phosphates in 0.3%PBS T20 for a further incubation at room temperature for 1hr, and 1:2000 for IgG subclasses 1-4. The substrate solution, consisted of 5mg p-nitrophenylphosphate (PNPP) in diethanolamine buffer (pH 9.8) (3 tablets of PNPP in 15ml of diethanolamine buffer) was left to incubate at room temperature for 30min. The absorbance values, optical densities were measured at 405nm, using an automatic microplate reader (Thermo scientific multiskan FC). A dilution concentration of 1:100 was used for the LL antigenic material and sera, but a dilution concentration of 1:2000 was used for conjuagtes, whole IgG and IgG subclasses used for the purpose of this study. Absorbance values were read as above.

2.4 SDS-PAGE

The separating gel, (12.5µl of Acrylamide stock, 11.2µl of TrisHCl pH8.8 and 6.2µl of distilled water, 300µl or 0.3µl of 10% SDS, 100µl of 10% freshly made APS and 20µl of TEMED (which causes polymerization), was loaded on the electrophoresis gel kit, gels were covered with butanol after loading to allow the formation of a fine, clean and smooth edge and left to polymerize for 1hr 45min. The stacking gel (1.75µl of Tris pH 6.8, 2.33µl of Acrylamide stock and 5.682µl of distilled water) was loaded and combs were inserted carefully immediately and left to polymerize for 1hr.

Gels were transferred unto the electrophoresis kit, clamped tightly, connected to cold running tap and filled with running buffer, pH 8.3 to maintain the current. Combs were eased out carefully, and samples (equal amount of sample and sample cocktail, (in this case 50µl of each) that has been boiled for 5min) were loaded into wells. 20µl of the marker (MultiMark, Invitrogen) was loaded, as well as 20µl of each sample. To allow easy dissociation or separation of the proteins through the stacking gel, the gel was run at 100v at 20mA at the beginning, after which voltage was increased to 150v until the end of the electrophoresis.

At the end of the assay, the gel was transferred into a tray, covered with coomasie blue for staining for 1hr, and covered with cling film and left on a rocking platform. At the end of 1hr, the gel was washed with distilled water, transferred into another tray and submerged in de-stain for 1hr, after which it was washed and re-submerged in fresh de-stain and left overnight on the rocking platform. De-stain was discarded the next day, the gel was washed in distilled water and images were taken under the UV light.

2.5 Western Blot/ Immunoblot.

Extracts were fractionated by means of SDS-PAGE, after which gels were transferred carefully to an automatic blotting device (iBlot (Gel transfer device) by Invitrogen, and in 7min blotting was completed, the nitrocellulose paper was washed and blocked with 0.3%PBS Tween 20 and milk for 1hr after which it was washed three times with washing buffer, 0.1%PBS Tween 20, followed by incubation with human sera samples with concentration of 1:100 and diluted in 0.3% PBS T20 with skimmed milk at room temperature. At the end of 1hr, this was followed by incubation with IgG subclasses, or IgG whole molecule at 1:5000 and 1:10,000 respectively (for HCF), for 1hr at room temperature. Washing was done three times for 1min each at every stage of the assay. The substrate solution consisted of 1 tablet of BCIP/NBT (Sigma) in 10mls distilled water and left to incubate at room temperature for 30min, after which photographs were taken under the UV light.

2.6 Lectin Assay

The lectin binding assay of the extracts of the laminated layer of *E. granulosus* was carried out according to Shimizu et al (1982) with little modifications as follows. The crude LL was electrophoresed as above on 12% polyacrylamide gel and transferred onto nitrocellulose papers (NCP). Proteins were transferred using iBlot (gel transfer device) by Invitrogen. After washing with PBS, the NCP was cut into strips and each strip was treated with 0.15M NaCl, 0.01M Tris-HCl, 0.5mM CaCl2 (pH 7.4) containing 1% bovine serum albumin (BSA) at RT for 1hour. The NCP strips were

washed with PBS afterwards for few minutes. Different peroxidase labelled lectins was dissolved in the above buffer at a concentration of 50µg/ml for 1hour. After incubation, the strips were washed again with the same buffer, but without BSA. The presence of lectin conjugates was visualised by the incubation of the NCP strips in 10ml of 0.05% diaminobenzidine (DAB) and 0.01% H2O2 in 0.1M Tris- HCl (pH 7.4) at RT for 1-2min. The following peroxidase labelled lectins were used; Concanavalin A (Con A), Soybean agglutinin (SBA), Wheat germ agglutinin (WGA) and Horse gram agglutinin (DBA). (See table 3.1 for sugar specificities).

2.7 Affinity Chromatography

The work of Taherkhani *et al* (2007) indicated that the most significant immunoreactive bands in the laminated layer showed good binding with the WGA lectin. It was therefore decided to try to affinity purify these components from the crude extract, using WGA sepharose 4B column (Pharmacia, Germany). The choice of column was mainly because as described by Taherkhani et al (2007), WGA binds with the components on the LL and also because it was readily available at the time of this study. A 2 ml column was packed into a syringe according to the instruction of the manufacturer and equilibrated with 20 mMTris-HCl, pH 7.4 containing 0.5 M NaCl. The column was connected to a peristaltic pump and a UVI Cord optical density monitor also connected to a chard recorder. 0.5ml of laminated layer extract was run through the column and followed by 20 mMTris-HCl, pH 7.4,
containing 0.5 M NaCl until the chart recorder reached base level. Adherent molecules were then eluted from the column using 0.2M α-D-methylglucoside. The

elution fraction was collected into eppendof tubes by observing the OD trace on the chart recorder and stored frozen until further use.

CHAPTER 3

CHARACTERIZATION OF LAMINATED LAYER EXTRACTS

3.1 Introduction

Larval echinococcosis (traditionally referred to as hydatid disease) is caused by the larval stages of the cestodes belonging to the genus *Echinococcus* in mammals, including humans (Thompson, R.C.A.1995). Basically; they have a bladder-like morphology and are established in the parenchymas of internal organs, especially liver and lungs. They are protected by a layer of extracellular, carbohydrate-rich material, termed the laminated layer (LL), which is syntheized by the underlying cellular germinal layer (GL). The LL is fundamentally a meshwork formed by higly O-glycosylated glycoproteins of the mucin type. The glycans decorating these mucins

are hugely based on galactose and the structure is now partly known, even though the sequences of the corresponding peptide backbones are not (Diaz *et. al*, 2011).

Evidently, the mucin meshwork accounts for the LL in *E. multilocularis*, and possibly the LL of a minor species, *E. vogeli*. In comparison, the LL of *E.granulosus* contains in addition, nanodeposits of calcium myo-inositol hexakisphosphate (Diaz *et. al*, 2011). The LL is said to be the most important element of the host parasite interfaces in larval echinococcoses. Because it is the parasite structure that is exposed to the host, it is involved in multiple interactions with the immune system, and to a very large extent, it modifies the larval echinococcal infection immunology.

"The LL is a macroscopically coherent and elastic structure, based on a microscopic three-dimensional meshwork of hydrophilic, highly hydrated fibrils". Viewed under the transmission electron microscope, the fibrils are arranged irregularly and are approximately 10mm in diameter (Diaz *et. al*, 2011).In addition to the fibrillar meshwork, *E. granulosus* is composed naturally of electron- dense granules that occurs individually or in clusters (Morseth, 1967). According to Richards *et. al*, (1983), these granules were not only determined to have 41nm size, but were also determined to be composed of from 8nm electron-lucent spheres fused together. These granules, even though conspicuous in *E. granulosus*, have never been reported in *E. vogeli* or *E. multilocularis* (Sakamoto and Sugimura, 1969, Ingold, et*. al*, 2001).

The name laminated layer is got from the concentric laminations it shows under light microscope. Viewed under scanning electron microscope, the laminations give sectioned *E. vogeli* LL an open book appearance with pages that are very thin (Ingold *et. al*, 2001). In contrast, the *E*. *granulosus* LL appears as more compact

(Elissondo *et. al*, 2007).The origin of the laminations is unknown (Morseth, 1967), but by viewing under transmission electron microscope, they appear to be a resultant from different compaction degrees adopted by a single type of ultrastructure. The fibrillar meshwork is comprised of the abundant carbohyrates that characterize the LL (Richards *et. al*, 1983). Pioneering works by Kilejian *et. al*, (1962); Kilejian and Schwabe, (1971), and Russi *et. al*, (1974) determined that the carbohydrate component could not be separated away from proteins. Kilejian *et. al*, (1962) defined the carbohydrate-protein complex as a mucopolysaccharide. It was a correct description at that time, even though, today, the term mucopolysaccharides refer to proteoglycans, which the LL does not contain.

The LL meshwork is formed rather by the other major type of highly glycosylated glycoproteins, the mucins. According to kilejian *et. al*, 1962; Korc t al, 1967; Kilejian and Schwabe, 1971 ; Russi *et. al*, 1974 ; the monosaccharide composition of the LL, galactose only (Gal), N-acetylgalactosamine (GalNAc) and N-actylglucosamine (GlcNAc) is only compatible with mucin-type O- glycans among the forms of glycosylation known in animals. In addition to the O-glycans, animal mucins can possess limited numbers of N-glycans (Devine and McKenzie, 1992). Nonetheless, in the LL constituents this does not seem the case, as mannose (invariably present in N-glycans) is not detectable in the crude hydatid cyst wall, i.e the GL plus the LL (Diaz *et. al*, 2009). In 2002, a purified molecule from *E. multilocularis* metacestode using anti- carbohydrate monoclonal antibody reacted selectively with the LL (Em2(G11), (Deplazes and Gottstein, 1991; Dai *et. al*, 2001), and based on its high molecular weight, high threonine content, and decoration with mucin type O-glycans was defined as a mucin (Hulsmeier *et. al*, 2002). The glycome of the *E. granulosus*

LL was recently tackled and mucin-type O-glycans that are related to those described in the *E. multilocularis* mucin were revealed (Hulsmeier *et. al*, 2002), but reaching larger sizes (Diaz *et. al*, 2009).

According to Hokke *et. al*, (2007), the major features of the structure of the LL glycans are (1) the construction from cores 1 and 2 (2) the quantitative dominance of the non-decorated cores with respect to more elaborate glycans also present, (3) the lack of sialylation, expected in invertebrates; (4) the lack of fucosylation, which sharply contrasts with the glycobiology of schistosomes, (5) the elongation by (Galβ1-3)n , which was unknown previously; and (6) the capping of glycans by Galα 1-4 (which are thought to be also probably present in protoscolex glycoconjugates (Baz *et. al*, 1999). The non-decorated core 1 is probably a major LL glycan across the genus, as the structural data suggests (Hulsmeier *et. al*, 2002; Diaz, *et*. *al*, 2009), and by the binding of lectins specific for it (PNA, Jacalin) to the LL of *E. multilocularis*, *E. vogeli*, and *E. granulosus* (Ingold, *et. al*, 2001; Ingold, *et. al*, 2000; Casaravilla and Diaz, 2010). Also, probably shared with other species is the virtual absence of N-glycans in the *E. granulosus* LL, as conconavalin A, which binds N-glycans, labels the GL but not the LL of *E. multilocularis* (Ingold, *et. al*, 2000), with a similar, although less clear-cut result obtained for *E. vogeli* (Ingold, *et. al*, 2001). By contrast, some carbohydrates motifs present in the LL can be species specific. For example, neither the antibody Em2 (G11) nor a polyclonal antiserum against the *E. multilocularis* LL react with the *E.vogeli* LL (Deplazes and Gottstein, 1991; Ingold *et. al*, 2001). Also present in the *E.multilocularis* is the non-decorated O-linked GalNAc but not in the *E. granulosus* LL (Hulsmeier *et. al*, 2002 ; Diaz *et. al*, 2009). The deployment of a large huge LL especially in *E. granulosus* can be considered a

biosynthetic feat by the much thinner GL. Available microscopical evidence and biological common sense has made it clear that the GL carries out a polarized exocytic activity that results in LL build up (Rogan and Richards, 1989). More precisely, the GL syncytial tegument is responsible for this. There is similarity between this tegument and that present in the internal surface of the brood capsules and external surface of the protoscolices, it is syncytial and also microtriche bearing. Nonetheless, in comparison, the GL tegument is specialized, with that of the brood capsules being thicker and presenting numerous vesicles, and abundant, large mitochondria (Sakamoto and Sugimura, 1970), presumably, reflecting necessities associated with LL biosynthesis. The brood capsule functions to generate protoscoleces through inward budding. Nevertheless, brood capsules that are everted can synthesize an external LL, and probably give rise to daughter cysts (Rogan and Richards, 1986). Brood capsules that are intact can even synthesize an inward facing LL, in what is obviously an abnormal developmental pathway (Conchedda *et. al*, 2008).

Therefore, the normally inward- facing tegumental pole of the brood capsule, similar to the outward-facing tegumental pole of the GL has a quiescent but normally suppressed capacity to secrete LL components. Notice should be taken that the protoscolex tegument can differentiate into GL tegument and the synthesize LL during reverse development towards metacestode, as it is obtained in secondary infections (Diaz *et. al*, 2011). Soon after the beginning of cystic development, the synthesis of LL starts. As early as three days after activation, *E. granulosus* oncospheres developing in vitro secrete fibrillate material. Although, its not until day

six that the fist material resembling the mature LL meshwork appears (Harris *et. al*, 1989). A second wave of fibrillar material is secreted by day eight, which could be delineated outwardly and inwardly by particles possibly the $InsP_6$ deposits. The LL has been observed in vivo to appear 14 or 20 days after infection by *E.multilocularis* oncospheres (Rausch,1954; Sakamoto and Sugimura, 1970; Gottstein *et. al*, 1992). There is a probability, tendency that LL formation is delayed, appearing after approximately 28days in vitro (Heath and Osborn, 1976), or after 20-40 days in vivo (Rogan and Richards, 1989; Breijo, *et. al*, 2008) when metacestodes develop from protoscolices, which need to become re-programmed for reverse development. With every chance and possibility, the LL O-glycans are synthesized in the Golgi apparatus of the GL tegumentary cells. Somehow, the LL must be turned over or re-modeled. Subsequent and successive laminations are from the inside of pre-existing ones, pushing these towards the outside. The parasite can grow at the time of commencement of LL synthesis from approximately 30µm in diameter to tens of cm (for *E. granulosus*), the external strata of the LL must always be under tension (Harris *et. al*, 1989). This is manifested in the turgidity of normal *E. granulosus* cysts, and in solitary LL pieces curling up with the opposite concavity, hollowness to that found in the intact cyst (Richards *et. al*, 1983).

In spite of the fact that the LL is elastic, elasticity on its own cannot explain growth by up to six orders of magnitude in linear dimension, and therefore mechanical and/ or chemical loosening of the structure must take place (Diaz *et. al*, 2011). The need to (1) provide mechanical support for the turgidity of metacestode, possibly a contributory factor to parasite growth, and (2) protect GL cells from host immunity, might have influenced the main evolutionary pressures that gave rise to the LL.

Fulfilling the functions must be done in such a way that parasite nutrients and waste products are allowed passage, and parasite growth is permitted. The physically coherent, elastic, hydrophilic meshwork, that allows the diffusion of macromolecules to at least up to 150KDa has been the evolutionary answer to these requirements (Coltorti and Varela-Diaz, 1974), but protects the GL from host leukocytes. The parasite is not utterly made insensitive to host inflammation, this is attested for by the death of established metacestodes when inflammatory resolution fails. It instead bestows a partial protection against host effectors, but furthermore and more importantly, it appears to downregulate inflammation. The major source of *Echinococcus* molecules that the immune system of the infected host comes in contact with must be the massive LL. “This comprised the adhesion of leukocytes to LL external surface and the interaction, the relationship that is between soluble host recognition molecules and the large solvent exposed area represented by the entire thickness of the LL” (Diaz *et. al*, 2011), additionally, material shedding from the LL outer strata is a prerequisite for parasite growth. Recognised molecules by LL monoclonal reactive antibodies are released from *E. multilocularis* vesicles in vitro (Gottstein *et. al*, 1992; Walker *et. al*, 2004). Host macrophages, in experimental infections adhere to the LL outer surface and bring about the phagocytosis of LL-derived particles (Richards et al, 1983; Gottstein and Hemphill, 1997). Together, the large exposure of the host immune system to the LL and the complete profile of regulatory responses in larval echinococcoses suggest that the components of LL bring about regulation.

This view, widely held (Rogan, 1998; Conchedda *et. al*, 2004; Vuitton and Gottstein, 2010), is supported by observations that inflammatory resolution in *E. granulosus*

and *E. vogeli* infections correspond in time with LL deployment (Rausch, 1954; Breijo *et. al*, 2008), which, depending on the model, takes place two to six weeks post-infection (Diaz *et. al*, 2011). Currently, the immunological reasoning behind whether the LL induces regulation dictates that for it to be then the innate immune system must have interpreted it as a non- dangerous material, consequently initiating pathways that generate adaptive regulatory responses (Diaz, and Allen, 2007). Evasion of the immune system by chronic pathogens is now indeed believed to be dependent on expansion and/ or the recruitment of natural and/ or adaptive Treg cells locally (Grainger *et. al*, 2010), in addition to the induction of IL-10 expression by effector T-cells (Jankovic, *et. al*, 2010).

3.2 MATERIAL AND METHOD

The laminated layer was prepared as described by Taherkhani *et. al*, (2007). The laminated layer was carefully removed from the whole cyst under magnifying microscope using forceps and blades. After which the parasite layer was kept frozen, and then thawed and cut into 1cm strips, put in 1ml PBS and freeze-thawed twice. Upon thawing, the laminated layer was weighed, and for the purpose of this study 17g was used. The strips were cut into smaller pieces and ground to a pulp using mortar and pestle. The mixture, i.e.17g of laminated layer and 17mls of 10% PBS was transferred into a plastic beaker, put on ice, and sonicated in a 150W sonicator for 2minutes at 10secs on and 10secs off cycle. The resultant milky-like liquid was transferred to sterile 1.5ml eppendof tubes and centrifuged at 10,000 rpm for 15mins. The supernatant was transferred to new sterile tubes and stored at -20℃ until use.

3.3 RESULTS

Extracts of the laminated layer of *E. granulosus* were fractionated by SDS-PAGE under reducing conditions. Results obtained showed bands in the 8, 22, 55 and 98KDa regions.

3.3.1 SDS-PAGE

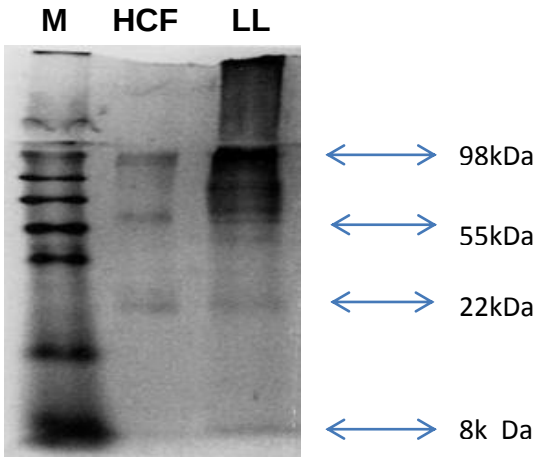


Figure 3.1: An SDS-PAGE Image showing the dissociation of the LL proteins as compared to HCF. Lane 1 (Marker), Lane 2 (HCF), Lane 3 (LL).

3.3.2 Reactivity of the Laminated layer in ELISA

To test whether the laminated layer extract was recognised as an antigen in ELISA, fourteen hydatid positive samples were tested for Total IgG and IgG1 and IgG4 subclass responses. These were compared with Sheep Hydatid Fluid antigen.

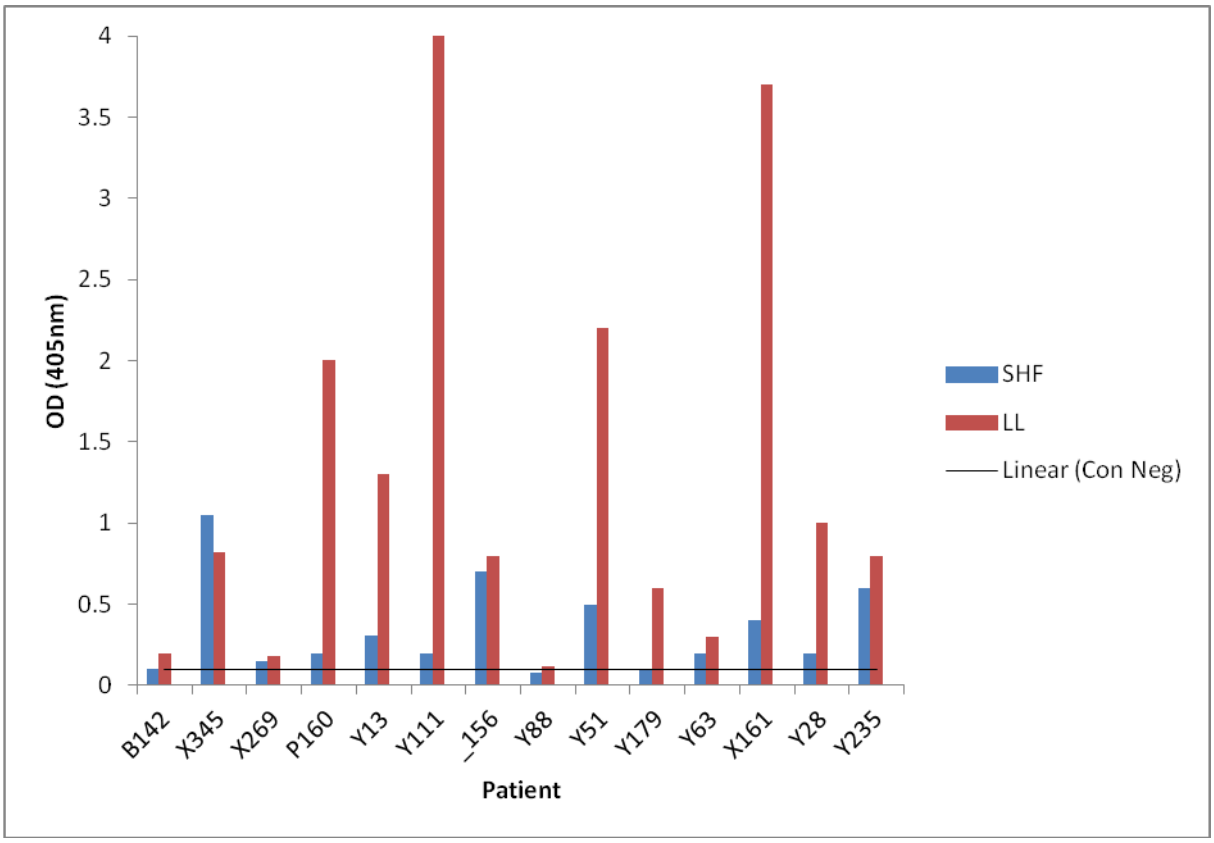


Figure 3. 2: Initial ELISA for fourteen confirmed Hydatid patients screened against cyst fluid (HCF) and Laminated layer (LL) antigens. The horizontal line represents the background level for clinically negative sera for both antigens.

3.3.3 Immunoblots

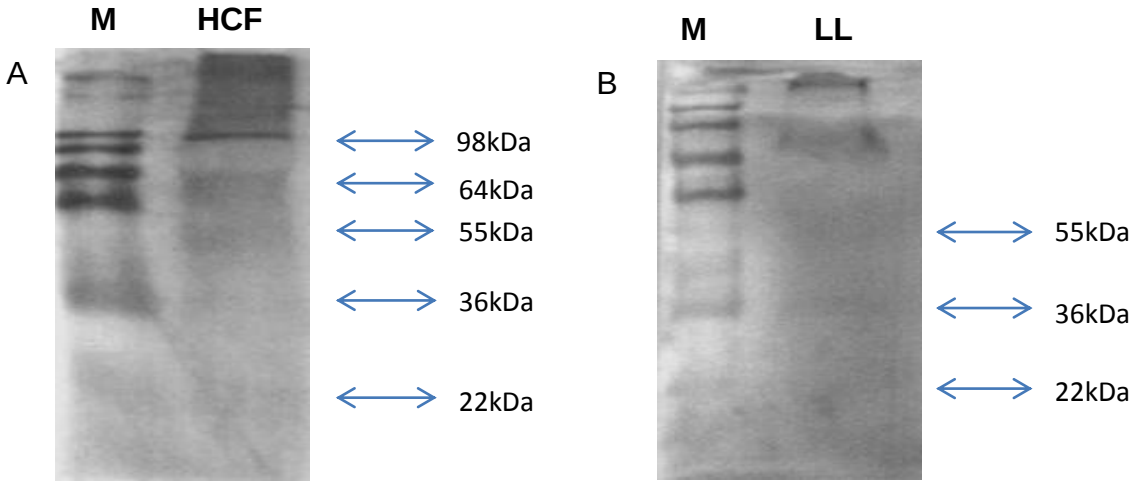


Figure 3.3: Immunoblot images of the reactivity of (A) Total IgG with HCF (Figure 3.3A), (B) Total IgG with extracts of LL (Figure 3.3B), both tested with pooled positive sera. Both samples recognise bands at 22 and 36KDa. Bands at 55KDa were also recognised by the LL.

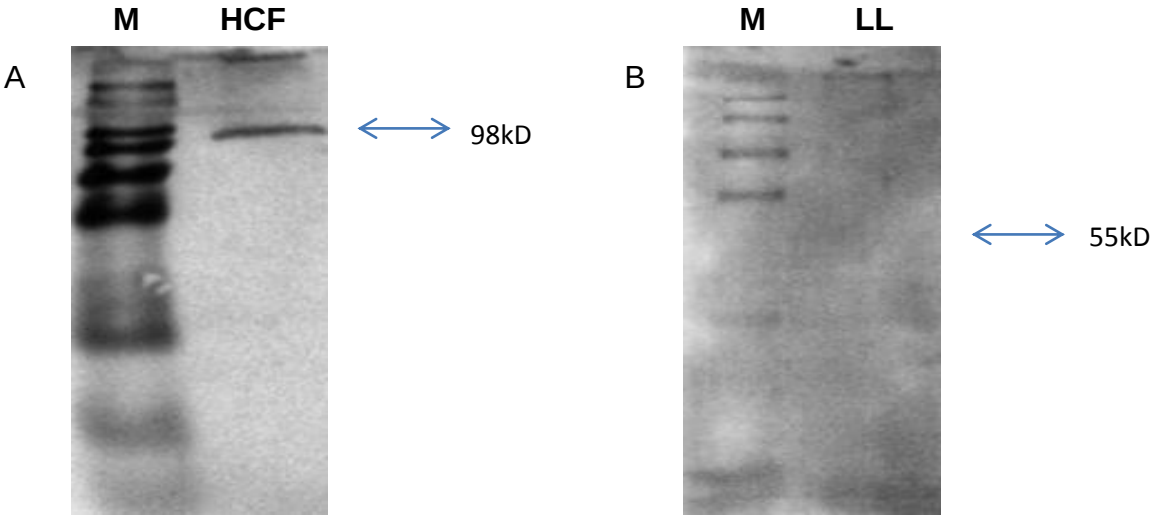


Figure 3.4: Blot images for the negative controls for the reactivity of (A) Total IgG with HCF (Figure 3.4A), (B) Total IgG with LL (Figure 3.4B). Both were tested with negative sera. There are no bands present on the LL but a band at 98KDa was recognised by HCF.

