SEROLOGICAL DIAGNOSIS OF HUMAN NEUROCYSTICERCOSIS CAUSED BY *Taenia solium* CYSTICERCI

M.Sc. Thesis

(2014)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfilment of the requirement for the
Master of Science in Biotechnology

Keshav G.C.

Roll No. : BT 065-068

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Keshav G. C.

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Abstract

SEROLOGICAL DIAGNOSIS OF HUMAN NEUROCYSTICERCOSIS CAUSED BY *Taenia solium* CYSTICERCI

Neurocysticercosis is the commonest parasitic neglected tropical disease of the human nervous system caused by larval stage or cysticercus of the pork tapeworm, *Taenia solium.* It is the major cause of epilepsy in ≥1% of the populations of the world's poorer and endemic countries including Nepal. Definitive diagnosis of NCC is performed in persons with characteristic neuroimaging findings, clinical symptoms and serological tests. Immunological techniques are directed towards detecting specific proteins secreted by *T. solium* and/or specific antibodies against *T. solium* cysticerci in serum and/or cerebrospinal fluid.

The CSA was prepared from the cysts of *Taenia solium* isolated from the local market of Naranghat, Chitwan, Nepal. The protein profiling of CSA revealed the polypeptide fractions of 158, 124, 86, 72, 58, 50, 44, 42, 38, 26, 24, 20, 17and 11 kDa as separated by SDS-PAGE. The immunoblotting of antigenic peptide transferred on PVDF membrane was interacted with different groups of sera. ELISA was performed using the CSA and the sera of the NCC patients visited to hospital (n=45) and 82.22% were found to be NCC positive. Male and female enrolled were found to be affected by 51.35% and 48.65% respectively. This finding suggests that the infection of the parasite does not discriminate male and female population. Age group of 20-30 i.e. 54.05% of total patients were found most infected. Clinical symptoms showed 51.35% patients had seizure.

In immunoblotting of NCC patients 100, 50, 39 and 24 kDa polypeptide produced immense reactivity. Among them, 39 and 24 kDa polypeptide protein fractions produced significant consistency with 100% (10/10) and 100 and 50 kDa fractions marked to 90% (9/10) consistency. Moreover, these bands were found completely absent in the healthy controls showing their greater application in diagnostic markers for NCC cases. Hence, the antigen having the size of 39 and 24 kDa possess higher potentiality on diagnostic application and future vaccine candidate development and production of synthetic peptides using molecular cloning techniques.

Key words*: Diagonsis, ELISA, Immunology, Immunoblotting, Neurocysticercosis, Taenia solium*

Glossary Acronyms

µg	Microgram
µl	Microliter
^{o}C	Degree Centigrade
Ab	Antibody
AEDs	Anti-Epileptic Drugs
Ag	Antigen
AID	Average Integrated Density
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
CC	Conjugate Control
CDC	Center for Disease Control and Prevention
CNS	Central Nervous System
CSA	Crude Soluble Antigen
CSF	Cerebrospinal Fluid
CT	Computed Tomography
DNA	Doxy-ribonucleic Acid
EITB	Enzyme-linked Immunoelectrotransfer Blot
ELISA	Enzyme-linked Immunosorbent Assay
ES	Excretion–Secretion
ESNCC	Epilepsy Secondary to NCC
HLA	Human Lymphocyte and Antigen

HRP	Horse Reddish Peroxidase
ICH	Intracranial Hypertension
IDV	Integrated Density Value
IEFE	Isoelectric Focusing Electrophoresis
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ITS	Internal Transcribed Spacer
kDa	Kilo Dalton
LAC	Latin American countries
LLGP	Lentil-Lectin Glycoprotein
m	Meter
M	Molarity
MAPIA	Multi-Antigen Printing Immunoassay
Mg	Milligram
Ml	Milliliter
mM	milimolar
MRI	Magnetic Resonance Imaging
N	Normalilty
NC	Negative Control
NCC	Neurocysticerocosis
nm	Nanometer
OD	Optical Density

p.i.	Post-Infection
PBS	Phosphate Buffered Saline
PC	Positive control
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene Difluoride
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RPM	Revolution Per Minute
rRNA	ribosomal Doxyribonucleci Acid
RT	Room Temperature
SC	Substrate Control
SD	Standard Deviation
SDS- PAGE	Sodium Dodecycle Sulphate Polyacrylamide Gel Electrophoresis
SSCP	Single-Strand Conformation Polymorphism
TDW	Triple Distilled Water
Th1/Th2	T helper 1/ T helper 2
TMB	Trimethyle Benzidine
TNF-α	Tumor Necrosis Factor-alpha
V	Volt
WB	Western Blotting
WHO	World Health Organization

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Chapter I

Introduction

1.1 Background

Human Neurocysticerocosis (NCC), also known as cerebral cysticercosis is the infection to the central nervous system with *Cysticercus cellulosae*, the larval stage of the pork tapeworm, *Taenia solium.* This is the most common helminth to produce Central Nervous System (CNS) infection in human beings and is the main cause of epilepsy in underdeveloped countries. People can be infected by eating uncooked or poorly cooked cysticercosis pork or by digesting eggs created by the adult tapeworm directly or regurgitating gravid proglottids from the human gut to the stomach. This disease is a serious public health problem in pigs and human beings in many developing areas or countries worldwide, and has been reported recently in some developed countries (Sciutto et al., 2000).

In general, the NCC is a disease closely related to poverty, and in particular with a poor personal hygiene and lack of food hygiene, socio-cultural and environmental factors, education for health in the community, and also very closely related to the sanitary conditions of each region. This is a preventable disease and able to eradicate (Sotelo, 2003), which currently affects more than 50 million people around the world, (Hotez et al., 2008). According to WHO, around 50 thousand people die each year as a result of the NCC and epilepsy secondary to NCC (ESNCC)(Roman et al., 2000). In this regard, WHO includes the NCC between neglected diseases or forgotten that cause a significant impact on the economy in several regions from all over the world. According to Sarti et al., 1992 it has been found that, it affects 4% of the population in endemic areas where hygiene, habits-food and sometimes religious trends can determine the incidence and prevalence of the disease (Savioli LS, 2010).

The infection by *T solium* produces two different diseases: taeniasis and/or cysticercosis. When humans eat eggs of *T. solium* they acquire cysticercosis (CC) that can be found in any tissue including heart, liver, lungs, and peritoneal cavity (Carpio, 2002). The location of the cysticercus in the CNS and in the retina (considering the retina as an extension of the CNS) is called NCC and is considered the most important neurological disease of parasitic origin in humans and the main cause of late-onset epilepsy (White Jr, 2000). Cysts of NCC are located mainly at the distal vascular territory in the cortical gray substance or at the level of the connection between substance gray and white where

the blood supply is remarkable including in HIV patients (Foyaca-Sibat and Ibañez-Valdés, 2002). More importantly, recent studies suggest that NCC is also associated with human cancer (Brutto et al., 2000).

1.1.1 Historical brief and nomenclature of *Taenia solium*

The earliest reference to tapeworms were found in the works of ancient Egyptians that date back to almost 2000 BC. The history of human taeniids; apparently, encysted larvae in pigs -cysticerci of *T. solium*- were well known to the ancient Greeks, referred to by Aristotle (384 to 322 BC). In addition, Hippocrates (460 to 377 BC) suggested that the Greek physicians knew that human harboured such cysts in the brain or suffered from any condition associated with them(Cox, 2002; Wadia and Singh, 2002). It was also known to Jewish and later to early Muslim physicians and has been proposed as one of the reasons for pork being forbidden by Jewish and Islamic dietary laws. Recent examination of evolutionary histories of hosts and parasites and DNA evidence show that over 10,000 years ago, ancestors of modern humans in Africa became exposed to tapeworm when they scavenged for food or preyed on antelopes and bovids, and later passed the infection on to domestic animals such as pigs (Brutto et al., 1998).

Human cysticercosis was first described by Johannes Udalric Rumler in 1555; however, the connection between tapeworms and cysticercosis had not been recognized at that time. Edward Tyson (1650 to 1708) was the first person to recognize the “head” (scolex) in the 17th century. These worms continued to be confused long after the work of Tyson, and although Goeze in 1782 had suspected that there were two separate species, the distinction between *T. solium* and *T. saginata* was not obvious until the middle of the 19th century when Küchenmeinster recognized the differences between both tapeworms based on the morphology of the scolex (Cox, 2002). The demonstration of the life cycle of *T. solium*, in 1784, shed new light on the nature of human cysticercosis. Around 1850, Friedrich Küchenmeister fed pork containing cysticerci of *T. solium* to humans awaiting execution in a prison, and after they had been executed, he recovered the developing and adult tapeworms in their intestines. By the middle of the 19th century, it was established that cysticercosis was caused by the ingestion of the eggs of *T. solium* (Wadia and Singh, 2002).

Before the relationship between *Taenia* and their cysticerci was understood, the larval stages were described with their own scientific name, as if they were separate species/genera. This unfortunate situation still exists today (Acha and Szyfres, 2003). Previously, *T. solium* has also been described as *Taenia cucurbitina* (Muller, 1975) while in Ecuador and other Latin American countries adult *Taenia* are commonly known as “solitaria”, “tallarines” and “Tenia solitaria”. On the other hand, the metacestode of

Taenia spp. bears names like “granizo”, “granillo”, “zahuate”, “pepa”, “tomatillo”, “ladilla”, “quinua”, “coscoja”, “grano” and “triquina” in different endemic areas from Mexico, Colombia, Peru, Chile and Ecuador (Camacho et al., 1991; Garcia and Brutto , 2000).

In 1536, Paracelsus first suspected that epilepsy of a sick priest derived from the presence of brain cysts. After Paracelsus first suspicion of epilepsy secondary to parasitic disease of the brain, it is not well elucidated exactly when the NCC was really confirmed for the first time in the history of medicine. As a general agreement, it is accepted that NCC is a millennial disease about which there is an early description made Paracelsus and another three centuries later on its relationship with the parasitic infection by *T. solium* (Del Brutto and Sotelo, 1988).

The first pathological descriptions and their respective clinical correlations were made by Virchow in 1860, who also identified the location basal meningeal of this parasite calling cysticerci multilocularis". Sotelo (1985) in Mexico publishes a new classification for the NCC based on the presence of active and inactive forms. In 1987- 1989, Sotelo proposes a plan for management of NCC according to the characteristics and tomographic studies of cerebrospinal fluid (CSF). An episode recorded in 1934 enabled to establish the time of appearance of the symptoms of the disease. In that year, British soldiers stationed in India returned to their country and some of these soldiers were diagnosed as NCC, for which they were subjected to a strict medical follow-up. Seizures, as the main symptom, took on average two years to appear, although there were cases where the epilepsy has lasted up to 20 or more years since their return (Flisser, 1988).

1.1.2 Systematic position

Kingdom:	Animalia
Phylum:	Platyhelminthes
Class:	Cestoda
Subclass:	Eucestoda
Order:	Cyclophyllidea
Family:	Taeniidae
Genus:	*Taenia*
Species:	*solium*

1.1.3 Morphology and ultrastructure of the parasite

Adult *T. solium* is a flat, segmented and hermaphrodite parasite, usually 1-5m long and the adult stages are located in the small intestine of humans. They are composed of a head or scolex (**Figure 1.1.**) with a diameter of approximately 1 mm bearing four muscular suckers for fixation and allowing some form of locomotion (Bowman, 1999). *T. solium* has an armed rostellum, bearing between 22 and 36 hooks ordered in two rows. An unsegmented narrow neck measuring approximately 5 to 10 mm, constitutes the portion with most of the biokinetic activity, (Naquira, 1999), from this part the entire body, called strobila is formed (Brutto et al., 1998).

The strobila constitutes of between 800 and 4000 proglottids or segments (1000 on average), which can be grouped as immature, mature and gravid segments. Immature segments are wider transversely than longitudinally; mature segments are square with primary sexual organs completely formed (**Figure 1.2.**); gravid segments have a rectangular shape with longitudinally the longest axis with most of the primary genital organs atrophied but almost entirely filled with a branched uterus packed with oncospheres.

Taeniidae possess neither a coelomic cavity nor a digestive system; exchange of nutritients and metabolites occurs mostly through the tegument. An excretory system is formed by longitudinal lateral channels running through each segment with transverse connections. Each segment has an osmo-regulatory system and a nervous system and muscular fibres, allowing rhythmic and co-ordinated movements (Naquira, 1999; Bowman, 1999).

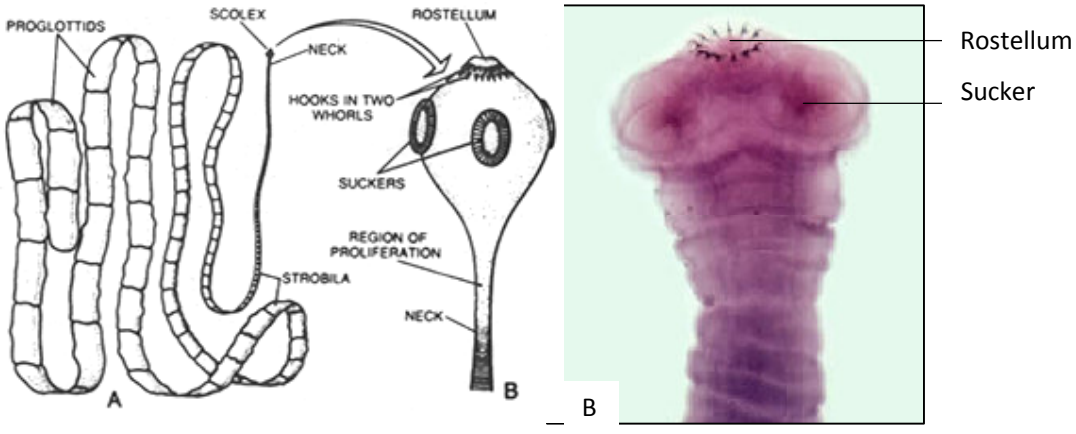


Figure 1.1. *T. solium*: A. whole body, B. its scolex [Source: http://www.infectionlandscapes.org/2012/08/tapeworms.html and ww.cdc.gov/dpdx/taeniasis/gallery.html#scoleces]

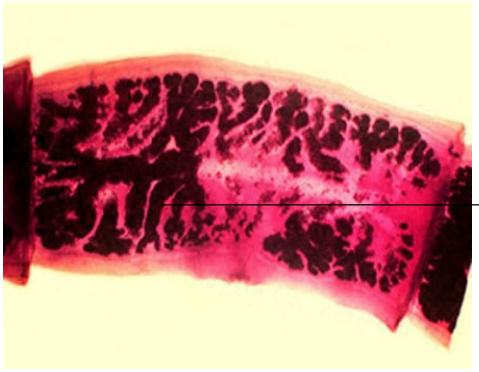


Figure 1.2. Mature proglottid of *T. solium* [Source: http://www.cdc.gov/dpdx/taeniasis/gallery.html#proglottids]

1.1.3.1 Oncospheres

As the parasite grows, new proglottids are formed behind the neck, and mature as they are moved toward the posterior end. Genitalia in the proglottids at the posterior end of the tapeworm have completed copulation and are gravid, containing up to 60,000 eggs (Flisser, 1994). The terminal gravid proglottids detach, and are shed sporadically from the host in fecal material.

Although many eggs are discharged from the proglottid through an anterior pore, some remain within the uteri and can survive for many months protected by the surrounding tegument and feces (Pawlowski, 2002). A single tapeworm can shed as many as 300,000 eggs per day (Lawson and Gemmell 1983).

The uterus contains spherical eggs or oncospheres (**Figure 1.3.**), measuring between 29 and 77µm (Muller, 1975). The eggs of *Taenia spp.* are composed of a chorionic membrane, a thick and grooved embryophore, which is formed by embryophoric blocks (constituted of a keratin-like protein), an embryophoral membrane, two oncospheral membranes surrounding the oncosphere (also named hexacanth embryo) bearing three pairs of hooks (**Figure 1.4.**) (Pawlowski and Schultz, 1972; Pawlowski and Schultz, 1972).The embryophore protects the onchosphere from environmental conditions, but disintegrates upon contact with gastric fluids (Lawson and Gemmell, 1983). The eggs of *Taenia* spp. are fairly resistant to external adverse environmental conditions and to common disinfectants (Bowman, 1999). It is believed that eggs can remain viable for up to two months in water, soil, traditional systems for treating wastewater and vegetation in a humid and warm environment (Brutto et al., 1998).

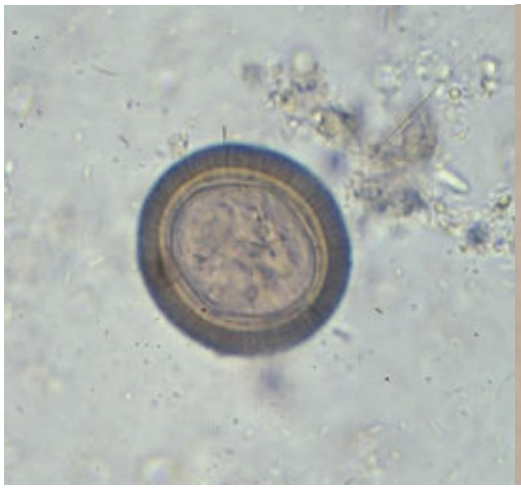


Figure 1.3. Oncosphere

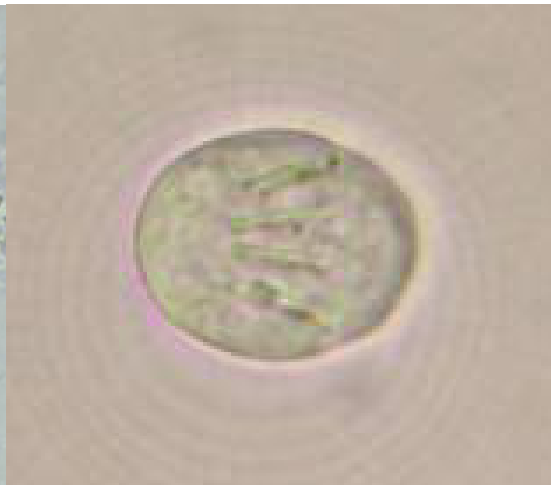


Figure 1.4. Hexacanth embryo

[Source: http://www.dpd.cdc.gov/dpdx]

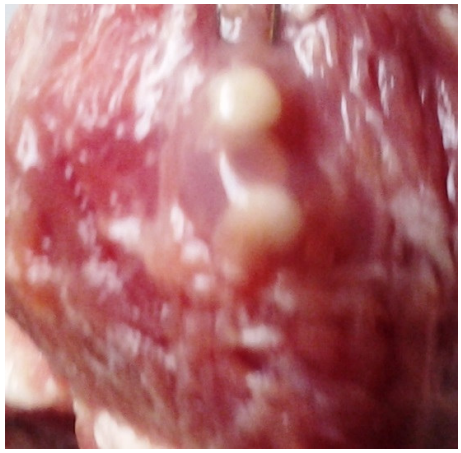


Figure 1.5. Cysticercus of *T. solium*

1.1.3.2 Cysticercus

Once the onchospheres have migrated, they encyst within the host tissue and develop into the metacestode stage, cysticercus of the parasite (**Figure 1.5.**). Onchosphere invasion into host tissue causes the formation of a cavity that is lined with epithelioid cells originating from small blood vessels (Pawlowski, 2002). The onchosphere then differentiates from a solid larva into a vesicular bladderworm surrounded by a layered membrane (Laclette et al., 1982). The scolex forms within the bladder on a germinal inner membrane layer (Schmidt et al., 1996), while the cuticular layer provides protection and acts as a trophoblast, absorbing nutrients and excreting waste by diffusion (Ramirez-Bon et al. 1982). These metamorphic processes occur very quickly; the metacestode reaches its full size within 70 days (Pawlowski, 2002).

A cysticercus is an ovoid vesicle of approximately 5-15mm diameter, with a transparent membrane. It contains an invaginated scolex and is filled with in a colourless liquid (**Figure 4.2.**). Although it does not constitute an independent entity, the larval stage is

known as Cysticercus spp.; it should be preferably named “metacestode of *Taenia* spp.”. A wild growing *T. solium* cysticercus in man is often referred to as “cysticercus racemosus”, which is a degenerative form of the metacestode, with the appearance of a cluster of lobes located in the meninges and the ventricular system of the brain. The old nomenclature has definitely some advantages, specifically with reference to the pathological effects i.e. the cysticercus stage refers to the fully developed infective larvae, whereas a metacestode can be of any larval stage.

