OCCURRENCE OF ECHINOCOCCOSIS/HYDATIDOSIS IN SLAUGHTER BUFFALOES AND *ECHINOCOCCUS GRANULOSUS* IN STRAY DOGS IN KATHMANDU VALLEY, NEPAL

SALINA MANANDHAR

MASTER OF SCIENCE

IN VETERINARY PUBLIC HEALTH

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Thesis title Occurrence of Echinococcosis/Hydatidosis in Slaughter Buffaloes and *Echinococcus granulosus* in Stray Dogs in Kathmandu Valley, Nepal

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ABSTRACT

The study aimed at identifying the occurrence of hydatidosis in slaughter buffaloes and *E. granulosus* in stray dogs' faecal samples in Kathmandu Valley, Nepal. A total of 500 buffalo carcasses in a slaughterhouse, riversides and individual butchers' sites were examined for the presence of hydatid cysts. The cyst fluid was collected and microscopically examined to determine if it was fertile or not. Of the 500 carcasses examined, 10.6% had hydatid cysts. Specifically, prevalence of hydatidosis in slaughterhouse carcasses was 6.7%. Whereas, those examined at riversides and individual butcher places had occurrences of 10.0% and 12.7%, respectively. These proportions were not significantly (p= 0.2013) different. Distributions of the hydatid cysts by specific organs showed singly occurrence of 6.4% in lungs, 2.4% in livers and 1.8% in both, in livers and lungs. These distributions were significantly (p= 0.0001) different. In a total of 53 hydatid cysts, 58.5% were fertile and 41.5% were sterile. These two proportions were not significantly (p= 0.076) different.

Overall, 366 faecal samples of stray dogs were collected from around buffalo slaughtering areas (slaughterhouse and individual butcher places), riversides, garbage sites and temple sites. These samples were examined by microscope to investigate the presence of *Taenia* eggs. Further, PCR analysis was performed to differentiate the

E. granulosus from other *Taenia* eggs and to determine the strains. The total prevalence of *Taenia* eggs in the faecal samples was 12.8%. Whereas, around the slaughterhouse and individual butcher places was high (17.6%) followed by riversides (14.1%), garbage sites (9.6%), and temple sites (7.6%). However, the percent distribution of *Taenia* egg-positives among different sites was not significantly ($p= 0.2148$) different.

PCR was performed on 47 faecal samples found microscopically positive for *Taenia* eggs, using mitochondrial cytochrome C Oxidase (CO1) gene. Eleven samples (23.4%) were PCR positive. A pooled sample of hydatid cyst protoscolices was also PCR positive. This pooled sample and one faecal sample were found highly homologous (99.7% and 99.2% respectively) with genotype G1 (sheep strain) of *E. granulosus.*

Apart from *Taenia* eggs, different other helminth eggs including zoonotic *Toxocara* and hookworm eggs were also found in dogs' faecal samples.

Questionnaire survey from butchers revealed that the waste disposal practices were highly undesirable. Dogs had free access to condemned meat. The infected meat was used for human or animal consumption. There was lack of awareness about hydatidosis among them.

High occurrence of hydatidosis in buffaloes, the contamination of the environment around the slaughter places and riversides, the unhindered excess of stray dogs to the waste enhance the infection risk of humans with cystic echinococcosis in the Kathmandu Valley. These findings clearly justify that there is an urgent need of introducing proper slaughterhouses and meat inspection systems in the country.

ชื่อเรื่องวิทยานิพนธ์ การพบโรคเอไคโนค๊อกโคซิสหรือโรคไฮดาติดในกระบือชำแหละและพยาธิ *Echinococcus granulosus* ในสุนัขจรจัดในกาฐมาณฑุแวลลีย์ ประเทศเนปาล

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บทคัดย่อ

การศึกษานี้มุ่งค้นหาโรคไฮดาติโดซิสในกระบือชำแหละ และ *E. granulosus* ในอุจจาระสุนัขจรจัดในเขตกาฐมาณฑุ แวลลีย์ เนปาล ทำการศึกษาระหว่างเดือนพฤศจิกายน พ.ศ. 2547 ถึงเมษายน พ.ศ.2548 โดยตรวจหาไฮดาติดซิสต์ในกระบือ 500 ตัว ที่ชำแหละ ในโรงฆ่าสัตว์ ริมฝั่งน้ำ และสถานที่ชำแหละของผู้ขายเนื้อแต่ละคน เก็บน้ำในซิสต์มาตรวจด้วยกล้องจุลทรรศน์ว่าเป็นซิสต์เจริญพันธุ์หรือไม่ จากการตรวจกระบือชำแหละ 500 ตัว พบไฮดาติดซิสต์ร้อยละ 10.6 เมื่อแยกเฉพาะแห่งพบความชุกในกระบือชำแหละที่โรงฆ่าสัตว์ร้อยละ 6.7 ในขณะที่พบในกระบือชำแหละที่ริมฝั่งน้ำและสถานที่คนขายเนื้อ ร้อยละ 10.0 และ 12.7 ตามลำดับ อัตราการตรวจพบไม่แตกต่างกันอย่างมีนัยสำคัญ (p=0.2013) การกระจายของไฮดาติดซิสต์ในอวัยวะจำเพาะ พบซิสต์ในอวัยวะเดี่ยว คือปอดร้อยละ 58.5 ตับร้อยละ 2.4 และพบทั้งในตับและปอดร้อยละ 1.8 การกระจายนี้มีความแตกต่างกันอย่างมีนัยสำคัญ (p=0.0001) ในจำนวน 53 ไฮดาติดซิสต์ พบว่าเป็นซิสต์เจริญพันธุ์ร้อยละ 58.5 และซิสต์หมัน ร้อยละ 41.5 สัดส่วนทั้งสองชนิดไม่แตกต่างกันอย่างมีนัยสำคัญ (p=0.076)

อุจจาระจากสุนัขจรจัดทั้งหมด 366 ตัวอย่าง เก็บจากบริเวณรอบพื้นที่ฆ่าสัตว์ (โรงฆ่าสัตว์และสถานที่ผู้ขายเนื้อฆ่ากระบือ) ริมฝั่งน้ำ ที่ทิ้งขยะ และวัด นำไปตรวจโดยวิธีเข้มข้น ฟอร์มาลินอีเทอร์ หาไข่พยาธิ *Taenia* จากนั้นนำไปแยกว่าเป็นไข่ *E. granulosus* หรือไข่ *Taenia* และบ่งชี้สายพันธุ์โดยวิธี PCR ผลการตรวจอุจจาระพบความชุกของไข่ *Taenia* ในอุจจาระตัวอย่างร้อยละ 12.8 โดยแยกเป็นความชุกสูงในที่รอบโรงฆ่าสัตว์ (ร้อยละ 17.6) ตามด้วยสถานที่ผู้ขายเนื้อ (ร้อยละ

14.1) ที่ทิ้งขยะ (ร้อยละ 9.6) และ วัด (ร้อยละ 7.6) อย่างไรก็ตาม ร้อยละของการกระจายของไข่ *Taenia* ในพื้นที่ต่าง ๆ ไม่แตกต่างกันอย่างมีนัยสำคัญ (p=0.2148)

ได้ทำ PCR กับอุจจาระ 47 ตัวอย่างที่ตรวจพบไข่ *Taenia* ด้วยกล้องจุลทรรศน์ โดยใช้จีนไซโทโครมซีออกซิเดสของไมโทคอนเดรีย (CO1) พบให้ผลบวก 11 ตัวอย่าง (ร้อยละ 23.4) และเมื่อทำกับโปรโตสโคเล็กซ์ที่รวบรวมเข้าด้วยกันจากไฮดาติดซิสต์ก็ให้ผลบวกเช่นเดียวกัน โปรโตสโคเล็กซ์รวมจากไฮดาติดซิสต์และอุจจาระ 1 ตัวอย่าง พบมีความเหมือน (ร้อยละ 99.7 และ 99.2 ตามลำดับ) กับ *E. granunosus* จีโนไทป์ G1 (สายพันธุ์แกะ)

นอกจากพบไข่พยาธิ *Taenia* แล้ว ยังพบไข่หนอนพยาธิอื่น รวมทั้งไข่ *Toxocara* และพยาธิปากขอในตัวอย่างอุจจาระสุนัขด้วย

การสำรวจผู้ขายเนื้อโดยใช้แบบสอบถามพบว่าวิธีการทิ้งปฏิกูลเป็นแบบไม่พึงประสงค์ สุนัขเข้าถึงเนื้อที่กักเก็บไว้ได้อย่างเสรี มีการนำเนื้อติดโรคให้คนหรือสัตว์บริโภค และผู้ขายเนื้อขาดความตระหนักเกี่ยวกับโรค

การพบโรคไฮดาติดในกระบือในจำวนมาก การปนเปื้อนสิ่งแวดล้อมรอบบริเวณโรงฆ่าสัตว์และริมฝั่งน้ำ การที่สุนัขจรจัดเข้าถึงสิ่งปฏิกูลอย่างเสรี ส่งเสริมความเสี่ยงต่อการติดโรคซิสติดเอไคโนค็อกโคซิสในคน โดยเฉพาะเมื่อ *E. granulosus* สายพันธุ์แกะแพร่กระจายในกาฐมาณฑุแวลลีย์ ดังนั้นจึงมีความจำเป็นเร่งด่วนที่ต้องพัฒนาแนวปฏิบัติในการฆ่าสัตว์ที่เหมาะสม โดยเฉพาะกระบือโดยการนำระบบตรวจสอบโรงฆ่าสัตว์และเนื้อสัตว์มาใช้ และคงการใช้วิธีทิ้งปฏิกูลที่เหมาะสมในประเทศ