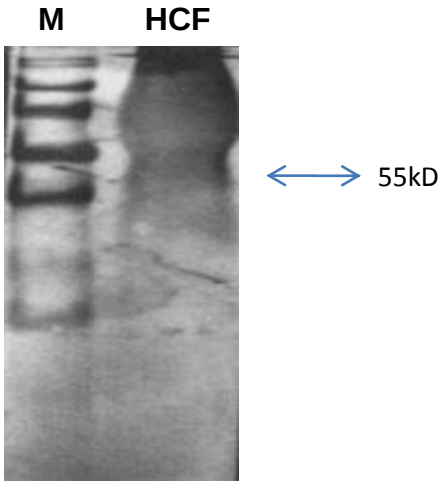


Figure 3.5: Blot Images for the reactivity of IgG1 with HCF with pooled positive sera. The band recognised is the one at 55KDa by HCF. No visible bands with the LL and so images were not included.

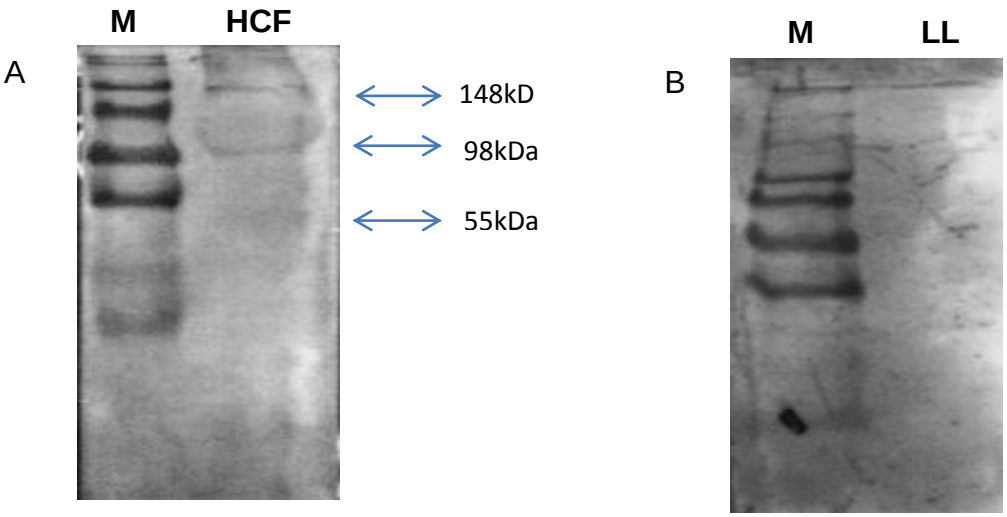


Figure 3.6 Blot images showing positive results of the reactivity of (A) HCF with IgG4 (Figure 3.7A) and (B) LL with IgG4 (Figure 3.7B). Both were tested with pooled positive sera. Again, as with positive IgG1, there were no bands recognised with the LL, while HCF, though not strongly have bands recognition at the 55, 98 and 148KDa regions. Images for the negative controls were not included as there were no apparent bands observed.

3.4 LECTIN BINDING ANALYSIS

Analysis of the laminated layer was carried out using series of lectins to investigate and characterise the carbohydrate components, since, according to Walker, (1994), laminated layer contains a considerable amount of carbohydrate components. Lectins are protein molecules that bind carbohydrates and each lectin have different sugar specificities (Walker, 1994).

Table 3.1 Table showing the different carbohydrates used in the analysis of lectin binding and their carbohydrate specificities (Major and Minor).

Lectin	Major	Minor
Concanavalin A (Con A) (Canavalia ensiformis)	A-Methyl-D-Mannoside	A-D-Glucose, N-Acetyl-α-D-Glucosamine
Soybean agglutinin (SBA) (Glycine max)	N-Acetyl-α-D-Galactosamine N-Acetyl-β-D-Galactosamine	None
Wheat germ agglutinin (WGA) (Triticum vulgaris)	N-Acetyl-β-D-Glucosamine	None
Horse gram agglutinin (DBA) (Dolichos biflorus)	N-Acteyl-α-D-Galactosamine	α-D-Galactose

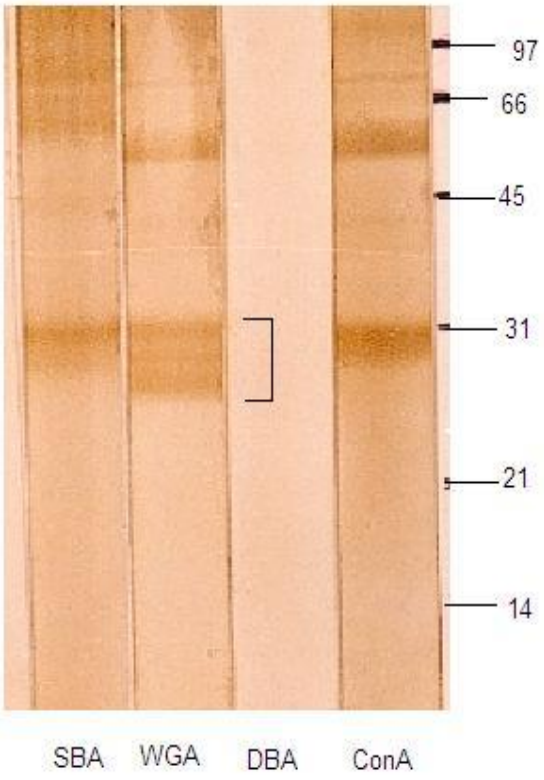


Figure 3.7 Glycoprotein patterns of the laminated layer antigen as detected by lectin. Major bands have been detected by SBA,WGA and ConA at the 31KDa region.

3.5 DISCUSSION

There have been no detailed studies on the analysis of the laminated layer of *E. granulosus* with SDS-PAGE, ELISA and Immunoblotting. The laminated layer contains various host and parasite molecules (Walker, 1994), it is therefore important to characterize each component.

In this cuurent study, extracts of crude laminated layer were analyzed using SDS-PAGE (under reducing conditions), ELISA, Immunoblotting and lectin asssay. An SDS-PAGE analysis stained in coomasie blue was done under reducing conditions to compare the extracts of laminated layer with HCF. Results show both antigenic materials with similar profiles, detecting bands at the 8, 22 and 55KDa (Figure 3.1). This assay was directly followed by an intial ELISA to confirm results obtained from the SDS analysis (see Figure 3.2). All fourteen confirmed patient samples gave positive response to HCF and the LL, with the LL having an overall greater response with all samples tested**.**

Analysis of the HCF and the LL by immunoblotting was carried out to further analyse the laminated layer and identify specific bands that can be used in the purpose of post-treatment follow-up. Reactivity of HCF and the LL with total IgG gave a common band recognition in the regions of 22, 36 and 55KDa (see Figure 3.3) and differently, HCF went on to detect bands at 64 and 98KDa (Figure 3.3). There were no bands detected with the LL for the negative controls, but a band at 98KDa was detected for HCF (Figure 3.4).

With IgG1, bands were only detected at the 55KDa region, there were no bands detected by the LL, therefore images were not included (Figure 3.5). The results obtained for negative controls show no apparent bands were detected by both HCF and the LL, therefore, images were not included . Bands at the 55, 98 and 148KDa regions were detected by HCF with IgG4, no bands were detected by the LL (Figure 3.6). Results and images for negative controls have not been included as there were no apparent bands showing.

In order to analyse and visualise the carbohydrate component of glycoprotein bands in the laminated layer, the crude LL was probed with various peroxidase labelled lectin conjugates (Table 3.1). Results (Figure 3.9) showed that the ConA, SBA and WGA all recognise major bands at the 31KDa region. Bands were also recognised by WGA and ConA at the 50KDa region. No bands were recognised by DBA.

The ability of these lectins; ConA, WGA, and SBA to have recognised bands on the surface of the extracts of the crude laminated layer of *E. granulosus* suggests the presence of α-Methyl-D-Mannoside, N-Acetyl-β-D-Glucosamine and N-Acetyl-β-D-Galactosamine in the laminated layer. The bands at the 31KDa region stained heavily for the presence of ConA, WGA and SBA.

Of particular interests are the results obtained with the reactivity of the laminated layer of *E. granulosus* with IgG1 and IgG4 as there were no bands detected when tested with these antibody isotypes (Figures 3.5 and 3.6 respectively). This is in agreement with the findings of Daeki et al, (2000) who stated that low concentration or reactivity of IgG1 and IgG4 is associated with disease regression or possible

calcification and again indicates that IgG4 is a good tool marker that can be used in the post-treatment surveillance of hydatid disease. The recognised bands by HCF and the laminated layer at 98 and 148KDa (Figures 3.3A, 3.4A, 3.6A, respectively) are probably host globulins and not parasitic in origin. According to Papadea and Check, (1989) the monomeric IgG had molecular weight of approximately 150KDa which dissociated into two groups of 22KDa light chains polypeptides and five types of heavy chains with molecular weights around 50-70KDa. This study is therefore in agreement as bands have been detected by HCF and the laminated layer at 148KDa region.

Bands recognised by HCF and the LL for positive assays at 22, 36 , 55KDa confirms the presence of antigen B (AgB) and antigen 5 (Ag5) in the HCF and LL antigenic materials. Carbohydrate antigens with molecular weights higher than 45KDa were reported by Miguez et al, (1996) in *E. granulosus* protoscolex using antisera raised from a carbohydrate enriched soluble fraction. There is a similarity between this result and the current study where the presence of carbohydrates were detected on bands in the 50-55KDa (N-Acetyl-β-D-glucosamine and/ or α—Methyl-D-Mannoside), and in the 60-66KDa (N-Acetyl-β-D-Galactosamine and/ or β-D-Galactose-(1-3)-N-Acetyl-Galactosmine linkage).

CHAPTER 4

LECTIN AFFINITY PURIFICATION

4.1 INTRODUCTION

Cystic hydatid disease is caused by *E. granulosus* in humans and transmission of the parasites naturally occurs between carnivorous as definitive hosts and herbivorous as intermediate hosts. Human infections occur accidentally via hand to mouth when comimg into contact with infected faecal matter contaminated with eggs e.g when petting dogs (CITE).

The cyst wall of metacestodes consists of inner, middle and external layers. The middle layer, otherwise known as the laminated layer is unique to the genus *Echinococcus* in comparison with other larval cestodes. Young cysts do not possess this layer and it is not until about 14-18days old when the first appearance is noticed as a thin, clear layer on its outer margin. This layer is an acellular, polysaccharide protein complex that stains strongly by periodic acid, Schiff's reagent (PAS) and equally provides a useful diagnostic marker in histological studies (Kilejian et al., 1962; Craig et al., 1995).

The laminated layer is given rise to by the inner germinal layer (Ortoletti and Ferretti., 1978; Harris et al., 1989; Holcman et al., 1994). Host material may be contributory to its structure (Kilejian and Schwabe , 1971; Pezzella et al., 1984). It has been shown the laminated layer (LL) of *E. granulosus* contains more of galactosamine than glucosamine. However, in protscolices (Px) and hydatid cyst fluid (HCF) glucosamine is more abundant than galactosamine. Also exhibited in the laminated layer is acid muco-polysaccharide (Richards, 1984).

In terms of antigenicity, Gottstein et al, (1983) isolated a lectin-binding carbohydrate antigen (Em2) of a specific fraction from crude metacestode of the *E. multilocularis* by immunoaffinity chromatography against anti *E*. *granulosus* hydatid fluid IgG coupled to CNBr-sepharose 4B. The molecular mass of this antigen is 54KDa (Gottstein, 1985; Furuya et al., 1989; Gottstein, 1992; Gottstein and Felleisen, 1995). In their study of carbohydates on the surface of *E. granulosus* protoscolices (Px) in mice, Migues et al, (1996) showed that carbohydrates were largely bound to parasite surfaces and were highly immunogenic. In spite of the fact that the laminated layer of *E. granulosus* is largely made up of carbohydrate components, little attention has been received by the role of these components in immunogenicity.

Taherkhani and Rogan, (2000), reported following SDS-PAGE and Westernblot analysis, that the most important antigenic molecules of the laminated layer of *E. granulosus* are those confined to these two regions (50-66KDa and 25-29KDa).
Since lectins are carbohydrate binding protein that interacts with specific sugar moieties, a further characterisation of the carbohydrate components in these regions using a series of lectins to detect whether these molecues are glycoprotein was carried out by Taherkhani and Rogan, (2000).

Over the years, attempts to isolate glycoproteins from organic specimens have been made resulting in the development of a wide range of chemical and biological assays currently used for separating target proteins from crude mixtures (CITE), one of which is the lectin affinity chromatography assay (CITE).
Lectins or agglutinins as they are also known , are able to recognise and bind reversibly to specific sugar moieties of polysaccharides, glycoproteins and

glycolipids found in biological systems in the form of either serum proteins or as membrane-associated proteins (Hart, 1980). They have previously been used for separating glycosylated proteins from non-glycosylated proteins (Sharon, 1993; Ling et al, 2012). Proteins were first separated using a Concanavalin-A lectin/Sepharose 4B assay using cyanogen bromide (CNBr) as a means of immobilization for coupling proteins to the matrix, a method developed by Axen *et.al* (1967), however, due to the toxic gases that can be produced fom CNBr, an alternative modified assay using affinity chromatography on Con A-Sepharose using DEAE (Diethylethanolamine) as the immobilizer has been successfully used to purify alkaline phosphatase from human liver (Trepanier *et al,* 1976)

The work of Taherkhani et al (2007) indicated that the most significant immunoreactive bands in the laminated layer showed good binding with the WGA lectin. It was therefore decided to try to affinity purify these components from the crude extract, using WGA Sepharose 4B column (Pharmacia, Germany).

4.2 AIM

The aim of this particular assay was purify the extracts of the laminated layer of E. granulosus with the propsect of the using the purified extracts as antigenic material for further serological investigations with the hope of finding a serological parameter that can be useful in the diagnosis and follow-up of cystic hydatid disease.

4.3 RESULTS

A 2ml column was packed into a syringe according to the manufacturers instructions and equilibrated with 20mM Tris-Hcl (pH 7.4) containing 0.5M NaCl. The column was connected to a peristaltic pump and a UVI cord optical density monitor and also connected to a chart recorder. Extracts of laminated layer (0.5ml) was run through the column and followed by 20mM Tris-HCl (pH 7.4) containing 0.5M NaCl until the chart recorder reached base level. Adherent molecules were then eluted from the column using 0.2M α-D-methylglucoside. The elution fraction was collected in eppendof tubes by observing the OD trace on the chart recorder and stored frozen until further use.

The obtained purified extracts were subjected to further analysis using pooled hydatid sera and normal sera in ELISA. Results show greater reactivity of total IgG towards antigens (Figure 4.2a). The runs tested with IgG, IgG1 and IgG4 (Figure 4.2 A, B, and C) have given higher results and reactivity than the obtained eluates.

The eluates, which represent the bound / adherent proteins have not given good results when tested with total IgG and IgG subclasses 1 and 4.

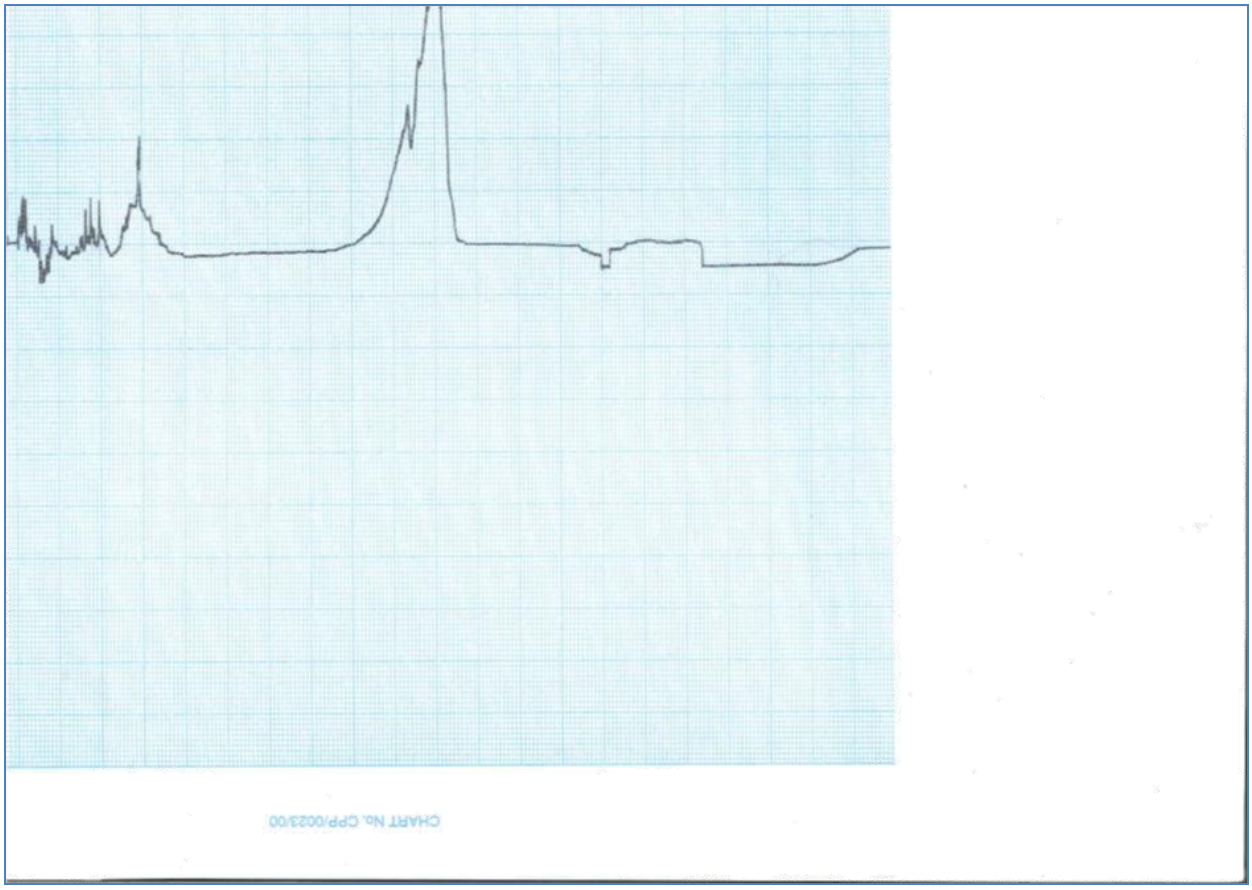
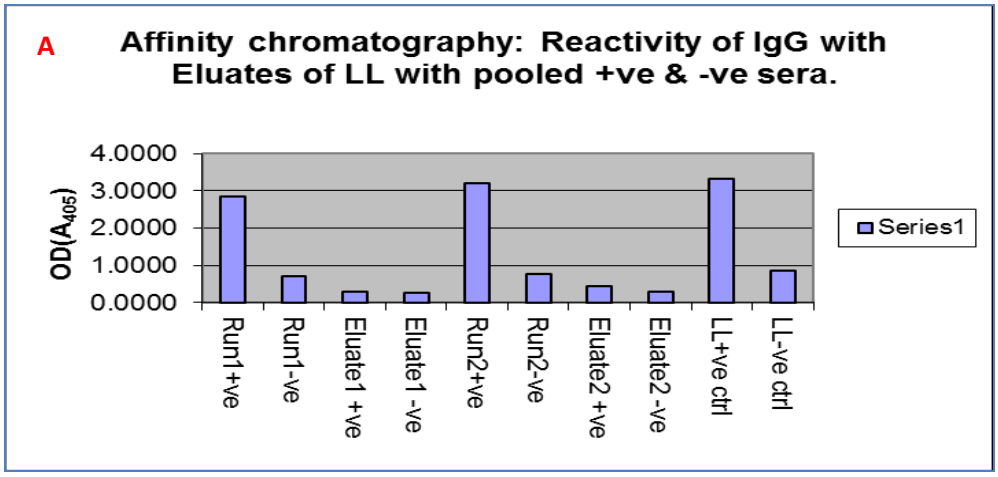
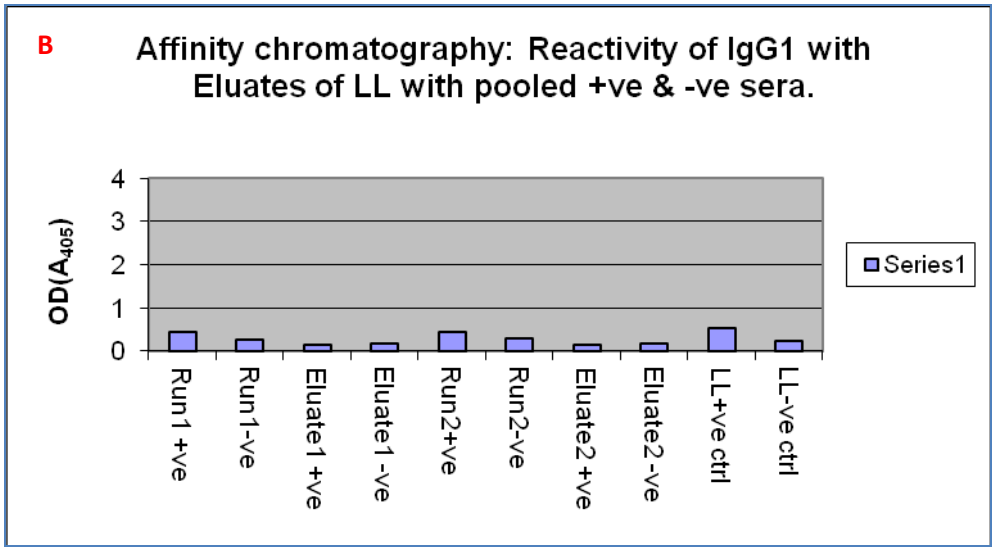


Figure 4.1
The chart recorder showing run through and elution peaks. Crude extract of laminated layer was run through the column and was recorded by the chart recorder before been eluted using 0.2M α-D-methylglucoside. Peak on
column indicate elution points.





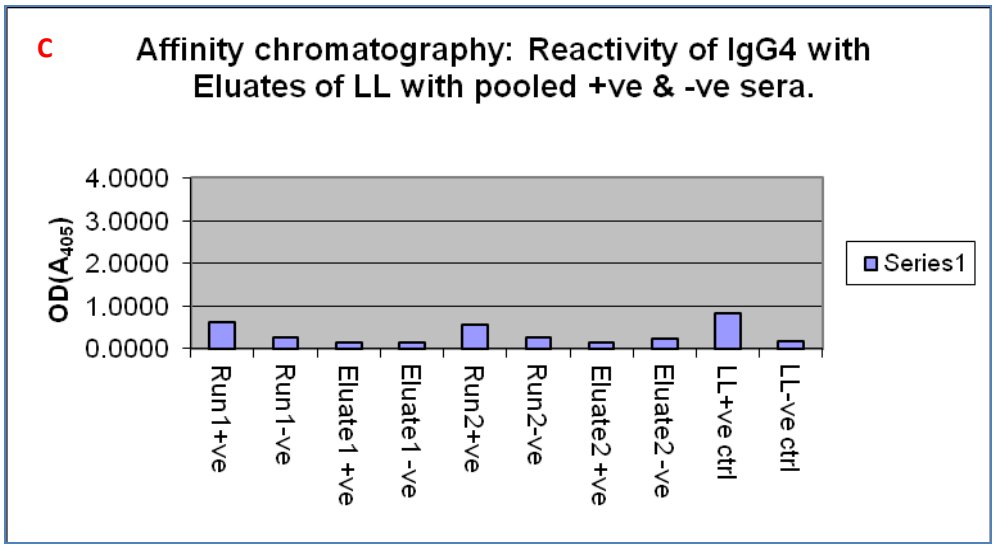


Fig 4.2: Graphs showing ELISA results of obtained eluates of the LL in reactivity with IgG, IgG1 and IgG4. They are shown to have low reactivity against total IgG Figure 4.2A), IgG1 (Figure 4.2B) and IgG4 (Figure 4.2C). In all three graphs, especially with total IgG (Figure 4.2A), the runs are seen to have a higher response.

4.3 DISCUSSION

Affinity chromatograpghy was first recognised as a seperation technique by Axen et al (1967). It is a technique that can be used for the purification of substances from complex biological mixtures and for the removal of small amounts of biological material from large amounts of contaminating substances. Therefore, in order to purify polyclonal antibody or antigen complex, the affinity columns were employed which are consisted of antibody or antigen fixed onto sepharose beads. One of the advantages of the affinity chromatography technique is its ability to isolate a solute from a biological material selectively. Nevertheless, the slow adsorption-desorption kinetics of many proteins usually causes contamination of the eluted protein, suggesting a draw back in the use of the technique for the preparative work as well as for the quantitative analysis purposes.

The use of affinity chromatography as a technique, a molecular investigative tool has been employed by so many and in so many laboratories around the globe. Fornstedt (1984) employed the technique when he did studies on the dissociation of Antigen-Antibody.

The technique was also used as a tool by Schiel and Hage (2009) in their study to examine the kinetics of biologicall interactions. In their approach, referred to as biointeraction chromatography, they used a column with an immobilized binding agent to examine association or dissociation of this agent with other compound.

Stewart and Doherty, (1973) also adopted the affinity chromatography technique when they selected bovine-serum albumin, known to have antipodal specificity in the binding of tryptophan as the affinity chromatographic matrix for the attempted chromatographic resolution of DL- tryptophan. They accomplished a complete

resolution when DL-tryptophan was chromatographed on bovine-serum albumin succinoylaminoethyl-sepharose.

The use of the affinity chromatography can not be over emphasized.

The aim and objective of this study and the purpose for employing the use of this assay was to separate host serum components from the crude laminated layer so as to obtain a purified laminated layer that can be used as an antigenic material with a view to obtaining distinct and reliable results upon testing with sera from our panel of confirmed hydatid disease patients.