1.1.4 Life cycle of *Taenia solium*

The cestode tapeworm, *T. solium*, requires both human and porcine hosts to complete its life cycle. The adult worm is found in the small intestines of humans where it attaches to the intestinal wall by its suckers and hooks. Infected humans excrete thousands of eggs and gravid proglottids, which are very resistant to adverse environmental conditions.

Pigs, the intermediate hosts, become infected when they ingest eggs of *T. solium* that are shed in the feces of infected humans. Ingested larvae hatch in the intestine of the pig, penetrate the intestinal mucosa, reach the blood stream and migrate to tissues including muscle (Brutto et al., 1998). Pigs acquire cysticercosis mainly in endemic areas where they have access to human feces (Sarti et al., 1992). Because the lifespan of domestic pigs is ordinarily around one year, metacestodes do not reach the degeneration stage in these animals (García et al., 2003).

Humans can become infected with *T. solium* in two ways (**Figure 1.6.**). First, a person may act as a definitive host when ingesting raw or undercooked pork infected with viable cysticerci. The scolex of the larva emerges from the cyst and attaches to the wall of the small intestine. Within three months, an adult tapeworm develops and starts to produce proglottids. Humans can also act as intermediate hosts if they ingest the eggs of *T. solium* via contaminated food. After reaching the small intestine, eggs hatch and the embryos (oncospheres) migrate through the mucosa to enter the circulation which then carries the larvae to various tissues, including the Central Nervous System (CNS), eyes, liver, lungs and striated muscle where severe pathology may occur. After reaching these sites, oncospheres evolve to the vesicular stage and are called cysticerci (Sotelo and Del Brutto, 2000). The metacestodes are generally tolerated by the host for several years, but eventually begin to degenerate (Dixon and Lipscomb, 1961), at which point they come under attack by the host immune system, causing inflammation.

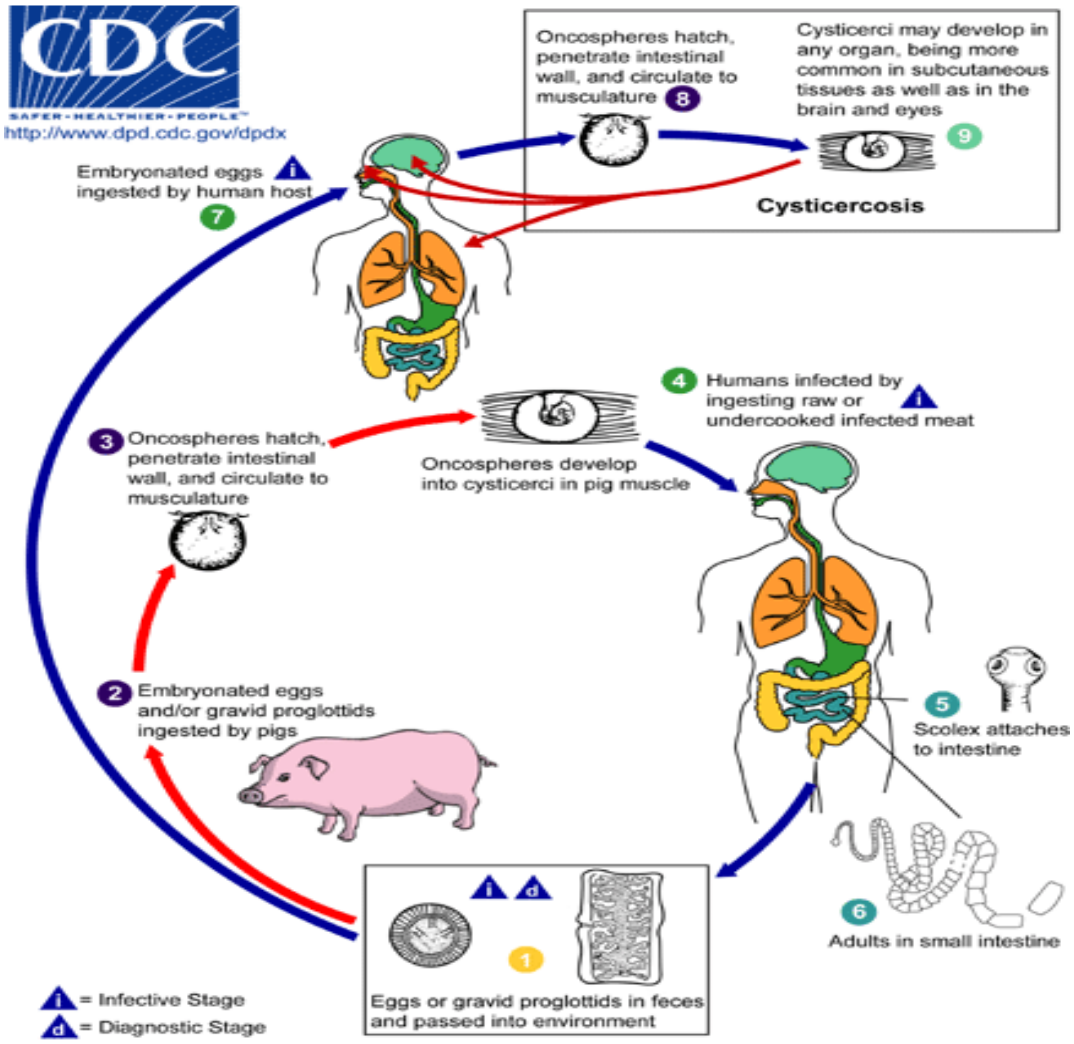


Figure 1.6. Life cycle of *T. solium* [Source: CDC]

1.1.5 Neurocysticerocosis

Neurocysticercosis (NCC), the most common parasitic brain disease worldwide, is endemic in countries with poor sanitation, and is increasingly being reported in developed countries due to globalization and immigration. It has been typical to affirm that clinical manifestations are extremely heterogeneous because they depend on the number, location, size, viability, or evolutionary phase of the parasite, as well as on the immunological response of the patient (Carpio, 2002). One of the most intriguing aspects of NCC is that presumably a high percentage of the individuals harboring NCC remain asymptomatic (Fleury et al., 2010); however, among the symptomatic group, the only clinical manifestation in most patients with parenchymal NCC is seizures, and their neurological status is usually normal (Carpio, 2002). In some patients NCC develops clinical manifestations many years after the parasite lodges in the central nervous

system, as either brain inflammation around the parasite or mass effect. It has also been stated that almost any neurological symptomatology may be found, ranging from mild headache or treatable acute seizures to very severe neurological manifestations, such as intracranial hypertension (ICH), dementia, or even death (Cardenas et al., 2010). These assumptions are probably true, since empirical observation has shown that many of these factors are related to a specific clinical manifestation; however, to date there is no definitive study, using appropriate methodology, designed to address the precise role of each of these factors, or the interaction among them, on the development of symptoms or signs due to NCC (Carabin et al., 2011).

1.1.6 Epidemiology of Neurocysticerocosis

1.1.6.1 Worldwide scenario of NCC

Neurocysticercosis (NCC) is endemic in countries with poor sanitation, and is increasingly being reported in developed countries such as the United States, Canada and Spain due to globalization and immigration. Although the real prevalence of NCC remains largely unknown, it had been estimated that 5 to 6 million humans have cysticercosis, with approximately 400,000 patients having symptomatic disease and 50,000 dying each year because of NCC (Craig et al., 1996; Crompton, 1999). Although, *T. solium* cysticercosis had been proposed to be declared an international reportable disease (Roman et al., 2000), cysticercosis do not lead to sudden large-scale international outbreaks of disease and therefore would not seem to constitute an appropriate subject for international notification (WHO, 2003).

NCC is prevalent in rural populations of developing nations where pigs dwell in close proximity to humans. Endemic areas include Latin America, China, India, Nepal, Southeast Asia and Sub-Saharan Africa (WHO, 2002). Serological data from surveys performed in rural communities of Mexico, Bolivia and Peru show that prevalence of human cysticercosis in these areas is between 8 and 12% (Garcia et al., 1991), yet up to 40% of seropositive individuals will become sero-negative within one year, complicating prevalence estimates. Infected individuals may also remain asymptomatic for years, possibly throughout the duration of viable infection, leading to underreporting of infected households.

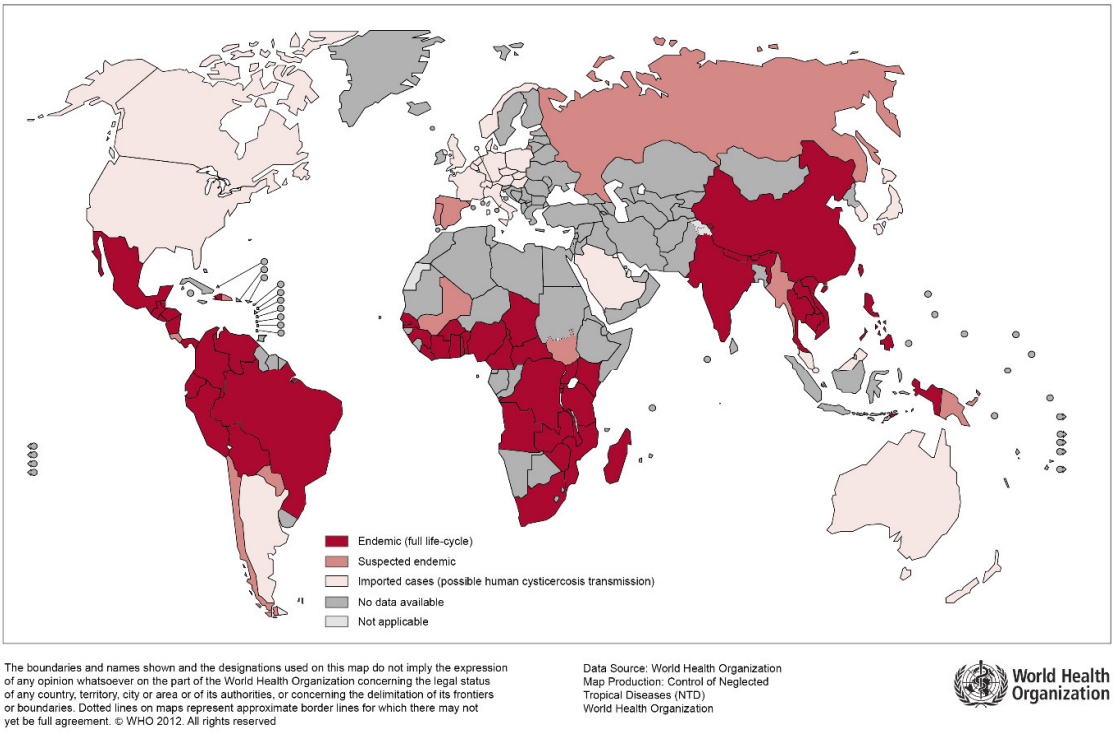


Figure 1.7. Worldwide scenario of cysticecosis/neurocysticercosis [Source: WHO, 2011]

Although the infection disappeared in many European countries during the nineteenth century, in some Eastern European countries control was not achieved until the beginning of the twentieth century, mainly due to the improvement of their political, social and economic status. Alarming recent reports show the persistence of the endemic in Africa, as well as in the Americas and in Asia. None of the endemic countries has been able to eradicate *Taenia solium´*s Taeniosis/Cysticercosis. Similarly, the frequency of human cases of NCC is increasing in some industrialized countries, such as the United States, Canada and Spain, due mostly to migrant workers, although some autochthonous cases have also occurred (Del Brutto, 2012).

1.1.6.2 NCC scenario in Nepal

There are very limited data on epidemiology of cysticercosis from Nepal. Heap (1990) reported that NCC is a common cause of epilepsy among Nepalese soldiers stationed with British Army in Hongkong (Brutto et al., 1998). The available data suggest that the prevalence ranges from 0.002 -0.1 % in general population in Nepal (Bista, 2006). Many clinical studies reported from Nepal had variable presentations, outcome in terms of diagnosis and treatment response (Panta, 2006; Amatya, 1999). Most of the studies were hospital based retrospective and diagnosis was based mainly on clinical presentation and neuroimaging.

For examples, in 2012, the neurology service unit of Tribhuvan University teaching hospital, Kathmandu, Nepal, reported 66 (36 male and 30 female) cases of NCC (Agrawal, 2012). Among these 77.2% of the patients presented seizures. In western region of Nepal, Basu and associates in 2007 had observed that the mean age at which NCC occurs was at 10.8 years (range 11 months - 15 years, most commonly affected age group 10 - 12 years; 8.9% patients were below 2 years of age) (Basu et al., 2007). In another study Ellis and associates examined 93 patients (age range: 2-14 years; 10.3% patients <6 years of age) presenting new onset seizures from Lumbini region; 73% of the patients had NCC (Ellis et al., 2000). In a study by Joshi and associates, EITB was utilized to screen for NCC using blood samples collected from patients (n= 60) that underwent CT-scanning. Most of the patients with multiple cysts showed a positive EITB test. Out of 60 samples collected from patients with NCC, as suggested by CT-scan/Magnetic Resonance Imaging (MRI), EITB test was found to be positive in 18 cases (Joshi et al., 2014).

1.1.7 Disease diagnosis

Definitive diagnosis of NCC is performed in humans with characteristic neuroimaging findings, clinical symptoms, histological techniques, serological (immunological) assay positive for *T. solium*, or exposure to a person with *T. solium* infection or an area where *T. solium* infection is endemic. Because the important features are the number, size and location of lesions, radiography has always been crucial for diagnosis of NCC (Mac Arthur, 1934). Currently used techniques include computed tomography (CT) and magnetic resonance imaging (MRI). CT has been claimed to have sensitivity and specificity of over 95% for the diagnosis of NCC, although CT images are rarely pathognomonic for this disease. MRI is the most accurate technique to assess the degree of infection, the location and the evolutionary stage of the parasite (Nash and Neva, 1984).

A wide range of serological tests have been used in diagnostic and epidemiological studies of cysticercosis. The EITB assay is nearly 100% specific and 98% sensitive for patients with either multiple active parenchymal cysts or extraparenchymal neurocysticercosis (Brand and Tsang, 1989). The quantitative immunological assay, the enzyme-linked immunosorbent assay (ELISA) have been employed for NCC diagnosis due to their high sensitivity for which cerebrospinal fluid (CSF) and/or serum may be used as samples. Furthermore, a monoclonal antibody-based sandwich ELISA is used for the detection of viable metacestodes of *Taenia* spp. in human, porcine and bovine serum samples. Monoclonal antibody-based antigen detection ELISA uses two monoclonal antibodies to "trap" the antigen circulating in blood, CSF or urine (Correa et al., 1989;

Castillo et al., 2009). These assays give a positive result when viable and well-established cysts are present.

For a solitary CNS lesion that presents a diagnostic dilemma by neuroimaging and serologic assays, advanced molecular approaches targeting genetic sequences of *Taenia* species have been used to detect NCC in CSF. Global DNA screening platforms using ribosomal RNA (rRNA) gene sequencing can be as a screening tool, the presence of *T. solium* is subsequently confirmed in a secondary analysis using *Taenia* species–specific primers in Polymerase Chain Reaction (PCR) technique (Harrington et al., 2009).

1.1.8 Treatment therapy for NCC

It is well proven that both Albendazole, an Imidazole, and Praziquantel, an isoquinolone, are cysticidal and result in the resolution of *T. solium* cysts (Sotelo et al., 1990; Gonzalez et al., 1995). Although the clinical benefits of cysticidal treatment continue to be controversial, there is an increasing body of evidence pointing to the clinical benefits of treatment of some forms of NCC. There is no standard regimen for the treatment of NCC but the treatment varies with the type of involvement and the presence of other factors. Management of NCC includes the use of symptomatic medication (including anticonvulsants), anti-inflammatory drugs, anti-parasitic drugs, or surgery. The treatment of NCC should be individualized and it should be based on the disease pathogenesis in any given patient. At the same time there are some of the guiding treatment of NCC (**Table 1.1.**) which will help to recover the different forms of NCC (Del Brutto et al., 2001).

Table 1.1. Guidelines for treatment of NCC

Parenchymal neurocysticercosis	Treatment strategy
Viable cysts	Cysticidal treatment + steroids
Calcified	Anti-epileptic drugs (AEDs); No cysticidal therapy
Enhancing lesions	
Single	AED; Cysticidal drugs if persistent
Multiple	Anticonvulsant + cysticidal and steroids
Extraparenchymal neurocysticercosis	
Intraventricular cyst	Neuroendoscopic removal

Subarachnoid cyst	Cysticidal treatment + steroids
Spinal cysticercosis	Cysticidal treatment + steroids

1.1.9 Transmission

T. solium transmission occurs via the acquisition of the adult worm infection and the dispersal of the tapeworm's eggs from feces to pigs and humans. Transmission of the disease is perpetuated by socioeconomic conditions, as pigs raised by individual households for sustenance or sales are allowed to forage freely feeding on human refuse. Consumption of uninspected pig meat is the major source of human *T. solium* taeniosis, and consequently, a major risk factor for human and pig cysticercosis (Zoli et al., 2003). Few pigs are taken to commercial abattoirs, and those that receive only a perfunctory lingual examination for disease (Gonzalez et al., 1997)). Often, pigs with known cysticercosis infections are sold on the black market to prevent economic loss (García et al., 2003). The absence of health care infrastructure and lack of education regarding preventative personal hygiene also greatly contribute to transmission of NCC in these areas. Inadequate latrine facilities and sewage disposal, as well as the use of wastewater for irrigation of crops, ensure host availability for the parasite (Pawlowski, 2002).

The transmission of *T. solium* eggs to pigs, the essential link in the pig-man-pig cycle, requires that pigs have access to human feces and that people consume improperly cooked infected intermediate host (pork) (Pawlowski and Murrell, 2000). Both the very high fecundity of the tapeworm and the various behavioral patterns of humans conspire to greatly facilitate egg transmission. Coprophagy is a normal activity of all free ranging and scavenging pigs. Indeed, in some parts of the world, pigs may be kept for the purpose of removing human feces and in others they may be fed feces deliberately as a cheap feed. Consequently, pigs may ingest whole proglottids along with a massive numbers of eggs (Dorny et al 2004; Phiri et al 2002).

NCC occurs following ingestion of eggs in human feces. It is likely that person to person transmission can occur by: (i) the ingestion of eggs in contaminated food and water; or (ii) the introduction of eggs from feces into the mouth by contaminated hands (Pawlowski, 2002). Outbreaks of cysticercosis among people who stringently avoid pork for religious or dietary reasons underscores the importance of person-to-person transmission (Schantz et al., 1992). Airborne infection by wind and through the ingestion of infected insects has also been suggested but this remains unproven. Other novel modes may sometimes occur; in some societies, for example the Bantu people, massive

infection may be caused by using local medicine prepared from proglottids (WHO, 1983).

Auto-infection by reverse peristalsis of the intestine, once considered to be an important source of infection (WHO, 1983) particularly during treatment, is now largely discounted. External auto-infection, however, by eggs transmitted from anus to mouth through dirty hands or contaminated food seems to be a more probable way of contacting *T. solium* NCC, than internal auto-infection (Webbe G., 1994).

1.1.10 Preventive and control measures

Control measures of NCC have been formulated by the World Health Organization in 2002 (WHO, 2003) based on four facts, i.e. cysticercosis is caused by the larval stage (cysticerci) of the pork tapeworm *T. solium*; cysticercosis is the most important neurological disease of parasitic origin in man; human cysticercosis is associated with poverty in areas where people eat pork and traditional pig husbandry is practiced and cysticercosis of the CNS is an important cause of chronic epilepsy, which places particular demands on health services. Hence, the measures currently available to control cysticercosis i.e. elimination of infected pigs/carcasses (meat inspection), improved sanitation, hygiene and pig husbandry, health education, treatment of intestinal taeniasis, chemotherapy of infected swine and mass treatment in humans and pigs have been proposed (Gonzalez, 2002; WHO, 2002). In this context, the main strategies available are directed towards elimination of infected pig carcasses (meat inspection), underpinned by improved sanitation, hygiene, pig husbandry and most importantly, proper health education.