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LIST OF ABBREVIATIONS AND SYMBOLS

A	Adenine
bp	base pair
C	Cytosine
CBS	Central Bureau of Statistics
CE	Cystic Echinococcosis
CO1	Cytochrome c oxidase
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguanosinetriphosphate.
dNTP	deoxynucleotidetriphosphate
DNA	Deoxyribonucleic acid.
E. granulosus	*Echinococcus granulosus*
E. multilocularis	*Echinococcus multilocularis*
E. oligarthrus	*Echinococcus oligarthrus*
E. vogeli	*Echinococcus vogeli*
ELISA	Enzyme-linked immunosorbent assay
EITB	Enzyme-linked immunoelectrotransfer blot
G	Guanine
g	gram
×g	gravity
GDP	Gross Domestic Product
HCl	Hydrochloric acid
KTM	Kathmandu
KOH	Potassium hydroxide
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PSC	Protoscolices
RAPD	Random Amplified Polymorphic DNA

RFLP.....Restriction Fragment Length Polymorphism

T. hydatigena .....*Taenia hydatigena*

T .....Thyamine

TLDP .....Third Livestock Development Project

µM .....micromolar

mM .....millimolar

µl .....microliter

µm.....micrometer

mg.....milligram

ml.....milliliter

ng.....nanogram

$ZnCl_2$.....Zinc chloride

1. INTRODUCTION AND OBJECTIVES

1.1 General Background

Nepal is a landlocked country. It is located in Southern Asia. It lies in between the two most populous nations of the world, China and India. The total area of the nation is 147,181 sq. km. (CBS, 2004). Ecologically, it can be divided into three climatic zones i.e. high mountains, hills, and the Terai. Its northern part is covered by the Himalayas. The middle part consists of hills called mid- hills and the southern-most part consists of a low lying fertile area called the Terai. Nepal is one of the least developed countries. The per capita income is US $ 250 per year (World Bank, 2004). The population of Nepal as per the census of 2001 is 23.2 million, which is growing at 2.25 percent per annum (CBS, 2004).

Economically, the nation is still poor because of the slow pace of development activities. More than 40 percent of the people are below poverty level. Agriculture is the predominant activity with 81% of the population engaged in it. Under such circumstances, the major concern of the nation is to raise the standard of living of the people by improving the agriculture sector. In agriculture, livestock is an integral part of the complex farming system (livelihood, food security, nutrition, agricultural operation and transport, soil fertility etc). Contribution of agriculture to the Gross Domestic Product (GDP) is estimated around 40%. Livestock contributes about 30% of Agriculture Gross Domestic Product (AGDP). The contribution of livestock may be further subdivided as 62.6% from dairy, 32.4% from meat and 5% from eggs (Ministry of Finance, 2004).

The present health indicators of Nepal do not reveal an encouraging scenario despite continued efforts to improve the health situation. Life expectancy at birth is 60.98 years. Infant mortality rate (IMR) is 68.51%. For 1500 people there is only one doctor. Safe water is available to 71% of the population and sanitation to 39.22%, and these indicators are clear manifestations of poor hygiene and health conditions in the

country (UNDP, 2004). Therefore, to address these issues and to improve the basic living conditions in the country, adequate and sufficient emphasis is required on health issues both with direct and indirect implications. The hygiene and sanitation situation is poor due to a low level of awareness among others. This is one of the major causes of parasitic diseases to both humans and livestock. Among the parasitic diseases, echinococcosis/hydatidosis is one of the major problems, which affect livestock farming as well as cause serious health risks to the human population.

1.2 Problem Statement

Echinococcosis/hydatidosis is a parasitic disease. It is caused by the dog tapeworm *Echinococcus granulosus*. Its infectious larval stage, the metacestode is called *Echinococcus cysticus* or *E. hydatidosus*. *E. granulosus* causing cystic echinococcosis (CE) or cystic hydatidosis in humans and livestock, and is an important zoonotic disease with worldwide distribution (Craig *et al*., 1996). It causes serious public health problems in certain parts of the world (Schantz, 1990). So enquiry in this area is important from the public and veterinary point of view. In addition there are economic losses from the condemnation of infected livestock organs. The economic loss is measured in terms of lowered meat, milk and wool production.

This parasite is found worldwide especially in areas where hygienic conditions and education are poor. Another cause is uncontrolled stray dogs, especially in underdeveloped countries, taking over scavenger function. There is some evidence that the disease is spreading because of a lack of meat control, dog management and appropriate legislation (Schwabe, 1986).

In India, extensive occurrence of hydatidosis has been reported in humans as well as in livestock. The socio-economic, cultural and religious factors have frequently played an important role in the transmission of infection to human. *E. granulosus* infections have been reported also in dogs (Bhatia and Pathak, 1990).

In Nepal, the infection of hydatid disease is increasing day by day. Research has been done in this disease area, particularly in Nepal, by Joshi *et al.* (1995 and 1996b) but there is a definitive scope for undertaking a representative study. It is also important to understand the problem after a decade of previous research. The up-to-date status of this disease was necessary.

This disease may be emerging as one of the major health problems in Nepal. Due to lack of awareness, some people and butchers are still giving raw livestock meat to domestic and stray dogs. Also the slaughtering of buffaloes takes place in open air. The intestinal contents and waste meat are routinely dumped in water where people bathe and draw their drinking water. Also at riversides, stray dogs roam freely and vultures perch in the trees, waiting to feed from bits of carcasses. These are the major sources of infection of this disease. The main cause of this disease is due to a low level of awareness, lack of legislation, lack of meat inspection, water pollution and also uncontrolled stray dogs. The present research takes up this issue for further research linking the analysis of fecal samples from dogs along with exploration of hydatid cysts among buffaloes.

1.3 Objectives of Study

This study was initiated with the following objectives,

i. To find out occurrence of hydatidosis in buffaloes.

ii To determine whether hydatid cysts are fertile or sterile and differentiate hydatid strains.

iii To find out the prevalence of taeniosis in stray dogs' faecal samples.

iv To differentiate the eggs of *Echinococcus granulosus* from other *Taenia* species by Polymerase Chain Reaction (PCR) in stray dog's faecal samples.

2. LITERATURE REVIEW OF *ECHINOCOCCUS GRANULOSUS*/HYDATIDOSIS

2.1 Introduction

Hydatidosis, a problem of worldwide importance, is caused by adult or larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* of the family *Taeniidae*. At present, taxonomically it has four valid species, namely: *E. granulosus*, *E. multilocularis*, *E. oligarthrus* and *E. vogeli* (FAO/UNEP/WHO, 1981; Soulsby, 1982; Thompson and McManus, 2001). These four species are morphologically distinct in both adult and larval stages (OIE, 2004).

Adult parasites are usually attached to the mucosa of the anterior part of the small intestine of definitive hosts. The larval stages are found in the inner organs of a wide variety of domestic and wild intermediate hosts (Thompson and Lymbery, 1988; Thompson, 1995). Among these four species *E. granulosus* and *E. multilocularis* are the most important from a public health and economic point of view (McManus, 1996). *E. oligarthrus* and *E. vogeli* are restricted to the South American and Arctic regions in sylvatic areas, respectively.

E. granulosus is cosmopolitan in distribution. The parasite is adapted to definitive hosts of the family canidae and a wide range of intermediate hosts (humans, domestic and wild herbivores/omnivores) (Thompson and McManus, 2001). It is widely spread in sheep-rearing areas and where definitive hosts occur (Torgerson and Health, 2003). The adult parasite hardly causes disease in the definitive host (Thompson, 1995). The clinical and economic, as well as zoonotic, significances of these parasites are almost completely confined to infection of intermediate hosts.

E. multilocularis, which causes alveolar or multilocular echinococcosis, has principally a holarctic (Eurasia and North America) distribution (Lightowlers, 1990). The *E. multilocularis* has a sylvatic cycle, which is linked via infected small mammals to different wild and domestic carnivores. In the sylvatic cycle, the arctic

fox (*Alopex lagopus*) and red fox (*Vulpes vulpes*) play a key role as definitive hosts and small mammals, mainly rodents, are the intermediate hosts. In some areas, other wild canids, such as coyotes (*Canis latrans*), and wild felidae (*wild cats*) can also serve as definitive hosts. Aberrant hosts, both animals and humans can also become infected with metacestodes, which have the potential to cause alveolar echinococcosis (AE) (Eckert and Deplazes, 2004). It is relatively less widespread and prevalent than *E. granulosus* and more often a fatal, zoonotic disease (Schantz *et al*., 2003).

2.2 Morphology of the Adult Parasite

The adult *E. granulosus* is only a few millimeters long, varying between 2-7 mm in length with 3-4 segments (rarely up to six segments) (Eckert and Deplazes, 2004; OIE, 2004). Anteriorly, the adult parasites possess a scolex, which has four muscular suckers and two rows of sickle shaped hooks, one large and one small, on the rostellum. The mean length of the large hook ranges from 22-49 µm, and that of the small hook ranges from 17-39 µm (Thompson and Lymbery, 1988; OIE, 2004).

The adult worm is hermaphrodite. The body or strobila has a number of reproductive units (proglottids) (Chatterjee, 1980; Smyth, 1994; Hendrix, 1998), the mature penultimate proglottid and the terminal proglottid. The latter is gravid and is usually more than half the length of the worm (Soulsby, 1982). This gravid proglottid/uterus has 12-15 short lateral diverticuli (Bhatia and Pathak, 1990) and, is usually filled with 100-1500 thick-shelled eggs (Thompson, 1995). The ratio of the anterior part of the strobilar to the gravid segment ranges between 1:0.86-1:1.30 (FAO/UNEP/WHO, 1981; Thompson, 1995).