After the crude extracts of laminated layer was run through the affinity chromatography column, eluates obtained were further subjected to ELISA in order to ascertain their level of antigenicity. Results showed poor reactivity of IgG1 and IgG4 subclass antibodies towards the LL antigen (Figure 4.2B and C), however, serum IgG antibody showed a much higher response towards the same antigen (Figure 4. 2 A). This could be because the laminated layer extracts used was not contained of viable antigenic materials or binding was poor or because nothing bound to the WGA Sepharose column used as WGA is known to be specific for N-Acetyl-β-D-Glucosamine, and maybe this carbohydrate was not present on the surface of the laminated layer used and therefore not present on its extracts or not present in enough quantity as to be able to cause a reaction. Another reason for the poor results obtained in general from this assay could be because there were not enough antigenic proteins released from the laminated layer. The same laminated layer source was used all through this present study and encouraging results were obtained using the same laminated in SDS-PAGE, ELISA and Immunoblots. This could be because, the adherence in the ELISA plates are higher and therefore boost the ability of our antigenic material (LL) to adhere and cause a reaction. Regarding

SDS-PAGE, it could be as a result of the boiling before use and the addition of sample cocktails used to break down into reducing conditions that helped in the dissociation of the LL giving rise to release of proteins and ultimately the bands obtained. All of these of course would need to be substantiated by further studies.

It is rather unfortunate that no encouraging results were obtained from affinity chromatography assay, but it can not be over emphasized that it is an assay that has proved its worth and usefulness over time and this is again depicted by the study of Abdi et al (2010). Abdi et al, (2010) cloned a 12KDa subunit of antigen B from *E. granulosus*, expressed and used it in diagnostic ELISA to test human sera for evidence of cystic echinococcosis. They compared the performance of the ELISA based on the recombinant antigen (rAgB) with that of similar assays based on native antigen B (nAgB) or hydatid cyst fluid. They further purified the recombinant protein using affinity chromatography. According to their results, the performance of the ELISA based on cyst fluid appeared identical to that of the assay based on the recombinant antigen.

CHAPTER 5

USE OF LAMINATED LAYER IN FOLLOW UP AFTER TREATMENT

5.1 INTRODUCTION

Continuous, repeated assessments of hydatid cyst viability are commonly made using serial radiology with or without serial serology. Nevertheless, there is no test for cure and therefore follow-up surveillance is required for many years. Lawn et al, (2004) reported that an enzyme linked immunosobent assay (ELISA) measuring CE-specific total IgG is the main serologic test used for diagnosis and follow-up at the Hospital for Tropical Diseases (HTD) in London. However, they stated that their experience with the test is that although a gradual decrease in optical density (OD) overtime following treatment maybe recorded, rarely does the test result become negative even after many years, even in those in whom it is apparent that the disease is cured. Likewise, serial radiology is often unreliable in demonstrating or establishing parasitologic cure; cysts killed by chemotherapy may persist and post-operative defects cannot always be distinguished/ separated from viable cysts.

Studies have reported previously that IgG subclasses 1-4 are expressed in different degrees in patients with chronic helminthic infections (Boctor and Peter, 1990, Short *et al*, 1990, Kurniawan *et al*, 1993). Likewise, the use of an ELISA incorporating partially purified hydatid cyst fluid antigen B to do cross-sectional studies of patients with CE have found a predominance of IgG1 and IgG4 expression in serum (Aceti et al, 1993; Wen and Craig, 1994; Shambesh et al, 1997; Daeki et al, 2000). Furthermore, compared with those with asymptomatic disease, serum concentrations of IgG4 are greater in patients with symptomatic CE (Shambesh et al,

1997). Therefore, suggestions have been made that measurements of IgG sbclass may provide a more sensitive index of disease activity than CE-specific total IgG.

Owing to the frequent lack of sensitivity of immunodiagnosis, diagnosis of CE remains highly dependent on imaging techniques (Zhang and McManus, 2006) with about 20% of clinically or surgically confirmed CE cases, and negative serology being presented by up to 50% of community-detected patients (Verastegui et al, 1992; Moro and Garcia, 2005). The most commonly used imaging techniques include magnetic resonance imaging (MRI), Ultrasonography (US) or radiography, for detection of characteristic space-occupying cysts (McManus et al, 2003; Eckert and Deplazes, 2004). MRI shows highly specific features of CE, but it is prohibitively expensive and not readily available in rural areas of many endemic countries. Ultrasound is contrastingly more accessible, much less expensive, and can identify hydatid cyst pathological type CE1 to CE5 (Brunetti and Junghanss, 2007).

According to Brunetti and Junghanss (2007), surgery, percutaneous techniques, antiparasitic treatment for active cysts, and the so- called “watch and wait” approach for the inactive cysts are some of the approaches employed in the clinical management of CE. Surgery currently remains the commonest approach for CE treatment that possess the potential to remove cyst and lead to absolute cure, but risks are involved, including those associated with any surgical intervention, anaphylactic reactions, and secondary CE due to spillage of viable parasite (protoscolices) material (WHO, 1996; Junghanss et al, 2008; Brunetti et al, 2010). The use of drug therapy, especially with benzimidazoles (albendazole or mebendazole) has been on the increase with CE treatment, and efficacy against the parasite in humans have been proven, with about 30% of patients cured and 30-50%

of cases improved after 12months followoow-up (WHO, 1996). However, the response to drug therapy can be unpredictable, and the optimum not been determined definitively (Junghanss et al, 2008; Stojkovic et al, 2009). Furthermore, recurrence risk remains the major problem in surgical or medical treatment (WHO, 1996; Stojkovic et al, 2009; Brunetti et al, 2010). Therefore, post- treatment or post-surgical follow-up of CE patients for several years is usually suggested.

Table 5.1 SUMMARY OF PATIENTS INFORMATION

Patient ID	**Gender/ Age**	**Cyst series**	**Organ Involvement**	**ALB Chemotherapy (mg/kg body weight)/ surgery**	**Sera Timeline**
B142	Female 6	CE1, CE4	R/ liver	Untreated	0-42.7months
P160	Female 16	CE2, CE3	R/ liver	Untreated	0-33.9months
X345	Female 20	CE2, CE3	Omentum/ mesentery	Untreated	0-3.6months
X161	Female 17	CE3, CE4	R/ liver	27.6mths ALB (20mg).	0-31.6months
Y88	Female 5	CE1	R/ liver	4.8mths 2 courses ALB (20mg); 17.8mths ALB (20mg).	0-8.4months
Y13	Female 20	CE1, CE3, CE4, CE5	R/ liver, lower abdomen	3.8mths 3 courses ALB (20mg); 22.2mths ALB (20mg); 58.7mths ALB (20mg); 62.3mths endocystectomy.	0-209.8months

Y28	Female 20	CE1, CE3	R/ liver	5.5mths 3 courses ALB (20mg); 41.6mths cystectomy and 2 courses ALB (20mg).	0-46.9months
Y51	Female 10	CE1, CE3, CE4	R and L/liver, Omentum/mesentery	1.6mths 1 course ALB (20mg).	0-10.5months
X269	Female	Data not available	Kidney	Untreated	Data not available
Y63	Female 25	CE1, CE3	R/ liver	At point "0" 1 course ALB (20mg).	0-47.3months
Y179	Female 22	CE2, CE£	R/ liver	0.1mths 3 courses ALB (20mg); 15.1mths ALB (20mg); 23.9mths ALB (20mg).	0-42.7months
Y235	Female 46	CE2, CE3	R/ liver	2.2mths ALB (20mg).	0-6.2months
Y111	Female 30	CE1, CE2, CE4	R and L/ liver	0.1mths 3 courses ALB (20mg); 15.1mths ALB (20mg); 23.9mths ALB (20mg).	0-46.2months
156	Female 33	CE2	R and L/ liver, Omentum/ mesentery	1.8mths 2 courses ALB (20mg); 4.2mths had endocystectomy.	0-4.1months

5.2 RESULTS

Extracts of laminated layer was tested and compared to HCF in an ELISA assay in order to determine the levels of sensitivity between the two when tested against total IgG and IgG1/IgG4 subclass antibodies.

All samples, untreated (table 5. 2, n =4) and treated (table 5. 3, n =10) were tested with ELISA to determine levels of total IgG, IgG1 and IgG4 subclasses in response to HCF and LL antigens. There are two tables below, table 5.2 is a summary of the antibody responses obtained for both laminated layer and HCF for untreated patients, while table 5.3 shows summary of antibody response for treated patients.

In determining whether the antibody responses obtained are low, medium or high, the OD values were taken into consideration and was decided that any response between OD value 0 and 1 is considered low and between 1 and up to 2 is medium, while anything higher than 2 is high (as evident on graphs).

Table 5.2 ANTIBODY RESPONSE (UNTREATED PATIENTS)

SAMPLE ID	IgG-HCF	IgG-LL	IgG1-HCF	IgG1-LL	IgG4-HCF	IgG4-LL
B142	low	low	low	low	low	low
P160	low	high	med	med	low	low
X269	low	low	low	low	low	low
X345	med	med	high	low	low	low

B142: Patient B142 is a low responder, and in all cases little reactivity was seen of serum IgG, IgG1 and IgG4 towards all antigens (Table 5.2, Figure 5.1)

P160: With Patient P160, a different trend was seen. Even though the same line trend was obtained for both LL and HCF in reaction with IgG, LL gave a higher response (Table 5.2, Figure 5.2A). A similar result to that obtained for total IgG was obtained for IgG1. Again, LL was shown to have a higher response (Table 5.2, Fig 5.2B). Interestingly, response of both antigenic samples, LL and HCF to IgG4 was on the same level and both have shown similarity in trends (Table 5.2, Figure 5.2C).

X269: Patient X269, like B142 is a low responder and has shown very low reactivity with neither of total IgG or immunogloblulin subclasses 1 and 4 (Table 5.2, Figure 5.3).

X345: Patient X345 in reactivity with total IgG. Both antigenic samples are low responders; both have displayed similar reactivity/ response and significant peaks at 0 and 1.55months respectively (Table 5.2, Figure 5.4A). Antibody response with IgG1 are noticeably very different. The LL is obviously a low responder while HCF responses are higher, peaking twice at 0 and 1.55months respectively and then experiencing sharp falls after reaching these peaks (Table 5.2, Figure 5.4B) .Again, with IgG4, both antigenic samples are low responders, antibody response patterns here are similar. Reactivity recorded for both samples was very low (Table 5.2, Fig 5.4c).

Table 5. 3 ANTIBODY RESPONSE(TREATED PATIENTS/ SAMPLES)

SAMPLE ID	IgG-HCF	IgG-LL	IgG1-HCF	IgG1-LL	IgG4-HCF	IgG4-LL
X161	low	high	low	med	low	low
Y13	low	low	low	High/low	low	low
Y28	low	low	low	low	low	low
Y51	low	high	low	high	med	low
Y63	med	low	Low/high	low	low	low
Y88	low	high	low	High/med	low	High/low
Y111	low	High/low	low	low	low	low
Y179	low	med	low	low	low	Low/high
Y235	low	low	low	low	high	low
156	med	med	med	low	med	low

Patient **X161**. Figure 5.5A is the result obtained for reactivity with total IgG where it is shown that there is not so much activity going on with HCF. It is noticeably a low responder (Table 5.3); the LL is displaying a much higher response, although there are no major peaks observed. Similarly, with IgG1, HCF again gives a lower response when compared to LL with a medium response. No major peaks observed here and the antigenic samples have shown variations in their line trend (Figure 5.5B). A slightly different result was obtained for reactivity with IgG4. Both LL and HCF show same line trend and are both low responders with HCF seen to be picking up (Figure 5.5C). With Patient X161, the LL seems to have greater serum response and activity with total IgG displaying the highest level of antibody activity.

Y13: Patient Y13 is an interesting one, as observed in Figure 5.6A, antibody response and reactivity is similar for both LL and HCF and both are low responders. An almost but slightly different result is recorded for the same samples in reactivity with IgG1 (Table 5.3, Figure 5.6B) where HCF remains a low responder but LL starts as a high responder then becoming a low responder with almost no reactivity going

on. Same line trend observed from 20.24months. A completely different result was obtained for the reactivity of these samples with IgG4. From the graph (Figure 5.6C), it was obvious that both are low responders.

Y28: Patient Y28. With this patient, both samples gave very low responses in reactivity to total IgG and the subclass isotypes IgG1 and IgG4. The reactivity with total IgG, IgG1 and IgG4 have displayed responses that are similar except for the little variation displayed with reactivity with IgG4 (Figure 5.7C).

Y51: Observable with patient Y51 is the higher response to total IgG in serum antibody level given by LL than towards HCF (Table 5.3, Figure 5.8A). No major peaks are observed. A different result was obtained for reactivity with IgG1 (Table 5.3, Figure 5.8B). Although they have both responded similarlys, both started off as low responders before they both rise at 2.27months (LL rising significantly higher) and peaking at 2.56months and then experiencing a fall. A variation in response to antibody was observed for reactivity with subclass IgG4 (Figure 5.8C). HCF peaked significantly at 5.49months (Figure 5.8C).

Y63: Patient Y6; here both antigenic samples gave low response to total IgG, although response to antibodies was similar. A slightly raised activity was observed between 0.49-34.68months (Table 5.3, Figure 5.9A). In response to subclass IgG1, both antigenic samples started off as low responders before HCF experienced a rise in activity at just after 0.49months and then peaking at 2.82months before experiencing a sharp fall (Table 5.3, Figure 5.9B). Result obtained for reactivity with subclass IgG4 are similar to that obtained for subclass IgG1. Both antigenic samples are considered low responders, but again between 0.95 and 34.68months, HCF experienced a rise in activity so also LL with a noticeable slight raise in activity (Table5. 3, Figure 5.9C).

Y88: Patient Y88 showed some interesting trends as serum antibody levels in response to the LL antigen had greater activity than towards HCF antigen (Table 5. 3). Total IgG antibody displayed the highest level of antibody activity, noticeably peaking at 2.6months followed by a decrease in reactivity after treatment with ALB (20mg/kg) just over 2months later. Antibody level then rises slightly higher at 5.7months then begins to drop continuing to drop significantly after treatment (20mg/kg) at 7.2months (Figure 5.10A). A sharp rise is then seen with IgG activity peaking at about 8.4months. In contrast, total IgG response towards HCF antigen shows a flat line response throughout. With IgG1 antibody, responses to the LL antigen (Figure 5.10B) show a very similar trend, with peaks corresponding to those shown by total IgG but at a lower level of reactivity. Similarly, a flat line response is also noticed with IgG1 towards HCF antigen. A similar set of results is demonstrated for reactivity of IgG subclass 4 antibody (Figure 5.10C) in response to both antigens and again, a flat line response is seen towards HCF antigen. However, with this subclass- antibody, only one significant peak is noticed towards the LL antigen occurring at 5.7months.

Y111: Patient Y111 in reactivity with total IgG showed an interesting trend also as serum antibody levels in response to the LL antigen had greater activity than towards HCF antigens (Table5. 3, Figure 5.11A); response from HCF was almost a flat line. With subclass IgG1, a contrasting result to that obtained for total IgG was obtained for both antigenic samples, they both showed a flat line response (Figure 5.11B). A completely different result was obtained for reactivity with subclass IgG4. A flat line response was obtained for LL antigen while a rising and falling trend was obtained for HCF with significant peaks at 0, 0.78, just before 23.07, and just after

23.07months (Figure 5.1C). With this patient, the LL was found to give greater response with total IgG, and HCF gave higher response with IgG4.

Y179: Patient Y179. With this patient, a rising and falling trend was observed for LL antigen in response to total IgG, while a flat line trend is exhibited by HCF, only showed a raised activity from 12.23momths through to 42.71months. The LL antigen showed peaks at 0.33, 14.89 and 42.71months respectively (Figure 5.12A). Figure 5.12B shows reactivity with IgG subclass 1, and with both antigenic samples, LL and HCF, a flat line trend was observed in contrast to response to total IgG (Figure 5.12A). Results obtained for reactivity with IgG subclass 4 is slightly similar to that obtained for IgG subclass 1 in that both antigenic samples responded with a flat line before both rising at 14.89months and both peaking at 20.15months and then experiencing a sharp fall (Figure 5.12C).

Y235: Patient Y235. With this patient, reactivity of the two antigenic samples, LL and HCF with total IgG and IgG subclasses 1 and 4 have exhibited low responses. HCF shows a flat line response with total IgG while LL displayed a rising and falling pattern, peaking significantly at 1.02 and 5.19months (Figure 5.13A). In contrast, with subclass IgG 1, LL displayed a flat line response while HCF showed a rising and falling pattern, peaking significantly at 5.19months (Figure 5.13B). HCF continued with its display of rising and falling pattern in response to IgG subclass 4, peaking significantly at 0 and 5.29months while a flat line response was maintained by the LL (Figure 5.13C).

156: Patient 156. Here, we see the LL performing and responding better to the total IgG antibody, peaking at just after 0 and 1.02months significantly. Both antigenic samples almost exhibited the same response and are considered medium responders (Figure 5.14A). With IgG subclass 1, again HCF is observed to continue

in showing the rising and falling pattern, peaking significantly at just after 1.02 and 2.3months. The LL shows a flat line response with a not so significant peak (Figure 5.14B). Both antigenic samples display the same response to immunoglobulin isotype 4 . Both are considered low responders, although HCF has a higher response compared to LL, both sample show a peak at just after 0months before falling, and then HCF is seen to rise again at 0.56months (Figure 5.14C).

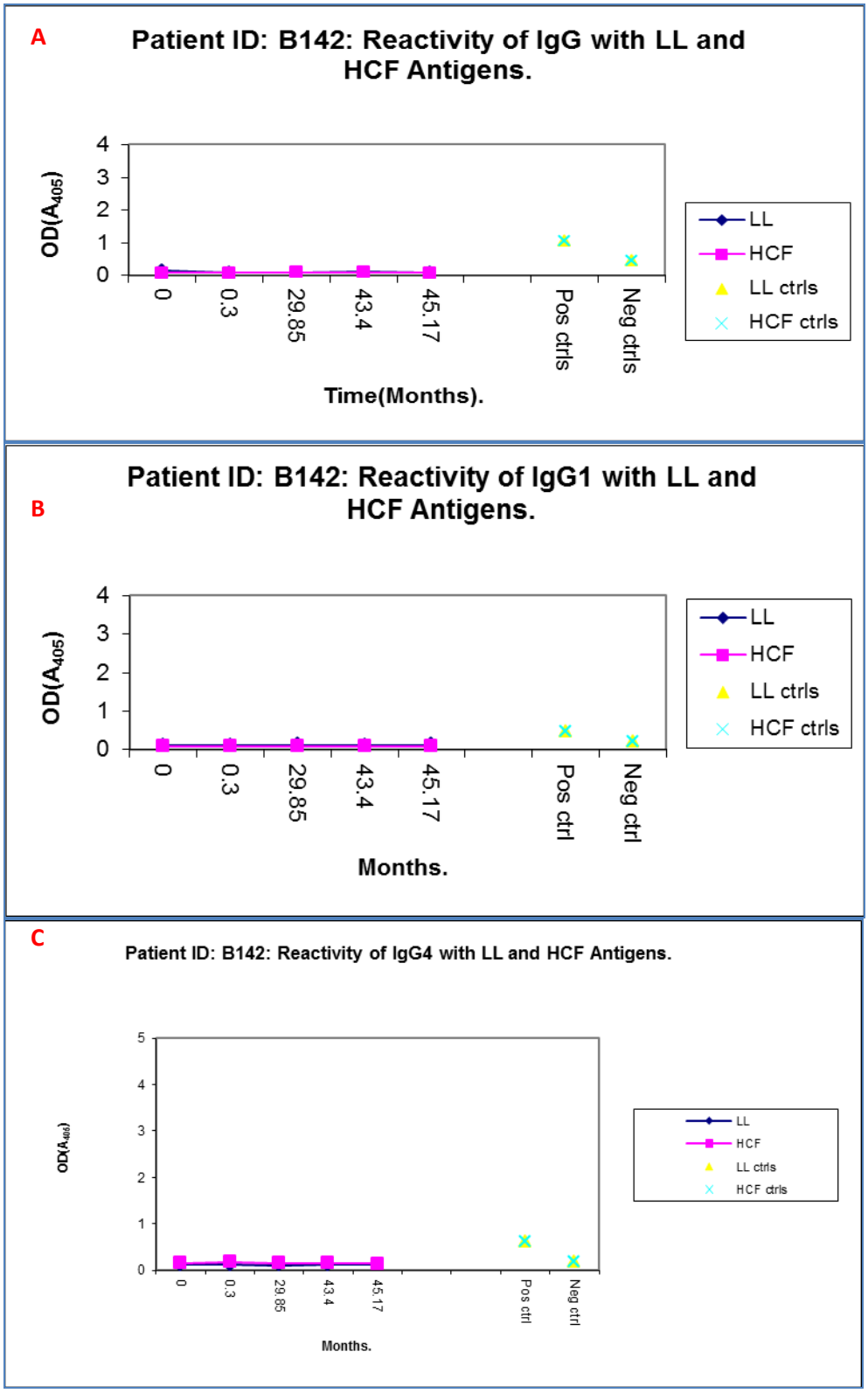


Figure 5.1: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). As evident on graphs, a flat line reaction was displayed by both samples.

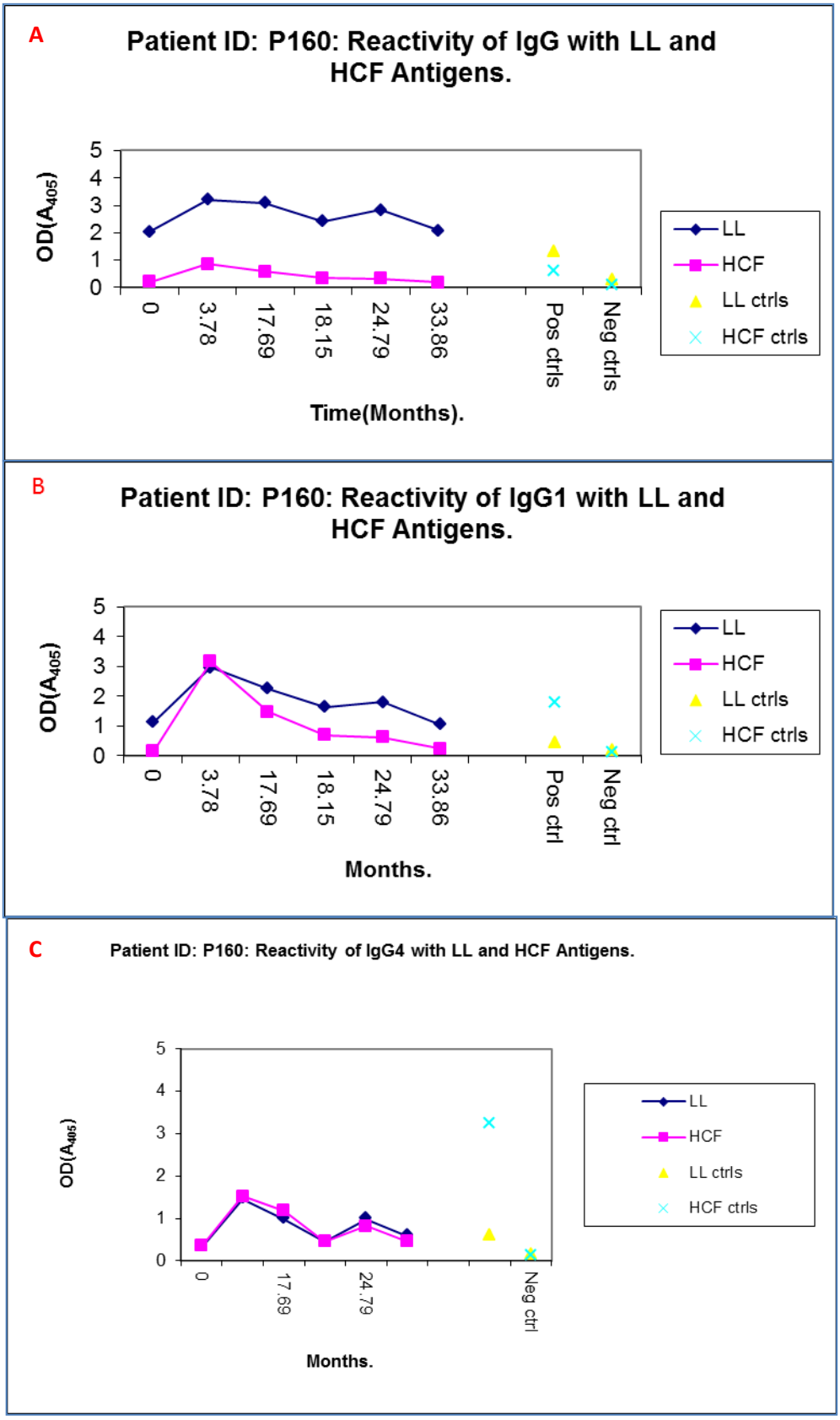


Figure 5.2: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). Trend lines are not the same and the LL appears to be reacting well as HCF or even better as depicted in Fig 5.2a.

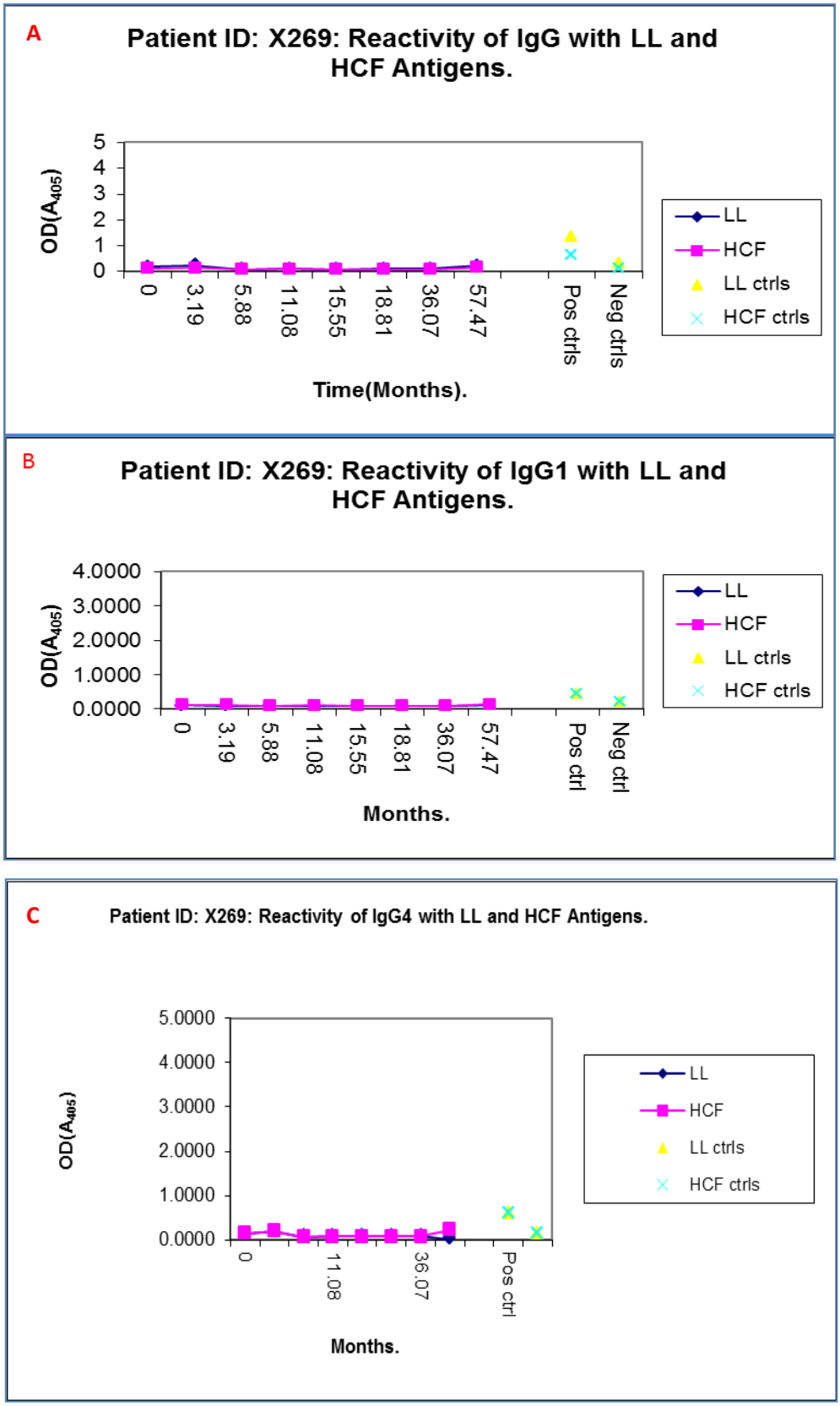


Figure 5.3: Graphs showing reactivity of HCF and LL with total IgG (a), IgG1 (b) and IgG4 (c). A flat line reaction is recorded for both samples in reactivity with these antibody molecules.