International Task Force for Disease Eradication in 1992 reported that cysticercosis is potentially eradicable (CDC, 1992). It is feasible because there are no animal reservoirs besides humans and pigs. The only source of *T. solium* infection for pigs is from humans, a definite host. Theoretically, breaking the life cycle seems easy by doing intervention strategies from various stages in the life cycle (Schantz, 2002). *T. solium* is a potentially eradicable disease, for the following reasons: (i) the life cycle requires humans as definitive hosts; (ii) tapeworm infection in humans are the only source of infection for pigs, the natural intermediate host; (iii) the transmission of infection from pigs to man is controllable; (iv) no reservoir for infection exists in wildlife; (v) there are practical methods for surveillance in pigs (visual inspection of the tongue), in humans (EITB and copro-antigens) and, (vi) safe and effective drugs for mass chemotherapy of taeniasis in humans and cysticercosis in pigs do exist (WHO, 2003).

Other measures include treatment of intestinal taeniasis, vaccination of pigs and chemotherapy of infected animals (Engels et al., 2003). Agro-industrialisation has led indirectly, even unintentionally, to the eradication of the parasite in industrialised

countries. However, as long as traditional pig husbandry is practised in developing countries, the life cycle of *T. solium* is likely to persist (Gonzalez et al., 2003).

1.2 Research plan and design

1.2.1 Research hypothesis

Some antigenic proteins of cysticercus larvae of *Taenia solium* parasite would be specific markers for the diagnosis of Neurocysticercus by western blotting and ELISA.

1.2.2 Research objectives

General objective

To define the marker for serological diagnosis of human NCC caused by cysticercus larvae of *T. solium*.

Specific objectives

1. Quantification of the crude soluble antigen (CSA) from the cysticercus larvae of *T. solium*.
2. Collection of secondary data of NCC patients from hospitals.
3. Analysis of complete protein profile of the cysticercus larvae of *T. solium* using SDS PAGE.
4. Determining the antigenic proteins from the cysticercus larvae of *T. solium* by comparing Western blot profiles of NCC patients and healthy controls.
5. Diagnosis of the suspected NCC patients using the antibody based ELISA.
6. Perform the seroepidemiological analysis.

1.2.3 Design of research plan

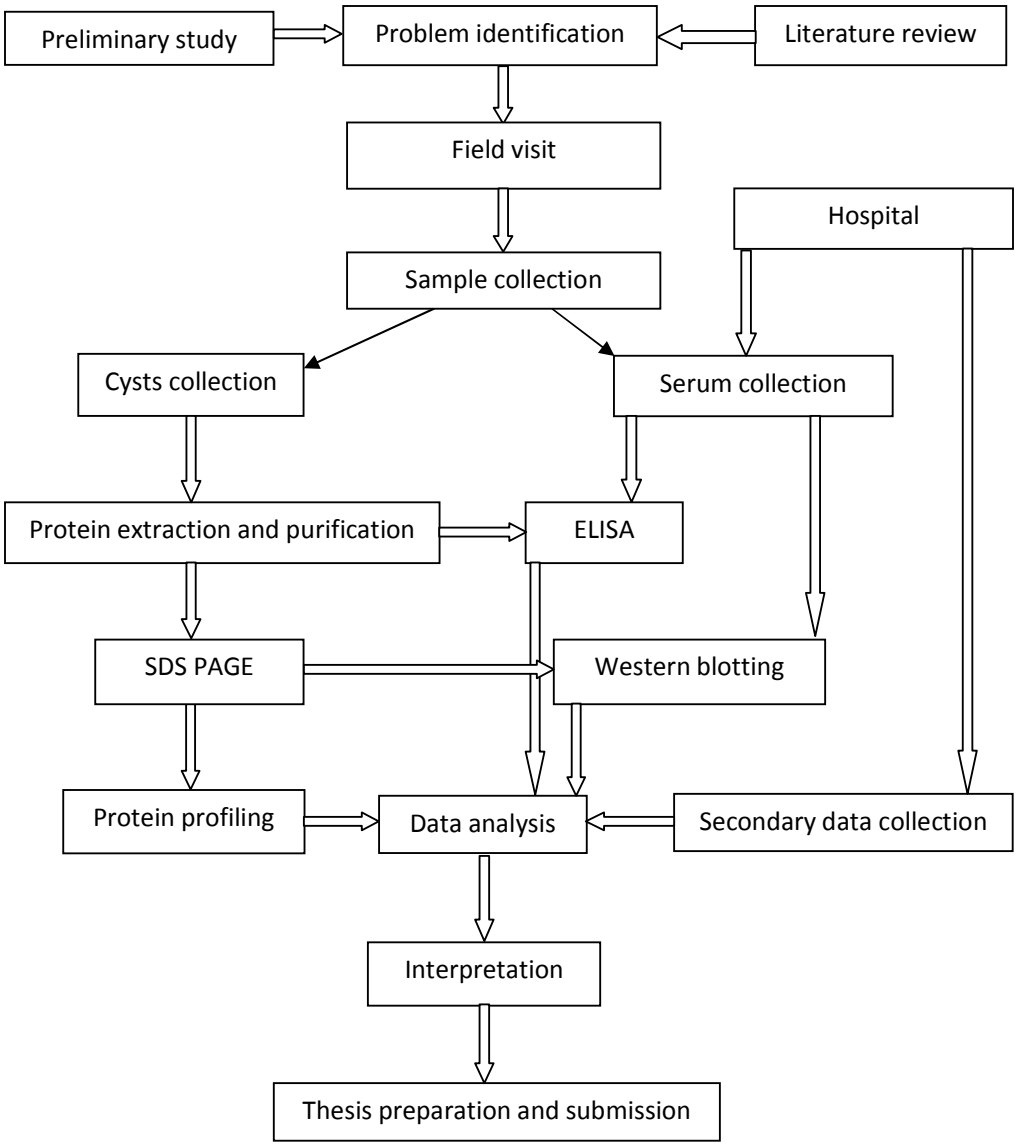


Figure 1.8. Research Design

1.2.4 Rationale

Neurocysticercosis (NCC), the infection of human Central Nervous System (CNS) by larval stage of *Taenia solium*, is one of the major neglected tropical diseases as recognized by WHO. Infection occurs following accidental ingestion of tapeworm ova found in human feces. NCC is the major cause of epilepsy in ≥1% of the populations (Savioli LS, 2010) and disability in many of the world's poorer and endemic countries including Nepal where families raise free roaming pigs that are able to ingest human feces. It is also frequently diagnosed in immigrants' population in developed countries like United States and Canada (Wallin and Kurtzke, 2004). Although parenchymal cysts are the most common location in the brain and cause seizures, cysts may also be present in the ventricles, meninges, spinal cord, eye, and subarachnoid spaces. Involvement in these other sites may result in aberrant growth (racemose cysts) and complicated disease that is difficult to treat and may cause increased morbidity and mortality (Coyle *et al*., 2012).

NCC is difficult to identify and treat. The conventional diagnosis of NCC requires a compatible clinical history, positive serology and typical computed tomography (CT) or magnetic resonance imaging (MRI) (Del Brutto et al., 2001). Since the diagnosis requires brain imaging, a technology commonly unavailable in resource-poor areas and clinical diagnosis is unreliable as the manifestations are diverse and non-specific (Coyle et al., 2012). So, it is a significant health problem in Latin American countries (LAC), Haiti, Sub-Saharan Africa, India, Nepal, Southeast Asia, China, Indonesia and other regions. Although accurate estimates of the extent of infection and disease are not available for most endemic regions including Nepal many studies suggest a higher prevalence of infection and disease burden.

In this study, we carried out sero-diagnosis using the cysts from the local communities where the disease is endemic which may increase the sensitivity of the test. The sample can be collected from the field and the test carried out in a nearby lab ultimately becomes cost effective. This type of study is required not only for the diagnosis but also to estimate the prevalence rate in the endemic and non-endemic regions of Nepal. The studies carried out in Nepal till now are based on the conventional diagnosis techniques in hospitals and the EITB strips that are manufactured by foreign companies. No such attempts have been performed in our own country to diagnose serologically the NCC cases using the cysts from the local communities. The sensitivity of the techniques used in this study would be very high as the antigen is from the local communities. Hence, the study is indispensable to carry out in own country, Nepal, for the better diagnostic purpose and for the estimation of prevalence rate of the infection in endemic and non-endemic regions of Nepal.

Chapter II

Literature Review

2.1 Neurocysticercosis

The clinical manifestations of NCC are extremely heterogeneous because they depend on the number, location, size, viability, or evolutionary phase of the parasite, as well as on the immunological response of the patient. One of the most intriguing aspects of NCC is that presumably a high percentage of the individuals harboring NCC remain asymptomatic; however, among the symptomatic group, the only clinical manifestation in most patients with parenchymal NCC is seizures, and their neurological status is usually normal (Carpio, 2002). In some patients NCC develops clinical manifestations many years after the parasite lodges in the central nervous system, as either brain inflammation around the parasite or mass effect. It has also been stated that almost any neurological symptomatology may be found, ranging from mild headache or treatable acute seizures to very severe neurological manifestations, such as intracranial hypertension (ICH), dementia, or even death (Cardenas et al., 2010; Rodrigues et al., 2012).

Regarding the parasite evolution phase, the transitional or degenerative phase develops clinical manifestations due to the inflammatory reaction of the brain. NCC predominantly affects adults in their third and fourth decade of life, and is relatively uncommon in children and the elderly (Carpio, 2002) Reports of NCC are very rarely in children younger than 2 years of age because of the prolonged incubation period of *T solium*. Most often, the disease is recognized in children older than 7, due to this incubation period.

There is also clinical heterogeneity across geographical areas; most cases from the Indian subcontinent present single degenerative lesions, whereas those from Latin America present few viable cysts (Singh, 1997). These differences are probably due to complex interactions between the host, parasite, and environmental factors (Fleury et al., 2010). Genetic differences in *T. solium* cysticerci have been reported from different countries (Maravilla et al., 2008) and may contribute towards the clinical variations among countries. A genetic susceptibility to NCC has been suggested by a reported positive association of HLA-DRBII 13 with single, contrast enhancing CT lesions (Jain et al., 1999).

2.1.1. Parenchymal neurocysticercosis

The most common clinical manifestation of parenchymal NCC is epileptic seizure, which occurs in 60-90% of cases; similarly, focal deficits or motor abnormalities are commonest in the parenchymal location. Focal neurological deficits, when present, are usually transient, over a period of a few days, weeks, or months, with periods of remission and relapse, most likely due to different evolutionary stages of the parasite (Carpio et al., 1994).

Symptomatology of altered mental state, psychiatric manifestations and depression are also reported in some patients. Inflammation surrounding parenchymal cysticerci is more intense in women and multiple degenerating parasites localized in the parenchyma are more frequently reported in young women (Kelvin et al., 2009). Regardless of the localization of the parasite, the inflammatory response, as expressed by cerebrospinal fluid (CSF) cellularity is also more intense in women (Fleury et al., 2010). Therefore, there are significant gender and age differences in the local immune response, even after adjusting for differences in healthcare access.

2.1.2. Extraparenchymal neurocysticercosis

The extraparenchymal location (around 15–30% of cases) develops different clinical manifestations. Headache and signs of intracranial pressure are more frequent in the extraparenchymal location (88%), in comparison with the parenchymal location (10%) (Carpio et al., 1994). When cysticerci are located inside the ventricular system, life-threatening acute intracranial hypertension as a result of hydrocephalus may occur; as a consequence, severe headache, dizziness and consciousness alteration are the predominant clinical manifestations (Citow et al., 2002).

Cysts in the subarachnoid space may lodge in the Sylvian fissure or basal cisterns and grow to a big size (racemose form), causing intracranial hypertension. This is associated with an intense inflammatory reaction, and fibrosis and progressive thickening of the leptomeninges at the base of the brain. There is an obstruction of the CSF circulation, resulting in hydrocephalus and progressive intracranial hypertension. Inflammation of meninges, cranial nerve involvement, chiasmatic syndrome, and cerebral infarcts secondary to vasculitis may develop (Cardenas et al., 2010). When hydrocephalus due to cysticercotic meningitis is present, the mortality rate is high (50%), and most patients die within 2 years after CSF shunting (Sotelo and Marin, 1987). Therefore, ventricular and basal cisternal locations are considered to be malignant forms of NCC (Estanol et al., 1986). In cases with NCC meningitis, cerebrospinal fluid (CSF) may show mild elevation

of protein, hypoglycorrhachia and lymphocytic pleocytosis, which is similar to other chronic basal meningitis, such as tuberculosis and mycosis (Carpio, 2002).

Spinal cord cysticercosis is rare. Patients experience nonspecific clinical manifestations, such as nerve root pain or spinal cord compression syndromes, according to the level of the lesion (Alsina et al., 2002). Severe forms of NCC may exceptionally occur, including cysticercotic encephalitis, which result in permanent neurologic sequel, such as amaurosis. Hydrocephalus and intraventricular NCC is extremely rare in children (Loureiro et al., 2003).

2.2 Immunology

2.2.1. Host immune responses

The immunology of human neurocysticercosis is particularly important because of its paradoxical relationship with disease pathogenesis. One of the most interesting phenomena in immunoparasitology is the evasion of the host immune response by the parasite (White, 1997). Cysticerci are capable of surviving in the human host for several years before their degeneration. Viable cysticerci are associated with little surrounding inflammation. This allows for the maintenance of the parasite. The mechanisms underlying the survival of parasites lodged in immunologically privileged sites is undoubtedly complex and may involve masking of cysticercus antigens by host immunoglobulins, concomitant immunity, molecular mimicry (synthesis of host- like antigens by the parasite) and suppression or deviation of host responses (Arechavaleta et al., 1998; Sciutto, 1995). In contrast, the immune mediated inflammation around one or more degenerating cysts may precipitate symptomatic disease. When the parasite begins to die, either naturally or following treatment with cestocidal drugs a surrounding granulomatous inflammatory response develops in human infections (Aluja et al., 1989). Predominant components of this inflammatory response include plasma cells, lymphocytes, eosinophils and macrophages. The latter engulf parasite remnants, eventually leaving a gliotic scar with calcification (Aluja et al., 1998; Rodriguez et al., 1978).

The inflammatory reaction is protective in the sense that it leads to destruction of parasite and transformation into a calcified nodule. The degree of inflammation depends on degree of the immune response to cysticerci infestation. The spectrum of inflammation varies from intense immune reaction and immune tolerance. Correa et al studied the presence of Human Lymphocyte and Antigen (HLA)–related antigens on the surface of cysticerci. They found a direct relation between microscopic signs of damage to cysticerci and presence of HLA attached to their surface (Correa et al., 1989). The

depressed immunity in patients with NCC may be the cause of the reported association of this parasitic disease with conditions resulting from immunological disturbances, such as hematological malignant diseases and conditions leading to the development of cerebral tumors (Herrera et al., 1999). During the immune response, both humoral and cell mediated immune responses have been detected.

2.2.1.1 Humoral immune responses

The humoral immune response is better understood than the cellular one. The fact that humans respond immunologically to antigens of *T. solium* cysticerci is evident from the number of immunodiagnostic assays that have been developed using different types of antigens (Correa et al., 1989). Several immunoglobulin (Ig) classes are produced as specific antibodies against the parasite. The most frequent is IgG, which can be detected in serum, cerebrospinal fluid (CSF) and saliva, the suggestive to long duration infection. There is a correlation between the presence of antibodies and the intensity of infection, as well as the viability of the parasite. Antibodies are most frequent in cases with live or dying parasites, and rarely in cases with calcified cysts (Sotelo and Brutto, 2000). There are also differences between benign and malignant cysticercosis, with cysticercotic encephalitis being more immunogenic. The humoral immune response in patients with NCC is quite heterogeneous as evidenced by the number of antigens recognized. Patients' antibodies may react with one to eight antigens in immunoelectrophoresis and up to 30 antigens in western blot (Correa et al., 1989; Proano-Narvaez et al., 1989). It has also been shown that the immune response may be transient in the households of patients with NCC and in apparently healthy individuals in the general population (Garcia et al., 2001).

2.2.1.2 Cellular immune responses

Precise patterns and pathways of the cellular responses in human NCC have only recently been investigated. Studies so far have addressed molecular components in the CSF, serum and the granuloma itself. Increased levels of interleukin (IL) 1 and IL-6 have been reported in the CSF of patients with inflammatory NCC. High levels of IL-6 in the CSF of patients with subarachnoid NCC have also been reported, suggesting acute phase response (Ostrosky-Zeichner et al., 1996). In addition, high levels of tumor necrosis factor-alpha (TNF-α) have also been noted in the CSF of children with active NCC, which is undetectable in controls and children with inactive NCC (Aguilar-Rebolledo et al., 2001).

Increased levels of eotaxin and IL-5, both eosinophil-selective mediators, have been found in the sera of patients with NCC. These cytokines are involved in recruiting eosinophils locally as well as systemically (Evans et at., 1998). The presence of eosinophils as the first attack cells was reported in porcine cysticercosis after cestocidal treatment and after vaccination. This suggests that eosinophils may play an important role in the degenerative phase in this parasitic infection. Another study showed that IL-2 was synthesized by the peripheral blood cells of 58% human beings with untreated, active and recently diagnosed NCC, while interferon-gamma (IFN-γ), IL-4 and IL-10, were only found in 11%, 10% and 14% respectively (Medina-Escutia et al., 2001). Interestingly, only IFN-γ was increased in the group of patients as compared to controls. The macroscopic disappearance of killed cysticerci takes about two months, but the immunological processes that occur within the dying granulomas are poorly understood. Very few immunohistochemical studies of the inflammatory response within cysticercus granulomas located in the CNS are available, mainly due to limited tissue specimens available. The reports suggest a mixture of Th1 and Th2 responses in human brain granulomas caused by cysticerci (Restrepo et al., 2001; Rangel et al., 1987).

An immunological study of patients with NCC treated with praziquantel (without major adverse effects) reported elevated soluble IL-2 in the CSF suggesting a Th1-type immune response to therapy, in contrast to the Th2-type immune response characteristic in animal models with viable cysticerci (Cruz-Revilla et al., 2000). It was therefore hypothesized that living cysticerci facilitate immune evasion by inducing a Th2-type immune response until the death of the larval parasite following Th1-mediated inflammatory response to develop (Flisser et al., 2002). This model however, is not consistent with other reports and it seems likely that the regulation of immunity in *T. solium* cysticercosis is a more complex phenomenon than a simple Th1/Th2 shift.

2.3 Disease diagnosis

Taenia solium neurocysticercosis (NCC) is recognized as an important parasitic disease of CNS, and research priorities include the development of more sensitive and specific diagnostic tests (Eddi et al., 2003). Taeniosis in humans can be caused by *T. solium, Taenia saginata* or *T. saginata asiatica*, but only infection with adult *T. solium* poses a risk for acquiring NCC. Accurate diagnosis and species differentiation is therefore preferable. Diagnostic criteria for NCC are based on histological, neuroimaging, immunological and epidemiological data. Absolute criteria for identification of *T. solium* are either by direct evidence through biopsy (CNS) or funduscopy (eye), or by visualization of scoleces on CT or MRI. All other criteria are ordered in descending fashion according to their diagnostic strengths into major, minor and epidemiological

categories. If no absolute criteria have been met, evidence of two major, one minor and one epidemiological criterion are required to give definitive diagnosis of NCC. A probable diagnosis of NCC is made when patients exhibit a combination of three or four criteria of lesser diagnostic strength (Del Brutto et al., 2001). Diagnosis of NCC on clinical grounds is difficult, because signs and symptoms are non-specific. Some neuroimaging findings are not pathognomonic either, and the serological tests have low sensitivity and specificity (White, 2000).

2.3.1. Diagnosis by Neuroimaging techniques

There are 3 clinical stages of NCC given by Sotelo & Carpio namely active, transitional and inactive NCC. Escobar defined 4 pathological stages of NCC. They are (i) Vesicular (ii) Colloidal vesicular (iii) Granular nodular and (iv) Nodular calcified. Vesicular is active form, colloidal vesicular and granular nodular represent transitional stage while nodular calcified stage is inactive stage of NCC (Carpio et al., 1994).