The gravid proglottids and or eggs are shed in the faeces (McManus *et al.*, 2003). The eggs are brown in colour and morphologically indistinguishable from those of other tapeworms of the genus *Taenia* (Smyth, 1994; Eckert *et al*., 2001b). The size of the eggs ranges from 30-40 µm and they have a thick radially striated shell. The egg

has a single hexacanth embryo, the oncosphere, which has three pairs of hooks (Chatterjee, 1980; Kassai, 1999; Thompson and McManus, 2001).

The adults of *E. multilocularis* are characterized by a smaller size (length of up to 4.5 mm), a mean number of five segments, and a sack–like uterus (Eckert and Deplazes, 2004).

2.3 Life Cycle

E. granulosus is an obligatory heterogeneous parasite with a complex life cycle. It requires two mammalian hosts to complete its life cycle. This involves the definitive hosts (for example, domestic dogs and wild canids) and the intermediate hosts (for example domestic and wild ungulates, humans) (OIE, 2004). The definitive host is infected by ingestion of offals containing fertile hydatid cysts (i.e. cysts with viable protoscolices). The protoscolices evaginate and attach to the intestinal mucosa and develop into adult stages (McManus *et al.*, 2003). The pre-patent period of *E. granulosus* in the definitive host ranges from 34-58 days (Thompson, 1995). The adult worm passes out gravid proglottids containing eggs, or free eggs are passed out with the faeces. These gravid proglottids, or eggs, are dispersed and contaminate the environment, feed, grass or water, etc, which are sources of infection to many intermediate hosts, including humans, over a wide area (Soulsby, 1982; Gemmell and Lawson, 1986; Thompson, 1995).

The infective eggs in grass, feed or in water are ingested by the intermediate hosts (Horton, 2003), and hatch into oncospheres (larvae) inside the stomach and intestines. The liberated larvae penetrate the small intestine and reach their final localization passing through vascular and lymphatic systems to the liver and lungs. They rarely spread to other organs (Soulsby, 1982).

Humans are normally accidental intermediate hosts because they are rarely involved in the transmission cycle. They can be considered as ecological aberrant hosts (Torgerson and Heath, 2003).

Once the oncosphere has reached its final location (liver and lungs), it develops into the metacestode stage (primary cyst). The time for development of the cysts is variable and may take several months (6-12 months) (Thompson, 1995). The developed cyst is unilocular, thick-walled, spherical in shape and fluid-filled. Its size ranges from 2 to 30 cm in diameter. But, in areas where there is unrestricted growth, the cyst may be very large and contains several liters of fluid (Urquhart *et al*., 1988). It is lined with an inner germinal membrane that produces brood capsules. On the inner wall of the brood capsules, an asexual budding process of protoscolices enhances the infectivity and compensates for low sexual reproduction. That produces thousands of protoscolices within a single cyst. Each single protoscolex is capable of developing into a sexually mature adult worm. Once the definitive hosts consume the organs with fertile and viable cysts they become infected and the life cycle is completed (Thompson and McManus, 2001).

The metacestode of *E. multilocularis* cysts are located exclusively in the liver and are not enveloped by host connective tissue. Therefore, it occurs as cancer-like growths in the liver parenchyma of intermediate hosts (mainly rodents and aberrant host animals and humans) (Hendrix, 1998; Eckert and Deplazes, 2004).

2.4 Pathogenicity and Clinical Signs

There are no pathogenic effects in definitive hosts even if the animals are heavily infected with *E. granulosus* (Eckert and Deplazes, 2004). Therefore final hosts, (mostly dogs), infected with *E. granulosus* show no clinical signs except itching on the back (sledge-like position) but if a large number of parasites are present, they may have diarrhoea.

The pathogenicity of the hydatid cyst depends on the severity of the infection and the organs involved. The clinical signs are not obvious in intermediate hosts (sheep, goats, cattle, horses, etc.) (Eckert and Deplazes, 2004). The disease is rarely diagnosed before slaughter of the animals. Sometimes animals show clinical symptoms, such as bronchopneumonia, hepatic disorders leading to ascitis; jaundice; heart failure; slow growth; weakness and lameness, but symptoms depend on the location of the cysts (OIE, 2005).

Most humans infected with hydatidosis are asymptomatic. In general, more than 90% of the cysts are found in the liver, lungs, or both, but are rarely (2-3% each) located in the brain, bone, heart, kidney and other tissues (McManus *et al*., 2003). These cysts are not destroyed by the body's defense mechanism, and may develop into hydatid cysts. Clinical signs can take months to years to develop and become more apparent as the cysts grow. As the cysts increase they can cause pain in the upper abdominal region, occlusion or dysfunction according to the function or area of the affected organs. The most serious development is if the cyst ruptures. The cysts may rupture into the thoracic or peritoneal cavity, causing anaphylaxis or secondary cystic echinococcosis, or into the biliary free, leading to cholangitis and cholestasis (Eckert and Deplazes, 2004).

2.5 Epidemiology and Geographical Distribution

Hydatidosis has a cosmopolitan distribution. The different patterns of distribution and transmission of this disease depend on a multiplicity of the hosts, agent and environment factors in different areas (Bhatia and Pathak, 1990).

E. granulosus is a silent cyclozoonotic infection of high global prevalence showing several regional peculiarities (Bhatia and Pathak, 1990). It is primarily maintained through domestic and sylvatic life cycles. The transmission dynamics of the parasites are determined by the interaction of factors associated with the two hosts (definitive and intermediate) and with the external environment (Permin and Hansen,

2004). The epidemiological factors and the transmission dynamics of *E. granulosus* and other members of the family *Taeniidae* (notably *Taenia hydatigena* and *Taenia ovis*) are (i) the biotic potential of the parasite, (ii) the immunity acquired by the intermediate host, and (iii) environmental factors (Eckert and Deplazes, 2004).

The biotic potential can be defined as "a potential number of viable cysts which can be established in an intermediate host by an individual definitive host per day" (Gemmell *et al.*, 2001). The parameters that determine the biotic potential are the proportion of larvae transforming into tapeworms, the number of worms per infected host, the mean number of eggs per proglottid, the mean number of proglottids shed per worm per day, the number of eggs shed from the average infected dog per day and the proportion of eggs transforming into viable cysts (Gemmell and Roberts, 1995). Thus biotic potential may vary widely in different ecological situations and climatic zones (Gemmell *et al*., 2001; Eckert and Deplazes, 2004).

The incidence and prevalence of the disease in intermediate hosts are affected by, among other factors, the age of the host upon infection and the immune response of the host (Soulsby, 1982). Acquired immunity of both the definitive and intermediate hosts plays an important role in regulating the transmission dynamics of *E. granulosus* (Gemmell and Roberts, 1995). Strong immunity can also be experimentally induced against *E. granulosus*, but it requires much larger numbers of eggs (approximately 50,000 eggs per animal) (Eckert and Deplazes, 2004). The infected definitive host passes thousands of eggs daily through faeces. These eggs are free-living and can be adversely affected by the external environment. The survival of the infective eggs is influenced by environmental factors, such as humidity, temperature, vegetation cover and soil types (Soulsby, 1982; Wachira *et al*., 1991). High temperatures and desiccation are the most important factors limiting the survival time of *Taenia* eggs in nature (Schantz, 1996). In contrast, the eggs are highly resistant and can remain infective for many months or up to about one year at lower ranges of temperatures (about 4^0C to 15^0 C) (Thompson and McManus, 2001; Eckert and Deplazes, 2004). On the other hand, *E. granulosus* eggs can survive freezing temperatures (Gemmell and Lawson, 1986; Veit *et al*., 1995), and for a few hours in

hot, dry climates such as in the Turkana area in Kenya (Wachira *et al*., 1991). These eggs may be dispersed over considerable distances from the faeces and may be transmitted to the intermediate host via pasture or contaminated water. This has important epidemiological implications since a single dog can thus infect many sheep over a wide area (Gemmell and Lawson, 1986).

It has also been shown that flies and possibly other insects may mechanically transport eggs over considerable distances, having been contaminated during feeding or egg-laying activities in or on faeces (Gemmell and Lawson, 1986). The intermediate hosts include most herbivores and omnivores sheep, goats, all antelopes, donkeys, buffaloes, cattle, horses, camels, pigs, marsupials (kangaroos), cervids, zebra, warthogs, and also humans. The level of infection in the intermediate host is determined by the number of viable eggs and the different animal species.

Also, abattoirs can play an important role in the spreading of a number of zoonotic diseases including echinococcosis/hydatidosis (Wachira *et al*., 1991). The occurrence of hydatidosis can be affected by factors such as poorly equipped slaughterhouses, home and open slaughtering, improper disposal of infected organs, and large populations of stray dogs especially around slaughtering areas. These factors are influenced by the type of legislation in place and the level of awareness of the human population (Gemmell, 1987; Dar and Alkarmi, 1997).

An additional factor in the epidemiology of *E. granulosus* is the phylogenetic adoption of *E. granulosus* between final hosts and intermediate hosts. This non-immune factor leads to the development of different species-strains of *E. granulosus* according to Thompson and McManus (2002) are given below.