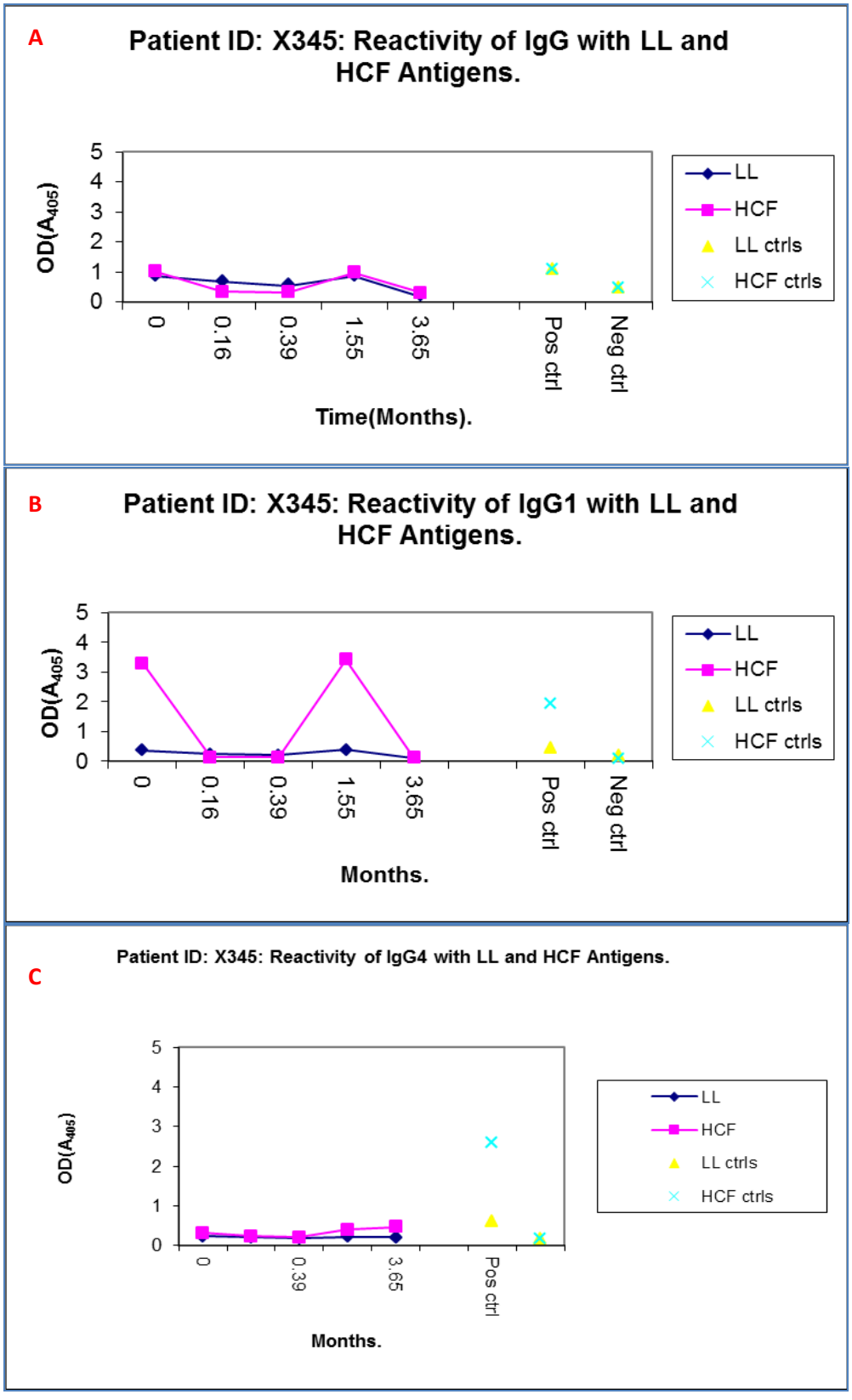


Figure 5.4: Graph showing reactivity of HCF and LL with IgG (a), IgG1 (b) and IgG4 (c). The lines obtained here are varied. The most reaction going on is with HCF and IgG1.

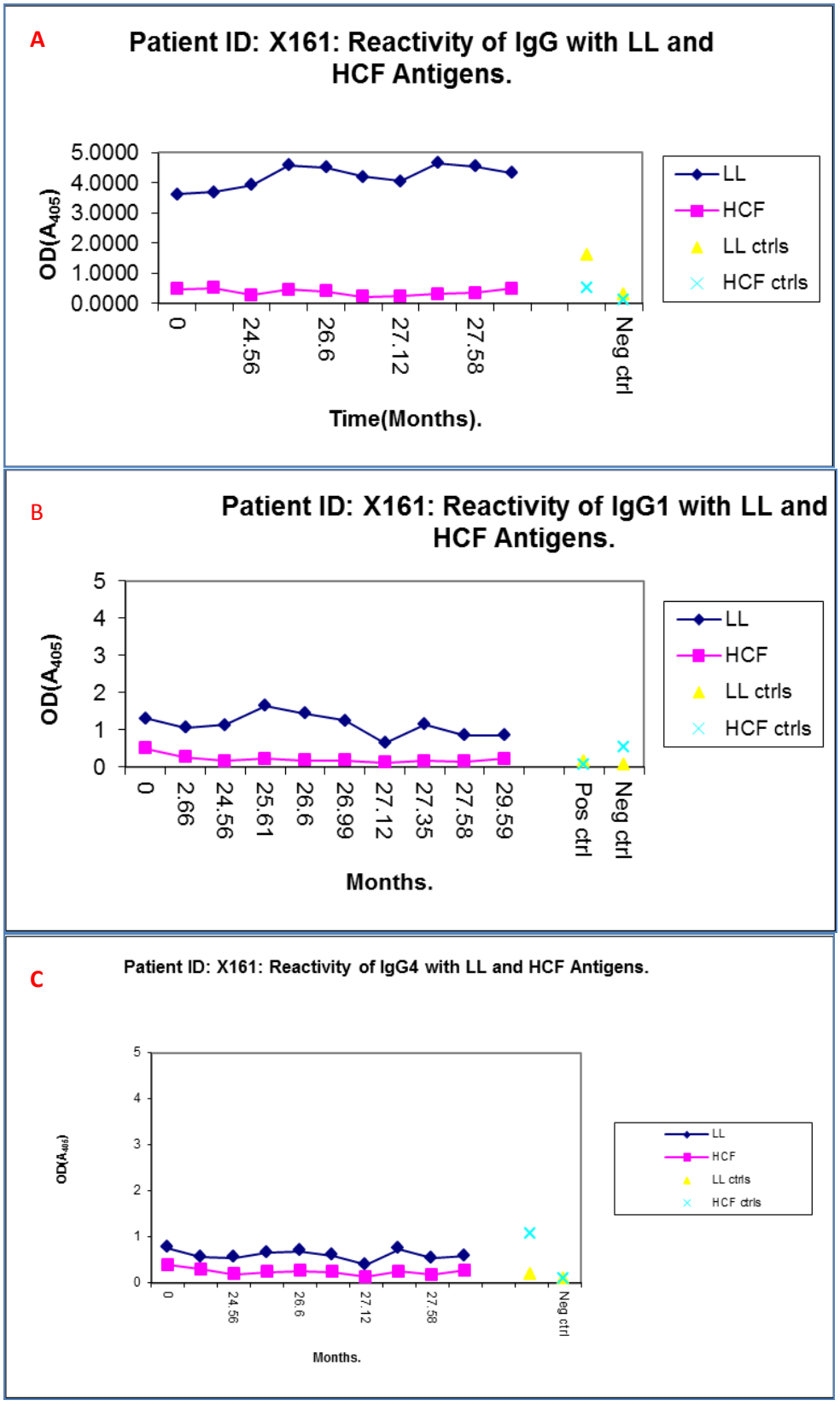


Figure 5.5: Graph showing reactivity of HCF and LL with total IgG (a), IgG1 (b) and IgG4 (c). Both samples show the same pattern, although LL gives a higher response with all three antibody molecules particularly with total IgG (Figure 5.5a).

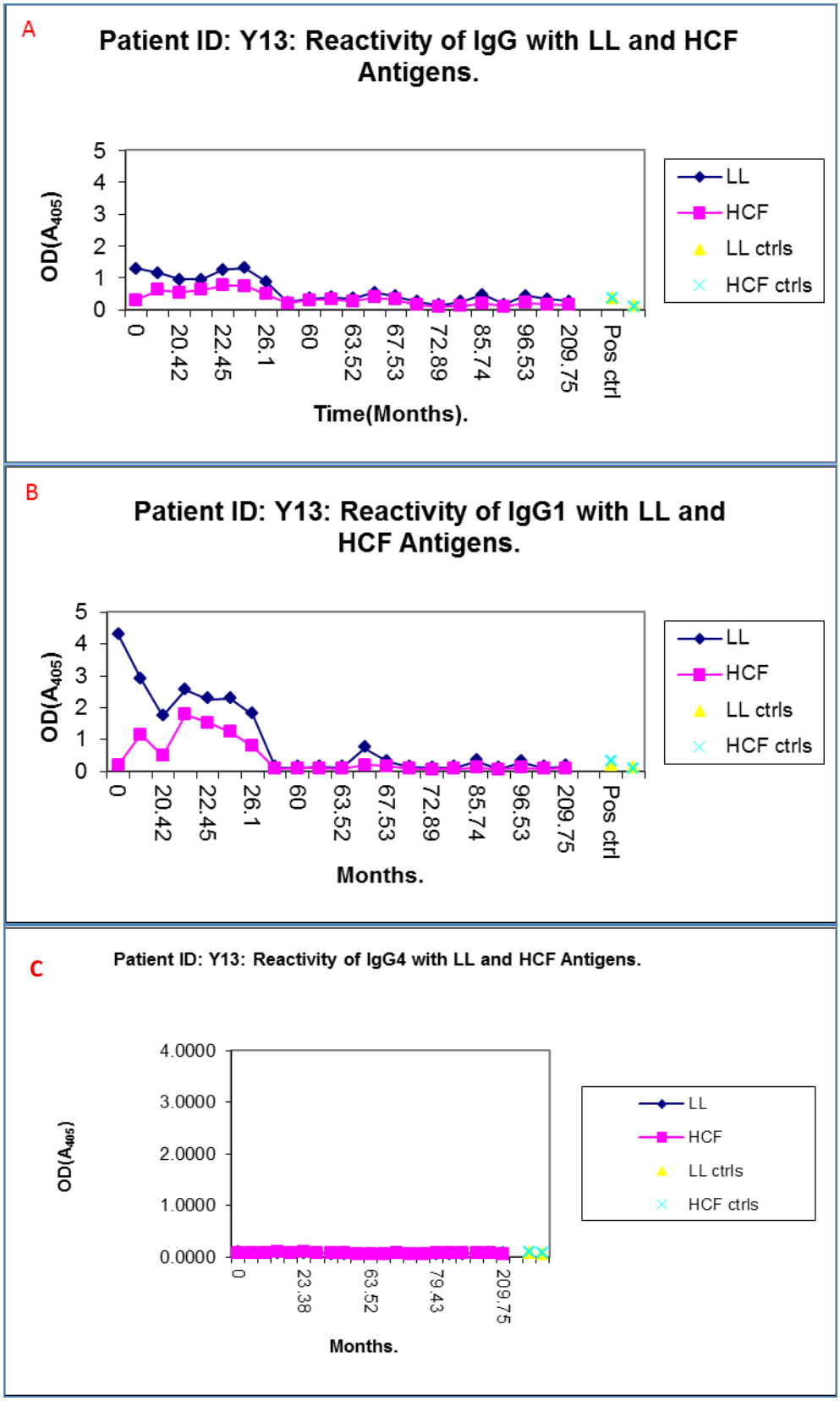


Figure 5.6: Graph showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). The same trends are displayed by IgG (A) and IgG1 (B) and a completely different, almost negative flat line observed with IgG4 (C).

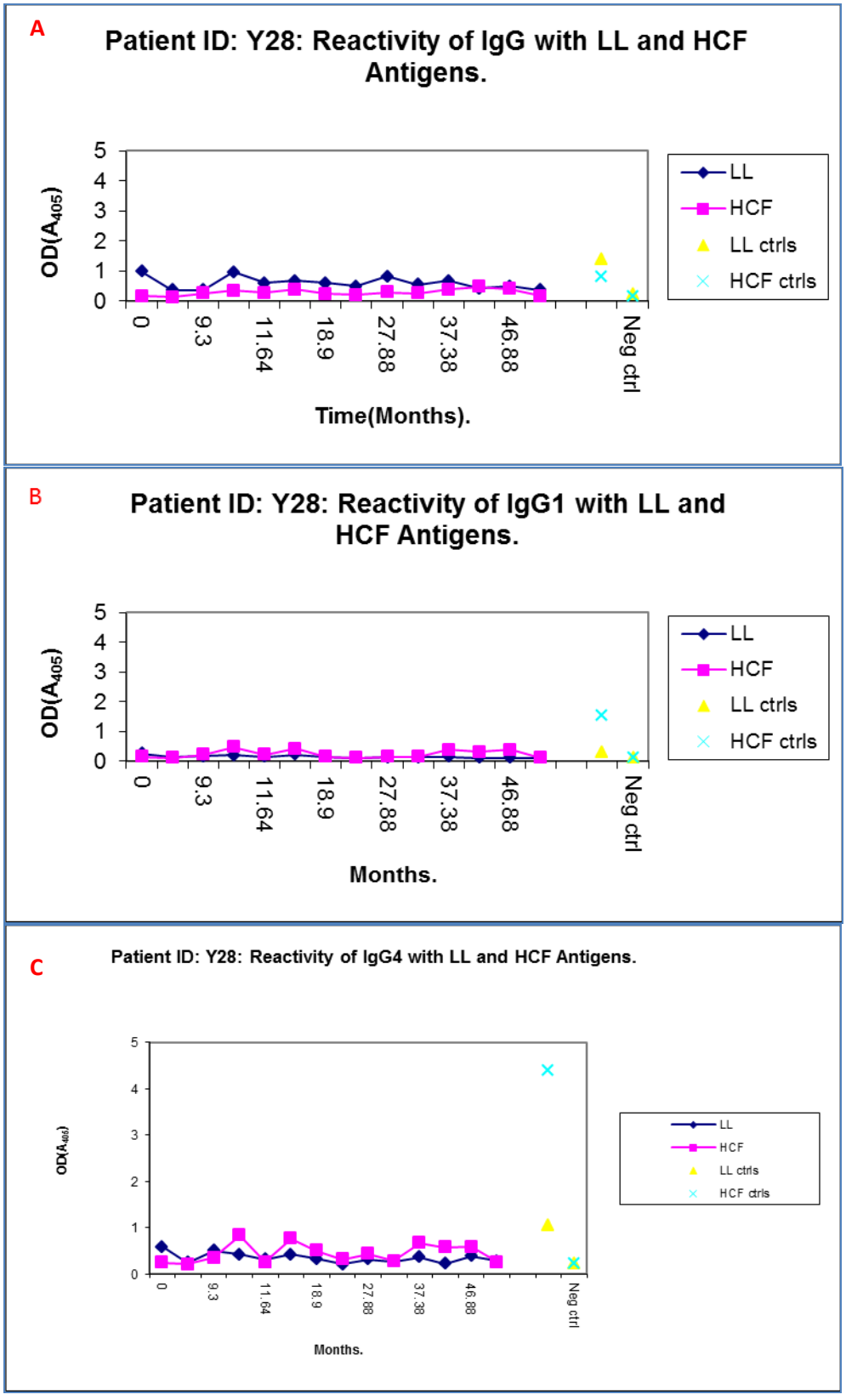


Figure 5.7: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). Very low reaction obtained for both samples with all three antibody molecules.

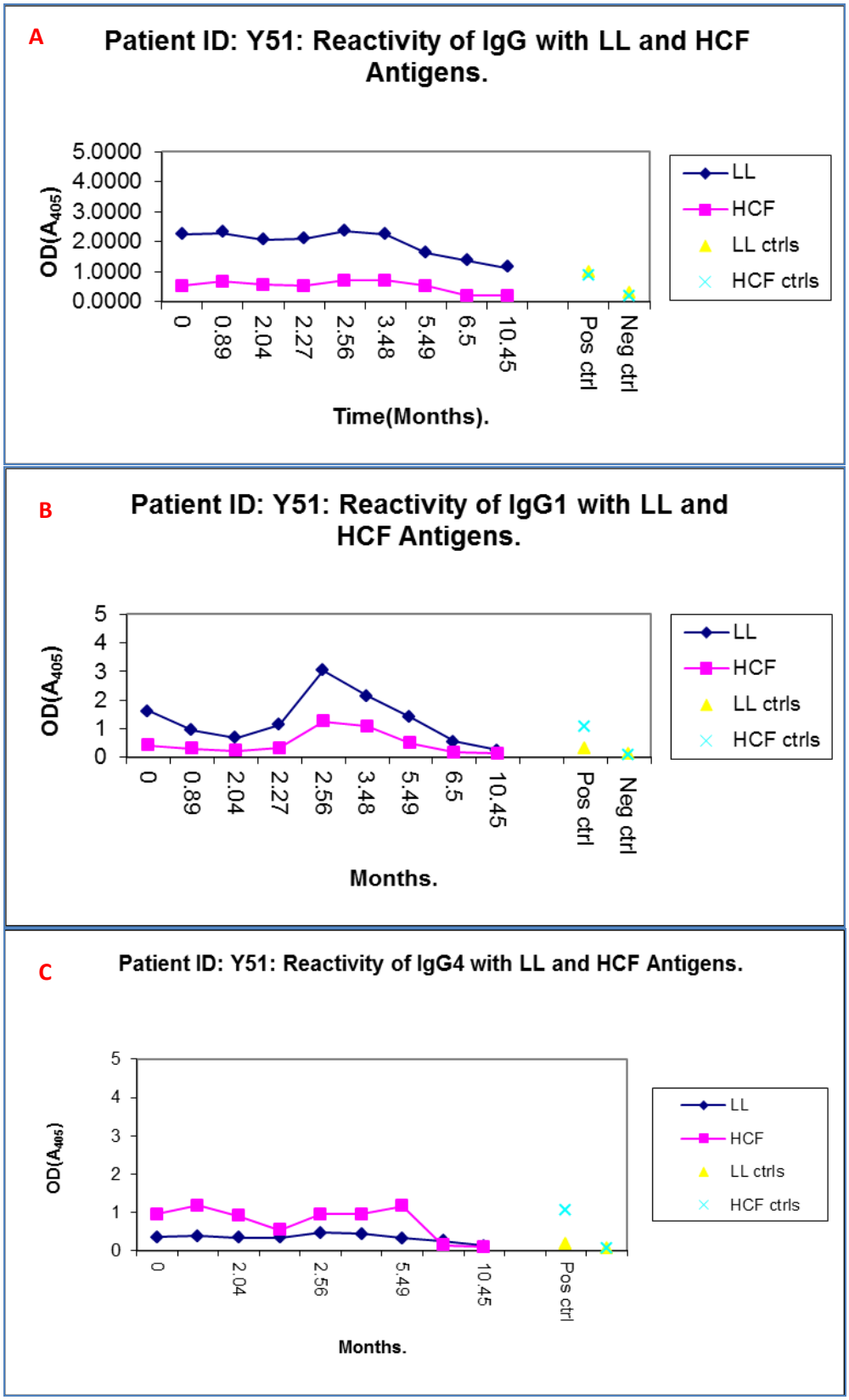


Fig 5.8: Graph showing the reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). The LL is seen to have the higher response with total IgG (A), IgG1 (C), although both samples display same pattern of response.

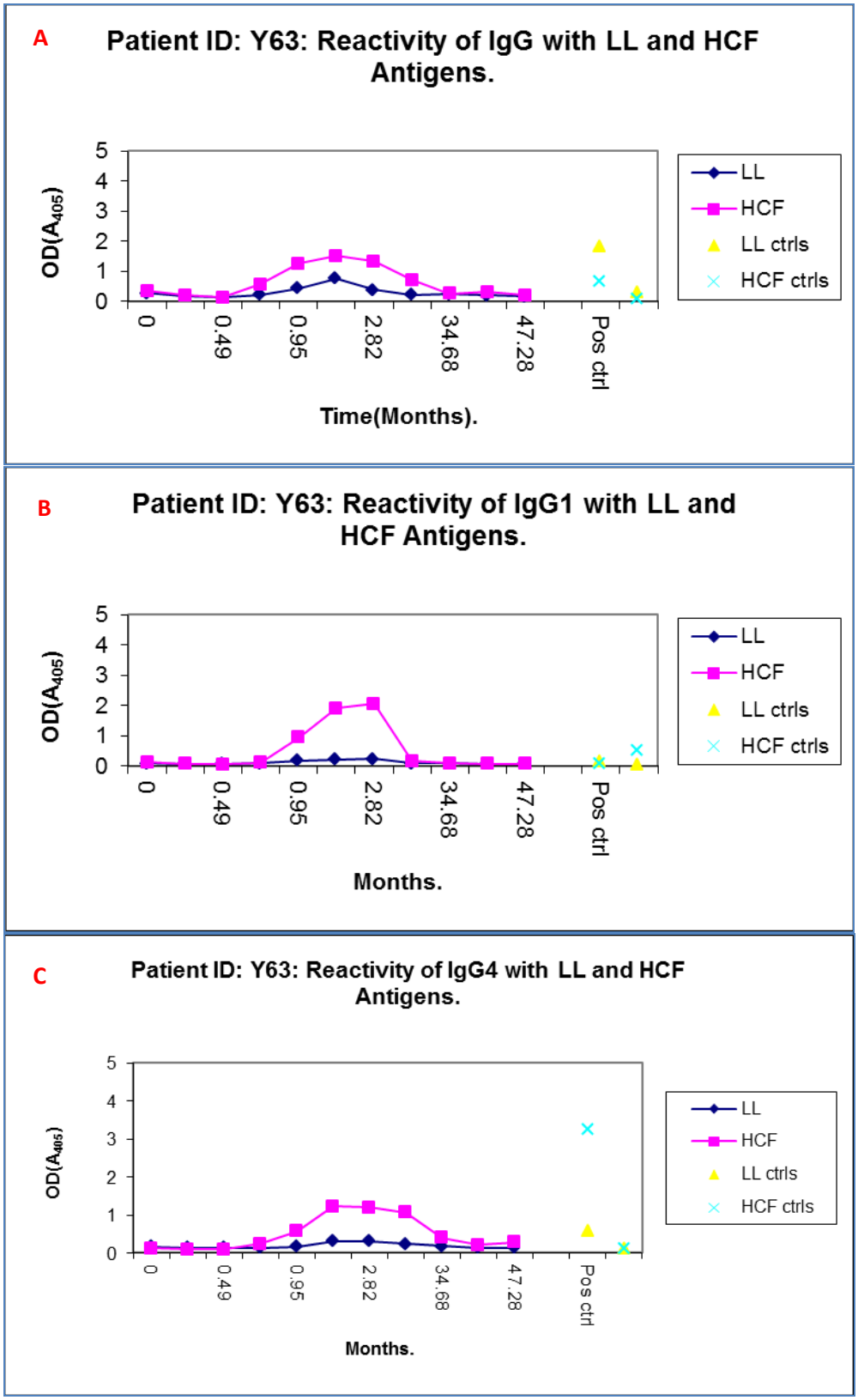


Fig 5.9: Graphs showing the reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). Here, HCF is seen to have given the higher response. Reactivity with LL is very low.