Although to date there are no trustworthy data on the specificity and sensitivity of neuroimaging diagnostic studies, computed tomography (CT) and magnetic resonance imaging (MRI) are the main tools in NCC diagnosis (Carpio et al., 1998). Demonstration of viable cystic lesions with a mural nodule (i.e., the invaginated scolex), associated with transitional or degenerative cysts and calcifications is a typical image of cysticercosis (Carpio et al., 1994). However, this typical image is not necessarily the most common. Single enhancing lesions are probably more common, especially in children, though in these cases some other aetiologies should be considered in the differential diagnosis to avoid unnecessary neurosurgical interventions (Carpio and Placencia ,1992).

MRI is superior for showing intraventricular or subarachnoid cysts, but CT is better for show in the calcification of inactive lesions (Carpio et al., 1998). CT is still most useful diagnostic tool for the diagnosis of NCC (Brutto and Sotelo, 1988). CT provides reliable information about the topography of the lesions and disease activity. MRI provides useful information in the evaluation of NCC patients, especially when CT findings are not conclusive (Brutto et al., 1989). MRI permits precise characterization of NCC in terms of disease activity and its location. These advantages have important therapeutic implications. The scolex is visualized within the cyst as a high intensity nodule by MRI; this “hole-with-dot” imaging is characteristic of NCC (Suss et al., 1986). Noninvasive diagnosis of intraventricular cysticerci is probably one of the best advantages of MRI. Cysticerci within the spinal cord are easily detected by MRI (Dorny et al., 2003). Although some authors have considered MRI the best diagnostic method for patients with NCC (Martinez et al., 1989). CT scan is still of paramount importance. Both CT and MRI are mutually complementary in providing optimal non-invasive diagnosis.

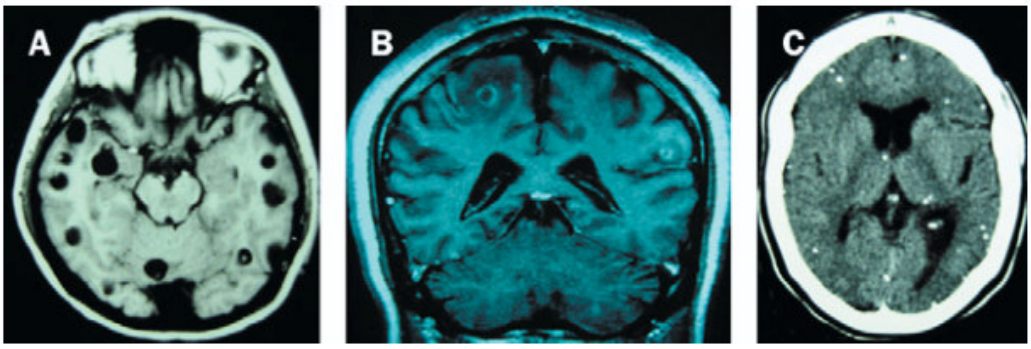


Figure 2.1. Neuroimaging; A. MRI of viable cysts, B. MRI of degenerating cysts and C. CT of calcified cysticerci [Source: Garcia et al., 2003]

2.3.2 Immunological diagnosis

Immunodiagnostic assays are available to detect antibodies that indicate present or past infection, as well as circulating antigens that indicate current infection (Bueno et al., 2000). Complement fixation, indirect haemagglutination, ELISA, and EITB are among the types of immunodiagnostic assays that have been developed. Nowadays, the EITB and ELISA are the tests most frequently used for diagnosis of human cysticercosis. These assays are classical tools in epidemiological studies to estimate the prevalence and identify the risk factors associated with transmission of *T. solium*. They also offer the possibility of surveillance of the infection during and after control programs (Sarti et al., 2000). Ideally, a combination of both (antibody and antigen detection) tests is best for seroepidemiological studies and for supporting diagnosis of NCC by neuro-imaging techniques. Serological tests can be very useful for confirmation of imaging techniques for differential diagnosis of other 'cyst-forming conditions,' including echinococcosis, brain tumors and tuberculosis (Dorny et al., 2003). Since clinical symptoms of NCC generally occur as a result of inflammatory reactions around degenerating cysticerci, early diagnosis of NCC by serology provides opportunities for the prevention of clinical symptoms.

Serological tests using a variety of crude antigen preparations from cyst material are still put forward as a useful tool for serological screening. The variety of methods used to prepare the soluble antigen makes comparison between test results difficult. It has already been demonstrated by several research groups that immunodiagnostic tests based on such crude antigen preparations have, at best, moderate sensitivity and specificity (Dorny et al., 2003). Using (semi)purified antigens could constitute a better alternative, but purification procedures are often complex and require considerable technical expertise. Moreover, data on sensitivity and specificity are often calculated

using a small number of clinically based serum and/or CSF samples. The test performances are calculated based on these restricted datasets which should be interpreted with caution and should not be generalized. Variations can be expected in test performances (related to factors such as the criteria used for the selection of NCC patients and healthy controls) (Arruda et al., 2005).

The improvement of immunodiagnostic tests for NCC has contributed greatly to a better understanding of the prevalence and epidemiology of the infection. Immunodiagnostic tests are also a valuable tool in assessing the disease burden and for clinical diagnosis and follow-up of NCC. However, no test on its own provides unequivocal proof. Antibody detection only reflects exposure to the parasite, whereas antigen detection reflects the presence of viable parasites. Antibody detection test results are sometimes misinterpreted leading to, for example, overestimation of the prevalence and/or unnecessary (and sometimes even hazardous) anthelmintic treatment of suspected NCC patients. When interpreting test characteristics (sensitivity and specificity), one should take into account the test population used to calculate these test characteristics. Only a few immunodiagnostic tests were validated on a large scale and in different populations, and, thus, provide reliable estimates of test sensitivity and specificity.

2.3.2.1 Antibody-detection methods

Infection with *T. solium* results in a specific antibody response. These antibodies can be detected in serum and in cerebrospinal fluid (CSF) in the case of NCC (Arruda et al., 2006). Several techniques have been described to detect antibodies to *T. solium* infections in humans, such as radioimmunoassay, hemagglutination, the complement fixation test, dipstick assay, latex agglutination, enzyme-linked immunosorbent assay (ELISA) and immunoblot techniques (Dorny et al., 2003). Initially, antigens used in antibody-detection assays were either cyst fluid, excretory–secretory (ES) products or crude homogenates from cysticerci from either *T. solium* or the related parasites *Taenia crassiceps* or *T. saginata* (Arruda et al., 2005). However, unpurified antigens have moderate sensitivities and relatively poor specificities (Dorny et al., 2003). Improved protein-purification techniques and research on antigenic properties of cyst fluid and surface proteins has led to the development of better serological tools (Ito et al., 1998).

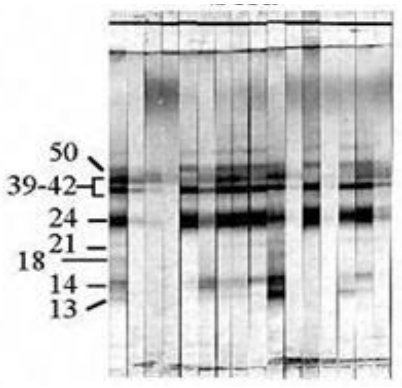


Figure 2.2. Typical antibody reactions in CDC's immunoblot for cysticercosis, where 50, 39-42, 24, 21, 18, 14 and 13 kDa protein fractions are noted as marker for NCC diagnosis. [Source: http://www.dpd.cdc.gov/dpdx/IMAGES/ParasiteImages/A-F/Cysticercosis/CysticerWB.JPG]

EITB

The most specific test so far is the enzyme-linked immunoelectrotransfer blot (EITB) with an initially reported specificity of 100%, and sensitivity of 98% when tested in humans (Tsang et al., 1989). This immunoblot uses an enriched fraction of glycoproteins obtained by purifying a raw cysticercus extract through chromatography with lentil-lectin (LLGP). Reaction with any one of seven specific bands is regarded as diagnostic for cysticercosis (**Figure 2.2.**). However, the sensitivity of this assay drops dramatically in cases with single cysts in the brain (Singh et al., 1999). Recently, Prabhakaran et al. reported that using conformation-sensitive immunoassays (with urea-induced tertiary conformations of the lentil lectin glycoproteins) detect antibodies in 46% of 60 patients with solitary cysticercus granuloma who were serologically negative on standard immunoblot (Prabhakaran et al., 2007).

The EITB test has been extensively used for the diagnosis of human and porcine cysticercosis (Tsang et al., 1991) and is commercially available. The assay, however, has some drawbacks. It depends on infected pigs for supplying the source material and, nowadays, strict regulations are in place for (international) transport of biological specimens. Preparation of the antigen and performance of the western blot require considerable technical expertise. Furthermore, batch differences can exist between different antigen preparations, and the antigen mixture is not suitable for use in an ELISA format due to the presence of non-specific fractions. Finally, a western blot assay is not suitable for field studies, nor is it a suitable or affordable assay for diagnosis in countries where cysticercosis is endemic (Hancock et al., 2003).

To address these issues, the seven diagnostic LLGP antigens have been identified, characterized and produced as either recombinant or synthetic proteins (Hancock et al.,

2003; Hancock et al. 2006). These proteins have been tested in ELISA and western blot assays for the diagnosis of cysticercosis (Bueno et al., 2005). When tested in ELISA, the sensitivities and specificities of the individual proteins were well below that of the corresponding native LLGP proteins in the EITB, whereas results for western blot format closely mirrored those of the LLGP EITB.

Recombinant protein

A multi-antigen printing immunoassay (MAPIA) was recently developed to compare the performance of multiple recombinant *T. solium* proteins (Handali, S. et al. 2009). Of six recombinant cysticercus proteins tested, the T24 antigen performed well in detecting cases with two or more viable cysts in the brain (97% sensitivity and 99.4%, specificity). Combining different cysticercus antigens did not improve the sensitivity of the test.

Glycoproteins were also purified from cyst fluid using isoelectric focusing electrophoresis (IEFE) (Ito et al., 1998). These antigens, applied both in immunoblot and ELISA, were highly specific and sensitive for differential serodiagnosis of NCC. Later, sera from rabbits immunized with these immunodiagnostic antigens were used to screen a *T. solium* metacestode cDNA library (Sako et al., 2000). Four clones (Ag1, Ag1V1, Ag2, Ag2V1) were characterized and the recombinant proteins were first tested in immunoblot. Ag1V1 and Ag2 were chosen as ELISA antigens, and the Ag1V1/Ag2 chimeric protein was expressed. With this ELISA using either IEFE purified glycoproteins or the chimeric protein, it was possible to detect antibody responses in pigs harboring 16 or more cysts from 30 days post-infection (p.i.); also, no cross-reactions were observed with pigs infected with *Taenia* hydatigena (Sato et al., 2003).

Recently, many other researchers reported the use of recombinant or synthetic proteins in immunodiagnosis of cysticercosis (Hell et al., 2009). A Pubmed Protein database search (http://www.ncbi.nlm.nih.gov/pubmed/) on *T. solium* resulted in 327 listed proteins, of which several are used in cysticercosis immunodiagnosis. The related parasites *T. crassiceps* and *T. saginata* are also the source of recombinant and synthetic proteins used for diagnosis of *T. solium* cysticercosis (Fleury et al., 2003).

Antibody detection in CSF

Recent studies, however, aimed at serologically distinguishing between patients with active and inactive cysticercosis by detecting IgG antibodies in either serum or cerebrospinal fluid (CSF) samples (Sahu et al., 2009). Barcelos et al. could distinguish between patients with active and inactive cysticercosis with the detection of immunodominant peptides in total saline extract of *T. solium* cysticerci only in CSF, not

serum. Because of the location of the cysts in the brain, serological testing of CSF (rather than serum) may be more appropriate for diagnosis; however, sampling of CSF is more cumbersome than is blood sampling (Barcelos et al., 2007).

Furthermore, the results of these studies are based on only a small subset of samples (10–30) and should therefore not be generalized. Therefore, antigen detection techniques are better suited for detecting viable cysts and especially for real-time monitoring of disease progression after anthelminthic treatment, because antibody-detection assays can remain positive for up to one year after curative surgical treatment (Ito et al., 1999).

Regardless of the technique used, detection of *T. solium* specific antibodies in serum only indicates exposure to the parasite and not necessarily established infection, resulting in a transient antibody response. In this regard, human taeniosis/cysticercosis is said to resemble an 'iceberg' with three tiers: the tip of this iceberg comprises people who harbor established symptomatic CNS disease, i.e. NCC. Clinical diagnosis of symptomatic NCC cases is often not possible due to the non-specific symptoms with seizures and headache being the most common. Below this first tier, there is a larger population with established cysticercosis outside the CNS or in the CNS but without discernable symptoms. The third and largest tier comprises people who were exposed to failed infections, but became seropositive (Garcia et al., 2001). Furthermore, antibodies may persist long after the parasite has been eliminated by immune mechanisms and/or antiparasitic therapy (Dorny et al., 2003).

Detection of anti-parasite antibodies in a population in an endemic village does not necessarily reflect the true prevalence, leading to misdiagnosis of a proportion of neurological cases (Bern et al., 1999). It can also lead to superfluous use of antiparasitic therapy in a patient where the parasites are not viable (Garcia et al., 2000).

2.3.2.2 Antigen-detection methods

Considering the drawbacks of antibody detection in clinical settings, antigen detection can provide a valuable alternative as it reflects the presence of viable parasites. In this respect, antigen detection can also provide a tool for serological monitoring of antiparasitic therapy: antigen levels drop rapidly after successful anthelminthic treatment (Garcia et al., 2000; Nguekam et al., 2003). Several assays have been developed to detect parasite antigens in serum, CSF or urine using either polyclonal or monoclonal antibodies (mAbs) (Parija et al., 2004).

Two mAb-based tests (B158/B60 Ag-ELISA and HP10 Ag-ELISA) have been validated and are used routinely for the detection of parasite antigens (Dorny et al., 2004; Rodriguez et al., 2009). The HP10 mAbs are reactive with a repetitive carbohydrate epitope on lentil–lectin adherent glycoproteins present on the surface and in the secretions of *T. saginata* cysticerci (Harrison et al., 1989). The antigens detected by the B158/B60 Ag-ELISA have not been identified, but partial characterization of the epitopes demonstrate that they are present on the tegument and on the ES-products of *T. saginata* metacestodes, and that they are either carbohydrate or partly carbohydrate/partly protein in nature (Draelants et al., 1995). The sensitivity of Ag-ELISA in humans is reported to be high: >80% for serum and even >90% for CSF (Garcia et al., 1998).

Unlike antibody detection, measurement of circulating antigen levels allows differentiation of NCC cases with viable parasites, with antigen levels correlating to the numbers and size of lesions. For instance, antigen levels were 10 times higher in extraparenchymal NCC (particularly subarachnoid NCC) than in intraparenchymal NCC, therefore high antigen levels should lead one to suspect the presence of extraparenchymal NCC (Rodriguez et al., 2009). In the same respect, detection of HP10 antigen in CSF could be correlated to the location of the cysticerci in the CNS: antigen could be detected in cases with cysticerci located in the subarachnoideal space and ventricles, but not in cases with parenchymal cysticerci (Bobes et al., 2006).

When applied to rurally reared pigs with low cyst burdens, these well-established and reliable serodiagnostic procedures seem to be less sensitive (Sciutto et al., 1998), although other authors were able to detect pigs harboring one single cyst (Dorny et al., 2004). In addition, the genus specificity of these tests does not allow the differentiation between infections of *T. solium* and *T. hydatigena* in pigs. Recently, *T. solium*-specific nanobodies (recombinant single domain antibodies derived from camelid heavy chain antibodies) have been isolated which showed great potential for developing a species-specific antigen detection test for diagnosis of porcine cysticercosis (Deckers et al., 2009).

In a recent community-based study in pigs, the B158/B60 and HP10 Ag-ELISAs were used in a Bayesian approach (together with tongue inspection and EITB) to calculate prevalence and sensitivity and specificity of each test (Krecek et al., 2008). This resulted in sensitivity and specificity (assuming absence of *T. hydatigena*) of 76% and 84%, respectively, for the B158/B60 Ag-ELISA and 55% and 83%, respectively, for the HP10 Ag-ELISA. The B158/B60 Ag-ELISA has also good diagnostic performance for urine antigen detection in humans and as such could be of great value in the clinical field (Castillo et al., 2009). The test had an overall sensitivity of 92% for viable parasites (this dropped to 62.5% in cases with only one viable cyst). Most patients with only calcified cysticerci

(83%) were urine antigen negative. There was a strong correlation between antigen levels in paired serum/urine samples. The use of urine or even saliva as diagnostic samples would be very convenient, especially in remote rural areas where *T. solium* is endemic, as they are easy to obtain in contrast to CSF or serum samples for which invasive and sterile prelevation techniques are necessary.

2.3.2.3 Cell proliferation assay

Recently, a new immunodiagnostic tool was described for the diagnosis of NCC. Instead of measuring antibody responses resulting from the humoral immune response against the parasite, a cyst fluid antigen-based lymphocyte proliferation test was developed to measure the cellular immune response. The researchers reported a sensitivity and specificity of 93.8% and 96.2%, respectively, when tested on 48 symptomatic NCC patients and 79 controls in India (Prasad et al., 2008). However, these results still need to be validated in other endemic areas.

2.3.2. Molecular diagnosis

In clinical practice, a solitary CNS lesion presents a diagnostic dilemma and may be difficult to differentiate by neuroimaging and serologic assays. Molecular approaches targeting genetic sequences of *Taenia* species have been used to detect NCC in CSF and other tissues and for geographic genotyping; however, these assays typically require a priori suspicion of *T. solium* infection (Yamasaki et al., 2005; Almeida et al., 2006). Although the internal transcribed spacer (ITS) region could screen for parasitic DNA, as well, this technique as a screening tool still need to be validated. Harrington et al reported 2 patients with single enhancing CNS lesions who had *T. solium* detected in their brain tissue samples by ITS rRNA gene sequencing in a global screening platform; the presence of *T. solium* was subsequently confirmed in a secondary analysis using *Taenia* species–specific primers in Polymerase Chain Reaction (PCR) technique. Global screening is a promising tool for the diagnosis of CNS infection, especially when traditional diagnostic tools are insensitive (Harrington et al., 2009).

In order to improve methods for identifying taeniid cestodes, various molecular approaches have been developed, including the use of DNA probes (Rishi et al., 1987; Gonzalez et al., 2000), polymerase chain reaction (PCR) coupled to restriction fragment length polymorphism (RFLP) (Gasser et al., 1995), single-strand conformation polymorphism (SSCP) (Gasser et al., 1999), PCR-amplified DNA sequences (Gonzalez, 2002), and random amplified polymorphic DNA (RAPD)-PCR (Vega et al., 2003). Each of these techniques has advantages and disadvantages, e.g., the use of DNA probes, PCR-RFLP and SSCP are relatively time-consuming; however, PCR using species-specific

primers provides rapid and sensitive and reliable diagnostic results. Most of these studies have been focused on the differentiation of *T. solium* from *T. saginata* and intraspecific genetic polymorphism.

Recently, mitochondrial DNA analysis of *T. solium* revealed the presence of two distinct Asian and American/African genotypes (Okamoto et al., 2001). Therefore, a comprehensive differential diagnosis based on mitochondrial DNA for *T. saginata*, *T. asiatica* and two genotypes of *T. solium* parasites has been established (Yamasaki et al., 2004). DNA-based differential diagnosis is also indispensable for definitive diagnosis of cysticercosis cases in which histopathological findings are not confirmatory (Yamasaki et al., 2005).

Chapter III

Materials and Methods

3.1 Study area

Chitwan and Kathmandu districts of the Central Development Region of Nepal were selected as the study area as these areas include both the endemic and non-endemic regions of NCC. The laboratory work was conducted in the Central Department of Biotechnology, Tribhuvan University and Annapurna Neurological Institute and Allied Sciences, Kathmandu, Nepal.

3.2 Sample collection

3.2.1 Cysts sample collection

The cysticercosis infected pig flesh was collected from the local market (Naranghat) of Chitwan district of central development region of Nepal. The collected flesh was taken to the Central Department of Biotechnology laboratory in cold chain and was stored at -20^{o}C in deep freeze until further procedure. The cysts were isolated from the infected flesh and stored in phosphate buffered saline (PBS) in eppendroff tubes at -20^{o}C in deep freeze until further procedure.