Species strain/isolate (genotype)	Intermediate hosts
Sheep (G1)	Sheep, cattle, pig, camel, goat, macropod
Camel (G6)	Camel, goat, cattle
Cattle (G5)	Cattle, buffalo, sheep, goat
Buffalo (G3?)	Buffalo, Cattle (Asia)
Horse (G4)	Horse, other equines
Pig (G7)	Pig
Tasmania sheep strain (G2)	Sheep, Cattle

In general, an intermediate host-adapted strain can not develop hydatid cysts in other intermediate hosts or grow to sterile cysts lacking protoscolices. The variability of 'strain' may be based on differences in nucleic sequences, and reflected in phenotypic characters that affect the life cycle pattern, host specificity, development rate, reproductive biology, antigenic diversity, metabolism, pathogenicity, transmission dynamics, in-vitro growth, morphology, and epidemiology (McManus, 1996; Gemmell *et al*., 2001).

Also, the transmission dynamics of *E. multilocularis* are based on the predator-prey relationship between carnivores and small mammals and depend on a large number of interacting factors such as geographic and spatial distribution, population size and dynamics, feeding habits, behavior, relation to human habits, dispersal and survival of eggs in environment, condition of climate and weather, biology and longevity, animal species, susceptibility, resistance, and immunity etc (Eckert and Deplazes, 2004).

The geographical distribution of hydatidosis in intermediate hosts has been extensively studied and is present in Asia, Africa, South and Central America and the Mediterranean region (McManus and Smyth, 1986). The adult *E. granulosus* has a world-wide geographic range and occurs on all continents including the circumpolar, temperate, subtropical and tropical zones (Bhatia and Pathak, 1990; Schantz *et al*., 1995; Craig *et al*., 1996).

The different strains occurring in intermediate host species are; the sheep strain (G1) in Europe, the Middle East, Africa, India, Nepal, China, Russia, the Australian mainland, Tasmania, New Zealand, the United states and South America; the camel strain (G6) in the Middle East, Iran, Africa, China, Nepal and Argentina; the cattle strain (G5) in Europe, South Africa, India, Nepal, Sri Lanka, Russia and South America; the pig strain (G7) in Poland, Slovakia, Ukraine, Russia and Argentina; the horse strain (G4) in Europe, Tasmania and South Africa. The Tasmanian sheep strain (G2) is found in Tasmania, and Argentina. The buffalo strain (G3?) has not yet been found in Asia (Pearson, *et al*., 2002; Thompson and McManus, 2001; Thompson and McManus, 2002).

2.5.1 Europe

In Europe, *E. granulosus* has an uneven geographical distribution with very low prevalence in some of the northern and central countries, but with medium or high prevalence in southern, south-eastern and eastern regions. Iceland and Greenland are free of the parasite (Schantz *et al*., 1995; Eckert, 1996).

The highest prevalence of cystic echinococcosis in humans and livestock and *E. granulosus* in dogs has been reported from countries adjacent to the Mediterranean region and the former Soviet block and adjacent countries (Eckert and Deplazes, 2004). In most of Europe, *E. granulosus* is perpetuated in synanthropic (domestic) cycles with the dog as the final host and domestic ungulates (cattle, sheep, goat, pig, horse, and donkey) as intermediate hosts (Schantz, 1996).

The intermediate hosts of *E. multilocularis* are rodents, but pigs, monkeys, dogs and man are paratenic intermediate hosts (Eckert and Deplazes, 2004). Alveolar hydatidosis is endemic in seven European countries. These are Austria, Belgium, France, Germany, Liechtenstein, Luxembourg, and Switzerland. Isolated cases have also been reported from Sweden, Great Britain, the Czech Republic, Hungary, Slovenia, Bosnia, Bulgaria, Poland and Greece (Schantz *et al*., 1995).

2.5.2 America

In Central America, such as Mexico, Guatemala, El Salvador and Honduras, the cysts of *E. granulosus* are known to occur in animal hosts and sporadically in humans (Thakur, 1999). In North, South and Central America the disease is reported to occur mainly in dog-sheep and dog-pig cycles (Schantz, 1996).

In humans, alveolar echinococcosis is also found in parts of North America too (Eckert and Deplazes, 2004).

2.5.3 Africa

E. granulosus has been reported from most African countries. Previous and recent reports describe the endemic occurrence of *E. granulosus* in dogs and livestock, and human cases of cystic echinococcosis in all North African countries bordering the Mediterranean Sea, including Morocco, Algeria, Tunisia, Libya and Egypt (Ibraham and Gusbi, 1997; Kachani *et al*., 1997; Ouhelli *et al*., 1997; Shambesh, 1997). In north and sub-Saharan Africa, the parasites are transmitted in a synanthropic cycle involving dogs (domestic and stray dogs) and various livestock animals (sheep, goats, cattle, and camels) are most important, but wild-life cycles exist involving a number of wild carnivores (jackal species, hyena, lion etc), wild ruminants and pigs. About 22-36% of dogs were found infected with *E. granulosus* and about 3-48% livestock were infected with hydatidosis in different north African countries. Human-life cycles involving wild carnivores: Golden jackal (*Canis aureus*) in Algeria and the fox species (*Vulpus rueppelli*) in Egypt can be hosts of *E. granulosus*. The prevalence rate of CE in humans is higher in Morocco, Algeria, Tunisia, Libya and certain parts of sub-Saharan Africa (Eckert *et al*., 2001b).

In Africa, *E. multilocularis* has been reported in Zimbabwe (Bordon *et al*., 1989) and northern Tunisia (Schantz, *et al*., 1995).

2.5.4 Australia

E. granulosus is widely distributed in the mainland of Australia, as reports showed on the occurrence of the parasites in domestic or wild animal hosts in Western Australia. There are several transmission cycles: a) the synanthropic cycle, involving domestic dogs and domestic herbivores, with predominantly sheep as intermediate hosts, b) the sylvatic cycle with wild dogs (dingoes, feral dogs or dingo/feral dogs hybrids) and red foxes (*Vulpes vulpes*) as definitive hosts and macropod marsupials (kangaroos) as intermediate hosts, and c) the mixed synanthropic/sylvatic cycle (Eckert *et al*., 2001 b).

2.5.5 China

According to data collected in 1985, *E. granulosus* is present in 87% of China's territory (Craig *et al*., 1991). The principal animal intermediate host in northern China is the sheep. The infection rates in sheep were high (> 50%) in all endemic provinces or autonomous regions (Zhu, 1985). Also high rates, 7-71%, 7-17%, 56%, 21% and 19-83% of infection with *E. granulosus* were found in the definitive host (domestic dog) in Xinjiang, Gansu, Ningxia, Sichuan and Qinghai endemic regions respectively. The average prevalence rates for dogs are usually 15-30% (Craig, 2004). Echinococcosis/hydatidosis has emerged as a major public health problem. Data presented at the meeting in Urumqi 1999 indicated there was probably a conservatively estimated 500,000 existing human cases of echinococcosis requiring treatment, since the disease has emerged as a public health problem. This figure had increased to 600,000 to 1.3 million people at the meeting at Dunghuang in 2,000 (Ito *et al*., 2003). CE is probably the most frequent echinococcosis, with nomad communities in ethnic minority populations of mountainous areas in the western Provinces and Autonomous Regions, (such as Tibetans, Kazakhs, and Mongols) (Wang *et al*., 2001), being most at risk (Shi, 1997; Wen and Yang, 1997). Recently, they identified *Echinococcus shiquicus n. sp.* in Tibetan foxes, and yaks as intermediate hosts and are additional risks for human infections (Xiao *et al*., 2005).

Apart from echinococcosis/hydatidosis *E. multilocularis* were found in Tibetan areas. In the northern part of China in the Gansu province ultrasonographic and immunodiagnostic surveys (1991-1997) have revealed a very high prevalence (4%) of examined persons had been documented with alveolar echinococcosis (AE) (Eckert and Deplazes, 2004).

2.5.6 South Asia

Cystic hydatid disease has been reported to occur in South Asian countries, i.e. in India, Nepal, Bhutan, Pakistan, Sri Lanka and Afganistan (Schantz *et al*., 1995; Schantz, 1996). A great variety of species serve as intermediate hosts: buffalo (bos bubalis), cattle, sheep, goat, swine and camel. In most countries stray and semi-domesticated dogs are the principal definitive hosts, and are abundant and readily become infected from home-slaughtered livestock and from scavenging offals disposed carelessly at commercial abattoirs (Schantz, 1996).

E. granulosus infection in definitive hosts has been reported as endemic in the southern and northern regions of India (Bhatia and Pathak, 1990). In these regions, prevalence of hydatidosis was found at 7.0% in sheep, 7.1% in cattle, 9.5% in water buffaloes, and 11.5% in pigs (Hafeez, 1997). A large number of hydatidosis in humans has also been reported from different parts of the India.

2.5.7 Nepal

In Nepal, the infection of hydatid disease is increasing. Only limited research has been done on this disease. During 1993-1995 in Kathmandu Valley, hydatid cysts were found in different slaughtered animals. Joshi *et al*. (1995) showed that 5% of the carcasses of water buffalo, 3% of goat, 8% of sheep, and 7% of pig were found positive for hydatid cysts by studying 17 different slaughtering sites in Kathmandu. Latter on Joshi *et al*. (1996 a) also showed that from the total examined livestock, 18% buffaloes, 9% sheep, 4% goats and 9% pigs were found to be positive for hydatid cyst. Further, a case study by Daley (2003) in Kathmandu showed that 26% of

slaughtered buffaloes were found positive for hydatid cysts. Khatri (2003) found a 22.98% occurrence of hydatidosis in buffaloes in three adjoining towns of Kathmandu Valley viz. Banepa, Panauti and Dhulikhel. Bajagai (2004) found 12.35% positive cases of hydatidosis in buffaloes in a study conducted in two wards (number 19 and 20) of Kathmandu municipality.