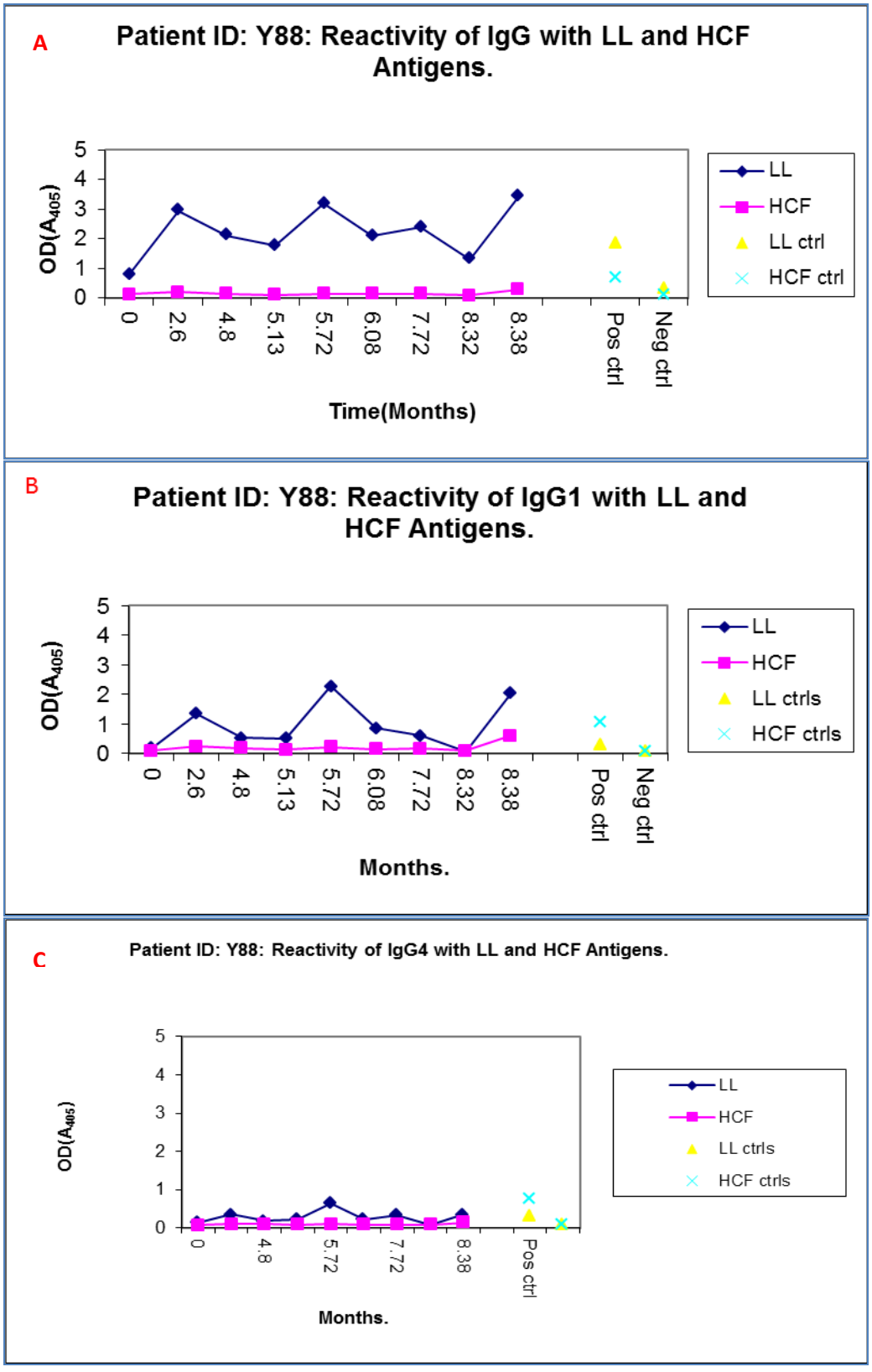


Figure 5.10: Graphs showing reactivity of HCF and LL with total IgG (a), IgG1 (b) and IgG4 (c). Here, LL is observed showing a rising and falling pattern of response (Fig 5.10a, b). HCF displays a flat line response with all three antibody molecules.

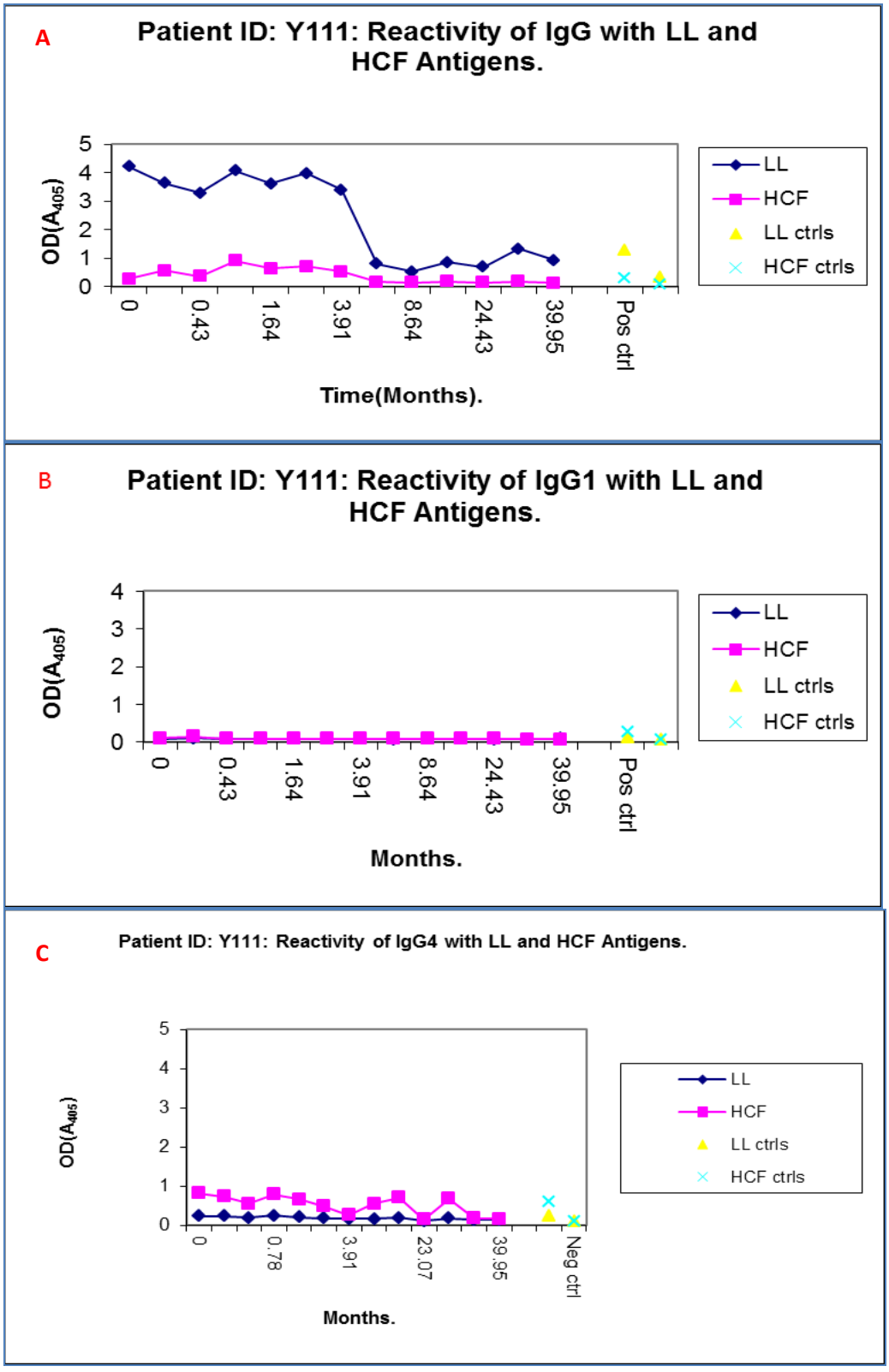


Figure 5.11: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). LL gave a high response with total IgG (Fig 5.11a), low response for both samples with IgG1 and IgG4 (Fig 5.11b and c, respectively).

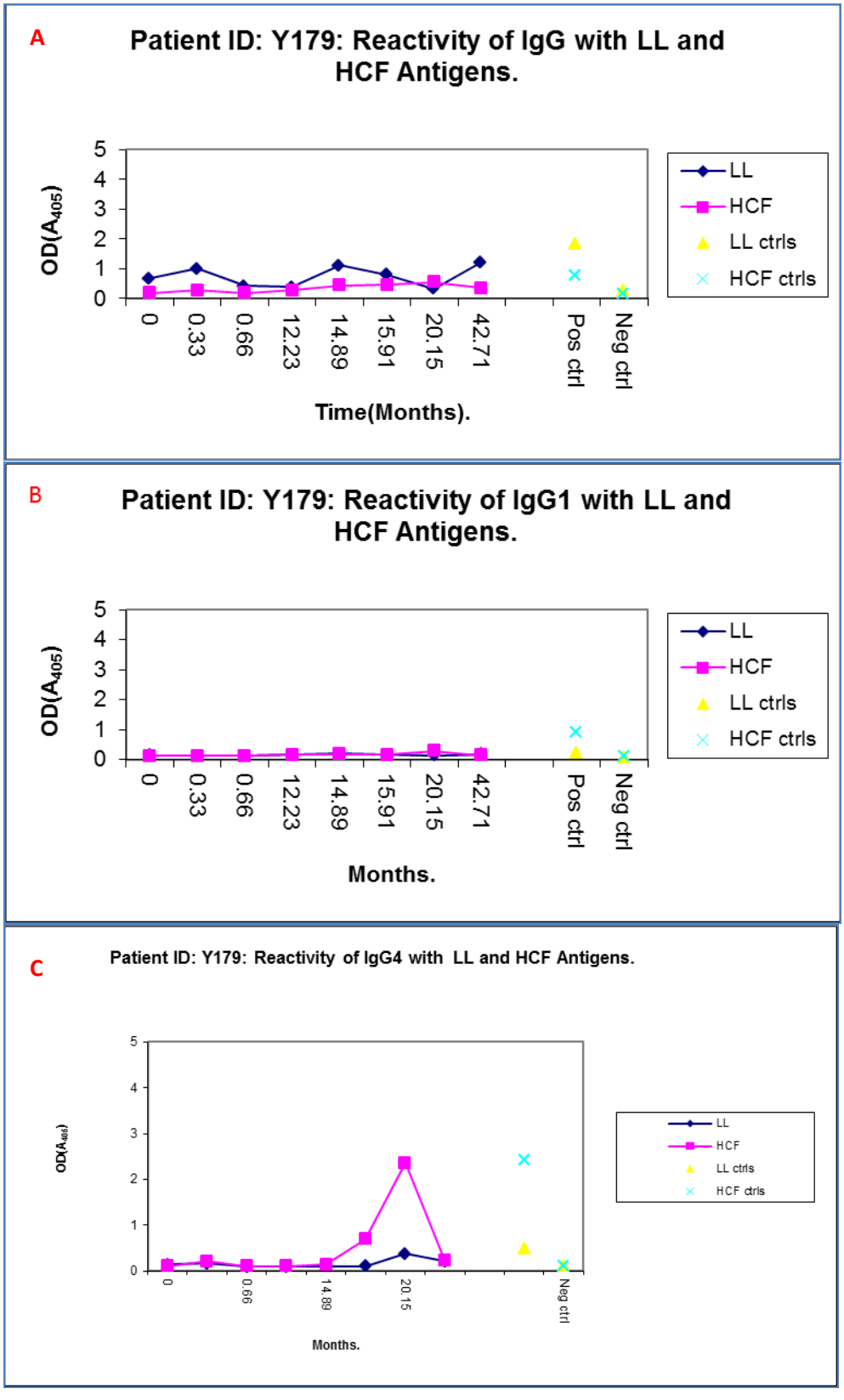


Figure 5.12: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). A very low response is observed for both samples here. HCF (Fig 5.12c) is seen exhibiting a sharp peak at 20.15months before falling.

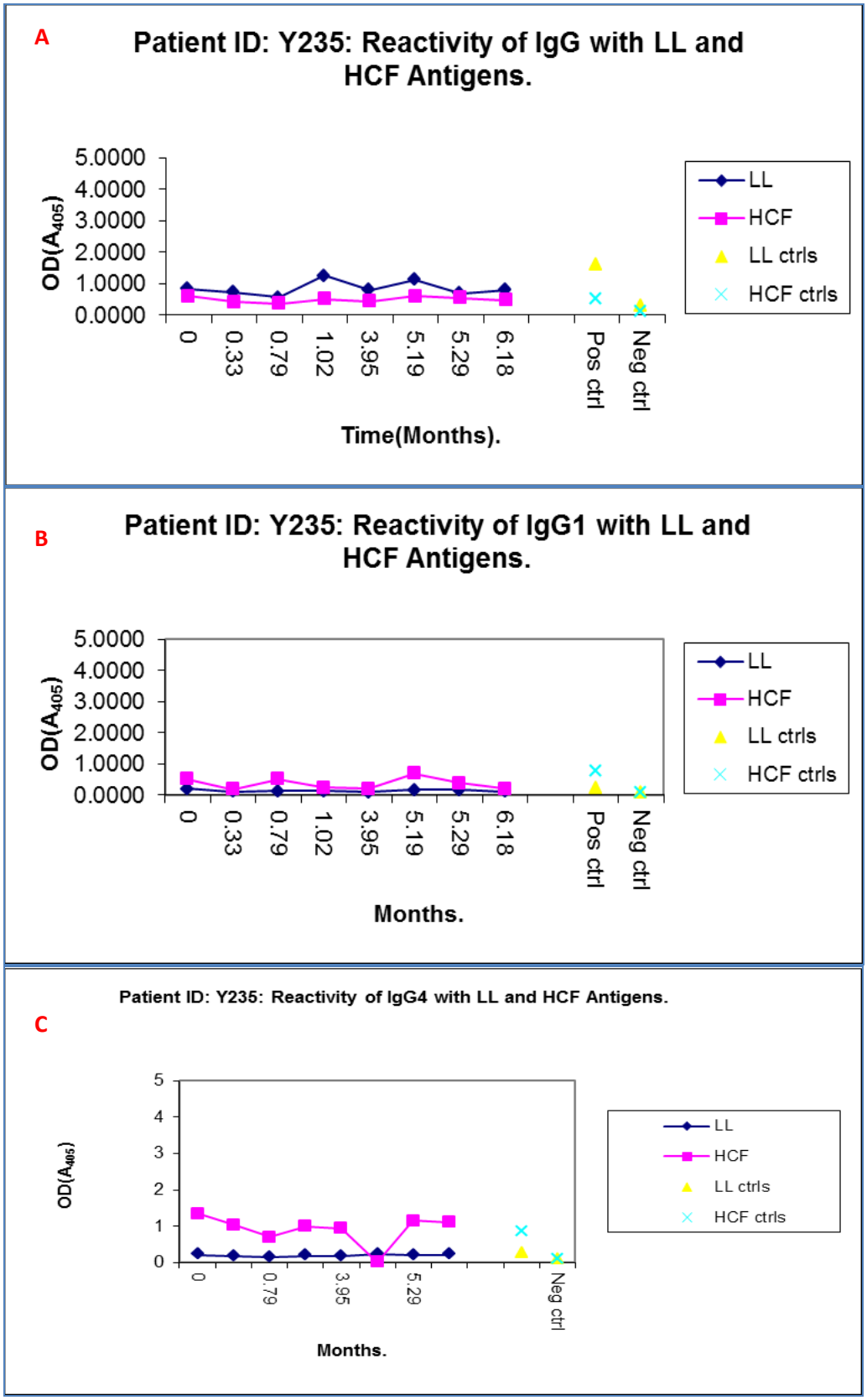


Figure 5.13: Graphs showing reactivity of HCF and LL with total IgG (A), IgG1 (B) and IgG4 (C). Line trends are similar for both samples particularly with total IgG and IgG1 (Fig 5.13a and b, respectively). HCF is a higher responder with IgG4.

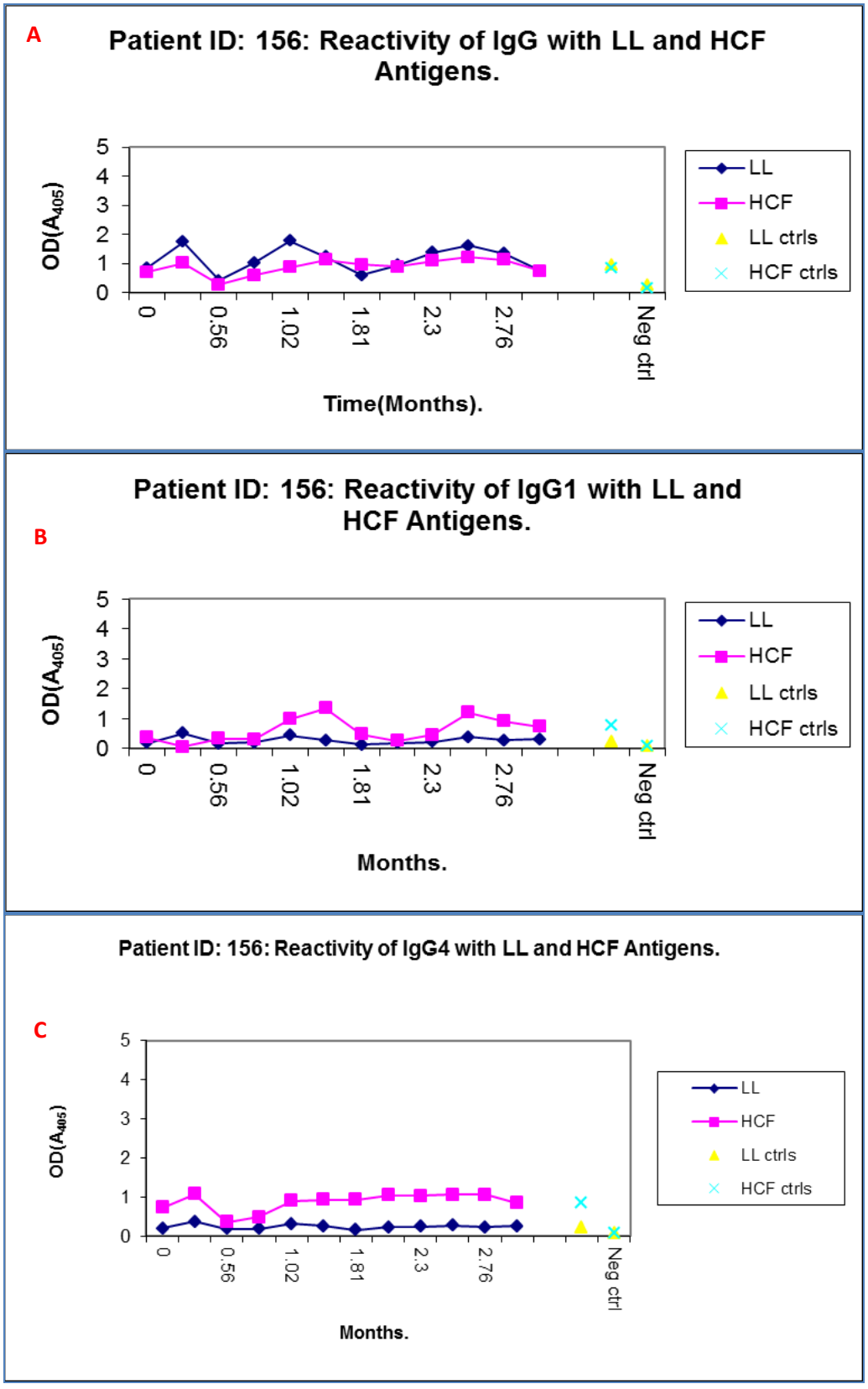


Figure 5.14: Graphs showing the reactivity of HCF and LL with total IgG (a), IgG1 (b) and IgG4 (c). With total IgG (Fig 5.14a), both samples appear to have responded equally, while with IgG1 (Fig 5.14b) and IgG4 (Fig 5.14c) HCF is seen to have slightly higher than the LL.

5.3 DISCUSSION

The usefulness of imaging techniques such as MRI, X-ray or Ultrasonography for follow-up of CE patients cannot be over emphasized. However, these techniques pose a difficulty in detecting the newly growing small cyst and also to discriminate between dead and viable cysts (Wen et al, 1993). Amongst the tests commonly used for the follow-up of CE patients are the enzyme-linked immunosorbent assay (ELISA) and immunoblotting and are used conventionally with the inclusion of crude hydatid cyst fluid, which so far has become the major source of antigen used for this purpose (Force et al, 1992; Ravinder et al, 1997; Poretti et al, 1999; Zarzosa et al, 1999; Gadea et al, 2000; Nouir et al, 2008). Efforts have therefore been directed at applying immunological tests of significantly diagnostic and prognostic values. ELISA and immunoblotting for serum antibody detection making use of various antigen preparations, including crude hydatid cyst fluid, purified fractions of antigen B and/ or 5, and *E. granulosus* protoscolex soluble extract have all been applied to follow-up CE patients (Sbihi et al, 1996; Gadea et al, 2000; Doiz et al, 2002; Rigano et al, 2002; Lawn et al, 2004; Ben Nouir et al, 2008).

Nevertheless, problems mainly related to temporally delayed reactions to clinical changes were displayed by all of these tests (Rigano et al, 2002; Nouir et al, 2008). Recombinant antigen B (rAgB) proves to have similar diagnostic value to native antigen B in CE patients (McVie et al, 1997; Mamuti et al, 2004). Nevertheless, little or no appliaction of rAgB has been used for post-treatment follow-up of CE patients. A lot substantiated when Nouir et al, (2008) did their study on the assessment of *E. granulosus* protoscolex soluble somatic antigens (PSSAs). Their prognostic value in the serological follow-up of young patients treated for cystic echinococcosis (CE),

compared to conventional hydatid fluid (HF) antigen was assessed. They recorded a gradual decrease in antibody levels in CE cases were shown by an Immunoglobulin G (IgG)- PSSA enyme-linked immunosorbent assay (ELISA). At least, seronegativity was reached in 20% of cases within 5 years post surgery in comparison to the standard IgG-HF ELISA which displayed a significantly lower progressive decrease in antibody levels, with serology only becoming negative in 15% of CCE patients at the endpoint of work has been done in trying to find the antigens other than native hydatid cyst fluid suitable for follow-up of CE patients, and this was of the follow-up period. They therefore came to a conclusion that PSSA represents a useful candidate to carry out a serologic follow-up of CE subsequent to treatment and deserves further respective evaluation for other age groups of CE patients.

The purpose of this study is to assess the potential usefulness of *E.granulosus* LL antigen as a marker in post treatment follow-up. In this study, 14 samples i.e only 14 sera samples from 14 confirmed hydatid disease patients were tested, 4 untreated and 10 treated samples. They were tested using the enzyme-linked immunosorbent assay for follow-up after treatment. In contrast to *E. multilocularis* and the Em2 antigen preparation Gottstein et al, (1992) little work has been carried out using the LL of *E*. *granulosus.*

Results in the current study show that the ELISA assay is a sensitive robust immunological tool for use in follow-up surveillance of CE patients. As already established, predominance of IgG1 and IgG4 are associated with seropositive individuals (Aceti et al, 1993; Wen and Craig, 1994; Ioppolo et al, 1996; Shambesh et al, 1997; Sterla et al, 1999; Daeki et al, 2000). In this study, results show that

reactivity of serum antibodies show a similar level of response to both the LL and HCF antigens, however, in some instances levels appeared higher towards the LL antigen.

A very high reactivity and predominance of IgG1 and IgG4 would have been expected from our study, especially for all 4 of our untreated patients since predominance of IgG1 and IgG4 is associated with disease progression and development (Daeki et al, 2000), however, results show that reactivity was low and in some cases a near negative result was recorded. One reason for this may be that the cysts of these individuals have deteriorated slowly over time to a state of calcification and had died a natural death (Romig et al, 1986; Morris, 1986; Pawlowski, 1997; Wang et al, 2003; Wang et al, 2006). According to Levine and Grantham (1992), cyst calcification is possible in patients who suffer also from autosomal dominant polycystic kidney disease (ADPKD), are older, have larger kidneys and worse renal function.

Again, there is not much discrepancy with results obtained for our treated patients/ samples. Results obtained here are very much in line with desired outcomes. A decline in the concentration of IgG1 and IgG4 is expected with cases characterised by cyst infiltration and/ or calcification (Daeki et al, 2000), our results agree with this as with all our treated patient samples. The reactivity of IgG1 and IgG4 especially with the laminated layer of *E. granulosus* showed very little reactivity. Low response was recorded for these IgG subclasses except for when a higher response was also recorded for the laminated layer of *E. granulosus* in reactivity with IgG1 with patient Y88 and for HCF with patients Y13 and Y51. A higher response was also recorded with patient Y88 with IgG4 and with patient Y235 both HCF and laminated layer of *E. granulosus* gave a higher response with IgG4. Patient Y13 was treated at 2.8months

with 3 courses of albendazole (ALB), one course at 22.2months, and another 1 course at 58.7months and had cystectomy at 62.3months. Even though patient Y13 was treated, the patient sample still gave a reaction with IgG1 up until 26months before exhibiting a flat line, which at this time could be said to have been cured.

The concentration of IgG1 was high for patient sample Y51 too. Again, this patient was treated with one course of albendazole (ALB) at 1.6months and went from a CE1 to CE3 and then to CE4. The reaction though, could be false positive. Subclass IgG1 and IgG4 responded high to the LL antigen with patient Y88. Patient Y88 was treated with 2 courses of ALB at 4.8 months, one course at 7.8 months. According to notes that were recorded by the ultrasonographer (AMREF database), the cyst in this patient remained as a CE1 type which most likely explains the high response in obtained results**.** Both HCF and laminated layer are high for patient sample Y235. The patient was treated with one course of ALB at 2.2months and went from a CE2 cyst to a CE3.

The current study, as stated earlier was carried out in order to investigate the possibility of the use of the laminated layer of *E. granulosus* in post-treatment follow-up of CE patients. Our results show decline in the concentration of IgG1 and IgG4 especially which correlates to regression of disease. Antibody response, particularly IgG4 is associated with cystic development, progression and growth (Daeki et al, 2000). Results presented here agree with those of Guerri et al, (2000); Rigano et al, (1995); and Rigano et al, (1996) that IgG4 subclass is a good marker for follow-up surveillance of CE even in this case with the laminated layer of *E.granulosus*.

CHAPTER 6

GENERAL DISCUSSION

Very little attention has been given to the immunological analysis of the laminated layer of E. *granulosus* considering the significance of this structure and the importance of the diagnostic antigens in the laminated layer of *E.multilocularis*.

The current study was carried out to investigate and analyse the laminated layer of *E. granulosus* with a view to the potential for its use in the post-treatment follow-up of CE.

Immunodiagnosis of hydatid disease in the past has always adopted the use of native hydatid cyst fluid as the antigenic material. Little attention has been given to the immunoreactivity of human cystic hydatid sera against the laminated layer of *E*. *granulosus*. The presence of large carbohydrates on the surface of the laminated layer perhaps make it less suitable for solid phase immunoassays such as the ELISA. However, the present study indicates that, with the use of crude sonicate, the laminated layer would as well as or even yield a higher response as the case may be.

Lectins have been used in the past and have been involved in the general molecular characterization of the laminated layer of the *Echinococcus* species. Kouguchi et al, (2011) Showed how a conventionally purified glycoprotein component of *Echinococcus multiloularis* protoscolex , designated as Emgp-89, may be useful as a serodiagnostic antigen for detecting *E. multilocularis* infection in dogs domesticated in endemic areas. They obtained Emgp-89 from the parasite material by a simple procedure using ConA-agarose and subsequent gel filtration chromatography. A

molecular weight of >4000KDa was showed by the purified fraction upon gel filtration and reacted with a series of lectins that specifically bind to mannose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. Evaluation of the serodiagnostic performance of Emgp-89 was through enzyme-linked immunosorbent asssays (ELISAs) by using sera from normal, domestic dogs and dogs infected with other helminths. Emgp-89 positively reacted with all 16 serum samples from *E.multilocularis*- infected dogs, thus showing that this antigen is highly sensitive. Although, they didn't break down their obtained molecular weight, but the result of this present agrees with results obtained by Kouguchi et al, (2011) in that mannose, N-acetylglucosamine and N-acetylgalactosamine were recognised by our lectin samples too.