3.2.2 Serum samples collection

Serum samples of the individuals were collected from the patients visited to Annapurna Neurological Institute and Allied Sciences, Kathmandu, Nepal. Random sampling was carried out for the sampling procedure based on their health complain. During sample collection, the background information of the individuals selected was. The collected serum sample was taken to the Central Department of Biotechnology, Tribhuvan University laboratory in cold chain and was stored at -20^{o}C in a deep freeze until further procedure.

3.3 Preparation of Crude Soluble Antigen (CSA)

The collected 4-6 cysts in eppendroff tube were thawed in room temperature and were washed with PBS 4-5 times and then crushed manually in morter and pestle using 500 µl

of PBS and collected in the 2ml eppendroff tube. Approximately equal volume of lysis buffer prepared from complete protease inhibitor cocktail (Roche) was added to the above tube. The lysis was done by four alternative cycles of freezing (-20^{o}C) and thawing (at room temperature) that is followed by sonication (sonicator-LOBA life) for 8 cycles for 30 sec each in cold chain. The suspension was then centrifuged at 3,000 rpm at 4^{o}C for 20 minutes. Finally, the supernatant, crude soluble antigen (CSA), was collected and stored at -20^{o}C until use.

3.4 Protein quantification

Bradford assay (Bradford, 1976) was performed to determine the concentration of crude protein in crude soluble antigen extract. In this method the concentration of protein was estimated in reference to the standard stock solution of Bovine Serum Albumin (BSA) at concentration of 1 mg/ml. Standard solution of BSA (100µg/ml) was prepared using the BSA stock solution. Based on protein binding principle to Coomassie Brilliant Blue G-250 (CBB) that produces maximum absorbance at 595 nm for protein dye complex; the protein concentration was measured using spectrophotometer at 595 nm. For this assay test tubes were labeled and added 100µg/ml standard solution of BSA and PBS as per tabulation below.

Table 3.1 Plan chart for BSA concentration standard curve determination

Test tube label	BSA (µg)	BSA-Standard (µl)	PBS (µl)
1	0	0	100
2	1	10	90
3	2	20	80
4	4	40	60
5	6	60	40
6	8	80	20
7	10	100	0

Bradford reagent at the rate of 1 ml in each labeled test tube was dispensed and measured the absorbance within 2 minutes at 595 nm keeping the solution in cuvette. In same pattern, 5 µl of CSA was used to find its OD using 95 µl PBS and 1 ml Bradford reagent. The assay was performed in duplicate to minimize the error.

3.5 Sodium Dodecycle Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Protein concentration of CSA was estimated and mixed with equal volume of Gel loading dye/buffer (Genei, India, cat. # 105350) and was allowed to boil for 5 minutes. The CSA (25 µg/per well) was loaded in the wells casted on polyacrylamide gel of 5% stacking gel which was casted vertically on gel apparatus (Cleaver Scientific Ltd.). The molecular marker, Page Ruler^{TM} Prestained Protein Ladder (Fermentas, cat. # SM0671) was loaded along with the sample in the gel. The set up was electrophoresed using running buffer (39 mM Tris, 48 mM Glycine, 0.1% SDS) at constant voltage of 100V for about 4 hour i.e. until the dye appeared to nearly run out of the gel as described by Laemmli (Laemmli, 1970) with certain modifications. The gel was taken out of the apparatus and washed once with distilled water and stained with Coomassie Brilliant Blue (CBB) for study of protin profile of cysticercus of *T. solium*. Similarly, SDS-PAGE was run loading at 45 µg/well for immunoblotting.

3.6 Immunoblotting

Immunoblotting was carried out based on Towbin et al with few modifications (Towbin et al., 1979). The SDS-PAGE gel was transferred to chilled Transfer buffer (Tris base 25 mM, glycine 192 mM, methanol 5%) for 10 minutes. Eight equal sized blotting papers of the gel size were cut and soaked for same time in same buffer. Exactly the same sized polyvinylidene difluoride (PVDF) membrane (Millipore, pore size, 0.45µm) was also cut and soaked in 100% methanol for 20 seconds and dipped in Transfer buffer for 5 minutes. For the protein transfer the sandwich of PVDF membrane, blotting paper and gel was arranged as PVDF membrane keeping towards Anode (+ charge) after placing four blotting paper and gel towards cathode (- charge) and four blotting paper on the top. The protein transfer was carried out by placing the sandwich at constant voltage of 100 V for about one hour in cold chain using wet (tank) transfer apparatus (Cleaver Scientific Ltd.). The pre-stained molecular weight marker was used for protein transfer confirmation. The PVDF membrane was then incubated in blocking buffer (1% BSA, 0.1% Tween-20 in PBS) for overnight at 4^{o}C. The next day, the molecular marker containing lane was separated and remaining membrane was cut longitudinally into strips of nearly 5mm. Each strip was then allowed to interact with sera (1:100 in PBS) separately for 2 hours at room temperature shaking at 100 rpm. Using washing solution (0.1% BSA, 0.1% Tween-20 in PBS) the strips were washed for three times each for 15 minutes. The alkaline phosphatase conjugated goat antihuman IgG (Santa Cruz, Lot # F0410) (1:1200 in PBS) was allowed to interact with antibodies in the strip by shaking for 2 hours at 100 rpm in room temperature. The strips were washed again; twice with washing buffer and

once with PBS, each for 15 minutes. Finally, color development was carried out by reacting with substrate BCIP-NBT (GeNei, Bangalore) until bands were distinctly appeared. And, the reaction was stopped with PBS and it was dried in room temperature. The strips were stored at RT wrapping in filter paper with labeling after taking the photograph.

3.6.1 Analysis of Protein bands in SDS-PAGE and immunoblot

The protein bands appeared in SDS-PAGE were captured and documented with the help of camera (Sony). The bands developed in SDS-PAGE and immunoblotting were imaged and analyzed for the unknown molecular weight with the help of Alpha Imager ^{TM} 2200 using Point to Point fit calculation method. Integrated Density Value (IDV) was calculated based on Alpha Imager ^{TM} 2200 by setting the highest value of total black as 255 unit and lowest value of white as 0 as per software applies. The area selected was 1179 unit area for IDV calculation in protein bands of CSA. Microsoft excel 2010 software was used for the statistical analysis of the bands of the CSA and immunoblots.

3.7 Enzyme Link Immunosorbent Assay

Assay was done as described by (Hommel et al., 1978) with some modifications. The CSA protein (100 ng/well) was coated in 96 well ELISA plate (NUNC, USA) in carbonate buffer (0.1M $NaCO_3$, 0.1M $NaHCO_3$, pH 9.6) and incubated overnight at 4^{o}C. The plates were aspirated and blocked with 125 µl/well blocking buffer (1% BSA, 0.1% Tween-20 in PBS) for 2 hours at room temperature. Washing was done for 5 times with washing buffer (0.1% BSA, 0.1% T-20 in PBS) and then incubated with 100 µl serum at dilution of 1:100 in dilution buffer (0.1 % BSA in PBS) in duplicate for ½ hour. One hundred microlitres (100µl) of goat antihuman immunoglobulin G (IgG) conjugated with horse reddish peroxidase (HRP) in dilution at 1:10000 were dispensed after washing as previous step and plates were incubated for ½ hour. Washing was repeated as in previous step followed by addition of 100 µl trimethyle benzidine (TMB) substrate and incubated for 15 minutes at dark in room temperature. The reaction was stopped by addition of 1N H_2SO_4 and OD was taken at 450 nm in ELISA plate reader (Spectromax 190). Positive controls (PC), negative controls (NC), conjugate control (CC) and substrate control (SC) along with the samples were incorporated in experiment to avoid error (**Table 3.2**).

Table 3.2 ELISA steps for PC, NC, SC and CC controls

ELISA steps	PC	NC	CC	SC
Coating Ag	Coating Ag	Coating Ag	Only coating buffer	Only coating buffer
Blocking	Blocking buffer	Blocking buffer	Blocking buffer	Blocking buffer
Samples	pc	nc	Capturing antibody	Blocking buffer
Detecting antibody	Detecting antibody	Detecting antibody	Detecting antibody	Blocking buffer
Substrate	Substrate	Substrate	Substrate	Substrate
Stop solution	Stop solution	Stop solution	Stop solution	Stop solution

PC, positive control; NC, negative control; CC, conjugate control; SC, substrate control

3.7.1 Determination of Cut-off value

Cut-off value was determined by running negative controls in the experiment. The mean and standard deviation of negative controls were calculated. Three times of the SD added to the mean value was taken as cutoff point.

Cut off value (OD) = Mean OD + 3 × SD value

3.7.2 Data analysis

Prism GraphPad5 software and Microsoft Excel 2010 were used for statistical analysis. Unpaired two-tailed t-test was applied to analyze the data. Similarly, correlation coefficients and P-values of different variables of the data were calculated to find out the statistical correlation and significance of the data respectively. Specificity was calculated as the percentage of negativity shown by a diagnostic tool from negative controls and the sensitivity as the percentage of positivity shown by a diagnostic tool from confirmed NCC cases (parasitologically positive). The specificity and sensitivity of the test was calculated by using the following formulae.

$$\text{Specificity} = \frac{\text{Total negative} - \text{False negative}}{\text{Total positive}} \times 100\%$$

$$\text{Sensitivity} = \frac{\text{Total positive} - \text{False positive}}{\text{Total positive}} \times 100\%$$

3.8 Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) scan

The neuroimaging of the patients was carried out in the hospital and the data of the positive samples were collected for the study.

3.9 Clinical symptoms

The clinical symptoms of all the test samples included in the research work were collected from the hospital for comparative analysis.

Chapter IV

Result

4.1 Morphology of cyst

Cysts were found intact into the host (pig) tissues.When the cysts were isolated from the meat sample, they were found asovoid vesicles of approximately 8-15mm diameter, with a transparent membrane (**Figure 4.1.**). It contained an invaginated scolex and was filled with in a colourless liquid when the cyst was observed under microscope (40x) after splashing it with coverslip (**Figure 4.2.**)

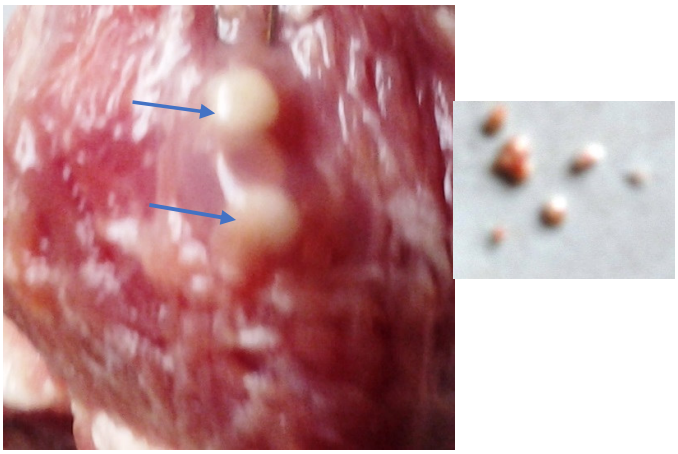


Figure 4.1.Cysticercus of *T. solium* from pig flesh

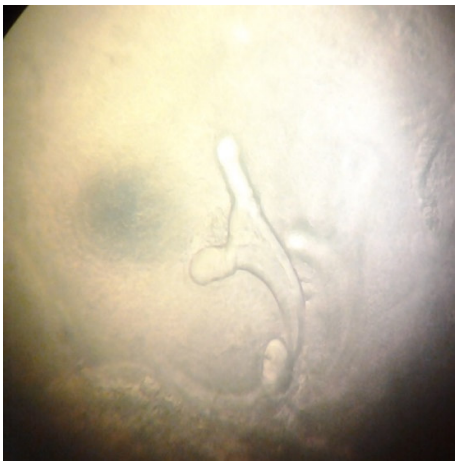


Figure 4.2. Microscopic view of cyst of *T. solium* showing invaginated scolex (40x)

4.2 CSA collection and determination of protein concentration

Crude soluble antigen (CSA) was prepared from 6-8 cysts at a time. The volume obtained was 1.5 ml from each batch. In total 4.5 ml of sample (CSA) was prepared in three times during the thesis work. The protein concentration was determined from the calibration curve of BSA standard solution (y = 0.0229x, R^2 = 0.9976) obtained from Microsoft Excel 2010 **(Figure 4.3.)**. The OD value of 0.217 at 595 nm using 5 µl from CSA valued the concentration of 1.89 µg/µl protein in CSA preparation of cysts of *Taenia solium*.

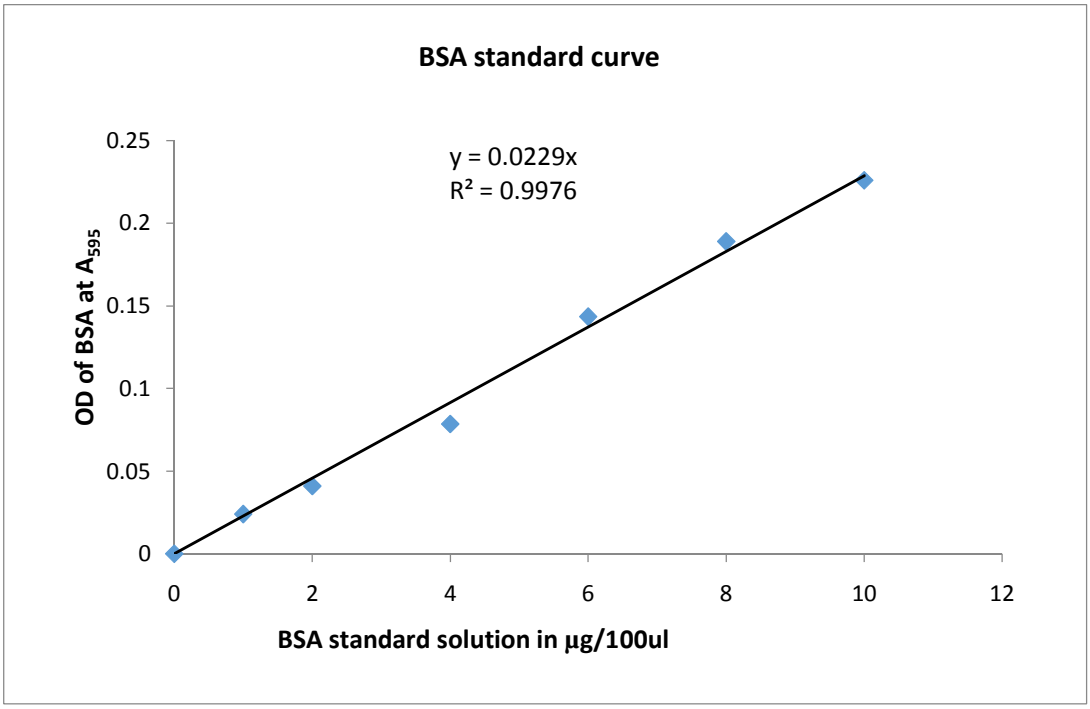
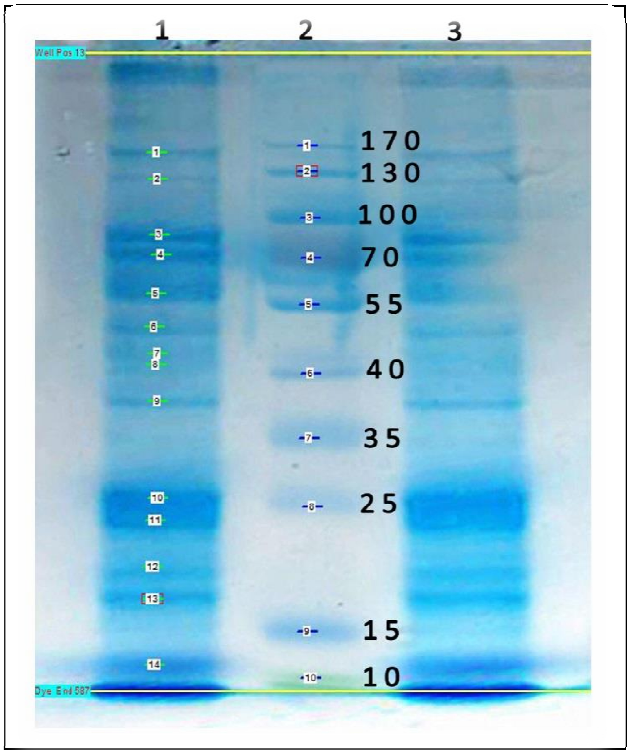


Figure 4.3. Calibration curve of BSA standard solution for protein estimation in CSA

4.3 Protein profile of CSA of cysticerci of *T. solium*

Many protein fractions were seen on the gel after running crude soluble antigen of cysticerci of *T. solium* in SDS-PAGE and stained with CBB. A number of protein fractions were found remarkably of higher intensity. Most of the gel had about 14 clearly distinguishable protein fractions. The CSA consisting various cytoplasmic and membrane bound components were found fractionated ranging from 11 to 158 kDa.The protein polypeptide fractions as analysed by using Alpha Imager ^{TM} 2200 were of 158, 124, 86, 72, 58, 50, 44, 42, 38, 26, 24, 20, 17and 11 kDa **(Figure 4.4., Table 4.1.)**.



olium; lane 2,
Digit in white
al line, line of

Table 4. 1. CSA profile of cysts of *T. solium* with markers obtained from Alpha Imager ™ 2200

----------MARKERS----------

Band	Position	Mol. Wt.	Rf
1	96	170	0.145
2	119	130	0.185
3	161	100	0.258
4	197	70	0.321
5	239	55	0.394
6	301	40	0.503
7	359	35	0.604
8	421	25	0.712
9	533	15	0.908
10	575	10	0.981

----------QUERIES----------

Band	Position	Mol. Wt.	Rf
1	102	158	0.155
2	126	124	0.197
3	176	86	0.284
4	194	72	0.316
5	229	58	0.377
6	259	50	0.429
7	283	44	0.471
8	293	42	0.489
9	326	38	0.546
10	413	26	0.698
11	433	24	0.733
12	475	20	0.806
13	504	17	0.857
14	563	11	0.96

The variation in intensities of these protein polypeptide fractions were further confirmed by integrated density value (IDV) analyzed by densitometry from Alpha Imager^{TM} 2200. The IDV of polypeptide fractions was found ranging from 130869 to 181566 taking the area of1179 unit. Thus the average integrated density (AID) values range from 111 to 154 when the highest value of total black was assigned as 255 units. The protein fractions having higher intensities and clearly distinguishable from other bands were 158, 124, 86, 72, 58, 50, 26 and 24 kDa. Among these the bands of 86, 72, 58, 26 and 24 kDa polypeptide fractions were having AID value of more than 130 **(Figure 4.5.)**.

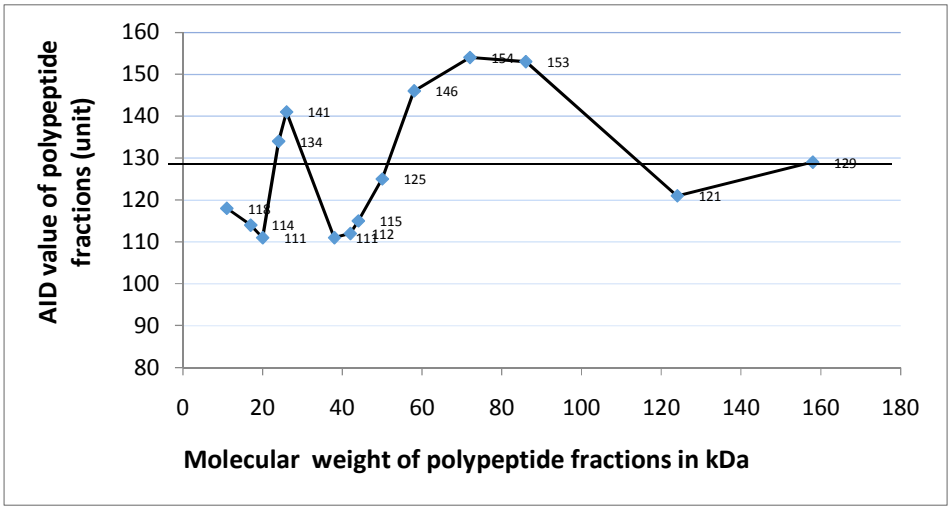


Figure 4.5. Average Integrated Density (AID) value of different protein polypeptide fractions calculated based on area selected (1179 units).