Joshi *et al*. (1995) showed that the highest prevalence of 5.7% *E. granulosus* was seen in domestic dogs from an area of Kathmandu city by using the coproantigen ELISA test. Also, Ratala *et al*. (1996) showed that dog fecal samples tested for *E. granulosus* by coproantigen ELISA technique were 4% positive. They also showed that out of 20 street dogs necropsies, 3 (15%) were infected with adult *E. granulosus*.

Joshi *et al*. (1995) further reported that during 1994/95 (up to July) a total of 120 human cases of hydatidosis were operated on in different hospitals in Kathmandu. Most of the patients had cysts in the liver (55%), lungs (43%), kidney (2%) and other organs (1%). Also 14% (115/831) of human sera samples were found to be positive for echinococcal antibodies using a commercially produced ELISA screening test (Joshi *et al*., 1996 b).

2.6 Diagnosis

The diagnosis of hydatidosis in humans is based on clinical, radiological, microscopic, computed axial tomography (CT scanning), immunological (Bhatia and Pathak, 1990; McManus *et al*., 2003) and magnetic resonance imaging (MRI) methods (Khuroo, 2002). Clinically it is difficult to diagnose without the aid of laboratory investigation. If the CT scan shows a cyst regardless of confirmation by serology, a diagnosis should be made (King, 2000).

The diagnosis of cystic echinococcosis in livestock of *E. granulosus* is mainly based on necropsy findings (Eckert *et al*., 2001 a). Diagnosis by clinical examination is rarely made. Ultrasound examination for cystic structures may be used for the

diagnosis in smaller animals, such as sheep and goats, but it has been also used in the horse (Eckert *et al*., 2001 a). Diagnosis of hydatidosis is by post mortem examination of tissues, especially livers and lungs. In post-mortem examination lesions are found to be fluid-filled cysts. The size of cysts may vary from about a ping-pong ball to a cyst comprising several liters of fluid. More than 90% of the cysts occur in the liver and lungs but may occasionally occur in other organs such as kidneys, spleen, omentum, heart etc. (McManus *et al*., 2003). Cysts may contain many protoscolices (fertile cyst) or only fluid (sterile cyst). These cysts are differentiated from other diseases like tuberculosis, tumors, pulmonary sequestration, which are seen as a parenchymatous opacity (Pawlowski *et al*., 2001) and can also be diagnosed by immunological tests and by molecular methods.

Immunological tests, although useful in humans, are less sensitive and specific in livestock and at present cannot replace necropsy (Craig *et al*., 2003; OIE, 2004). Clinical practice in humans should be noted that enzyme- linked immunosorbent assays (ELISA) using crude hydatid cyst fluid have a high sensitivity (over 90%) but specificity is often unsatisfactory (Eckert and Deplazes, 2004). Currently there is no suitably sensitive and specific serological test available for hydatidosis for any livestock species (Eckert *et al*., 2001 a). But a study by Kittelberger *et al*. (2002) evaluated three ELISA tests employing a purified Antigen B sub unit, a recombinant EG95 oncosphere protein (OncELISA) and a crude protoscolex antigen (ProtELISA) in naturally or experimentally infected sheep with *E. granulosus*. The highest diagnostic sensitivity was 63% with the protoscolex antigen, but specificities were high at around 96%. Although these sensitivities are relatively low in terms of individual cases, the assay could be beneficial if used on a flock basis. Also, recently Simsek and Koroglu (2004) investigated antigenic characteristics of hydatid cyst fluid in sheep by the SDS-PAGE method, to evaluate sensitivity and specificity of ELISA and Enzyme-Linked Immunoelectrotransfer Blot (EITB) assay for the diagnosis of sheep hydatidosis, and to determine seroprevalence of hydatidosis in the sheep population. The result showed that 116kDa band was specific in sheep tested by EITB by using the antigen prepared from sheep hydatid cyst fluid. Sensitivity and specificity of EITB assay were determined as 88% and 84% whereas corresponding

rates for ELISA were 60% and 94% respectively. Also seroprevalence of hydatidosis in sheep was found as 62% by ELISA and 66.4% by EITB. Recent studies suggest that EITB has potential to serve as an inexpensive diagnostic, surveillance and research tool in sheep. The cysts of different strains can be distinguished by molecular methods by PCR and different primers.

The disease in definitive hosts is mainly diagnosed by the presence of adult tapeworms attached to the dog's small intestine. But, in post-mortem examination of dogs, the small tapeworms lie deeply between the villi of the intestinal mucosa, and are often overlooked. In living dogs, examinations of faecal samples are done by coproscopy, but the *Taenia* eggs can not be differentiated, except excreted proglottids resembling rice-corn like. Nowadays, *Echinococcus sp*ecies can be diagnosed by several indirect methods such as: serum antibodies; copro-antigens and parasite DNA in fecal samples (OIE, 2004; *Craig et al*., 2003). ELISAs for specific copro-antigens have been developed, that have sufficient specificity and sensitivity for detecting *Echinococcus* species in faecal material from infected dogs and definitive hosts. Also, ELISA based on detection of antibodies has been developed for diagnosing *Echinococcus* species in the serum of infected final hosts. ELISA based on the detection of specific antibodies has not reached a practical stage as it does not differentiate between current and previous infections (OIE, 2004; Craig *et al*., 2003). Recently Benito and Carmena (2005) reported that double-antibody sandwich ELISA can be used for the detection of *E. granulosus*. Also DNA analytical techniques have been developed such as PCR-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA-PCR (RAPD-PCR), and southern blotting (SB) offering the most direct approach to the characterization of distinct species and strains of *Echinococcus* (McManus, 1996).

The copro PCR test is aimed at detecting *Echinococcus* DNA present in *Taenia* eggs of fecal samples. It gives more accurate estimation of the potential risk for human infection than ELISA-based methods. For the development of an *E. multilocularis*-specific PCR copro test two different genes have been used: the U1 snRNA gene (Bretagne *et al*., 1993) and the mitochondrial 12 sRNA gene (Dinkel *et*

al., 1998). Until now, no equivalent copro DNA test has been developed for *E. granulosus*. An *E. granulosus*-specific PCR was described that utilized the mitochondrial CO1 gene with reported sensitivity equivalent to the detection of 200 eggs and no cross-reactions were observed with DNA from *E. multilocularis, T. hydatigena, Taenia ovis* or *Dipylidium caninum* cestodes (Cabrera *et al*., 2002). Also, Abbasi *et al*. (2003) reported that this test enabled species-specific identification of *E. granulosus* isolated ova, but with a sensitivity of only several hundred eggs. The sensitivity limit was at least 100 eggs per gram of faeces. The reported sensitivity of the PCR was lower for *E. granulosus* than for the *E. multilocularis*.

2.7 Therapy and Treatment

2.7.1 Treatment against Tapeworms in Dogs

A wide variety of anthelmentics (arecolin, niclosamide, benzimidazole compounds and praziquantel) are available for the treatment of echinococcosis in dogs (Bhatia and Pathak, 1990). Praziquantel, the drug of choice, is effective against both juvenile and adult *Echinococcus* parasites. The dosage rate of praziquantel is 5 mg/kg (Eckert and Deplazes, 2004; OIE, 2005).

2.7.2 Treatment of Cystic Stages in Humans

In humans, surgical removal of the hydatid cyst is the treatment of choice for symptomatic cysts (Safioeas *et al*., 1999). Several of the benzimidazole compounds have been shown to have efficacy against hydatid cysts. Long term treatment with albendazole has a particularly marked effect on the cysts, and is used as pretreatment before surgery (Morris *et al*., 1990; Horton, 2003). The albendazole sulphoxide was shown to be an active anthelminthic (Horton, 2003). The current recommendation by the World Health Organization is percutaneous puncture under sonographic guidance, aspiration of cystic fluid, injection of a protoscolicidal agent (alcohol) and re-

aspiration of cyst content. This procedure needs to be further evaluated in larger studies (Riengchan *et al*., 2004).

2.7.3 Prevention and Vaccination

There is no drug treatment in herbivores or omnivores. The control procedure used to eliminate echinococcosis from Iceland, the Falkland Islands, Tasmania and New Zealand is not appropriate to continental environments. A vaccine to protect grazing animals against infection is an additional control method that focuses on grazing animals instead of the dog (Health *et al*., 2003). Vaccination with a recombinant oncospheral *E. granulosus* antigen (EG95) induces high degrees of protection, reducing the cyst numbers in vaccinated sheep by approximately 90-100% (Health *et al*., 2003). A high degree of immunity (about 80%), persists for six months (in absence of re-infection), and pregnant ewes vaccinated before lambing transfer high levels of antibody to their lambs (Health and Lightowlers, 1997). While considerable research has been undertaken with crude antigens to protect dogs from echinococcosis, no real success has been demonstrated so far (OIE, 2004).

3. MATERIALS AND METHODS

3.1 Study Area

The area selected for this study was Kathmandu Valley (Figure 1) in Nepal. Kathmandu is the capital city of Nepal. The valley covers about 530 sq. km. or 0.36% of the total land surface but has a population of about 1.645 million i.e. 8.6% of the total population of the country (CBS, 2004). Kathmandu is situated at 1320 meters above sea level and has a temperate climate. Being the capital city, it is also the main economic and business center of the country.

Samples for investigating hydatidosis of slaughtered buffaloes were collected at a buffalo slaughterhouse, private (individual) butchers' places and riversides where livestock are slaughtered.

Faecal samples from dogs for investigating *Echinococcus* were collected from compounds around the slaughterhouse, areas of private (individual) butchers' places, riversides, garbage sites and temple sites.