Arana et al, (2013) also carried out a study on the carbohydrate composition of *Taenia solium* whole oncosphere antigens (WOAs), in order to improve the understanding of the antigenicity of the *T. solium*. For accurate diagnosis of previous exposure to *T. solium* eggs and thus predict the development of cysticercosis, better knowledge of oncosphere is absolutely important. In their study, a set of 7 lectins conjugates with wide carbohydrate specificity were used on parasite fixations and some somatic extracts. According to them, lectin fluorescence revealed that D-mannose, D-glucose, D-galactose and N-acetyl-D-galactosamine residues were the most abundant constituents of carbohydrate chains on the surface of *T. solium* oncosphere. Their lectin blotting also showed that posttranslational modification with N-glycosylation was abundant while little evidence of O-linked carbohydrates was observed. They demonstrated, for the first time, that the presence of several lectins on the surface of the oncosphere was specific to carbohydrates found in intestinal mucus, suggesting a possible role in initial attachment of the parasite to host cells. Again, the result of the current study agrees with that of Arana et al, (2013) obtained

result. Carbohydrates such as mannose, N-acetylgalactosamine and galactose were recognised.

Results of SDS carried out show the fractions of LL recognising bands at the same regions as the HCF thereby suggesting the presence of AgB. Extracts of crude laminated layer were purified using the Affinity chromatography technique. The collected eluates were used in ELISA even though the assay worked, results show that higher response was derived when tested with total IgG while with the other two subclass immunoglobulins 1 and 4., the results show correlation with results obtained from the current study where with virtually all assays done, IgG1 and IgG4 is on the decline especially samples from treated patients.

For this part of the current study, the aim and ultimate plan was to purify the laminated layer of *E. granulosus* and then use the obtained purified extract in further serological investigations to be able to ascertain the serodiagnosis and general molecular capacities. As stated earlier, encouraging results were not obtained for the ELISA assay and therefore, it was decided not to go ahead with the other assays, but this does not negate the fact that the affinity chromatography is an established molecular tool that has proved very useful in molecular diagnosis and has been employed by various researchers in different laboratories around the globe.Isolation and characterization of cestode-derived antigens of infected hosts have been advocated with regard to elimination of cross-reactivity to improve immunodiagnostic tests (Ito and Smyth, 1987). In their study, Elayoubi and craig (2004) aimed at the prospect of the fractionation of immunodiagnostic-relevant *E*. *granulosus* antigens directly from faecal samples of infected dogs or from adult worm extracts using size-exclusion fast protein liquid chromatography (FPLC), and the subsequent biochemical characterization of the partially purified antigens.

The FPLC results indicated that at least 2 major antigenic components or fractions

(F1 and F2) with different large molecular weights (>670KDa and 146-440KDa respectively) were specifically present in faecal supernatants from *E. granulosus*-infected dogs as demonstrated by coproantigen capture ELISA. These 2 high molecular weight fractions probably represent the effective coproantigen activity that is detectable by the *Echinococcus* coproantigen ELISA (Elayoubi and Craig, 2004). The assy again has also been used by Gottstein et al, (1983) when they isolated a specific fraction (Em2) from crude metacestode of the *E.multilocularis* by immunoaffinity chromatography against anti-*E.granulosus* hydatid fluid IgG coupled to CNBr-Sepharose 4B. This antigen has a molecular mass of 54KDa and is located within the LL (Gottstein, 1985; Furuya et al, 1989; Gottstein, 1992). Em2 is now confirmed as species-specific antigen and WHO recommends the Em2-ELISA as a reference immunodiagnostic test for alveolar echinococcosis.
A partially purified Em 18/16 enriched fraction from *E. multilocularis* LL prepared by isoelectric focusing (Ito et al, 1997), may have a greater specificity than the Em2 (plus)-ELISA. Since cyst fluid contained two antigens (are 5 and antigen B), both antigens have shown to have cross reactions with other helminths in particular *E. multilocularis* and *Taenia solium* (Craig et al, 1995).

Taherkhani and Rogan, (2001) also decided to investigate the immunogenicity of the laminated layer of *E. granulosus* with respect to its role in immunodiagnosis. They attempted to remove host components such as sheep serum and / or sheep IgG, by preparation of a semi-purified LL fraction using an affinity chromatography column and to evaluate its antigenic reactivity by SDS-PAGE and immunoblotting. They found the normal LL showed bands in the 50-55KDa region and the 27-31KDa which presumably reflected the removal of heavy and light immunoglobulin chains.

However, the most useful aspect of the laminated layer in terms of serodiagnosis

was evident in immunoblotting. This indicated that the bands at 66, 55, 29 and 27KDa were significant molecules for diagnosis of human cystic hydatidosis patients. Since total IgG and IgG4 subclass antibody responses in positive sera recognised major bands in the 25 up to 29KDa region (much stronger at 29KDa) in unpurified LL, in the 25-31KDa (much stronger at 27KDa) in Px and one obvious band at 27KDa in semi-purified LL.

Extracts of laminated layer was immunoblotted against total IgG, IgG1 and I gG4, as evident on graphs (figure 3.5 and 3.6), where the LL especially detects no band against these two subclasses of IgG..

Again, the immunodiagnostic strength and capacity of the laminated layer of *E granulosus* was put to test when the LL was reacted with total IgG, IgG1 and IgG4 with sera from confirmed CE patients from Turkana , Kenya. Fourteen samples were tested in all, four untreated and 10 treated. Again, a decline in the response towards IgG1 and especially IgG was recorded. This could be due to the fact the cyst presented by these patients have undergone calcification over time and may have died a natural death. Follow-up after treatment of CE and AE is highly important and necessary to be able to monitor relapse, recurrence and/ or cure. This was shown in the work of Dreweck et al, (1997) when they evaluated Immunoglobulin subclass-specific antibody responses and isotype-specific recognition of *E. multilocularis* and *E. granulosus* antigens in both alveolar echinococcosis and cystic echinococcosis. AE patients were divided into 3 groups by clinical and therapeutic criteria according to their actual state of infection, i.e, elimination of parasite, and regression or progression of disease. CE patients were either before or after surgery, or in continuous chemotherapy due to parasite persistence. Total IgE was highly elevated

in progressive AE cases (7/11), but not in the cases with eliminated infection or regression. In patients with active AE disease, EmAg-specific IgE, total IgG, IgG1, IgG2 and IgG4 were particularly high. Similarly, total IgE was raised beyond reference values in 9 of 30 CE patients indicating progressive disease. CE patients sera antibody cross-reacted with crude EmAg, and detectable Ig levels of the same isotype were also measured by ELISA. In both AE and CE, parasite-specific antigen recognition was dominated by IgG1 and IgG4. In AE patients with progressive disease, IgG4 distinctively recognised low molecular weight EmAg of 26KDa, 18KDa, 16KDa and 12KDa. As prominent IgG4 and IgE responses develop with chronic helminth infections only, these serological parameters may indicate successful parasite infestation and severe outcome of disease. They submitted that analyses of immuogloblin isotype responses in AE patients by ELISA in combination with immunoblotting are a useful approach for post-treatment follow-up of patients at risk of developing recrudescent disease.

From the current study, results have shown that and agreed with the work carried out by Dreweck et al, (1997) by showing bands at and with low molecular weights in the region of 18KDa especially and also followed by the antibody specific response. IgG4 , especially with all our treated patients samples was on the decline, which could be pointing at the fact that treatment obtained and given to these patients have been successful as prominent IgG4 is only associated with chronic disease. Also, it shows that IgG4 is a reliable serological parameter in the diagnosis and follow-up of cysytic echinococcosis.

Overall, all of the assays employed in the current study have worked successfully to their optimum. Results obtained have not only shown the laminated layer of ***E.*** *granulosus* to be composed of antigenic materials but also it has indicated that the

laminated layer of *E. granulosus* can be employed as an antigenic material in the immunodiagnosis and post- treatment follow-up of CE. We would like to suggest that further work be carried out to substantiate this.

REFERENCES

Abdi, J., Kazemi, B., Mohebali, M., Bandehpour, M., Rahimi, M.T., Rokni, M.B. 2010. Gene Cloning, Expression and Serological Evaluation of the 12-KDa antigen-B Subunit from *Echinococcus granulosus*. Ann Trop Med Parasitol, 104(5): 399-407

Abu-Eshy, S.A. 1998. Some rare presentations of hydatid cyst (*Echinococcus granulosus*). *J. R. Coll. Surg. Edinb*. 43:347-352.

Aceti, A.A., Pennica, A., Teggi, L.M., Fondacaro, M., Caferro, O., Lecri, G., Tacchi, D., Celestino, G., Quaranta, F., De Rosa, and Sebastani, A. 1993. IgG subclasses in human hydatid disease: prominence of the IgG4 response. Int. Arch. Allergy Immunol. 102: 347-351.

Adjobimey, T. and Hoerauf, A. 2010. Induction of immunoglobulin G4 in human filariasis: an indicator of immunoregulation. Ann Trop. Med. Parasitol. 104, 455-464.

Akhan, O., Ozmen, M.N., Dincer, A., Sayek, I. and Gocmen, A. 1996. Liver hydatid disease: long-term results of percutaneous treatment. *Radiology*. 198:259-264.

Allan, D, Jenkins, P, Connor, R.J, Dixon, J.B. 1981. A study of immunoregulation of BALB/c mice by *Echinococcus granulosus equinus* during prolonged infection. Parasite Immunol. 3, 137-142.

Al-Ghoury, A.B., El-Hamshary, E.M., Azary, A.A, Hussein, E.M. Rayan, H.Z. 2010. HLA class II alleles: susceptibility or resistance to cystic echinococcosis in Yemeni patients. Parasitol. Res. 107, 355-361.

al-Yaman, F.M. and Knobloch, J. 1989. Isolation and partial characterization of species-specific and cross-reactive antigens of *Echinococcus granulosus* cyst fluid. *Mol. Biochem. Parasitol*. 37:101-107.

Ammann, R.W. and Eckert, J. 1996. Parasitic diseases of the liver and intestines: Cestodes; *Echinococcus*. *Gastroenterol Clin North Am*, 25:655-89.

Antoniou, M. and Tselentis, Y. 1993. Studies on *Echinococcus granulosus* using the scanning electron microscope. *Parasitol. Res*, 79:543-546.

Arana, Y., Verastegui, M., Tuero, I., Grandjean, L., Garcia, H.H., Gilman, R.H. 2013. Characterization of the carbohydrate components of *Taenia solium* oncosphere proteins and their role in the antigenicity. Parasitol Res, 112(10): 3569-78.

Arend, A.C., Zaha, A., Ayala, F.J., Haag, K.L. 2004. The *Echinococcus granulosus* antigen B shows a high degree of genetic variability. Experimental Parasitology, 108: 766-80.

Axen, R., Porath, J. and Ernback, S. 1967. 'Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides.'Nature (Lond.), 214, 1302.

Aydinli, B., Pirim I., Polat, K.Y., Gursan, N., Atamanalp, S.S., Ezer, M., Donmez, R. 2007. Association between hepatic alveolar echinococcosis and frequency of human leukocyte antigen class 1 and 2 alleles in Turkish patients. Hepatol. Res. 37, 806-810.

Aygun, E., Sahin, M., Odev, K, *et al*. 2001. The management of liver hydatid cysts by percutaneous drainage. *Can J Surg*, 44:203-09.

Barbieri, M., Sterla, S., Battistoni, J. and Nieto, A. 1993. High performance latex reagent for hydatid serology using *Echinococcus granulosus* lipoprotein antigen fraction purified from cyst fluid in one step. Int. J. Parasitol. 23, 565-572.

Barbieri, M., Saveri, M. A., Pirez, M. L., Battistoni, J. and Nietto, A. 1994. Use of specific antibody and circulating antigen levels in the hydatid immunodiagnosis of asymptomatic population. Int. J. Parasitol. 24, 937-942.

Barth, T.F.E., Hermann, T.S., Tappe, D., Stark, L., Gruner, B, et al. 2012. Sensitive and Specific Immunohistochemical Diagnosis of Human Alveolar Echinococcosis with the Monoclonal Antibody Em2G11. PLoS Negl Trop Dis 6(10): e1877.

Baz, A., Richieri, A, Puglia ,A, Nieto, A, Dematteis, S. 1999. Antibody response in CD4- depleted mice after immunization or during early infection with *Echinococcus granulosus*. Parasite Immunol. 21: 141-150.

Bayraktar, M.R., Mehmet, N., Durmaz, R. 2005. Th1 and Th2 inducing cytokines in cystic echinococcosis. Turkiye Parasitol. Derg. 29: 167-170.

Beggs, I. 1985. The radiology of hydatid disease. AJR, *Am J Roentgenol*, 145:639-48.

Ben Nouir, N., Nunez, S., Gianinazzi, C., Gorcii, M., Muller, N., Nouri, A.,Babba, H. and Gottstein, B. 2008. Assessment of *Echinococcus granulosus* Somatic Protoscolex Antigens for serological follow-up of Young Patients surgically treated for cystic Echinococcosis. J. Clin. Microbiol. 46(5): 1631- 1640.

Ben Nouir, N., Nunez, S., Frei, E., Gorcii, M., Muller, N., Gianinazzi, C., Mekki, M., Nouri, A., Babba, H. and Gottstein, B. 2008. Post-surgical follow-up (by ELISA and Immunoblotting) of cured versus non-cured cystic echinococcosis in young patients. Parasitology 135 (Pt 1) 105-114.

Beisel, W.R. (1982) Single nutrients and immunity. Am. J. Clin. Nutr. 35, 417-468.

Blanton, R.E., Gideon, B.A., Kijobe, J. and King, C. 1991. Antibody responses to in

vitro translation products following albendazole therapy for *Echinococcus granulosus. Antimicrob Agents Chemother*. 35:1674-76.

Boctor, F.N. and Peter, J.B. 1990. IgG subclasses in human chronic Schistosomiasis: over production of Schistosome specific and non- specific IgG4. Clin Exp Immunol 82: 574-578.

Bortoletti, G. and Ferretti, G. 1973. Investigation on larval forms of *Echinococcus granulosus* with electron microscope. *Riv. Parasitol*. 34:89-10.

Bortoletti, G. and Ferretti, G. 1978. Ultrastructural aspects of fertile and sterile cysts of *Echinococcus granulosus* developed in hosts of different species. Int. J. Parasitol. 8, 421-431.

Breijo, M., Anesetti, G., Martínez, L., Sim, R.B., Ferreira A.M . 2008. *Echinococcus granulosus*: the establishment of the metacestode is associated with control of complement-mediated early inflammation. Exp. Parasitol. 118, 188-196.

Bresson-Hadni, S, Liance, M. Meyer,J.P. Houin,R. Bresson,J.J. and Vuitton, D.A . 1990. Cellualr immunity in experimental *Echinococcus multilocularis* infection. II. Sequential and comparative phenotypic study of the periparasitic mononuclear cells in resistant and sensitive mice. Clin. Exp. Immunol. 82, 378-383.

Brunetti, E. Kern, P. Vuitton, D.A. 2010. Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans. Acta Trop. 114, 1-16.

Brunetti, E. and Junghanss, T. 2007. Update on ystic hydatid disease. Curr Opin Infect Dis 22: 497-502.

Budke, C.M. Deplazes, P. Torgerson, P.R 2006. Global socioeconomic impact of cystic echinococcosis. Emerg. Infect. Dis. 12, 296-303.

Caremani, M., Benci, A., Maestrini, R., Accorsi, A., Caremani, D. and Lapini, L. 1997. Ultrasound imaging in cystic echinococcosis. Proposal of a new sonographic classification. *Acta Tropica*. 67:91-105.

Carmena, D., Benito, A., Eraso, E. 2006. Antigens for the immunodiagnosis of *Echinococcus granulosus* infection: an update. Acta Trop. 98: 74-86.

Casado, N., Criado, A., Jimenez, A., De Armas, C., Brasa, C., Perez-serrano, J. and Rodriguez-Caabeiro, F. 1992. Viability of *Echinococcus granulosus* cysts in mice following cultivation in vitro. *Int. J. Parasitol*. 22:335-339.

Casaravilla, C. and Diaz, A. 2010. Studies on the structural mucins of the *Echinococcus granulosus* laminated layer. Mol. Biochem. Parasitol. 174, 132-136.

Chemale, G., Haag, K.L., Ferreira, H.B., Zaha, A. 2001. *Echinococcus granulosus* antigen B is encoded by a gene family. Molecular and Biochemical Parasitology, 116: 233-237.

Chordi, A. and Kagan, I.G. 1965. Identification and characterization of antigenic components of sheep hydatid cyst fluid by immunoelectrophoresis. *J. Parasitol*. 51:63-71.

Coltorti, E.A. and Varela-Diaz, V.M. 1974. *Echinococcus granulosus*: penetration of macromolecules and their localization in the parasite membranes of cysts. Exp. Parasitol. 35, 225-231.

Conchedda, M, Gabriele, F. Bortoletti, G . 2008. Morphological study of anomalous “laminated” brood capsules in cystic echinococcosis in humans and sheep. Acta Trop. 105, 215-221.

Conchedda, M., Gabriele, F., Bortoletti, G. 2004. Immunobiology of Cystic echinococcosis. Parasitologia. 46, 375-380.

Conder, G.A., Marchiondo, A.A., Williams, F. and Andersen, F.L. 1983. Freeze-tech characterization of the teguments of three metacestodes: *Echinococcus granulosus*, *Taenia crassiceps* and *Taenia taeniaeformis*. *J. Parasitol*. 69:539-548.

Craig, P.S. 1986. Detection of specific circulating antigen, immune complexes and antibodies in human hydatidosis from Turkana (Kenya) and Great Britain, by enzyme-immuno assay. Parasite Immunol. 8, 171-188.

Craig, P.S., Rogan, M.T. and Allan, C.J. 1995. Hydatidosis and cysticercosis larval cestodes. *In*: Medical Parasitology (Gillesrie, S.H. and Hawkey, P.M. *eds*), Oxford University, Oxford, UK. pp (209-239).

Craig, P.S. (1993) Immunodiagnosis of *Echinococcus granulosus*, p. 85-118. *In*: Andersen, F.L. *et al*. (*ed*), Compendium on cystic echinococcosis. Brigham Young University, Provo, Utah.

Czermark, B.V. *et al*. 2001. *Echinococcus garnulosus* revisited: radiologic patterns seen in paediatric and adult patients. *AJR Am. J. Roentgenol.* 177:1051-1056.

Daeki, A.O., Craig, P.S., Shambesh, M.K. 2000. IgG-subclass antibody responses and the natural history of hepatic cystic echinococcosis in asymptomatic patients. Ann. Trop. Med. Parasitol. 94, 319-328.

Dai, W.J.,Hemphill, A., Waldvogel, A., Ingold, K., Deplazes, P., Mossmann, H., Gottstein, B. 2001. Major carbohydrate antigen of *Echinococcus multilocularis* induces an immunoglobulin G response independent of alphabeta^{+} CD4^{+} T-cells. Infect. Immun. 69, 6074-6083.

Dai, W.J., Waldvogel, A. Siles-Lucas, M.,Gottstein, B. 2000. *Echinococcus multilocularis* proliferation in mice and respective parasite 14-3-3 gene expression is mainly controlled by an alphabeta CD4 T-cell mediated immune response. Immunology. 112, 481-488.

Dakkak, A. 2010. Echinococcosis/ hydatidosis: a severe threat in Mediterranean countries. Vet. Parasitol. 174, 2-11.

Dempster, R.P., Harrison, G.B., Berridge, M.V.,Heath, D.D. 1992. *Echinococcus granulosus*: use of an intermediate host mouse model to evaluate sources of protective antigens and a role for antibody in the immune response. Int. J. Parasitol. 22, 435-441.

Deplazes, P. and Gottstein, B. 1991. A monoclonal antibody against *Echinococcus multilocularis* Em2 antigen. Parasitology 103 (Pt.1), 41-49.

Dessaint, J.P., Bout, D., Wattre, P., and Capron, A. 1975. Quantitative determination of specific IgE antibodies to *Echinococcus granulosus* and IgE levels in sera from patients with hydatid disease. Immunology 29, 813-823.

Devine, P.L. and McKenzie, I.F. 1992. Mucins : structure, function, and associations with malignancy. Bioessays 14, 619-625.

Diaz, A., Ibarguren. S., Breijo, M., Willis,,A.C .,Sim, R.B. 2000. Host-derived annexin ∏ at the host-parasite interface of the *Echinococcus granulosus* hydatid cyst. Mol. Biochem. Parasitol. 110, 171-176.

Diaz, A. and Allen, J.E. 2007. Mapping immune response profiles: the scenario from helminth immunology. Eur. J. Immunol. 37, 3319-3326.

Diaz, A. et. al. 2009. The major surface of carbohydrates of the *Echinococcus granulosus* cyst: mucin- type O- glycans decorated by novel galactose- based structures. Biochemistry 48, 11678-11691.

Diaz, A., Willis, A.C. and Sim, R.B. 2000. Expression of the proteinase specialised in bone resorption, cathepsin K, in granulomatous inflammation. Mol.Med. 6, 648-659.

Diaz, A., Casaravilla, C., Allen, J.E., Sim ,R.B., Ferreira, A.M. 2011. Understanding the laminated layer of larval *Echinococcus* II Immunology. Trends. In. Parasitol. 27: 263-272.

Diaz, A.., Casaravilla, C. , Irigoín, F. , Lin, G., Previato, J.O., Ferreira, F. 2011. Understanding the laminated layer of larval *Echinococcus* 1: structure. Trends Parasitol. DOI: 10.1016/j.pt.2010.12.012.

Doiz, O., Benito, R., Sbihi, Y., Osuna, A., Clavel, A., Gómez-Lus, R. 2001. Western blot applied to the diagnosis and post-treatment monitoring of human hydatidosis. *Diagnostic Microbiology and Infectious Disease*. 41:139-142.

Doiz, O., Benito, R., Gil, R., Rojas, A., Rubio, M.C et al, 2002. Pre- and post-surgical detection of IgG, IgM, IgA specific to hydatidosis by ELISA with purified antigen enriched with the 5/B antigen complex. J Clin Lab Anal 16: 295- 298.

Dreweck, C. M., Lüder, C.G., Soboslay, P.T., Kern, P .1997. Subclass-specific serological reactivity and IgG4- specific antigen recognition in human echinococcosis. Trop. Med. Int. Health. 2, 779-787.

Eckert, J., Conraths, F.J., Tackmann, K. 2000. Echinococcosis: an emerging or re-emerging zoonosis? Int. J. Parasitol. 30, 1283-1294.

Eckert, J. and Deplazes, P. 2004. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin Microbiol 17: 107-135.

Eckert, J., Schantz, P., Gasser, R., *et al*. .2001. Geographic distribution and prevalence. *In*: Eckert, J., Gemmell, M.A., Meslin, F-X., Pawlowski, Z.S, *eds*. WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Paris: World Organisation for Animal Health, 100-41.

Eiermann, T.H. et. al. 1998. HLA and alveolar echinococcosis. Tissue Antigens 52, 124-129.

Elayoubi, F.A. and Craig, P.S. 2004. *Echinococcus granulosus* coproantigens: Chromatographic Fractionation and Characterization.Parasitology, 128: 455-465.

Elissondo, M. et. al. .2007. In vitro and in vivo ffects of flubendazole on *Ehinococcus granulosus* metacestodes. Parasitol. Res. 100, 1003-1009.

El-On, J. 2003. Benzimidazole treatment of cystic echinococcosis. *Acta Trop*. 85:243-252.

Emery, I. et. al. .1997. Lack of H-2 gene influence on mouse susceptibility to secondary alveolar echinococcosis. Int. J. Parasitol. 27, 1433-1436.

Emery, J. et. al. 1998. In vivo treatment with recombinant IL-12 protects C57BL/6J mice against secondary alveolar echinococcosis. Parasite. Immunol. 20, 81-91.

Erşahin, Y., Mutluer, S. and Güzelbağ, E. 1993. Intracranial hydatid cysts in children. *Neurosurgery*, 33:219-25.

Ferreira, A. M. et. al. 2000. How *Echinococcus granulosus* deals with complement. Parasitol Today. 16, 168-172.

Fernandez, V., Ferreira. Henrique, B., Fernandez, C., Zaha, A. and Nieto, A. 1996. Molecular characterisation of a novel 8-KDa subunit of *Echinococcus granulosus* antigen B. Molecular and Biochemical Parasitology (77) 247-250.

Filice, C., Pirola, F., Brunetti, E., Dughetti, S., Strosselli, M. and Foglieni, C.S. 1990. A new therapeutic approach for hydatid liver cysts. Aspiration and alcohol injection under sonographic guidance. *Gastroenterology*. 98:1366-1368.

Filice, C. *et al*. 1992. Ultrasound examination of hydatid cysts treated with albendaole*. J. Clin. Ultrasound*. 20:569-570.

Filice, C. and Brunetti, E. 1997. Use of PAIR in human cystic echinococcosis. *Acta Trop*, 64:95-107.

Filice, C., Brunetti, E., Bruno, R. and Crippa, F. 2000. Percutaneous drainage of echinococcal cysts (PAIR- puncture, aspiration, injectionn, reaspiration): results of a worldwide survey for assessment of its safety and efficacy. WHO- Informal Working Group on Echinococcosis- Pair Network. *Gut*, 47:156-57.

Force, L., Torres, J.M. and Carillo, A. 1992. Evaluation of eight serological tests in the diagnosis of human ehinococcosis and follow-up. Clin. Infect. Dis. 15473-480.
Frider, B., Larrieu, E. and Odriozola, M. 1999. Long-term outcome of asymptomatic liver hydatidosis. *J. Hepatol*. 30:228-31.

Frosch, P., Hartmann, M., Muhlschlegel, F., Frosch, M. 1994. Sequence heterogeneity of echinococcal antigen B. Mol. Biochem. Parasitol. 64: 171-175.

Furuya, K., Sasaki, S., Honma, H., Kumagal, M.,Sato, N., Takahashi, M. and Uchino, J. 1989. Serologic investigations of human alveolar hydatid disease by Western blotting and indirect histo-immunoperoxidase techniques. *Jpn. J. Parasitol. 38 (4): 184-193*. *Iranian Biomedical Journal 5 (1): 47-51 (January 2001)* 51.

Gadea, I., Ayala, G., Diago, M.T., Cunat, A. and Garcia De Lomas, J. 2000. Immunological diagnosis of human hydatid cyst relapse: utility of the enzyme-linked immunoelectrtransfer blot and discriminant analysis. Clin. Diagn. Lab. Immunol. 7549-552.

Gargouri, M., Amor, N.B., Chehida, F.B., *et al*. 1990. Percutaneous treatment of hydatid cysts (*Echinococcus granulosus*). *Cardiovasc Intervent Radiol*, 13: 167-73.