4.4 Immunoblot profiling

The serum sample from both categories, the patients and the healthy controls produced various banding patterns depending upon the antibodies present in it with the CSA of cysts of *T. solium* by antigen-antibody interaction. The grouping and detail study of such profiling patterns of bands helped for the identification of antigenic polypeptide fractions present on cysts of *T. solium*. The immunoblot profile of specific polypeptide fractions present in patients were found to be absent in the healthy controls as observed in this study **(Table4.2.)**.

4.4.1 Peptide bands in NCC patients

The immunoblot profile was carried out among 10 NCC patients with a range of age 8 to 51 years (mean 28 years).Among them male (n=6) subjects were higher by 60%. It showed interaction between antigenic fractions from CSA (prepared from cysts of *T. solium*) with different sera of NCC patients possessing IgG. The predominant IgG of active NCC patients exhibited different patterns of different bands while interaction with peptide fractions of cysts of *T. soilum*. WB profiles elicited bands ranging from 15 to 124 kDa in size **(Appendix I)**. The number of distinct polypeptide bands from each individual test sample varies ranging highest up to 25 and lowest to 20 averaging 22 bands. In total, 49 different distinct polypeptide fractions were identified from 10 test samples of NCC patients viz. 124, 120, 108, 100, 88, 86, 85, 82, 76, 72, 70, 68, 66, 65, 64, 63, 62, 60, 58, 56, 55, 51, 50, 46, 44, 42, 40, 39, 38, 37, 36, 35, 34, 32, 30, 29, 28, 27, 26, 24, 23, 22, 21, 20, 19, 18, 17, 16 and 15 kDa **(Figure 4.6.)**.

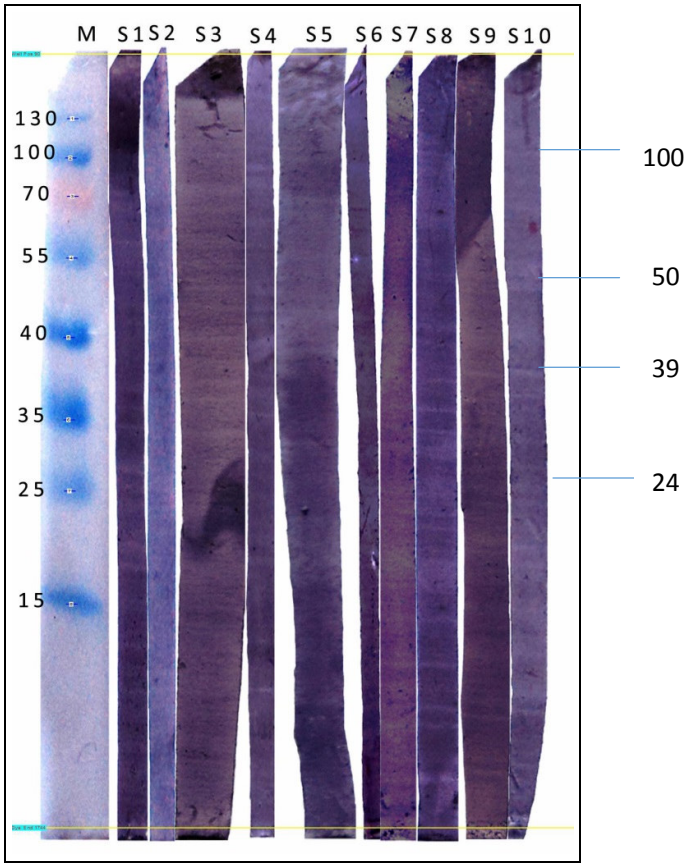


Figure 4.6. Western blot profiles of NCC patients. Lane M represents prestained mol. wt. marker and digits aside are mol. wt. of standard bands; lanes S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10 represent patient test samples; Digit in white blocks, numbering of bands assigned by Alpha Imager ™ 2200; Top horizontal line, line of initiation; Bottom horizontal line, dye front.

The immunoblotting profile of the polypeptide fraction produced from CSA with the sera of the patients resulted in various distribution patterns. The repetition of 39 bands was observed at least in 30% (3 out of 10 western blot strips). The repetition frequency increased to 40% i.e. 4 subjects out of 10 for 30 bands. The repetition frequency up to 50% was observed in 18 bands but the study was focused on the polypeptides that possessed repetition for more than 50%. These protein fractions include 120, 100, 50, 46, 40, 39, 38, 37, 35, 32, 26, 24, 22, 18, and 16 kDa **(Figure 4.7.)**. The immunoblot produced inconsistent bands that had no repetition in different immunoblot profiles since these polypeptides bands were not remarkable so excluded from the study.

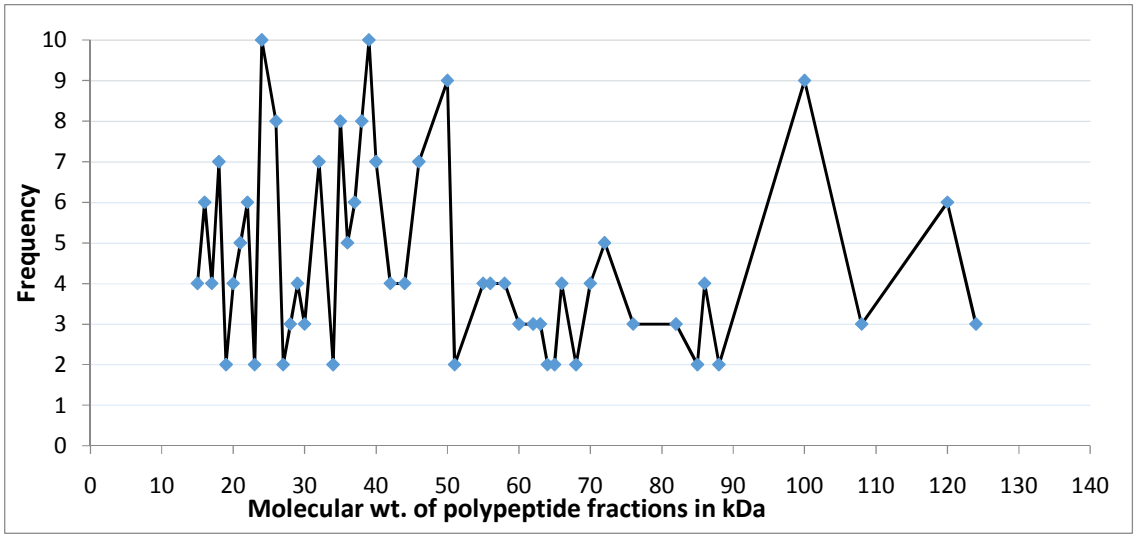


Figure 4.7. Frequency of major bands detected in NCC patient samples.

The thesis work pointed the remarkable figure for 100, 50, 39 and 24 kDa that produced immense result on reactivity. In NCC patient samples, 39 and 24 kDa polypeptide protein fractions produced significant consistency that figured to 100% (10/10) and 100 and 50 kDa fractions marked to 90% (9/10) consistency **(Figure 4.7.).** The sensitivity and specificity of WB analysis was calculated based on the 24 and 39 kDa polypeptide fractions and was found to be 100% (n=10) and 100% (n=6) respectively.

4.4.2 Peptide bands in healthy controls

The immunoblot profile was carried out among 6 healthy controls. Among them 4 were male subjects. The predominant IgG of healthy controls exhibited different patterns of different bands while interaction with peptide fractions of cysts of *T. soilum*. The bands ranging from 15 to 128 kDa in size were observed in the immunoblot profile of healthy controls **(Appendix III)**. The number of distinct polypeptide bands from each individual healthy control varies from 15 to 21 averaging 17 bands. In total, 48 different distinct polypeptide fractions were identified from 6 healthy controls viz. 128, 127, 124, 118,

112, 110, 108, 105, 95, 93, 92, 88, 85, 81, 78, 76, 72, 68, 66, 65, 62, 60, 56, 55, 54, 52, 48, 45, 44, 41, 38, 37, 36, 35, 34, 32, 29, 28, 27, 26, 25, 23, 22, 21, 20, 19, 17and 15 kDa **(Figure 4.8.)**.

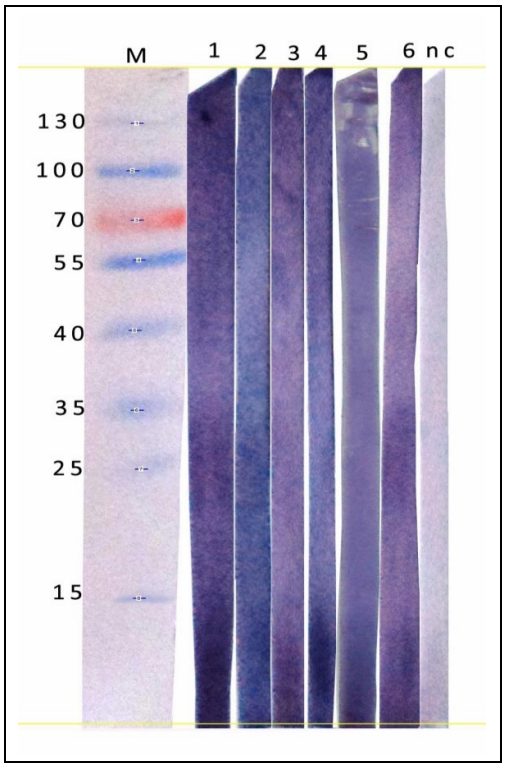


Figure 4.8. Western blot profiles of healthy controls. Lane M represents prestained mol. wt. marker and and digit aside are mol. wt. of standard bands; lanes 1, 2, 3, 4, 5 and 6 represent healthy control samples and nc represents negative control; Digit in white blocks, numbering of bands assigned by Alpha Imager ^{TM} 2200; Top horizontal line, line of initiation; Bottom horizontal line, dye front.

Among these 6 different immunoblot profiles at least 50% repetition was observed in 18 (37.5%) different polypeptide bands. The immunoblot profiles elicited more than 50% repetition of 3 different polypeptide fractions viz. 38, 36 and 15 kDa **(Table 4. 2.)**.

4.4.3 Selection of antigenic (immunogenic) protein fractions

The CSA fraction profiles from SDS-PAGE when transferred to PVDF membrane and immunoblotted revealed different bands in different study groups. The Western blotting of CSA fraction with sera of NCC patients revealed 49 fractions and that of healthy controls revealed 48 fractions. The average number of bands detected in patients was 22.4 and that of controls was 17(**Table 4.2.**).The decrease in average number of bands in patients and controls reflects the significance of the detected bands as their possibility for antigenic marker.

Table 4.2. Distribution frequency (%) of major bands detected in different study groups.

Bands (kDa)	Repetition frequency (%)	
	NCC Patient samples (n=10)	Healthy controls (n=6)
128	-	17
127	-	50
124	30	33
120	**60**	**-**
112	-	50
110	-	33
108	30	17
105	-	50
100	**90**	**-**
95	-	50
93	-	17
92	-	33
88	20	33
86	40	-
85	20	33
82	30	-
81	-	33
78	-	33
76	30	33
72	50	17
70	40	-
68	20	50
66	40	17
65	20	33
64	20	-
63	30	-
62	30	17
60	30	50
58	40	-
56	40	17
55	40	17
54	-	83
52	-	33
51	20	-

Bands (kDa)	Repetition frequency in (%)	
	NCC Patient samples (n=10)	Healthy controls (n=6)
50	**90**	**-**
48	-	50
46	**70**	**-**
44	40	50
42	40	-
41	-	50
40	70	-
39	**100**	**-**
38	80	83
37	60	50
36	50	83
35	80	33
34	20	33
32	70	50
30	30	-
29	40	17
28	30	50
27	20	17
26	80	17
24	**100**	**-**
23	20	33
22	60	50
21	50	33
20	40	50
19	20	50
18	**70**	**-**
17	40	50
16	**60**	**-**
15	40	67
Average no. of bands	22.4	17.33
Std. Dev.	1.84	2.80

CSA polypeptide bands after immunoblot were found to produce different intensities. The intensity determines the level of IgG production in the tested sample against the interacted antigenic fraction. Some remarkable bands were highly intense having higher integrated density value even though they were not consistent in different profile. Such highly intense fractions signify for the higher production of antibodies against those antigen and inconsistency refer for their low immunogenic value in NCC cases.

The repetition frequency of the polypeptide fractions up to 50% was observed in 18 bands of the NCC patients' immmunoblot profiles and the study was focused on the polypeptides that possessed repetition for more than 50%. There were 15 bands that had the repetition of more than 50% in the WB profiles of NCC patients. These protein fractions include 120, 100, 50, 46, 40, 39, 38, 37, 35, 32, 26, 24, 22, 18, and 16 kDa. Moreover the bands 120,100, 50, 46, 39, 24, 18, and 16 kDa were found completely absent in the healthy controls which were the important bands among those 15 bands in NCC patients. The bands of 100, 50, 39 and 24 kDa protein fractions were found present in 90% or more in NCC patients (**Table 4.2.**) showing their greater application in diagnostic markers for NCC cases.

The polypeptide fraction when grouped according to their corresponding size in the interval of 25 kDa size, showed maximum number of fraction lies within the range of 25-50 kDa group (**Figure 4.9.**). This signifies the immunogenic fraction obtained from CSA prepared lies in this portion. Hence, the antigen having the size in the range of 25-50 kDa possess higher potentiality on antigenicity and future vaccine candidate development.

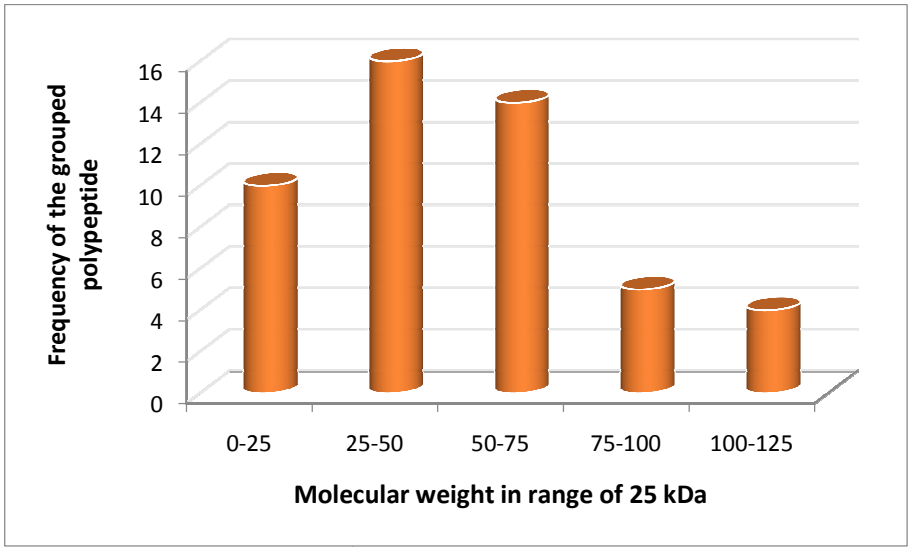


Figure 4.9.Total number of polypeptide bands observed in NCC patients' strips categorized with respect to their molecular weight at interval of 25 kDa.

4.5 Enzyme Linked Immuno-sorbent Assay (ELISA)

The assay was performed among 45 NCC patients with 2 positive controls and 8 negative controls along with substrate control and conjugate control. The OD readings at 450 nm of all the variables are given in **Table 4.3** and in **Appendix III.** Out of 45 NCC patients, 37 (82.22%) were found to be NCC positive and the rest 8 (17.78%) were negative.

Table 4.3. OD Readings at 450 nm of the study variables in ELISA.

	Variables				
Parameters	**Substrate control (n=1)**	**Conjugate control (n=1)**	**Negative controls (n=8)**	**Positive controls (n=2)**	**Samples (n=45)**
Mean OD ± SD	0.8045± 0.135057	0.8945± 0.044548	1.0995± 0.132281	3.29325 ± 0.071771	2.9831 ± 0.62603

4.5.1 Cut-off value determination

The cut-off value was determined by running negative controls and calculating the mean and standard deviation. Three times of the SD added to the mean value was taken as cut-off point. The cut-off value was determined to be 1.496 (**Figure 4.10.**).

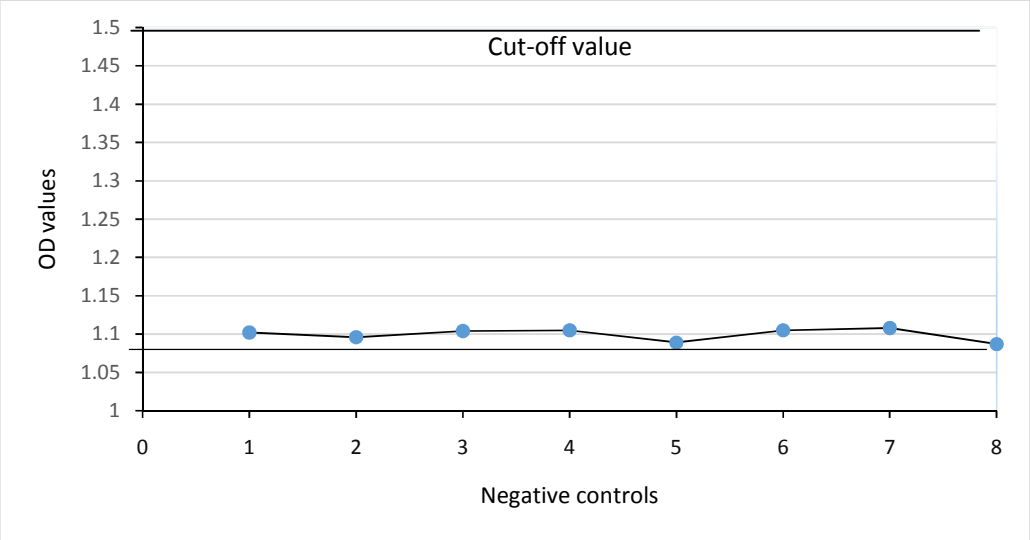


Figure 4.10. Cut-off value determination from negative controls.

4.5.2 ELISA on NCC cased visited to hospital

Antibody based ELISA was performed in total 45 NCC cases visited to hospital that were used as test samples. Besides test samples, positive controls and negative controls were used to calculate the sensitivity and specificity of the test and substrate control and conjugate control were used for the quality control of the test. Out of 45 NCC cases visited to hospital, 37 (82.22%) were found to be NCC positive and the rest 8 (17.78%) were negative by using the antibody based ELISA assay (**Figure 4.11.**).

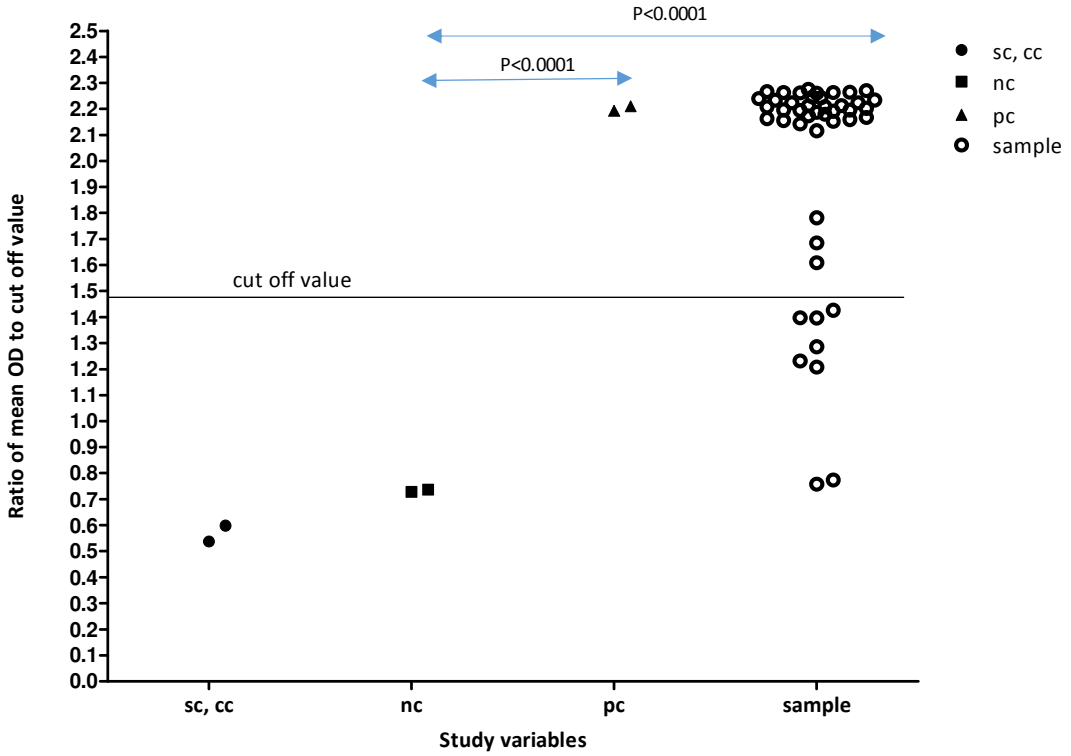


Figure 4.11. Immune response by sera of different study variables as expressed in ELISA with CSA of cysts of *T. solium*; sc, substrate control; cc, conjugate control; nc, negative control; pc, positive control and sample, patients visited to hospital.