The duration of the sampling period was from November, 2004 to April, 2005. Field work was performed in the Kathmandu Valley, Nepal. Laboratory diagnosis was performed partly in the Central Animal Disease Investigation Laboratory, Kathmandu, Nepal and partly in the Institute of Parasitology, Freie Universität Berlin.

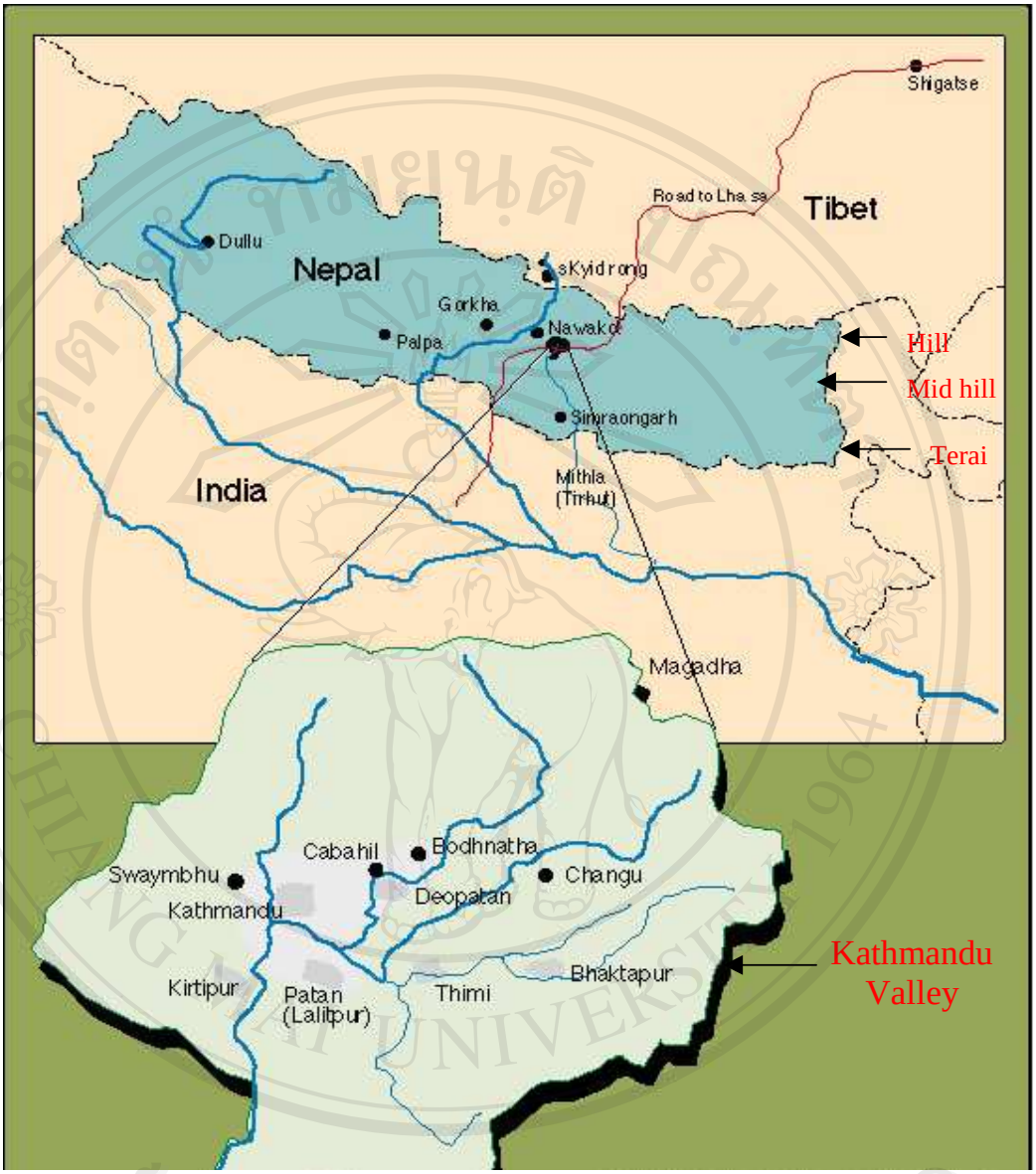

Figure 1. Map of Nepal and Kathmandu Valley

3.2 Justification for the Site Selection of the Study Area

Today, the Kathmandu Valley is polluted due to a large number of factors including a high density of population, contaminated water, lack of proper hygiene and sanitation facilities and an uncontrolled and unmanaged dog population etc. The dog population in Nepal is more than eighteen million (Joshi *et al*., 2003). Ruminants are slaughtered daily in one slaughterhouse, at riversides places, and in open areas. Butchers kill animals either in small courtyards or on the ground floors of their own houses. The fresh meat is distributed either through small outlets or directly to the customer. Nepal is dependent on the importation of animals from India, even though the States of India from which animals are obtained ban the export of animals for slaughter. This makes the meat sector dependent on clandestine trading activities (TLDP, 2002). Also the major buffaloes markets in Nepal are located in the Terai area where Indian and Nepalese buffaloes are mixed and flow into the marketing system (TLDP, 1999). Buffalo meat is in high demand in the Kathmandu Valley due to economic and cultural factors. Most of these buffaloes are brought to the Kathmandu Valley from the Terai region. Only a few people are raising ruminants in their own houses in the Kathmandu Valley. In this valley, there is inadequate water supply and waste disposal facilities. The institutions and governmental rules for slaughter are also weak, resulting in almost no monitoring. So, when people kill animals, they leave the leftover carcass on the ground. Dogs, pigs and birds come to these places for scavenging, leading to extended contamination. Rumen contents are thrown in the river, thus polluting the water. In turn people use river water for cleaning vegetables and washing clothes. Also children come to play around these places where dog's faeces are additionally present, at riversides and contaminated places. So there are high chances of occurrence and the spread of zoonotic diseases through these sources. Hence, an enquiry in this area is expected to be beneficial not only to identify disease occurrence, but also to initiate improvement measures.

3.3 Research Design and Study Process

The study was designed as a descriptive cross-sectional survey. The study is based on two categories of samples: buffaloes and stray dogs. However, poor hygiene and sanitation seriously raise questions as to how far there is a risk of hydatidosis due to contamination by stray dogs. The study, therefore, concentrated on surveying carcasses and collecting cysts from buffaloes after observing and identifying the infected carcasses. Similarly, in how far stray dogs were affected with *E. granulosus* was studied by collecting and examining their faeces. In how far dogs were affected was compared among stray dogs around the slaughtering places and garbage sites and those around temple sites. The collected samples were tested in laboratories.

Apart from these, an awareness survey was made with the butchers to understand their practices and collect opinions regarding the various aspects of slaughtering, knowledge and understanding of echinococcosis/hydatidosis and their behavior. The questionnaire for enquiry has been included in Annex I.

Besides, the butchers were also given information on good practices and health risks from unhygienic practices to enable them to understand the gravity of problems emanating from poor hygiene and lack of knowledge. This is expected to help significantly in generating awareness among butchers, vital for human and livestock health particularly in view of their poor awareness.

3.4 Sample Size and Sampling Method

There is no specific census of the population of butchers, number of buffaloes slaughtered and number of stray dogs in the Kathmandu Valley. Hence, it was difficult to make a proper sampling plan. Therefore, the following sampling processes were adopted.

- First of all, buffaloes slaughtering places were mapped in consultation with the Butchers' Association of Kathmandu.

- Secondly, the total number of slaughterhouse, riverside slaughter places, and butchers' individual slaughter places were estimated.

The association provided information that in a slaughterhouse and at four riverside areas, buffaloes are slaughtered, and 103 butchers in the valley were involved in slaughtering buffaloes as well. In view of these numbers provided by the Butchers' Association for this study, the slaughterhouse (100%) and 50% of the riverside places were selected for sampling. Similarly, 33% of the butchers were selected for sampling purposes. For the selection of the sample of slaughtered buffaloes, a convenient sampling method was applied. In view of the proposed sampling method, an attempt had been made to enhance the sample size to make the sampling more representatives.

The proposed sample size of the slaughtering area and the sample size of the buffaloes are listed in the following tables.

Table 1. Proposed sample size for buffaloes slaughtering areas

Sites	Number	Selected number	Percentages
Slaughterhouse	1	1	100
Riversides	4	2	50
Individual butchers	103	34	33

Table 2. Sample size of slaughtered buffaloes

Sites	Proposed sample size	Examined sample size	No. of visits days
Slaughterhouse	100	120	10
Riversides	96	120	24
Individual Butchers	204	260	34
Total	400	500	68

The total proposed sample size for buffaloes investigated for hydatidosis was 400 in 68 total visits. However, following the flexible strategy, in the same 68 visits, 500 buffaloes' samples were investigated in the following manner. The total of samples segregated for the slaughterhouse was 100. Ten sample visits were made in the slaughterhouse and on each occasion 12 buffaloes on an average were observed. From there total 120 slaughtered buffaloes were examined. In the same way, 12 visits each were made to two riversides places. Altogether, 120 slaughtered buffaloes were examined from there. Similarly, among 103 butchers places, 34 individual butchers' places were visited. The total proposed sample size in this category was 204, but on each occasion 5 to 7 butchers places were visited and altogether 260 samples were examined during 34 visits (Table 2).