Gharbi, H.A., Hassine, W., Brauner, M.W. and Dupuch, K. 1981. Ultrasound examination of the hydatid liver. *Radiology*, 139:459-463

Gharbi, H.A., Hammou, A. and Cerri, G.G. 1997. Diagnostic therapeutic role of ultrasound in tropical medicine. *In*: Current Trends in Digestive Ultrasonography, volume 24. Gandolfi, L. and Fukuda, M. (*eds*). Basel: Karger, pp (149-181).

Gilbert, D., Moellering, R. and Sande, M. 2001. Table 6: risk categories of antimicrobics in pregnancy. The Sanford Guide to antimicrobial therapy. 31st edn. Hyde Park: *Antimicrobial Therapy*, pp (55).

Godot, V. et. al. 1997. Increased basal production of interleukin-10 by peripheral blood mononuclear cells in human alveolar echinococcosis. Eur. Cytokine Netw. 8, 401-408.

Godot, V. et. al. 2000. Resistance/susceptibility to *Echinococcus multilocularis* infection and cytokine profile in humans. II. Influence of the HLA B8, DR3,DQ2 haplotype. Clin. Exp. Immunol. 121, 491-498.

Godot, V. et. al. 2000. Resistance/susceptibility to *Echinococcus mutilocularis* infection and cytokine profile in humnas. 1. Comparison of patients with progressive and abortive lesions. Clin. Exp. Immunol. 121, 484-490.

Gossios, K.J., Kontoyiannis, D.S., Dascaloogiannaki, M. and Gourtsoyiannis, N.C. 1997. Uncommon locations of hydatid disease: CT appearances. *Eur Radiol*, 7:1303-8.

Gonzalez-Sapienza, G., Lorenzo, C., Nieto, A. 2000. Improved immunodiagnosis of cystic hydatid disease by using a synthetic peptide with higher diagnostic value than that of its parent protein, *Echinococcus granulosus* antigen B. J. Clin. Microbiol. 38:

3979-3983.

Gottstein, B., Eckert, J. and Fey, H. 1983. Serological differentiation between *E. Granulosus* and *E. multi-locularis* in man. *Z.Parasitol.69: 347-356.*

Gottstein, B. 1992. *Echinococcus multilocularis* infection: Immunology and Immunodiagnosis*.* Advances in Parasitology, 31:321-380.

Gottstein, B. 1985. Purification and Characterization of a specific antigen from *Echinococcus multilocularis*. Parasite immunology, 7: 201-212.

Gottstein, B., Deplazes, P., Aubert, M. 1992. *Echinococcus multilocularis*: Immunological study on the “Em2-positive” laminated layer during in vitro and in vivo post-oncospheral and larval development. Parasitol Res 78(4): 291-297.

Gottstein, B., Jacquier, P., Bresson-Hadni, S., Eckert, J. 1993. Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus antigen. J Clin Microbiol 31(2): 373-376.

Gottstein, B. et. al. (1987) Sero-eepidemiological survey for alveolar echinococcosis (by Em2-ELISA) of blood donors in an endemic area of Switzerland. Trans. R. Soc. Trop. Med. Hyg. 81, 960-964.

Gottstein, B. and Felleisen, R. 1995. Protective immune mechanism against the metacestode of *Echinococcus multilocularis*. *Parasitol*. *Today*, 11:320-326.

Gottstein, B. et. al. (1996) Immunological parameters associated with susceptibility or resistance to alveolar hydatid disease in Yupiks/ Inupiats. Arctic Med. Res. 55, 14-19.

Gottstein, B. et. al. 1994 .*Echinococcus multilocularis*: parasite-specific humoral and cellular immune response subsets in mouse strains susceptible (AKR, C57B1/ 6J) or ‘resistant’ (C57B1/10) to secondary alveolar echinococcosis. Clin. Exp. Immunol. 96, 245-252.

Gottstein, B. and Hemphill, A.. 1997. Immunopathology of Echinococcosis. Chem. Immunol. 66, 177-208.

Grainger, J.R. et. al. 2010. Microbe- dendritic cell dialog controls regulatory T-cell fate. Immunol. Rev. 234, 305-316.

Grimm, F., Maly, F.E., Lu, J. and Llano, R. 2008. Analysis of Specific Immunoglobulin G Subclass Antibodies for Serological Diagnosis of Echinococcosis by a Standard Enzyme-Linked Immunosorbent Assay. *Am. Soc. Microbiol*. pp (613-

616).

Guerri, M.L., Davila, M., Rodriuez, M., Javier Nieto, F. and Ladron de Guevara, C. 2000. Utility of IgG subclasses in the diagnosis and follow up of hydatidosis. Enferm. Infect.Microbiol. Clin. 18: 262-2666 (In Spanish).

Haliloğlu, M., Saatçi, I., Akhan, O., Özmen, M.N. and Besim, A. 1997. Spectrum of imaging findings in pediatric hydatid disease. AJR, *Am J Roentgenol*, 169:1627-31.

Haag, K.L., Alves-Junior, L., Zaha, A., Ayala, F.J. 2004. Contingent, non-neutral evolution in a multicellular parasite: natural selection and gene conversion in the *Echinococcus granulosus* antigen B family. Gene 333: 157-167.

Harris, A. et. al. 1989. *Echinococcus granulosus*: ultrastructure of epithelial changes during the first 8 days of metacestode development in vitro. Int. J. Parasitol 19, 621-629.

Heath, D.D. and Smyth, J.D. 1970. In vitro cultivation of *Echinococcus granulosus, Taenia hydatigena, T. ovis, T. pisiformis* and *T. serialis* from oncosphere to cystic larva. *Parasitology*. 61:329-343.

Heath, D.D. and Osborn, P.J. 1976. Formation of *Echinococcus granulosus* laminated membrane in a defined medium. Int. J. Parasitol. 6, 467-471.
Heath, D.D. and Lawrence, S.B. 1976. *Echinococcus granulosus*: development in vitro from oncosphere to immature hydatid cyst. *Parasitology*. 73:417-423.

Heath, D.D. and Lawrence, S.B. 1981. *Echinococcus granulosus*: early development in vitro in the presence of serum from infected sheep. *Int. J. Parasitol*, 11:261-266.

Hokke, C.H. et. al. 2007. Glycomics- driven discoveries in schistosome research. Exp. Parasitol. 117, 275-283.

Holcman, B., Heath, D.D. and Shaw, R.J. 1994. Ultrastructure of oncosphere and early stages of metacestode development of *Echinococcus granulosus*. *Int. J. Parasitol.* 24:623-635.

Holcman, B. and Heath, D.D. 1997. The early stages of *Echinococcus granulosus* development. *Acta Tropica*, 64:5-17.

Horton, R. 1997. Albendazole in treatment of human cystic echinococcosis: 12 years of experience. *Acta Trop*, 64:79-93.

Hubner, M. P. et. al. 2006. *Echinococcus multilocularis* metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients. Clin. Exp. Immunol. 145, 243-251.

Hulsmeier, A.J., Gehrig, P.M., Geyer, R., Sack, R., Gottstein, B. et al. 2002. A major *Echinococcus multilocularis* antigen is a mucin-type glycoprotein. J. Biol. Chem 277, 5742-5748.

Ingold, K. et. al. 2001. Characterization of the laminated layer of in vitro cultivated *Echinococcus vogeli* metacestodes. J. Parasitol. 87, 55-64.

Ingold, K. et. al. 2000. High molecular mass glycans are major structural elements associated with the laminated layer of in vitro cultivated *Echinococcus multilocularis* metacestodes. Int. J. Parasitol. 30, 207-214.

Ioppolo, S., Notargiacomo, S., Profumo, E., Franchi, C., Ortona, E., Rigano, R. and Siracusano, A. 1996. Immunological responses to antigen B from *Echinococcus granulosus* cyst fluid in hydatid patients. Parsite Immunol. 18:571-578.

Irigoin, F. et. al. 1996. Comparison of complement activation in vitro by different *Echinococcus granulosus* extracts. Parasite Immunol. 18, 371-375.

Irigoin, F. et. al. 2008. Resistance of the *Echinococcus granulosus* cyst wall to complement activation: analysis of the role of $InsP_6$ deposits. Parasite Immunol. 30, 354-364.

Ito, A. and Smyth, J.D. 1987. Adult Cestodes- Immunology of the Lumen-dwelling Cestode infections. In Immune Response in Parasite Infections: Immunology, Immunopathology and Immunoprophylaxis. Vol. II: Trematodes and Cestodes (ed. Soulsby, E.J.L), pp115-163.

Ito, A., Urbani, C., Jiamin, Q., *et al*. 2003. Control of echinococcosis and cysticercosis: a public health challenge to international cooperation in China. *Acta Trop*, 86:3-17.

Jankovic, D. et. al. 2010. IL-10 production by $CD4^+$ effector T cells: a mechanism for self-regulation. Mucosal Immunol. 3, 239-246.

Jenkins, D.J. and Morris, B. 1995. Unusually heavy infection of *Echinococcus granulosus* in wild dogs in south eastern Australia. *Australian Veterinary Journal*, 66:36-37.

Jenkins, D.J. 2002. *Echinococcus* in Australia: the role of wildlife in transmission,

with particular reference to South Eastern Australia. *In*: Craig, P., Pawlowski, Z.S. (*eds*), Cestode Zoonoses: Echinococcosis and Cysticercosis an Emergent and Global problem. IOS Press, Amsterdam, pp (327-332).

Jenkins, D.J. et. al. 2005. Emergence/ re-emergence of *Echinococcus* spp- a global update. Int. J. Parasitol. 35, 1205-1219.

Jimenez, S., Perez, A., Gil, H., Schantz, P.M., Ramalle, E., Juste, R.A. 2002. Progress in control of cystic echinococcosis in La Rioja, Spain: decline in infection prevalences in human and animal hosts and economic costs and benefits. Acta Trop. 83: 23-221.

Jiang, L., Zhang, Y.G., Liu, M.X., Zheng, F. 2012. Analysis on the reactivity of five subunits of antigen B family in serodiagnosis of echinococcosis. Experimental Parasitology, 131: 85-91.

Junghanss, T., Da Silva, A.M., Horton, J., Chiodini, P.L., Brunetti, E. 2008. Clinical management of cystic echinococcosis: State of the Art, Problems, and Perspectives. Am J Trop Med HYg 79: 301-311.

Kacprzak, E. and Stefaniak, J. 1995. Evaluating the activity of liver cystic echinococcosis using the delayed-hypersensitivity skin reaction to common antigens. Ann. Trop. Med. Parasitol. 89, 25-29.

Kain, K.C. and Keystone, J.S. 1988. Recurrent hydatid disease during pregnancy. Am. J. Obstet. Gynecol. 159, 1216-1217.

Kamiya, M. and Sato, H. 1990. Survival, strobilation and sexual maturation of *Echinococcus multilocularis* in the small intestine of golden hamsters. *Parasitology*, 100:125-130.

Karimov Sh, I. et. al. 1998. Epidemiologic aspects of echinococcosis. Khirurgiia (Mosk) 98, 37-39 (in Russian).

Kern, P. et. al. 2003. European echinococcosis registry: human alveolar echinococcosis, Europe, 1982-2000. Emerg. Infect. Dis. 9, 343-349.

Kern, P. 2003. *Echinococcus granulosus* infection: Clinical presentation, Medical treatment and outcome. *Langenbecks Arch surg*. 388:413-420.

Khabiri, A. R. et. al. 2006. Analysis of specific IgE and IgG subclass antibodies for diagnosis of Echinococcus granulosus. Parasite Immunol. 28, 357-362.

Khuroo, M.S. 2002. Hydatid disease: current status and recent advances. *Ann Saudi Med*. 22:56-64.

Kilejian, A. et. al. 1962. Host-parasite relationship in Echinicoccosis. V111. Infrared spectra and chemical composition of the hydatid cyst. Exp. Parasitol 12, 377-392.

Kilejian, A. and Schwabe, C. W. 1971. Studies on the polysaccharides of the *Echinococcus granulosus* cyst, with observations on a possible mechanism for laminated membrane formation. Comp. Biocem. Physiol. B40, 25-36.

Kjossev, K.T. and Losanoff, J.E. 2005. Classification of hydatid liver cysts. *J. Gastroenterol. Hepatol*. 20:352-59.

Korc, I., Hierro, J., Lasalvia, E., Falco, M., Calcagno, M. 1967. Chemical characterization of the polysaccharide of the hydatid membrane of *Echinococcus granulosus*. Exp Parasitol, 20(2): 219-24.

Kouguchi, H., Matsumoto, J., Yamano, K., Katoh, Y., Oku, Y., Suzuki, T., Yagi, K. 2011. *Echinococcus multilocularis*: purification and characterization of glycoprotein antigens with serodiagnostic potential for canine infection. Exp Parasitol, 128(1): 50-6.

Kurniawan, A., Yazdanbakhsh, M., van Ree, R., Aalberse, R., Selkirk, M.E., Partono, F., Maizels, R.M. 1993. Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human Filariasis. J Immunol 150: 3941-3950.

Larrieu, E. and Frider, B. 2001. Human cystic echinococcosis: contributions to the natural history of the disease. *Ann. Trop. Med*. *Parasitol.* 95:679-87.

Larrieu, E. *et al*. 2004. Ultrasonographic diagnosis and medical treatment of human cystic echinococcosis in asymptomatic school age carriers: 5 years of follow-up. *Acta Trop*. 91:5-13.

Lascano, E.F. et. al. 1975. Fine structure of the germinal membrane of *Echinococcus granulosus* cysts. J. Parasitol. 61, 853-860.

Lawn, S.D., Bligh, J., Craig, P.S., Chi Odni, P.L. 2004. Human cystic echinococcosis: evaluation of post-treatment serologic follow-up by IgG subclass antibody detection. Am J. Trop Med Hyg 70: 329-335.

Le, T.H., Pearson, M.S., Blair, D., Dai, N., Zhang, L.H. and McManus, D.P. 2002.

Complete mitochondrial genomes confirm the distinctiveness of the horse-dog and sheep-dog strains of *Echinococcus granulosus*. *Parasitology*, 124: 97-112.

Leducq, R. and Gabrion, C. 1982. Developmental changes of *Echinococcus multilocularis* metacestodes revealed by tegumental ultrastructure and lectin-binding sites. *Parasitol*. 104:129-141.

Leggatt, G.R., Yang, W. and McManus, D.P. 1992. Serological evaluation of the 12KDa subunit of antigen B in *Echinococcus granulosus* cyst fluid by immunoblot analysis. *Trans. R. Soc. Trop*. *Med. Hyg*. 86:189-192.

Lewall, D.B. and McCorkell, S.J. 1985. Hepatic echinococcal cysts: sonographic appearance and classification. *Radiology*, 155:773-775.

Li, F. et. al. 2003. HLA-DRBI allele in 35 patients with alveolar echinococcosis in Gamsu province of China. Chin. Med. J. (Engl.) 116, 1557-1560.

Lightowlers, M.W. and Gottstein, B. 1995. Echinococcosis/hydatidosis: antigens, immunological and molecular diagnosis, pp (355-410). *In*: Thompson, R.C.A. and Lymbery, A.J. (*eds*), The biology of *Echinococcus* and hydatid disease. CAB International, Wallingford, United Kingdom.

Lightowlers, M.W., Liu, D.Y., Haralambous, A. and Rickard, M.D. 1989. Subunit composition and specificity of the major cyst fluid antigens of *Echinococcus granulosus*. *Mol. Biochem. Parasitol*. 37:171-182.

Liu, D., Rickard, M.D. and Lightowlers, M.W. 1993. Assessment of monoclonal antibodies to *Echinococcus granulosus* antigen 5 and *antigen B for detection of human hydatid circulating antigens. Parasitology*, 106:75-81

Ling, Y.L., Hincapie, M., Packer, N., Baker, M. S., Hancock, W.S., Fanayan, S. 2012. An optimized approach for enrichment of glycoproteins from cell culture lysates using native multi-lectin affinity chromatography. Journal of Separation of Science 35(18): 2445-2452.

Macpherson, C.N.L.. 1992. Use of ultrasoundin the diagnosis of parasitic disease. *Tropical Doctor*, 22:14-20.

Macpherson, C.N. and Milner, R. 2003. Performance characteristics and quality control of community based ultrasound surveys for cystic and alveolar echinococcosis. *Acta Trop*. 85:203-09.

Macpherson, C.N.L. *et al*. 2004. Cystic echinococcosis in the Berber of the Mid Atlas mountains. Morocco: new insights into the natural history of the disease in

humans. *Ann. Trop. Med. Parasitol*. 98:481-490.

Maizels, R.M. et. al. 2004. Helminth parasites-masters of regulation. Immunol. Rev. 201, 89-116.

Mamuti, W., Yamasaki, H., Sako, Y., Nakao, M., Xiao, N. et al, 2004. Molecular cloning, expression and serological elevation of an 8-Kilodalton subunit of antigen B from Echinococcus multilocularis. J Clin Microbiol 42:1082- 1088.

Mamuti, W., Sako, Y., Bart, J.M., Nakao, M., Yamasaki, H., Lightowlers, M.W., Craig, P.S., Ito, A. 2006. Echinococcus multilocualris: development stage-specific expression of antigen B 8-KDa subunits. Experimental Parasitology, 113: 75-82.

Mamuti, W., Sako, Y., Bart, J.M., Nakao, M., Ma, X., Wen, H., Ito, A. 2007. Molecular characterization of a novel gene encoding an 8-KDa subunit of antigen B from *Echinococcus granulosus* genotypes 1 and 6. Parasitology International, 56: 313-316.

Manfras, B. J. et. al. 2002. Increased activation and oligoclonality of peripheral $CD8^+$ T cells in the chronic human helminth infection alveolar echinococcosis. Infect. Immun. 70, 1168-1174.

Manfras, B. J. et. al. 2004. Impeded Th1 CD4 memory T cell generation in chronic-persisting liver infection with *Ehinococcus multilocularis*. Int Immunol. 16, 43-50.

Marty, A. M., Johnson, L. K., Neafie, R. G. 2000. Hydatidosis (echinococcosis). In: Meyers, W. M., Neafie, R. G., Marty, A. M., Wear, D.J., editors. Parasitolgy of Infectious Disease: Volume 1, Helminthiases. Washington, D.C: Armed Forces Institute of Pathology, American Registry of Pathology. Pp145-164.

McManus, D.P. and Bryant, C. 1986. Biochemistry and physiology of *Echinococcus*. *In*: The Biology of *Echinococcus* and Hydatid Disease (Thompson, R.C.A. *ed*), *Allen and Unwin*, London, pp (114-142).

McManus, D. P. and Bryant, C. 1995.. Biochemistry,physiology and molecular biology of *Echinococcus*. In *Echinococcus and* Hydatid Disease (*ed*. Thompson, R. C. A. & Lymbery, A. J.), pp. 135-181. *CAB International*, Wallingford, Oxon, UK.

McManus, D.P. 2002. The molecular epidemiology of *Echinococcus granulosus* and cystic hydatid disease. *Transactions of the Royal Society of Tropical Medicine* and *Hygiene* 96, s1/151-s1/157.

McManus, D.P., Zhang, W., Li, J., Bartley, P.B. 2003. Echinococcosis: Lancet 362, 1295-1304.

McVie, A., Ersfeld, K., Rogan, M.T, Craig, P.S. 1997. Expression and immunological characterisation of Ehinococcus granulosus recombinant antigen B for IgG4 subclass detection in human cystic echinococcosis. Acta Trop 67: 19-35.

Mejri, N. and Gottstein, B. 2006. Intraperitoneal *Echinococcus multilocularis* infection in C57BL/6 mice affects CD40 and B7 costimulator expression on peritoneal macrophages and impairs peritoneal T-cell activation. Parasite Immunol. 28, 373-385.

Mejri, N.J. et. al. 2011. Intraperitoneal murine *Echinococcus multilocularis* infection induces differentiation of TGF-beta-expressing DCs that remain immature. Parasite Immunol. 33, 471-482.

Mejri, N. et. al. 2011. Intraperitoneal *Echinococcus multilocularis* infection in mice modulates peritoneal $CD4^+$ and $CD8^+$ regulatory T cell development. Parasitol. Int. 60: 45-53.

Men, S., Hekimoglu, B., Yucesoy, C., Arda, I. and Baran, I. 1999. Percutaneous treatment of hepatic hydatid cysts: an alternative to surgery. *Am J Roentgenol*, 172:83-89.

Menghebat, L., Jiang, L. and Chai, J. (1993) A retrospective survey for surgical cases of cystic echinococcosis in the Xinjiang Uygur Autonomous Region, PRC (1951-90). *In*: Andersen, F., Chai, J., Liu, F. (*eds*). Compendium on cystic echinococcosis with special reference to the Xinjiang Uygur Autonomous Region, People's Republic of China. Provo: *Brigham Young University Print Services*, pp (135-45).

Meri, S. and Pangburn, M. K. 1990. Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. Proc. Natl. Acad. Sci. U.S.A. 87, 3982-3986.
Mezioug, D. and Touil-Boukoffa, C. 2009. Cyokine profile in human hydatidosis: possible role in the immunosurveillance of patients infected with *Echinococcus granulosus*. Parasite. 16, 57-64(in French).

Mohamed, A., Yasawy, M. and Karawi, M.A. 1998. Combined albendazole and praziquantel versus albendazole alone in the treatment of hydatid disease. *Hepatogastroenterology*. 45:1690-94.

Mondragón-de-la-Peña, C. et. al. 2002. *Echinococcus granulosus* down regulates the hepatic expression of inflammatory cytokines IL-6 and TNF alpha in BALB/c mice. Paraite. 9, 351-356.

Monteiro, K.M., Scapin, S.M., Navarro, M.V., Zanchin, N.I., Cardoso, M.B., da Silveira, N.P., Goncalves, P.F., Stassen, H.K., Zaha, A., Ferreira, H.B. 2007. Self-assembly and structural characterization of Echinococcus granulosus antigen B recombinant subunit oligomers. Biochimica Biophysica Acta, 1774: 278-285.

Moro, P.L. et. al. 1999. Human hydatidosis in the central Andes of Peru: evolution of the disease over 3 years. Clin. Infect. Dis. 29, 807-812.

Moro, P. and Garcia, H.H. 2005. Screening for cystic echinococcosis in an endemic region of Peru using portable ultrasonography and the enzyme-linked immunoelectrictransfer blot (EITB) assay. Parasitol Res 96: 242-246.

Morris, D.L. *et al*. 1984. Abdominal hydatid-disease – computed tomographic and ultrasound changes during albendazole therapy. *Clin. Radiol*. 35:297-300.

Morris, D.L. 1986. Spontaneous cure of hydatid cyst disease. *Lancet* 1, 1206.

Morseth, D.J. 1967. Fine structure of the hydatid cyst and protoscolex of *Echinococcus granulosus*. J. Parasitol. 53, 312-325.

Mourglia-Ettlin, G. et. al. 2011. *Echinococcus granulosus* glycoconjugates induce peritoneal B cell differentiation into antibody-secreting cells and cytokine production. Parasite Immunol. 33, 621-631.

Muzulin, P.M., Kamenetzky, L., Gutierrez, A.M., Guarnera, E.A., Rosenzvit, M.C. 2008. *Echinococcus granulosus* antigen B family: further studies of strain polymorphism at the genomic and transcriptional levels. Experimental Parasitology, 118: 156-164.

Nahmias, J. *et al*. 1994. 3-year to 7-year follow-up after albendazole treatment of 68 patients with cystic echinococcosis (hydatid-disease). *Ann. Trop. Med. Parasitol*. 88:295-304.

Nono, J.K. et. al. 2012. Excretory/ secretory-products of *Echinococcus multilocularis* larvae induce apoptosis and tolerogenic properties in dendritic cells in vitro. PLoS Negl. Trop. Dis. 6, e1516.

Oriol, R., Williams, J.F., Perez Esandi, M.V. and Oriol, C. 1971. Purification of lipoprotein antigens of *Echinococcus granulosus* from sheep hydatid fluid. *Am. J. Trop. Med. Hyg*. 20:569-574.

Oriol, C. and Oriol, R. 1975. Physiocochemical properties of a lipoprotein antigen of *Echinococcus granulosus*. *Am. J. Trop. Med. Hyg*. 24:96-100.

Ortona, E., Rigano, R., Margutti, P., Notargiacomo, S., Ioppolo, S., Vacari, S., Barca, S., Buttari, B., Profumo, E., Teggi, A. and Siracusano, A. 2000. Native and recombinant antigens in the immunodiagnosis of human cystic echinococcosis. *Parasite Immunol*. 22:553-559.

Pawlowski, Z.S. 1997. Critical points in clinical management of cystic echinococcosis: a revised review. *In*: Compendium on Cystic Echinococcosis in Africa and Middle Eastern Countries with Special Reference to Morocco (Anderson, F.L. *et al*., *eds*), pp (119-135), *Brigham Young University*.

Pawlowski, Z.S. et al. 2001. Echinococcosis in humans: clinical aspects, diagnosis and treatment. In: WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern (Eckert, J. *et al., eds*), pp (20-69), WHO/OIE.

Pelaez, V., Kugler, C., Correa, D. *et al*. 2000. PAIR as percutaneous treatment of hydatid liver cysts. *Acta Trop*, 75:197-202.

Pezzella, M., Galli, C., Delia, S., Vullo, V.,Zennaro, F., Lillini, E. and Sorice, F. 1984. Fractionation and characterization of hydatid fluid antigens with identification of an antigen similar to human serum albumin. *Trans. R. Soc.Trop. Med. Hyg. 78: 821-826.*

Polat, P., Kantarci, M., Alper, F., Suma, S., Koruyucu, M.B. and Okur, A. 2003. Hydatid disease from head to toe. *Radiographics*. 23:475-94.

Poretti, D., Felleisen, E., Grima, F., Pfisher, M., Teuscher, F., Zuercher, C., Reichen, J. and Gottstein, B. 1999. Differential immunodiagnosis of human cystic hydatid disease and other cross- reactive pathologies. Am. J. Trop. Med. Hyg. 60193-198.

Powanda, M.C. and Beisel, W.R. 1982. Hypothesis: leukocyte endogenous mediator/ endogenous pyrogen/ lymphocyte-activating factor modulates the development of nonspecific and specific immunity and affcets nutritional status. Am. J. Clin. Nutr. 35, 762-768.

Quintieri, F. et. al. 1994. Further evaluation of autoreactive T cells in hydatid patients. Immunol. Lett. 40, 59-63.

Raether, W. and Hänel, H. 2003. Epidemiology, clinical manifestations and

diagnosis of zoonotic cestode infections: an update. *Parasitol Res*. 91:412-38.

Rafiei, A., Jahanshahi, A. and Talaeizadeh, A. 2008. Evaluation of Specific IgG Antibody Detection in Diagnosis and Post Surgical Monitoring of Cystic Echinococcosis. *Iranian. J. Parasitol*. 3:10-14.