The comparative statistical analysis between different study variables was performed. The unpaired t-test was applied between negative control and positive control and p value obtained was <0.0001 which showed their means were significantly different. Similarly, p value obtained from the unpaired t-test between negative control and sample was also <0.0001 which indicated that their means were significantly different.

Out of 45 NCC cases visited to hospital, 37 cases were positive as displayed by antibody based ELISA. Since all the negative controls were negative in the antibody ELISA, so the specificity of the test was found to be 100%. The sensitivity of the antibody ELISA was found to be 82.22%.

4.6 Seroepidemiological study of confirmed patients

Seroepidemiological study was performed among the 37 NCC positive patients as depicted by ELISA assay. The seroepidemiological study was based on the different epidemiological variables such as gender, age groups and clinical symptoms. The study reflected the various prevalence rate among the different epidemiological variables under study.

4.6.1 Seroepidemiology based on gender and age groups

Among the 37 NCC positive patients analysed, 19 (51.35%) were male and 18 (48.65%) were female. The mean age of the patient was 26.67 years with the standard deviation of 11.0631 and the range of the patient age was 4-57 years. Out of 37 NCC patient 2 patients were of age less than 10 years among them one was male and one was female. Among 5 patients between the age group 10-20, male patient were 2 and female were 3 in number. Out of 20 patients in the age group 20-30, 9 were male and 11 were female and this group occupies 54.05% of total patients. Similarly, the age group 30-40, 40-50 and more than 50 have 5, 2 and 3 patients among them 4 and 1, 1 and 1, 2 and 1 were male and female patients respectively **(Figure 4.12.)**. The patients age group showed positive correlation with the gender of the patients and the correlation was found to be significant ($p<0.05$) **(Table 4.3.)**.

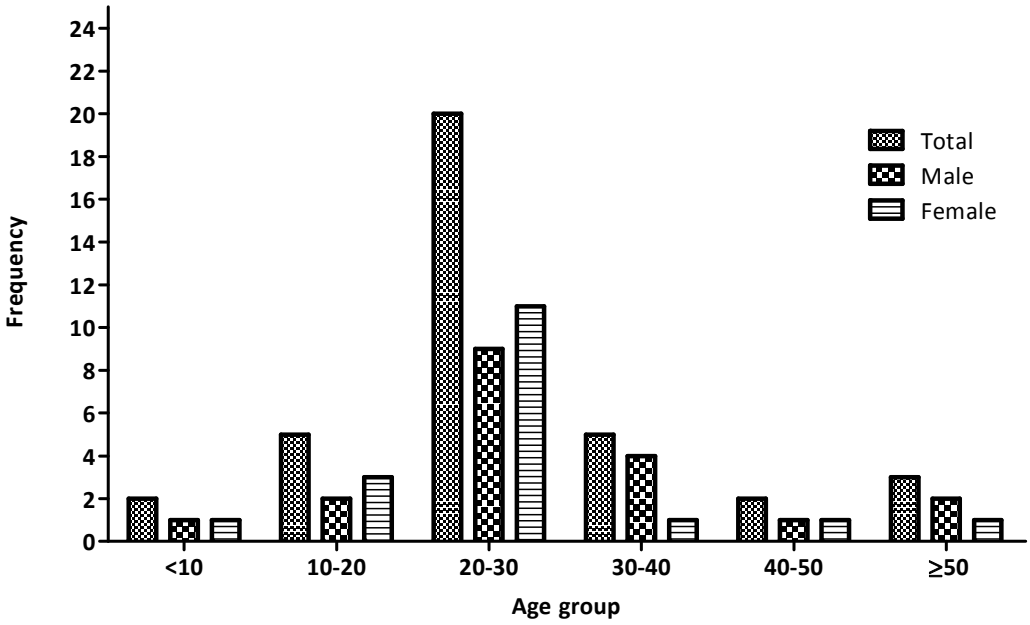


Figure 4.12. Relation between age group and gender of the patients.

Table 4.4. Correlation between age group and gender of patients with their P values.

Parameters	Total		Male - Female correlation
	Male	Female	
Correlation coefficients	0.9723	0.9838	0.9148
P values	0.0011*	0.0004*	0.0106*

* Significant ($p<0.05$)

4.6.2 Seroepidemiology based on clinical symptoms

Based on the clinical symptoms among the 37 NCC positive patients analyzed, 19 (51.35%) had Seizure. Similarly 16 (43.24%), 5(13.51%), 4(10.81%) and 3(8.11%) patients had Headache, Loss of conscious, Abnormal/jerky movements and Fainting attack respectively **(Figure 4.14.)**. The relation among various clinical symptoms of patients was found to be significant (p=0.0487).

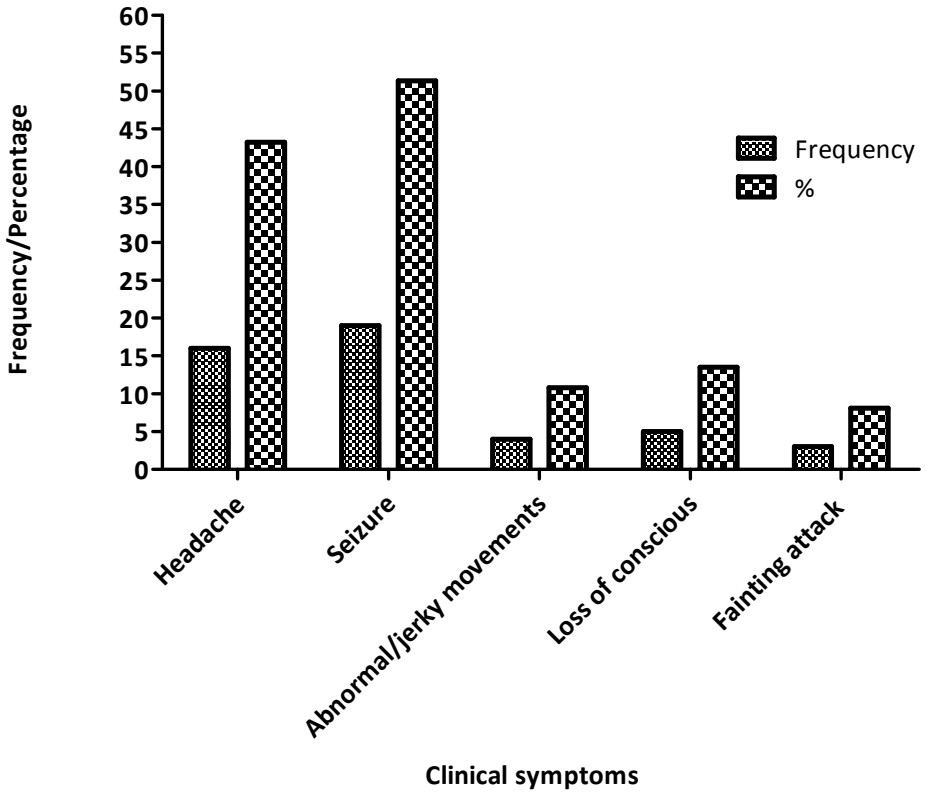


Figure 4.13. Relation among various clinical symptoms of patients.

4.7 CT and MRI of the positive cases

Out of 45 samples taken into consideration only 12 of them were confirmed as NCC positive patientsas confirmed by the neuroimaging of the brain followed by serological assay. Computed tomography (CT) scans and magnetic resonance imaging (MRI) of the brain was performed to confirm the cases by neuroimaging techniques (**Figure 4.15., Figure 4.16.**). The neuroimaging (CT and MRI) pictures were also used to detect the types and location of the cysts in the brain.

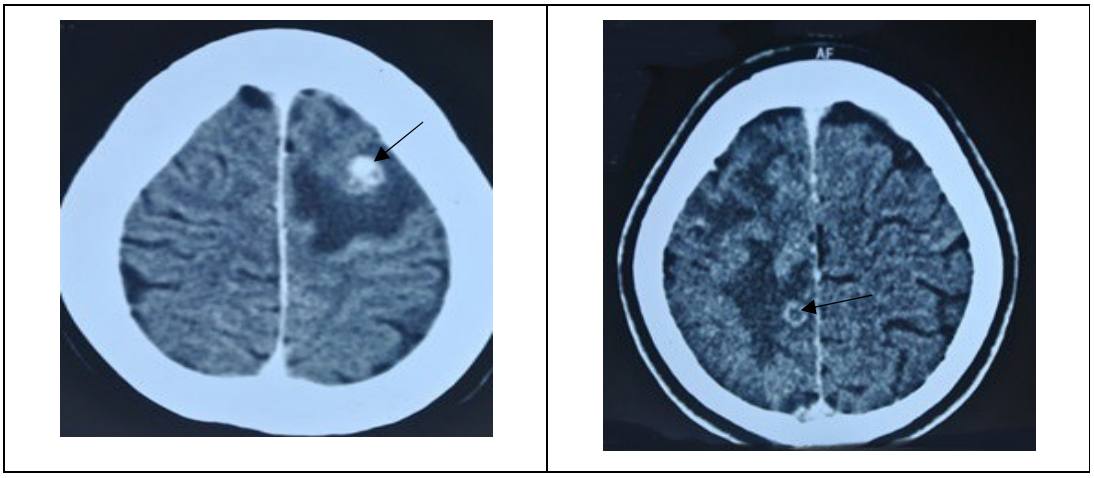


Figure 4.14. CT scans of brain showing cysts. [Source: Annapurna Neurological Hospital, Kathmandu, Nepal].

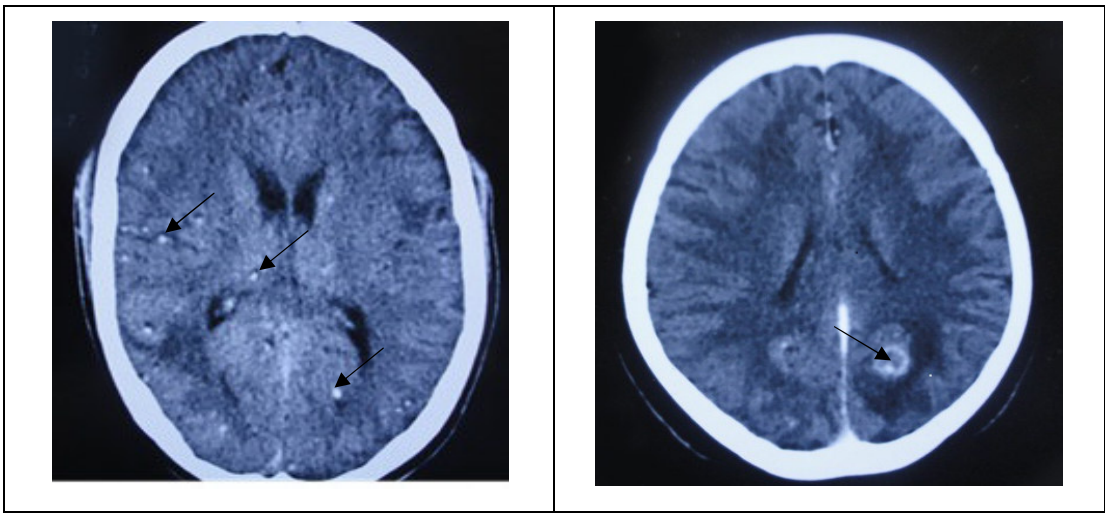


Figure 4.16.MRI of brain showing cysts. [Source: Annapurna Neurological Hospital, Kathmandu, Nepal].

Table 4.5. Co-relation of hospital diagnosed patients with WB and ELISA.

S. N.	Samples	Hospital diagnosed	WB analysis	ELISA analysis	S. N.	Samples	Hospital diagnosed	WB analysis	ELISA analysis
1	kb1	+	+	+	24	jd26	-	np	+
2	bs2	-	np	+	25	rr29	-	np	+
3	pb3	-	np	+	26	ms30	-	np	+
4	hb4	-	np	+	27	dp31	-	np	+
5	sa5	-	np	+	28	uj35	-	np	+
6	hs6	+	+	+	29	aa36	-	np	+
7	sp7	+	+	+	30	sg37	+	np	+
8	st8	+	+	+	31	sk38	-	np	+
9	np9	+	+	+	32	so39	-	np	+
10	bk10	+	+	+	33	rk41	-	np	+
11	bs11	+	+	+	34	rs42	-	np	-
12	al12	+	+	+	35	sg46	-	np	+
13	rn13	-	np	+	36	mk47	-	np	+
14	sk14	+	+	+	37	dc50	+	np	+
15	bs16	-	np	+	38	nc15	-	np	-
16	ar18	-	np	+	39	kp52	-	np	+
17	sp19	-	np	+	40	bk27	-	np	-
18	bg20	-	np	+	41	kb32	-	np	-
19	sp21	+	+	+	42	lb17	-	np	-
20	sp22	-	np	+	43	ja56	-	np	-
21	gn23	-	np	+	44	pk51	-	np	-
22	cp24	-	np	+	45	rm48	-	np	-
23	sg25	-	np	+	**Total positive**		**12**	**10**	**37**

+ Positive

- Negative

np Test not performed

4.8 Co-relation of hospital diagnosed patients with western blotting and ELISA diagnosis

The diagnosis of the test samples carried out using the antibody based ELISA was co-related with that of the WB analysis and the diagnosis performed in the hospital. Of 45 NCC patients visited to hospital, 12 were NCC positive cases as diagnosed in the hospital using clinical symptoms, neuroimaging, EITB and antigen ELISA. WB analysis was performed only in the 10 test samples and all were NCC positive cases. Antibody ELISA showed 37 NCC positive cases out of 45 test samples which suggested the low sensitivity of the assay. The negative NCC cases as depicted by antibody ELISA were also confirmed negative by hospital diagnosis which suggested the 100% specificity of the assay (**Table 4.6.**).

Chapter V
Discussion

Neurocysticercosis (NCC), the most important neurological disease of parasitic origin in humans (WHO, 2002) is a major cause of adult-onset epilepsy in areas where the pork tapeworm *T. solium* is endemic (García et al., 1997). NCC is characterized by a strong immune response surrounding infected neural tissues. The presence of excretory-secretory antigens and proteins released by the degeneration of the cysticerci in the host stimulates the production of specific immunoglobulins in which the isotopic predominance is related to the infection phase (Molinari et al., 2002; Abraham et al., 2004). Anti-cysticerci IgG is the one most detected in both the acute and the chronic phases of the disease. The best immunogenic proteins for NCC immunodiagnosis are in the cysticercus scolices, and these have been used to detect serum or CSF antibodies in patients with suspected NCC (Nascimento et al., 1987). Therefore, in addition to the CSA, human anti-IgG antibodies were used for the immunoassays (Lopez et al., 2004).

Neurocysticercosis is difficult to diagnose because of the dimensions, quantities and localization of the cysticerci in the CNS. The diagnosis of NCC is performed by histological techniques, neuroimaging followed by a confirmatory serological (immunological) assay. Computed tomography (CT) and magnetic resonance imaging (MRI) scans of the brain are formally indicated for diagnosing NCC, but the costs relating to these procedures make it difficult for most of the population with high infection rates in developing countries to have access to these services. Moreover, doubts concerning images may occur and auxiliary diagnostic methods are required (Del Brutto et al., 2001). The enzyme-linked immunoelectrotransfer blot (EITB), a western blot assay developed in 1989 that is 98% sensitive and 100% specific for detecting pathologically confirmed cases of NCC (Brand and Tsang, 1989) was recognized as the serological assay of choice for the detection of cysticercosis in humans and pigs by the WHO in 1995. Since the EITB is not quantitative, a quantitative assay that detects cysticercosis antibodies was developed as a means to monitor patient disease status and efficacy of chemotherapy. One quantitative assay format, the enzyme-linked immunosorbent assay (ELISA), performed in polystyrene plates, is particularly desirable, as it lends itself to high-throughput diagnostic applications. Immunoenzymatic tests like ELISA have been employed for NCC diagnosis due to their high sensitivity for which cerebrospinal fluid (CSF) may be used as samples. However, considering the difficulties in collecting CSF, which requires specialized physicians in special facilities, serum has been chosen as an

alternative source sample (Barcelos et al., 2005). Furthermore, a monoclonal antibody-based sandwich ELISA is used for the detection of viable metacestodes of *Taenia spp*. in human, porcine and bovine serum samples. Monoclonal antibody-based antigen detection ELISA uses two monoclonal antibodies to "trap" the antigen circulating in blood, CSF or urine (Correa et al., 1989; Garcia et al., 1998; Castillo et al., 2009). These assays gave a positive result when viable, well-established cysts are present. The monoclonal antibodies used in the test were originally prepared against antigens from *T. saginata*, and not only detect viable cysticerci in bovines, but also cysticerci of *T. solium* in pigs and humans. The cross-reactions between antigens produced by *T. solium* and *T. saginata* metacestodes are easily distinguished using this technique.

In this research work we identified immunogenic protein fractions which elicited high antibodies on infection and absence of antibodies against those protein fractions in healthy controls so the antigen candidates could exploit for immunodiagnostic tool of NCC. Furthermore, CSA of cysticerci tested by ELISA with sera from NCC suspected patients was used not only for the serological diagnosis but also for the seroepidemiological survey. We had applied SDS-PAGE, Western blot technique and ELISA to achieve the goal.

The CSA prepared from the parasite lysate was a heterogeneous mixture as the collection of lysate was from the centrifugation at 3,000 rpm (Manandhar, 2008) containing cytoplasmic and membrane (organelles) bound protein components. CSA preparation of cysts of *T. solium* had been found to contain total protein concentration of 1.89 µg/µL. CBB dye binds most readily to arginyl and lysyl residues of different proteins (not to the free amino acids).

Proteins or polypeptides present in CSA got denatured in the presence of a detergent like sodium dodecyl sulfate and reduce in the presence of beta-mercaptoethanol. The denatured protein complexes with SDS in the ratio of 1.4 gram SDS per gram of protein giving overall negative charge to the complex. The calculated amount of CSA loaded in SDS-PAGE when fractionated applying electric force, the complexes moved towards the anode separating the proteins based on difference in charge they possesses and their molecular weight. The polypeptide fractions separated by polyacrylamide gel electrophoresis were stained with CBB. Since the method has limited range of sensitivity for the detection, it produced the detectable band possessing at least 100ng of protein. CBB staining to CSA on gel produced 14 clearly distinguishable polpypetide fractions ranging from 11 to 158 kDa that include protein polypeptide fractions of 158, 124, 86, 72, 58, 50, 44, 42, 38, 26, 24, 20, 17and 11 kDa with certain bands showing higher intensity which represented for the higher amount of the particular polypeptide in the CSA. In a similar study the electrophoresis profile for crude antigens from scolices of *T.*

solium cysticerci showed at least 21 bands at 200, 180, 120, 100, 95, 80, 68, 65, 56, 53, 50, 45, 40, 38, 36, 34, 29, 26, 22, 20 and 15kDa of major proteins, for which the molecular weights ranged from 200 to 15kDa (Iudici Neto et al., 2007).