For the stray dogs too, a convenient sampling method was applied. The total proposed size was 300 in order to make the study representative. As in buffaloes, the sampling plan of dogs was also made flexible to enable the increase of size, so that a larger sample size would be feasible. The distribution of the samples was as follows:

Table 3. Sample size of dogs' faecal materials

Sites	Proposed sample size	Collected sample size	Percentage
Around buffaloes slaughtering areas (slaughterhouse, riversides, individual butchers' places) / garbage sites	200	261	71.3
Temple sites	100	105	28.7
Total	300	366	100

Three hundred sixty six dogs' faecal samples were collected, more than the proposed 300. About 71.3% dog faecal samples were collected from areas where the buffaloes are slaughtered (slaughterhouse, riversides, individual butchers' places and garbage sites) and 28.7% were collected from temple sites (Table 3).

3.5 Materials Collection

- Samples: Hydatidosis or infected organs of buffaloes (livers and lungs) were collected.
- Collected cysts materials differentiated as fertile and sterile cysts.
- Faecal samples of stray dogs were collected from the ground.

3.6 Methods

The methods for field survey and laboratory examinations are specified below:

3.6.1 Hydatidosis Survey

Different organs e.g. livers, lungs, spleens, heart and kidneys of slaughtered buffaloes were examined visually for hydatid cyst. The organs were palpated carefully for any swellings for detecting hydatid cyst in each slaughtered animal. Infected organs for hydatid cyst were collected in plastic bags for further laboratory examination.

3.6.1.1 Laboratory examination of the cysts

In the laboratory, the cysts were examined to differentiate the fertile (containing protoscolices) and sterile (without protoscolices) cysts under the microscope. The materials which looked like cysts but did not fit the above criteria were discarded. The cyst of *E. granulosus* was differentiated from the cyst of *Taenia hydatigena* by the presence of one scolex or many protoscolices. A cyst of *E. granulosus* may contain a hundred to a thousand protoscolices, whereas the cyst of *T. hydatigena* contains only one scolex. The latter cyst occurs predominantly in the viscera of the abdominal cavity, but also in livers. In the laboratory, the cysts were examined as follows:

First of all the cysts were separated from the organs. Then the cyst wall was split open by a scalpel blade and the cyst content was poured into a Petri dish. One drop of fluid containing protoscolices was placed on the slide, covered with a cover slip, and was examined under the microscope at 100 and 400 magnifications. Small amount of fluid containing protoscolices was stored at -20^0 C and was brought to Berlin for PCR test. The rest cyst and fluid materials were sterilized by formalin and were discarded.

3.6.1.2 Strain identification by PCR

In order to identify the strains of *E. granulosus* present in the protoscolices samples by comparison with published gene sequences of *E. granulosus* strains (M'rad *et al.*, 2005), we undertook the procedures for DNA extraction and

amplification described in section 3.6.3.2 and 3.6.3.3., using a pooled sample of fluid taken from protoscolices.

3.6.2 Collection of Faecal Samples of Stray Dogs

Three hundred sixty six faecal samples from stray dogs were kept in plastic bags. From these samples about one gram of faecal materials was separated and was mixed with 3ml of 10% buffered formalin. The remaining samples were stored at -20^0 C for PCR test.

3.6.2.1 Microscopic examination

The collected faecal samples were examined by concentration technique. The concentration procedure recommended was the formalin-ether method (WHO, 1991). All types of worm eggs (roundworms, tapeworms, schistosomes, and other fluke eggs), may be recovered by this method. The method is as follows: Ten milliliters of 10% formalin was added to approximately 1g of faeces and stirred using an applicator stick, until a slightly cloudy suspension was observed. Wet gauze (2 layers) was fitted on top of a wax paper cup. Then the faecal suspension was passed through the gauze into the cup and the content was transferred to a centrifuge tube. More formalin solution was added to reach the final volume of 10ml. Then 3ml of ether was added. The cap was locked and was shaken well for 30 seconds and centrifuged for 1 minute at 450×g. The fatty plug (debris) was discarded with an applicator stick, and the supernatant was poured away by quickly inverting the tube. The tube was replaced in its rack and the fluid was allowed to drain down to the sediment on the sides of the tube. Then the sediment was mixed well and a drop was transferred to a slide, covered with a cover slip and was examined under the microscope by 100 and 400 magnifications. All helminthes eggs were recorded.

It is only possible to differentiate *E. granulosus* eggs from other *Taenia* ones by copro-antigen ELISA and by PCR with specific primers. Because of that, all *Taenia* egg-positive faecal samples and some randomly selected negative samples were

brought to Berlin by keeping them in a cool box with dry ice pack. Then these samples were stored at -20^{0}C.

3.6.3 Differentiation of *Echinococcus granulosus* Eggs from other *Taenia* Eggs by Polymerase Chain Reaction (PCR) in Stray Dogs' Faecal Samples

Before collecting the *Taenia* eggs for DNA extraction, all samples were kept at -80^{0}C for 5 days to inactivate the eggs present in faecal samples. Thereafter, the samples were processed as follows:

3.6.3.1 Separation of eggs from faecal materials

Three grams of dog's faecal samples were mixed with 20-30ml of zinc chloride (300g $ZnCl_2$+ 450ml water) solution. The faecal samples were well mashed with $ZnCl_2$ solution and were passed through 100µm mesh sieve to remove undigested particles. The solution was centrifuged at 400×g for 30 minutes. The supernatant was passed through a 20µm mesh sieve. The sieve was washed thoroughly with distilled water supplemented with 0.2% Tween 20 (Mathis *et al*., 1996). The materials retained in the sieve were transferred into a tube with a flattened side and centrifuged for 5 minutes at 700×g. After that the supernatant was discarded. One drop of sediment was put on a glass slide to count the eggs under the microscope at 100 magnifications. The pellet (about 300µl) obtained was used for DNA extraction.

3.6.3.2 Alkaline lysis of eggs to yield DNA

The whole 300µl pellet was taken and 25µl 1M KOH and 7µl of 1M DTT (dithiothreitol) were added, mixed and incubated at 65^{0}C for 15 minutes (Bretagne *et al.*, 1993; Sherifi, 2005). Thereafter, the solution was neutralized by adding 60µl 2 M Tris-HCl, pH 8.4 and 2µl 32% HCl, centrifuged and mixed for 30 seconds. The DNA was purified by using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Deutschland) according to the manufacturer's recommendations. Two hundred microliters lysis-

buffer AL and 20µl proteinase K were added and incubated at 56^{0}C for 10 minutes and centrifuged in 1.5ml micro-centrifuge tubes to remove drops from the inside of the lid. After that 50µl Chelex 50% (Bio-Rad Laboratories®, Hercules, München, Deutchland) was added in order to remove PCR inhibitors. A 400µl sample was taken in a new micro-centrifuge tube and 200µl ethanol (96-100%) was added, mixed for 15 seconds and centrifuged at 6,000×g for 1 minute. QIAamp Spin Column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded. Five hundred micro-liters wash-buffer AW1 was added into the QIAamp Spin Column and centrifuged 6,000×g for 1 minute. Then QIAamp Spin Column was placed in a clean 2ml collection tube, and the tube containing the filtrate was discarded. This step was repeated. After that 500µl wash-buffer AW2 was added into the QIAamp Spin Column and centrifuged at 20,000×g for 3 minutes. This step was repeated one time again. Then 50µl buffer AE (Elution Buffer) was added into the QIAamp Spin Column and incubated at room temperature for 5 minutes in order to increase DNA yield. Then samples were centrifuged at 6,000×g for 1 minute and were stored at -20^{0}C.

3.6.3.3 DNA amplification

PCR amplifications were carried out according to Pauly (2003) in 25µl (for 1 sample) reaction volumes containing the following reactive:

Reaction mixtures	Volume	Concentration
$MgCl_2$	10 µl	2.5mM
10×PCR buffer	2.5µl	1×
dNTP's (dATP, dCTP, dGTP and dUTP)	0.25µl	1mM (250µM each nucleotide)
Primers (CO1-1)/ (CO1-2)	0.5µl/ 0.5µl	1µM
DNase/RNase free Aqua dest	6.05µl	
Ampli Taq Gold® Polymerase	0.2µl	1 Unit
Template DNA	5µl	

The primer pair used for the amplification of the mitochondrial cytochrome c oxidase (CO1) gene were JB3 (5' TTTTTTGGGCATCCTGAGGTTTAT 3') forward primer and JB4.5 (5' TAAAGAAAGAACATAATGAAAATG 3') reverse primer (Boweles and McManus, 1994). The reaction mixtures were overlaid with one drop of mineral oil and centrifuged at 10,000×g for 5 seconds. The tubes were taken to a thermal cycler with the following profile:

The PCR was carried out in Trio-Thermoblock™ Thermocycler (Biometra, Göttingen, Germany), programmed to perform a denaturation step at 95^0C for 15 minutes, at 50^0C for 30 seconds and at 72^0C for 2 minutes (to activate the Taq Gold® DNA Polymerase), followed by 39 cycles consisting of 30 seconds at 95^0C, 30 seconds at 50^0C and 2 minutes at 72^0C. The last extension step was 10 minutes longer. The samples were stored at 4^0C.

3.6.3.4 Gel electrophoreses

The PCR products were electrophoresed through 2% agarose. The 25 ml gel contained 2.5µl ethidium bromide (SIGMA®, Steinheim, Deutchland). The electrophoreses chamber was filled with a buffer solution (1x electrophoreses buffer) until the top surface of the gel submerged by approximately 1 mm. The 5µl of sample buffer was mixed with 25µl of PCR product and centrifuged at 10,000×g for 20 seconds. The 10µl samples and 10µl DNA Marker were placed into agarose gel wells. For each set of PCR reaction, negative controls (no DNA) and positive controls (*Taenia hydatigena*) were included. The bands were visualized under Ultra Violet (UV) light. The control target yields an amplicon of about 440 bp upon amplification.