Rausch, R. 1954. Studies on the helminth fauna of Alaska. XX. The histogenesis of the alveolar larvae of *Echinococcus* species. J. Infect. Dis. 94, 178-186.

Rausch, R.L. 1981. Characteristics of the larval *Echinococcus vogeli* Rausch and Bernstein, 1972 in the natural intermediate host, the paca, Cuniculus paca L. (Rodentia: Dasyproctidae). Am. J. Trop. Med. Hyg. 30, 1043-1052.

Rausch, R.L. et. al. 1987. Spontaneous death of *Echinococcus multilocularis*: cases diagnosed serologically (by Em2 ELISA) and clinical significance. Am. J. Trop. Med. Hyg. 36, 576-585.

Ravinder, P.T., Parija, S.C. and Rao, K.S. 1997. Evaluation of human hydatid disease before and after surgery and chemotherapy by demonstration of hydatid antigens and antibodies in serum. J. Med. Microbiol. 46859-864.

Reuben, J.M. et. al. 1978. Immunoprophylaxis with BCG of experimental *Echinococcus multilocularis* infections. Infect. Immun. 21, 135-139.

Richards, K. S. et. al. 1983. *Echinococcus granulosus equinus*: an ultrastructural study of the laminated layer, including changes on incubating cysts in various media. Parasitology 86, 399-405.

Richards, K.S. et. al. 1983. *Echinococcus granulosus equinus*: the histochemistry of the laminated layer of the hydatid cyst. Folia Histochem. Cytobiol. 22, 21-31.

Richards, K.S. et. al. 1983. *Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts. Parasitology. 86, 407-417.

Rigano, R., Profumo, E., Bruschi, F., Carulli, G., Azzara, A., Ioppolo, S., Buttari, B., Ortona, E., Margutti, P., Teggi, A. and Siracusano, A. 2001. Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defenses. *Infect. Immun*. 69:288-296.

Rigano, R. et. al. 2004. *Echinococcus granulosus*-specific T-cell lines derived from patients at various clinical stages of cystic echinococcosis. Parasite Immunol. 26, 45-52.

Rigano, R. et. al. 1998. Cytokine patterns in seropositive and seronegative patients with *Echinococcus granulosus* infection. Immunol. Lett. 64, 5-8.

Rigano, R. et. al. 2001. Modulation of human immune response by *Echinococcus granulosus* antigen B and its possible role in evading host defences. Infect. Immun. 69, 288-296.

Rigano, R. et. al. 1995. Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. Clin. Exp. Immunol. 102, 281-285.

Rigano, R. et. al. 1995. In vitro production of cytokines by peripheral blood mononuclear cells from hydatid patients. Clin. Exp. Immunol. 99, 433-439.

Rigano, R., Profumo, E., Ioppolo, S., Notargiacomo, S., Ortona, E., Teggi, A. and Siracusano, A. 1995. Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. Clin. Exp. Immunol. 102: 281-285.

Rigano, R., Profumo, E., Teggi, A. and Siracusano, A. 1996. Production of IL-5 and IL-6 by peripheral blood mononuclear cells (PBMC) from patients with *Echinococcus granulosus* infection. Clin. Exp. Immunol. 105: 456-459.

Rigano, R. et. al. 1999. Cytokine gene expression in peripheral blood mononuclear cells (PBMC) from patients with pharmacologically treated cystic echinococcosis. Clin. Exp. Immunol. 118, 95-101.

Rigano, R. et. al. 1999. Serum cytokine detection in the clinical follow up of patients with cystic echinococcosis. Clin. Exp. Immunol. 115, 503-507.

Rigano, R. et. al. 2004. Cytokine expression in the follow-up of patients with cystic echinococcosis. Parasitologia 46, 441-442.

Rigano, R., Ioppolo, S., Ortona, E., Margutti, P., Profumo, E. et al, 2002. Long-term serological evaluation of patients with cystic echinococcosis treated with benzimidazole carbamates. Clin Exp Immunol 129: 485-492.

Riley, E.M. et. al. 1986. *Echinococcus granulosus* infection in mice: host responses during primary and secondary infection. Parasitology 92(Pt 2), 391-403.

Rodriguez-Sosa, M. et. al. 2002. Cutting edge: susceptibility to the larval stage of the helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signalling. J. Immunol. 168, 3135-3139.

Rogan, M.T. and Richards, K.S. 1989. Development of the tegument of *Echinococcus granulosus* (Cestoda) protoscoleces during cystic differentiation in vivo. Parasitol. Res. 75,299-306.

Rogan, M.T. and Richards, K.S. 1986. In vitro development of hydatid cysts from posterior bladders and ruptured brood capsules of equine *Echinococcus granulosus*. Parasitology 92 (Pt 2), 379-390.

Rogan, M.T., Craig, P.S., Zeyhle, E., Romig, T., Lubano, G.M., Deshan, L. 1991. Evaluation of a rapid dot-ELISA as a field test for the diagnosis of cystic hydatid disease. Trans R Soc Trop Med Hyg, 85(6): 773-7.

Rogan, M.T. and Craig, P.S. 1997. Immunology of *Echinococcus granulosus* infections. Acta Trop. 67, 7-17.

Rogan, M.T. 1998. T-cell activity associated with secondary infections and implanted cysts of *Echinococcus granulosus* in BALB/c mice. Parasite Immunol. 20, 527-533.
Rogan, M.T. 1988. Observations on the origin of daughter cysts within hydatid cysts of *Echinococcus granulosus. Ann. Trop. Med. Parasitol*. 82:405-406.

Rogan, M.T., Hai, W.Y., Richardson, R., Zeyhle, E. and Craig, P.S. 2006. Hydatid cysts: does every picture tell a story?. *Trends in Parasitol*, 22:432-438.

Romig, T. et. al. 1986. Cyst growth and spontaneous cure in hydatid disease. Lancet 327, 861-862.

Rott, M.B., Fernandez, V., Farias, S., Ceni, J., Ferreira, H.B., Haag, K.L. and Zaha, A. 2000. Comparative analysis of two different subunits of antigen B from *Echinococcus granulosus*: gene sequences, expression in *Escherichia coli* and serological evaluation. Acta Trop. 75:331-340.

Russi, S. et. al. 1974. Isolation and characterization of a blood P1 active carbohydrate antigen of *Echinococcus granulosus* cyst membrane. J. immunol. 112, 1061-1069.

Sailer, M. et. al. 1997. Alveolar echinococcosis of the liver in a six –year old girl with acquired immunodeficiency syndrome. J. Pediatr. 130, 320-323.

Sakamoto, T. and Sugimura, M. 1969. Studies on echinococcosis. XX1. Electron microscopical observations on general structure of larval tissue of multilocular *Echinococcus*. Jpn. J. Vet. Res 17, 67-80.

Sakamoto, T. and Sugimura, M. 1970. Studies on Echinococcosis. 23. Electron microscopical observations on histogenesis of larval *Echinococcus multilocularis*. Jpn. J. Vet. Res. 18,131-144.

Sakamoto, T. and Cabrera, P.A. 2003. Immunohistochemical observations on cellular response in unilocular hydatid lesions and lymph nodes of cattle. Acta. Trop. 85, 271-279.

Santivanez, S. and Garcia, H.H. 2010. Pulmonary cystic echinococcosis. Curr. Opin. Pulm. Med. 16: 257-261.

Santivanez, S.J., Arias, P., Portocarrero, M., Rodriguez, S., Gonzalez, A. E., Gilman, R. H., Gavidia, C. M. and Garcia, H. H. 2012. Serological diagnosis of lung cystic hydatid disease using the synthetic p176 peptide. Clinical and vaccine immunology. 944- 947.

Sbihi, Y., Janssen, D., Osuna, A. 1996. Serologic recognition of hydatid cyst antigens using different different purification methods. Diagn Microbiol Infect Dis 24: 205- 211.

Schantz, P.M. and Gottstein, B. 1986. Echinococcosis (hydatidosis), p. 69-107. *In*: Walls, K.W. and Schantz, P.M. (*eds*), Immunodiagnosis of parasitic diseases, vol. 1. *Academic Press*, *Inc.* Orlando, Fla.

Schiel, E. J. and Hage, D.S. 2009. Kinetic studies of biological interactions by affinity chromatography. Journal of Separation of Science. 32(10): 1507-1522.

Schipper, H.G. et al. 2002. Percutaneous evacuation (PEVAC) of multivesicular echinococcal cysts with or without cystobiliary fistulas which contain non-drainable material : first results of a modified PAIR method. *Gut*, 50:718-723.

Schweiger, A., Grimm, F., Tanner, I., Mullhaupt, B., Bertogg, K, et al. 2011. Serological diagnosis of echinococcosis: The diagnostic potentila of native antigens. Infection 40(2): 139-152.

Shambesh, M.K., Craig, P.S., Wen, H., Rogan, M.T. and Paolillo, E. 1997. IgG1 and IgG4 serum antibody responses in asymptomatic and clinically expressed cystic echinococcosis patients. Acta Trop. 64: 53-63.

Shapiro, S.Z., Bahr, G.M. and Hira, P.R. 1992. Analysis of host components in hydatid cyst fluid and immunoblot diagnosis of human *Echinococcus granulosus* infection. *Ann. Trop. Med. Parasitol*. 86:503-509.

Sharon N. 1993. Lectin-carbohydrate complexes of plants and animals: An atomic view. *Trends in Biochemical Sciences*. 18:221–226.

Shepherd, J.C. and McManus, D.P. 1987. Specific and cross-reactive antigens of *Echinococcus granulosus* hydatid cyst fluid. *Mol. Biochem. Parasitol.* 25:143-154.

Shepherd, J.C., Atiken, A. and McManus, D.P. 1991. A protein secreted in vivo by *Echinococcus granulosus* inhibits elastase activity and neutrophil chemotaxis. *Mol. Biochem. Parasitol*. 44:81-90.

Short, J.A., Heiner, D.C., Hsiao, R.L., Andersen, F.L. 1990. Immunoglobulin E and G4 antibodies in cysticercosis. J Clin Microbiol 28: 1635-1639.

Sinner, V. W., te Strake, L., Clark, D. and Sharif, H. 1991. MR Imaging in Hydatid Disease. *American Roentgen Ray Society*, AJR 157:741-745.

Sinner, W.V. 1997. Advanced medical imaging and treatment of human cystic echinococcosis. *Semin Roentgenol*, 4:276-90.

Siracusano, A.E. et. al. 2002. Molecular and cellular tools in human cystic echinococcosis. Curr. DrugTargets immune Endocr. Metabol. Disord. 2, 235-245.
Siracusano, A. et. al. 1988. Cellular immune responses of hydatid patients to *Echinococcus granulosus* antigens. Clin. Exp. Immunol. 72, 400-405.

Siracusano, A. et. al. 2008. Molecular cross-talk in host-parasite relationships: the intriguing immunomodulatory role of *Echinococcus* antigen B in cystic echinococcosis. Int. J. Parasitol. 38, 1371- 1376.

Sjoberg, A. P. et. al. 2009. Complement activation and inhibition: a delicate balance. Trends Immunol. 30, 83-90.

Smyth, J.D. and Barrett, N. 1980. *In vitro* culture of *Echinococcus*. *Trans. R. Soc. Trop. Med. Hyg.* 74:117.

Smyth, J.D. and McManus, D.P. 1989. The physiology and biochemistry of Cestodes. *Cambridge University Press*, Cambridge.

Srours, S.F. and Sayfan, J. 2001. Echinococcosis of the spleen during pregnancy. Isr. Med. Assoc. J. 3, 290-291.

Sterla, S., Sato, H. and Mieto, A. 1999. *Echinococcus granulosus* human infection stimulates low avidity anticarbohydrate IgG2 and high avidity antipeptide IgG4 antibodies. Parasite Immunol. 21: 27-34.

Stewart, K.K. and Doherty, R.F. 1973. Resolution of DL- Tryptophan by Affinity Chromatography on Bovine-serum Albumin-Agarose Columns. Proc. Nat. Acad. Sci. USA. 70(10): 2850-2852.

Stojkovic, M., Zwahlen, M., Teggi, A., Vutova, K., Cretu, C.M. 2009. Treatment response of cystic echinococcosis to benzimidazole: a systematic review. PloS Negl Trop Dis 3: 1-10.

Suskind, R.M. 1980. Immune status of the malnourished host. J. Trop. Pediatr.26,1-6.

Swiderski, Z. 1983. *Echinococcus granulosus*: Hook muscle system and cellular organization of infective oncospheres. *Int. J. Parasitol*, 13:289-299.

Taherkhani, H. and Rogan, M.T. 2001. Preparation of semi-purified Laminated Layer of Hydatid Cyst for Diagnosis of Infection. Arch Razi Ins, 52: 61-74

Taherkhani, H., Zeyhle, E. and Rogan, M.T. 2007. Antibody responses in hunman cystic hydatid disease to the laminated layer of *Echinococcus granulosus*. *Parasitol Res*. 101:647-652.

Teggi, A. and Di Vico, B. 2001. The natural history of human cystic echinococcosis (CE) y imaging methods. *In*: Tapeworm Zoonoses, an Emergent and Global Problem (Craig, P.S., *ed*.), pp (125-134), NATO Science Series, *IOS Press*.

Thompson, R.C.A. 1995. Biology and systematics of Echinococcus. In *Echinococcus* and Hydatid disease (Thompson, R.C.A and Lymbery, A.J.,eds), pp. 1-50, CAB International.

Thompson, R.C.A. and McManus, D.P. 2001. Aetiology: parasites and life-cycles. *In*: Eckert, J., Gemmell, M., Meslin, F-X., Pawlowski, Z.S, *eds*. WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Paris: World Organisation for Animal Health, pp (1-19).

Thompson, R.C.A. and McManus, D.P. 2002. Towards a taxonomic revision of the genus *Echinococcus*. *Trends in Parasitology*, 18:452-457.

Tillgren, V. et. al. 2009. The tyrosine sulphate-rich domains of the LRR proteins fibromodulin and osteoadherin bind motifs of basic clusters in a variety of heparin-binding proteins, including bioactive factors. J. Biol. Chem. 284, 28543-28553.

Todorov, T. and Boeva, V. 1999. Human echinococcosis in Bulgaria: a comparative epidemiological analysis. Bulletin of the World Health Organization, 77:110-118.

Torgerson, P.R., Shaikenov, B., Bairtusinov, B. and Abdyybekova, A. 2002a. The emerging epidemic of echinococcosis in Kazakhstan. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 96:124-128.

Torgerson, P.R., Shaikenov, B., and Kuttybaev, O. 2002b. Cystic echinococcosis in Central Asia. New epidemic in Kazakhstan and Krygystan. *In*: Craig, P., Pawlowski, Z.S. (*eds*), Cestode zoonoses: echinococcosis and cysticercosis an Emergent and Global problem. *IOS Press*, Amsterdam, pp (99-105).

Torgerson, P.R. and Budke, C.M. 2003. Echinococcosis- an international public health challenge. *Research in Veterinary Science*, 74:191-202.

Togerson, P.R. et. al. 2008. Alveolar Echinococcosis: from a deadly disease to a well-controlled infection. Relative survival and economic analysis in Switzerland over the last 35 years. J. Hepatol. 49, 72-77.

Togerson, P.R. et. al. 2010. The global burden of alveolar echinococcosis. PLoS Negl. Trop. Dis. 4, e722.

Trepanier, J.M., Seargeant, L.E. and Stinson, R.A. 1976. Affinity purification and some molecular properties of human liver alkaline phosphatase. Biochem. J. 155 (653-0).

Tuxun, T. et. al. 2012. Th17/ Treg imbalance in patients with liver cystic echinococcosis. Parasite Immunol. 16, 1365-3024.

Tüzün, M. and Hekimoğlu, B. 1998. Hydatid disease of the CNS: imaging features. AJR, *Am J Roentgenol*, 171:1497-500.

Tüzün, M., Altinörs, N., Arda, I.S. and Hekimoğlu, B. 2002. Cerebral hydatid disease CT and MR findings. *Journal of Clinical Imaging*, 26:353-357.

Verastegui, M., Moro, P., Guevara, A., Rodriguez, T., Miranda, E. et al. 1992. Enzyme-linked immunoelectrictransfer blot test for diagnosis of human disease. J. Clin Microbiol 30: 1557-1561.

Vicary, F.R., Cusick, G., Shirley, I.M. and Blackwell, R.J. 1977. Ultrasound and abdominal hydatid disease. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 71:29-31.

Virginio, V.G. et al. 2003. A set of recombinant antigens from *Echinococcus granulosus* with potential for use in the immunodiagnosis of human cystic hydatid disease. Clin. Exp. Immunol. 132: 309-315.

Vuitton, D.A. et. al. 2006. Survival strategy of *Echinococcus multilocularis* in the human host. Parasitol. Int. 55(Suppl), S51-S55.

Vuitton, D. A. et. al. 2003. Epidemiology of alveolar echinococcosis with particular reference to China and Europe. Parasitology 127 (Suppl), S87-S107.

Vuitton, D.A. et. al. 1984. Humoral and cellular immunity in patients with hepatic alveolar echinococcosis. A 2 year follow-up with and witout flubendazole treatment. Parasite Immunol. 6, 329-340.

Vuitton, D.A. and Gottstein, B. 2010. *Echinococcus multilocularis* and its intermediate host: a model of parasite-host interplay. J. Biomed. Biotechnol. 2010, 923193.

Vuitton, D.A. 2003. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta trop. 85, 119-132.

Vuitton, D.A. 1997. The WHO informal working group on echinococcosis. The coordinating Board of the WHO-IWGE. Acta Trop. 67, 147-148.

Walker, M. J. 1994. Electrophoretic techniques. In: Principles and Techniques of Practical Biochemistry. (eds. Wilson, K. and Walker, M.J.). 4th ed. Chaptter 9, Cambridge University Press. UK.

Walker, M. et. al. 2004. Isolation and characterization of a secretory component of *Echinococcus multilocularis* metacestodes potentially involved in modulating the host-parasite interface. Infect. Immun. 72, 527-536.

Wang, X., Li, Y. and Feng, S. 1994. Clinical treatment of hepatic and abdominal hydatid cyst by percutaneous puncture, drainage and curettage. *Chung Kuo Chi Sheng Chung Hsueh Yu Chi Sheng Chung Ping Tsa Chih*, 12:285-287 [Chinese]

Wang, Y.H. , Rogan, M.T., Vuitton, D. A., Wen, H., Bartholomon, B., Macpherson, C. N. L., Zou, P. F., Ding, Z. X., Zhou, H. X., Zhang, X. F., Luo, J., Xiong, H. B., Fu, Y., McVie, A., Giraudoux, P., Yang, W. G., Craig, P. S. 2001. Cystic echinococcosis in semi-nomadic pastoral communities in north-west China. Trans. R. Soc.Trop.Med. Hyg. 95 (2): 153-58.

Wang, Y., Zhang, X., Bartholomot, B., Liu, B., Luo, J., Li, T., Wen, X., Zheng, H., Zhou, H., Wen, H., Davaadorj, N., Gambolt, L., Mukhar, T., Al-Qaoud, K., Abdel-Hafez, S., Giraudoux, P., Vuitton, D.A., Fraser, A., Rogan, M.T. and Craig, P.S. 2003. Classification, follow-up and recurrence of hepatic cystic echinococcosis

using ultrasound images. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 97:203-211.

Wang, Y. *et al.* 2006. Post-survey follow-up from human cystic echinococcosis in northwest China. *Acta Trop*. 98:43-51.

Wellinghausen, N. et. al. 1999. Zinc status in patients with alveolar echinococcosis is related to disease progression. Parasite Immunol. 21, 237-241.

Wen, H. and Yang, W.G. 1997. Public health importance of cystic echinococcosis in China. Acta Trop. 67, 133-145.

Wen, H. and Craig, P.S. 1994. Immunoglobulin G subclass responses in human cystic and alveolar echinococcosis. Am. J. Trop. Med. Hyg. 51: 741-748.

Wen, H., New, R.R., Craig, P.S. 1993. Diagnosis and treatment of human hydatidosis. Br J Clin Pharmacol, 35: 565-574.

Whang, Z. et. al. 2008 Echinococcosis in China, a review of the epidemiology of *Echinococcus* spp. Ecohealth 5, 115-126.

World Health Organization .1996. Guidelines for treatment of cystic and alveolar echinococcosis. WHO Informal Working Group on Echinococcosis. Bull World Health Organization 74: 231-242.

WHO/OIE .2001. Echinococcosis in humans: clinical aspects, diagnosis and treatment. *In*: *WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern*. Eckert, J., Gemmell, M.A., Meslin, F.X. and Pawlowski, Z.S. (*eds*). Geneva: World Health Organization, pp (20-66).

WHO Informal Working Group. 2003. International classification of ultrasound images in cystic echinococcosis for application in clinical and field epidemiological settings. *Acta Trop*. 85:253-61.

Yang, Y.R. et. al. 2005. Brain metastasis of alveolar echinococcosis in a hyperendemic focus of Echinococcus multilocularis infection. Trans. R. Soc. Trop. Med. Hyg. 99, 937-941.

Yang, Y.R. et. al. 2006. Simultaneous alveolar and cystic echinococcosis of the liver. Trans. R. Soc. Trop. Med. Hyg. 100, 597-600.

Yang, Y.R. et. al. 2006. Unique family clustering of human echinococcosis cases in a Chinese community. Am. J. Trop. Med. Hyg. 74, 487-494.

Yang, Y.R. et. al. 2006. Community surveys and risk factor analysis of human alveolar and cystic echinococcosis in Ningxia Hui Autonomous region, China. Bull. World Health Organ. 84, 714-721.

Yang, Y.R. et. al. 2009. Human cases of simultaneous echiococcosis and tuberculosis-significance and extent in China. Parasit. Vectors 2, e53.

YuRong, Y., Magda, K.E., McManus, D.P. 2012. Immunogenetics of human echinococcosis. Trends in Parasitology, 10: 447-454.

Zarzosa, M.P., Orduna Domingo, A., Gutierrez, P., Alonso, P., Cuervo, M., Prado, A., Bratos, M.A., Garcia-Yuste, M., Ramos, G. and Rodriguez Torres, A. 1999. Evaluation of six serological tests in diagnosis and postoperative control of pulmonary hydatid disease patients. Diagnostic and post-operative control of pulmonary hydatid disease patients. Diagn. Microbiol. Infect. Dis. 35255-262.

Zhang, W., Li, J. and McManus, D.P. 2003. Concepts in Immunology and Diagnosis of Hydatid Disease. *Clinical Microbiology Reviews*, pp (18-36).

Zhang, S. et. al. 2008. Expression of major histocompatibility complex class 1 chain related molecule A, NKG2D, and transformimg growth factor-beta in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites? J. Infect. Dis. 197, 1341-1349.

Zhang, W. et. al. 2012. Immunology and Immunodiagnosis of cystic ehinococcosis: an update. Clin. Dev. Immunol. 101895.

Zingg, W. et. al. 2004. Alveolar echinococcosis of the liver in an adult with human immunodeficiency virus type-1 infection. Infection 32, 299-302.

Zhang W.and McManus, D.P. 2006. Recent advances in the immunology and diagnosis of echinococcosis. FEMS Immunol Med Microbiol 47: 24-41.

http://images.google.co.uk/images?imgurl=http://www.biochemj.org/bj/362/0297/bj3620297f01.gif
accessed on 16th December, 2010.

http://images.google.co.uk/images?imgurl=http://pathmicro.med.sc.edu/parasitology/EchinococcusLifeCycle
accessed on 16th December, 2010.

APPENDIX

For SDS-PAGE:

Acrylamide

29.2g Acrylamide

0.8g N'N'-Bis methylene acrylamide

Make up 100ml ddH_2O

Filter and store at 4℃ in dark.

10% SDS

1g SDS in 10ml ddH_2O

TRIS BUFFERS:

1.5M Tris pH 8.8

Add 18.15g Tris base to 50ml ddw

To pH 8.8 with 1M HCl

100ml with ddH_2O

0.5M Tris pH 6.8

Add 6.0g Tris base to 50ml ddH_2O

Adjust to pH 6.8 with 1M HCl

Make up to100ml with ddH_2O

Running buffer: pH 8.3 for 1L

3.03g Tris base

14.42g glycine

Make up to 1L with ddH_2O

Dithiothreitol (DTT) 0.1%

Bpb stock

1.39g DTT in 6ml of 0.1% Bpb

10ml 0f 10% SDS stock

0.1% Bromophenyl blue (Bpb)

0.01g Bpb in 10ml ddH_2O

Coomassie Blue stain

1g Coomassie brilliant blue

450ml methanol

100ml acetic acid

Make up to 1L with ddH_2O

Ammonium peroxidise (APS)

0.1g APS in 1ml ddH_2O

Drying reagent

100ml acetic acid

10ml glycerol

Make up to 1L with ddH_2O

De-stain solution

35ml acetic acid

100ml methanol

365ml ddH_2O

Make up to 500ml with ddH_2O

12.5% 1mM Tris-glycine separating buffer

12.5ml acrylamide stock

11.2ml Tris HCl pH 8.8

6.2ml ddH_2O

Total volume 29.9ml

5% seperating gel

1.25ml Tris ph 6.8

1.67ml acrylamide stock

7.o3ml ddH_2O

Total volume 9.95ml

Sample cocktail

1.88ml Tris ph 6.8

6.0ml 10%SDS

3.0ml Glycerol

2.12ml ddH_2O

Total volume 13ml

FOR ELISA :

Diethanolamine buffer pH 9.8

10mg $MgCl_2$ $6H_2O$

9.7ml diethanolamine

Make up to 100ml with ddH_2O

Adjust pH using drops of 1M HCl to lower pH or 2M NaoH to raise pH.

Bicarbonate carbonate buffer

4 BCB capsules

400ml ddH_2O

Phosphate buffer saline (0.1% PBS T20 wash buffer)

4 PBS tablets

0.8ml Tween 20

800ml ddH_2O

Blocking buffer (0.3% PBS T20, 5% milk solution)

1 PBS tablet

0.6ml Tween 20

200ml ddH_2O

And from stock

100ml o.3% PBS T20

5g skimmed milk

For Western blot:

Bromochloroindolyl phosphate (BCIP)

1 BCIP tablet in 10ml ddH_2O

0.15M $NaCl_2$, 0.01M Tris-HCl containing 0.5mM $CaCl_2$ (pH 7.4)

C4H11O2 (Tris)	2.422g
$NaCl_2$	17.53g
$CaCl_2$	0.111g
DDW	1600ml

Adjust pH to 7.4 with 0.1M HCl2 and make up to 2litre with DDW.

Eluting Buffer (0.2M Glycine HCl, pH 3.0)

Glycine	1.5g
NaCl	2.94g
DDW	80ml

Adjust to pH 3.0 with 1M HCl, and make up to 100ml with DDW.