Integrated density value analyzed by densitometry from Alpha Imager ^{TM} 2200 resulted different polypeptides bands viz. 158, 124, 86, 72, 58, 50, 26 and 24 kDa in size having higher intensities and clearly distinguishable bands from other bands. Among these the bands of 86, 72, 58, 26 and 24 kDa polypeptide fractions were having AID value of nearly equal or higher than 130; the average value calculated by supposing the highest value of 255 units assigned for total black as from Alpha Imager ^{TM} 2200. This study revealed 14 clearly distinguishable protein fraction bands out of which only 5 polypeptide bands possesses remarkably high AIDV (above 130 AIDV). Similar type of work had been done in *Leishmania donovani* (Manandhar, 2008).

Polypeptide fractions separated from SDS-PAGE and immunoblotted by western blotting technique revealed various bands in NCC patients. In patients different antibodies produced in response to antigenic interaction from the parasite are high in number. So, NCC patient group produced 49 different distinct polypeptide fraction bands in 10 test samples that appeared variable when transferred to PVDF membrane and immunoblotted. Peptides fraction having molecular weight ranging from 15 to 124 kDa protein polypeptide were identified in this study. There were 15 major bands viz. 120, 100, 50, 46, 40, 39, 38, 37, 35, 32, 26, 24, 22, 18, and 16 kDa having the repetition frequency more than 50% in this study group. Maximum up to 25 interactive bands from NCC patient group reflected for various antigenic fragments found in parasite. The large number of bands from patients revealed a broad range of immune specificities resulting extensive heterogeneity of serological anti-cysticercal responses of individual patients.

Although 48 different distinct polypeptide fractions of molecular weight ranging from 128 to 15 kDa were identified from 6 healthy controls, only 3 different polypeptide fractions viz. 15, 36 and 38 kDa were found to have repetition frequency more than 50% and these bands were also present in the NCC patients. The immunoblot profiles of NCC patients showed the repetition frequency of the polypeptide fractions up to 50% in 18 bands. Since the study was focused on the polypeptides that possessed repetition for more than 50%, there were 15 bands that had the repetition of more than 50% in the WB profiles of NCC patients. Moreover the eight distinct antigenic bands 120, 100, 50, 46, 39, 24, 18, and 16 kDa were found completely absent in the healthy controls which were the important bands among those 15 bands in NCC patients. The bands of 100, 50, 39 and 24 kDa protein fractions were found present in 90% or more in NCC patients showing their greater application in diagnostic markers for NCC cases. The absence of

particular band from the sera of healthy controls clearly indicates for the absence of that antigen and its corresponding antibodies developed against that antigen.

Similar study in western blotting separation of lentil-lectin glycoproteins (LLGP) results in seven distinct antigenic bands at 13, 14, 18, 21, 24, 39-42, and 50 kDa (Brand and Tsang, 1989). Antibody reactivity with any one of these bands indicates exposure to cysticercosis. In the study done by Neto and his associates, immunogenic proteins with the molecular weights of 200, 180, 120, 100, 95, 68, 65 and 26kDa were the most reactive and among them 100, 95 and 26kDa proteins provided the best sensitivity and specificity indices when the appropriate dilutions of the sera were used (Neto et al., 2007). The 100 and 95kDa proteins are probably similar to the antigen B, which is important for inducing an immune response (Kojic and White, 2003). In a study performed by Joshi and his associates, *T. solium* specific antigenic bands corresponding to 13, 14, 16, 24, 42 and 50 kDa glycoprotein markers were obtained from the serum samples of the patients that were diagnosed with NCC by imaging techniques (Joshi et al., 2014).

Till date different antigenic polypeptides were identified with varying degree of reactivity but such cases were not reported from cysticerci isolated from Nepal and subject being used as Nepalese people. This study identified 100, 50, 39 and 24 kDa polypeptide having more than 90% reactivity. The importance of our study also preserved on 100% reactivity of 39 and 24 kDa. This particular result based on studied carried out on Nepalese population could reflect it as the regional marker for the endemic population.

The present study showed that most of the polypeptide bands from cysticerci did not showed good reactivity and just some could be indicated as antigens for NCC immunodiagnosis. The low reactivity found in proteins bands with different molecular weights possibly occurred due to the antigenic similarity between the cysticerci, and other antigens such as the adults of *T. solium* and other helminthes. This similarity might predict the occurrence of cross-reactions when sera from patients free from NCC, but with other parasitic diseases, including taeniasis, are tested. Studies using sera from patients with infections caused by viruses, bacteria or protozoa have also shown nonspecific reactions when tested against *T. solium* antigens (Ishida et al., 2003). These findings increase the difficulty of antigen evaluation and subsequent use for NCC immunodiagnosis, since sera from patients with different diseases should be analyzed. Despite the diversity of nonspecific reactions and cross-reactions, the present study has contributed towards elucidating the possibility of such events occurring between the CSA and the available sera from normal.

ELISA performed out of 45 NCC patients 37 (82.22%) were found to be NCC positive and the rest 8 (17.78%) were negative. The samples chosen were those suspected with NCC by clinical diagnosis and those subjects were from the endemic region and some may have recovered the disease but the antibodies may be present in their body which result in high positive cases in this antibody based ELISA. This may also be the probable reason for the low sensitivity of the test.

In a similar study carried out in Brazil, the sera from 88 patients with epileptic seizures who underwent cerebral computed tomography (CT) were analyzed for the detection of antibodies to *T. solium* cysticercus by ELISA with *T. solium* cysticercus total saline antigens showed 95% sensitivity and 68% specificity with 30% ELISA positivity (Ishida et al., 2006). In a study done in the endemic region of Nepal, the porcine cysticercosis seemed to be rather high i.e. the prevalence was 23.5% when the testing technique used was ELISA (Bista et al., 2006).

Based on gender and age group, among the 37 NCC positive patients analysed, about equal percent of male and female were found to be affected i.e. 51.35% and 48.65% were male and female respectively. In a study carried out in the neurology service unit of Tribhuvan University teaching hospital (Kathmandu Nepal), reported 66 cases of NCC and among them both sexes (36 male and 30 female) were almost equally infected (Agrawal, 2012).

The maximum number of patients were in the age group 20-30 that nearly covered about 54.05% of total patients involved in this research work. This was followed by the age group 10-20, 30-40, more than 50, less than 10 and 40-50. In each group nearly male and female population were found to be same. The mean age of the patient was 26.67 years with the standard deviation (SD) of ±11.0631 and the range of the patient age was 4-57 years. The patients age group showed positive correlation with the gender of the patients and the correlation was found to be significant ($p<0.05$) as well. Hence, this research work concludes that there is no discrimination in gender for the *T. solium* infections. In a study carried out in western region of Nepal, the researchers have observed that the mean age at which NCC occurs was at 10.8 years (range 11 months - 15 years, most commonly affected age group 10 - 12 years; 8.9% patients were below 2 years of age) (Basu et al., 2007). The deviation of findings from the study may be because of the age range of the subjects studied by Basu et al., 2007 who did not cover the age above 15 years.

Based on the clinical symptoms among the 37 NCC positive patients analyzed, 51.35% patients had Seizure. Similarly 43.24%, 13.51%, 10.81% and 8.11% patients had Headache, Loss of conscious, Abnormal/jerky movements and Fainting attack respectively. The relation among various clinical symptoms of patients was found to be

significant (p=0.0487). This finding is in accordance with the previous finding that the major cause of adult onset epilepsy is due to NCC in underdeveloped areas where pork tapeworm *T. solium* is endemic (García et al., 1997). In a study among NCC patients seen in neurology clinics, about 79% had seizures/epilepsy, 38% severe headaches, 16% focal deficits, and 12% signs of increased intracranial pressure. Several other symptoms were also reported in less than 10% of the patients. It is concluded that these estimates are only applicable to patients who are assisted in neurology clinics and likely overestimate the frequency of manifestations among all NCC cases (Carabin et al., 2011). In another study examined among 93 patients (age range: 2-14 years; 10.3% patients <6 years of age) presenting new onset seizures from Lumbini region, 73% of the patients had NCC. These observations were confirmed by radio imaging techniques (Ellis et al., 2000).

Limitation

This sound research work implies for removal of utmost possible hindrances. Despite all our endeavors, few parameters remain as limitation to this work. The time constraint is the major factor to limit my ambitious study. So different hurdles like irregular supply of different reagent and short of various equipment are the major drawback that hinders for the best result. The better result of the study lies on the parameters like the tests of cross reactivity with diseases including taeniasis and the infections caused by viruses, bacteria or protozoa have also shown nonspecific reactions when tested against *T. solium* antigens. But lack of sufficient chemical components (e.g. secondary antibody, prestained protein ladder) caused the work in limited experiments albeit we had serum collected from such patients.

The one important hurdle of this work is low number of the tests performed because of small sample size in western blotting. The reason behind are many, like lack of other reagents required for the study because of which we were bound to conduct the limited number of test.

Difficulties in stabilizing proteins, in which their antigenic determinants are kept during the extraction stages and subsequent adsorption in PVDF membrane, have been reported by some investigators and may be important for maintaining antigen-antibody reactivity.

The imperative limitation of the research work is the lack of time and budget due to which molecular diagnosis of the test samples could not be performed.

Chapter VI

Conclusion

Neurocysticercosis is the commonest parasitic disease of the human nervous system caused by larvae of *Taenia solium*, where pigs are eaten and sanitation is poor, and also in the more developed countries as a result of increasing migration. It is the main cause of epilepsy in underdeveloped countries. Definitive diagnosis of NCC is performed in persons with characteristic neuroimaging findings, clinical symptoms and serological tests. Immunological techniques are directed towards detecting specific proteins secreted by *T. solium* and/or specific antibodies against *T. solium* cysticerci in serum.

From the present investigation, the serological diagnosis is found to be useful for the detection of specific antibodies in the serum of the patients against the specific antigenic cytoplasmic and membrane bound polypeptides of the cyst of *T. solium*. The protein profiling of CSA revealed the polypeptide fractions from 158 to 11 kDa. Among these the bands of 86, 72, 58, 26 and 24 kDa polypeptide fractions were having AID value of more than 130.

In immunoblotting of NCC patients, the thesis work pointed the remarkable figure for 100, 50, 39 and 24 kDa polypeptide that produced immense result on reactivity. In NCC patient samples, 39 and 24 kDa polypeptide protein fractions produced significant consistency that figured to 100% (10/10) and 100 and 50 kDa fractions marked to 90% (9/10) consistency. In addition, these bands were found completely absent in the healthy controls showing their greater application in diagnostic markers for NCC cases. The antigen having the size in the range of 25-50 kDa possess higher potentiality on antigenicity and future vaccine candidate development.

In ELISA, among 45 suspected NCC patients, 37(82.22%) were found to be NCC positive and the rest 8(17.78%) were negative. Based on gender and age group, about equal percent of male and female were found to be affected that is 51.35% and 48.65% were male and female respectively. The maximum number of patients were in the age group 20-30 that nearly covered about 54.05% of total patients in which male and female population were nearly same. The patients age group showed positive correlation with the gender of the patients and the correlation was found to be significant ($p<0.05$). These findings suggest that the infection of the parasite does not discriminate male and female population. Based on the clinical symptoms, 51.35% patients had Seizure. Similarly 43.24%, 13.51%, 10.81% and 8.11% patients had Headache, Loss of conscious,

Abnormal/jerky movements and Fainting attack respectively. The relation among various clinical symptoms of patients was found to be significant.

It is clear that the immunodiagnostic tools for NCC has greatly contributed to a better understanding of the prevalence and the epidemiology of the infection. Immunodiagnostic tests also provide a valuable tool in measuring the impact of the disease on human health, data that are still missing in most endemic areas. They as well contribute to the diagnosis of NCC and the follow up of treatment.

Recommendation

- Because the neuro-imaging methods are inaccessible and/or too expensive for the rural population at risk in many endemic countries where serology may provide the only tool for diagnosis of the infection, efforts should be made to make cheap, reliable and standardized tools more widely accessible.
- For future test evaluation, selection of NCC patients and controls (both healthy individuals and individuals with other parasitic diseases) should be performed in a standardized way so that test performances can be evaluated and compared in a reliable manner.
- Previous studies have already shown increased specificity for immunodiagnostic tests by using purified antigens instead of crude antigens. On the other hand, reports of false-positive results have also been registered, thus suggesting that the use of purified antigens for immunodiagnosis should be done with caution.
- Production of CSA from the cysts of *T. solium* is possible at low cost, therefore, CSA can be applied in ELISA tests as means of excluding negative cases, particularly for seroepidemiological surveys. Antigens of 100, 50, 39 and 24 kDa may be further purified used in ELISA reactions and/or immunoblotting to confirm positive results.
- The major problem involving crude soluble antigen production continues to be the small amounts of protein recovered after the whole process. Thus, further studies should be carried out, with the aims of producing proteins in satisfactory quantities and achieving their stabilization under conditions in which they remain immunogenic for long periods.

- Among these possibilities is the production of recombinant proteins by using molecular cloning techniques, which could also be used for characterizing the genes coding for proteins with molecular weights of 100, 50, 39 and 24 kDa from *T. solium* cysticerci, and for producing synthetic peptides that could be used in validating immunodiagnostic tests.
- For the NCC cases with low sensitivity of available diagnostic techniques, molecular diagnosis should be performed for accurate diagnosis of the disease along with the identification of the parasite.
- Analysis of sheath protein should be done to understand its peptide sequence and from there to the designing of degenerate primers to be used for isolation, cloning, sequencing and expression of sheath protein gene. This gene will help to validate the structure and secretion of sheath protein which will help to design DNA vaccine.

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Appendices

Appendix I

Polypeptide bands of different NCC patient test samples observed in WB

	NCC patients test samples									
	S 1	**S 2**	**S 3**	**S 4**	**S 5**	**S 6**	**S 7**	**S 8**	**S 9**	**S 10**
Bands (kDa)	120	124	120	120	120	108	120	124	124	120
	108	100	100	100	100	88	100	108	100	100
	100	82	82	86	86	76	86	100	85	86
	88	72	70	72	76	70	72	82	70	70
	85	66	65	66	68	65	66	72	63	64
	76	62	62	63	64	60	60	68	58	58
	72	56	56	56	58	55	55	62	55	55
	66	51	51	50	50	50	50	56	50	50
	63	46	50	46	46	44	46	50	44	46
	60	42	46	42	40	40	40	44	40	44
	58	39	42	40	39	39	39	42	39	40
	50	38	39	39	38	38	36	40	38	39
	48	36	37	38	37	37	35	39	37	38
	46	35	36	37	35	35	32	38	35	36
	39	32	34	35	32	32	28	37	29	35
	38	29	30	32	27	28	26	35	26	32
	36	26	26	29	24	26	24	32	24	30
	34	24	24	26	23	24	22	29	22	28
	30	23	18	24	20	19	20	27	21	26
	26	21	15	22	17	18	16	24	19	24
	24	18		21	15	16		22	18	21
	22	17		18				21	16	20
	20	15		17				18		17
	18			16				15		16
	16									
Total no. of bands	25	23	20	24	21	21	20	24	22	24

Appendix II

Table 1 Polypeptide bands of different healthy control samples observed in WB

	Healthy control samples					
	hc1	**hc2**	**hc3**	**hc4**	**hc5**	**hc6**
Bands (kDa)	124	127	127	127	128	124
	112	112	118	112	110	110
	105	105	108	105	95	88
	92	93	92	92	85	76
	78	85	81	81	78	66
	68	76	68	72	68	54
	60	65	62	65	60	45
	55	56	48	60	52	38
	48	41	38	52	44	36
	44	38	36	48	41	35
	41	37	35	44	38	32
	36	36	28	38	37	28
	32	34	22	37	36	25
	28	32	19	34	29	22
	22	27		26	15	20
	20	25		23		19
	17	23		21		
	15	21		19		
		20		17		
		17		15		
		15				
Total no. of bands	18	21	14	20	15	16

Appendix III

OD readings at 450 nm of all the variables measured in ELISA

A. OD readings at 450 nm of test samples

S. N.	Samples	OD	Mean OD	S. N.	Samples	OD	Mean OD	S. N.	Samples	OD	Mean OD
1	kb1	3.39	3.382	17	sp19	3.228	3.308	33	rk41	3.355	3.311
		3.374				3.256				3.267	
2	bs2	3.317	3.342	18	bg20	3.354	3.2605	34	rs42	2.123	2.0905
		3.367				3.262				2.058	
3	pb3	3.305	3.3265	19	sp21	3.238	3.166	35	sg46	3.267	3.295
		3.348				3.283				3.323	
4	hb4	3.413	3.3835	20	sp22	3.171	3.2515	36	mk47	3.291	3.3045
		3.354				3.161				3.318	
5	sa5	3.395	3.386	21	gn23	3.226	3.2775	37	dc50	2.438	2.521
		3.377				3.277				2.604	
6	hs6	3.325	3.3405	22	cp24	3.347	3.23	38	nc15	1.987	1.9245
		3.356				3.208				1.862	
7	sp7	3.306	3.326	23	sg25	3.26	3.235	39	nn52	2.401	2.407
		3.346				3.2				2.413	
8	st8	3.411	3.3855	24	jd26	3.212	3.303	40	bk27	2.075	2.0885
		3.36				3.258				2.102	
9	np9	3.39	3.387	25	rr29	3.295	3.2055	41	kb32	2.165	2.134
		3.384				3.311				2.103	
10	bk10	3.328	3.356	26	ms30	3.208	3.285	42	lb17	1.126	1.134
		3.384				3.203				1.142	
11	bs11	3.341	3.35	27	dp31	3.284	3.22	43	ja56	1.864	1.8425
		3.359				3.286				1.821	
12	al12	3.429	3.403	28	uj35	3.197	3.2705	44	pk51	1.187	1.1575
		3.377				3.243				1.128	
13	rn13	3.401	3.3915	29	aa36	3.317	2.6655	45	rm48	1.824	1.8065
		3.382				3.224				1.789	
14	sk14	3.336	3.359	30	sg37	2.684	3.2245				
		3.382				2.647					
15	bs16	3.421	3.396	31	sk38	3.239	3.283				
		3.371				3.21					
16	ar18	3.333	3.284	32	so39	3.286					
		3.235				3.28					

B. OD readings at 450 nm of positive controls

S.N.	Positive controls	OD	Mean
1	pc1	3.271	3.306
		3.341	
2	pc2	3.214	3.2805
		3.347	

C. OD readings at 450 nm of substrate control and conjugate control

	OD	Mean
sc	0.9	0.8045
	0.709	
cc	0.863	0.8945
	0.926	

D. OD readings at 450 nm of negative controls

S.N.	OD
nc1	1.102
nc2	1.096
nc3	1.104
nc4	1.105
nc5	1.089
nc6	1.105
nc7	1.108
nc8	1.087
mean	1.0995
s.d.	0.132281
cv	12.03101
cut off value	1.496343

Appendix IV

A. Bradford reagent composition

Reagents	Amounts (For 1000 ml)	For 500 ml	For 100 ml
Coommassie Brilliant Blue G-250 (CBB G-250)	100 mg	50 mg	10 mg
Ethanol (95%)	100 ml	50 ml	10 ml
Orthophosophoric acid (85%)	100 ml	50 ml	10 ml
Distilled water			
Bovine serum albumin (BSA)	Stock solution 1 mg/ml		
Brown bottle	(To keep the reagent protected from direct sunlight)		

B. SDS-PAGE composition

Gel composition	Solution components	Volume required (ml)			
12% Resolving gel	TDW	1.6	3.3	4.9	6.6
	30% Acrylamide	2	4	6	8
	1.5% Tris (pH 8.8)	1.3	2.5	3.8	5
	10% SDS	0.05	0.1	0.15	0.2
	10% $(NH_4)_2S_2O_8$	0.05	0.1	0.15	0.2
	TEMED	0.002	0.004	0.006	0.008
	Final volume (ml)	5	10	15	20
5% Stacking gel	TDW	0.68	1.4	2.1	2.7
	30% Acrylamide	0.17	0.33	0.5	0.67
	0.5% Tris (pH 6.8)	0.13	0.25	0.38	0.5
	10% SDS	0.01	0.02	0.03	0.04
	10% $(NH_4)_2S_2O_8$	0.01	0.02	0.03	0.04
	TEMED	0.001	0.002	0.003	0.004
	Final volume (ml)	1	2	3	4