3.6.3.5 Purification of PCR products

The PCR products were purified by using a commercial kit (High pure PCR product purification kit®, Roche Applied Science, Mannheim, Germany). Five hundred micro-liters of binding buffer was mixed with 100µl PCR sample. The mixture was placed into a high pure micro-centrifuge filter tube and centrifuged at

13,000×g for 30 seconds. The sample was washed with 500µl wash buffer and centrifuged at 13,000×g for 1 minute. Again the sample was washed with 200µl wash buffer and centrifuged at 13,000×g for an additional 1 minute to remove the residues. To elute the DNA sample, 50µl elution buffer was added into a high pure micro-centrifuge filter tube and centrifuged at 13,000×g for 1 minute. These micro-centrifuge tubes contained the purified DNA were stored at -20^{0}C.

3.6.3.6 DNA sequences

The DNA concentration was estimated by means of a spectrophotometer, the Gene Quant Calculator® (Amersham Pharmacia Biotech, Freiburg, Germany). Approximately 100ng of purified PCR products were sent for sequencing to Sequence Laboratories Göettingen GmbH using CO1-1 forward and CO1-2 reverse primers. Sequences were recorded as chromatographic files. Forward and reverse sequences were compared for each gene fragment, and sequences were manually edited and aligned using Genedoc® software (Nicholas *et al*., 2005). The verified double stranded CO1 sequences were compared to the following GenBank CO1 sequences: U50464 (G1), M84665 (G5) and M84666 (G6) (M'rad *et al*., 2005). Mutations found in CO1 sequences were compared to GenBank with the search tool 'Basic Local Alignment Search Tool' (BLAST) with default parameters.

3.7 Data Management and Analysis

Field and laboratory data were entered into Microsoft-Excel. Data and results from experiments were presented systematically in tabular forms. Wherever applicable, percentages were calculated and presented. Comparison of two or more strata or with theoretical basis was done by applying Chi-square, Kruskal-Wallis and Fisher's exact tests. Results were discussed against the theoretical proportions and findings of previous studies. The detailed methodology for testing and laboratory procedures has been specified above in the appropriate sections.

4. RESULTS

The basic objectives of this study were to identify the occurrence of hydatidosis in slaughtered buffaloes and the prevalence of echinococcosis in stray dogs. Following the objectives, 500 slaughtered buffaloes were investigated and similarly 366 dogs' faecal samples were collected and examined. In view of the extensive use of buffalo meat in Kathmandu Valley and existence of a large number of stray dogs, these problems are considered to be of serious public health importance. The results of the study are presented below.

4.1 Occurrence of Hydatidosis in Slaughtered Buffaloes

4.1.1 Occurrence by Sites

Occurrence of hydatidosis in slaughtered buffaloes at different places is shown in the following table:

Table 4. Occurrence of hydatid cysts in slaughtered buffaloes by sites in KTM valley, Nepal.

Sites	Buffaloes			
	No. examined	No. infected	% infected	95% CI
Slaughterhouse	120	8	6.7	2.9 - 12.7
Riversides	120	12	10.0	5.3 - 16.8
Individual butchers (n=34)	260	33	12.7	8.9 - 17.6
Total	500	53	10.6	8.0 - 13.6

χ^2= 3.21, 2df, p=0.2013; CI = Confidence Interval: n= number of individual butchers

Table 4 shows that out of 120 buffalo carcasses examined in the slaughterhouse, 6.7% were infected with hydatid cysts while in slaughter sites along the riversides 10.0% of the carcasses had hydatid cysts. Of those 260 buffaloes that were slaughtered by individual butchers, 12.7% of them were found with hydatid cysts.

Overall, 10.6% of the 500 buffalo carcasses examined had hydatid cysts However, there was no significantly (p= 0.2013) different in the occurrence of hydatid cysts among the different sites.

4.1.2 Occurrence by Organs

Occurrence of hydatid cysts were examined in various organs like lungs (Figure 5), liver (Figure 6) and others organs (spleen, heart and kidney). The results are shown in following Table 5.

Table 5. Distribution of hydatid cysts by organs in 500 slaughtered buffaloes in KTM valley, Nepal.

Organ affected	Buffaloes	
	No. infected (%)	95% CI
Lungs only	32 (6.4)	4.4- 8.9
Liver only	12 (2.4)	1.3- 4.2
Lungs and Liver	9 (1.8)	0.8- 3.4
Total	53 (10.6)	8.0- 13.6

H = 18.33, d. f. = 2, p = 0.0001

CI = Confidence Interval

Table 5 shows that 6.4% of buffaloes infected with hydatid cysts were found in the lungs only, and 2.4% in livers only. The occurrence in both organs, lungs and liver

was 1.8% while no cyst was found in the spleen, heart and kidneys. It showed that the occurrence of cysts was significantly (p= 0.0001) different among the organs.

4.1.3 Fertility Status of Hydatid Cysts

Cysts were examined to differentiate whether they were fertile or sterile (Table 6).

Table 6. Occurrence of fertile and sterile cysts by organs in 500 slaughtered buffaloes in KTM valley, Nepal.

Affected Organ	Animal infected with cysts	Animal infected with fertile cysts	Animal infected with sterile cysts	% infected with fertile cysts	% infected with sterile cysts
Lung only	32	19	13	59.4	40.6
Liver only	12	7	5	58.3	41.7
Liver + Lungs	9	5	4	55.6	44.4
Total	53 (10.6%)*	31 (6.2%)*	22 (4.4%)*	58.5	41.5

( )*, Percentage of each column total out of 500 examined buffaloes

The distribution proportions of fertile and sterile cysts by organs are shown in Table 6. In a total of 32 infected lungs only 59.4% were fertile cysts and 40.6% sterile cysts. These two proportions were not significantly (p= 0.127) different. However, in a total of 12 infected livers only 58.3% were fertile cysts and 41.7% sterile cysts. These two proportions were not significantly (p= 0.408) different. Whereas in a total of 9 combined infected livers and lungs 55.6% were fertile cysts and 44.4% sterile

cysts. These two proportions were not significantly (p= 0.635) different. Also in a total of 53 infected buffaloes, 58.5% were fertile cysts and 41.5% sterile cysts. These two proportions were not significantly (p= 0.076) different. In general out of 500 slaughtered buffaloes, 10.6% of the animals were infected with hydatid cysts. Among these, 6.2% were infected with fertile cysts and 4.4% with sterile cysts. These proportions were not significantly (p= 0.204) different. The fertility of cysts was proved by microscope where the protoscolices could be demonstrated as seen in figure (7).

4.2 Prevalence of *Taenia* Eggs in Stray Dogs' Faecal Samples

Dogs' faecal samples were examined for *Taenia* eggs by the ether formalin concentration method in different sites. The results of percentage of *Taenia* egg-positives (Figure 8) in stray dogs' faecal samples are given below in Table 7.

The Table 7 shows that out of 366 total stray dogs' faecal samples, 17.6%, 14.1%, 9.6% and 7.6% were found to be positive with *Taenia* eggs around slaughter sites (slaughterhouse and individual butchers' places), riversides, garbage sites, and temple sites respectively.

Overall, 12.8% of stray dogs' faecal samples were found to be positive with *Taenia* eggs. However, the percent distribution of *Taenia* eggs positive around different sites was not significantly (p= 0.2148) different.

Table 7. Distribution of *Taenia* egg positives in dogs' faecal samples in different area in KTM valley, Nepal

Area	No. of samples	No. of positive samples (%)	95% CI
Slaughterhouse and individuals' butchers places	131	23 (17.6)	11.5- 25.2
Riversides	78	11 (14.1)	7.3 - 23.8
Garbage	52	5 (9.6)	3.2 - 21.0
Temples	105	8 (7.6)	3.4 - 14.5
Total	366	47 (12.8)	9.6 - 16.7

$\chi 2$= 5.76, 3df, p= 0.2148; CI = Confidence Interval

4.3 Prevalence of Different Helminths Eggs other than *Taenia* Eggs in Stray Dogs' Faecal Samples

Apart from *Taenia,* other eggs from different helminths were also found in the faecal samples of dogs as shown below in Table 8. Some of the various eggs found were hookworms, *Tirchuris/Capillaria, Toxocara, Spirometra* and *Physaloptera*.

Table 8. Distribution of helminths eggs other than *Taenia* eggs in dogs' faecal samples in KTM valley, Nepal

Sites	No. of samples	% of Hook worm	% *Trichuris/ Capillaria*	% *Toxocara*	% *Spirometra*	% *Physaloptera*
Slaughterhouse and individual butchers' places	131	16.0	22.9	12.2	1.5	0.0
Riversides	78	14.1	15.4	14.1	0.0	0.0
Garbage	52	36.5	26.9	19.2	5.8	0.0
Temples	105	8.6	13.3	8.6	0.0	1.9
Total	366	18.3	19.1	12.6	1.4	1.9

4.4 Results from PCR

4.4.1 Strain Identification of *E. granulosus* Cysts by PCR

The pooled sample of protoscolices from buffalo cysts was subjected to PCR and was positive. The sample was next sequenced and aligned with the genotypes G1 (sheep strain), G5 (cattle strain) and G6 (camel strain). Based on gene alignment between the sequenced gene and published sequences (M'rad *et al*., 2005), the sample showed 99.7% homology with the genotype G1 (sheep strain), 91.3% homology with genotype G6 (camel strain) and 91.3% homology with genotype G5 (cattle strain), see Figure 2.

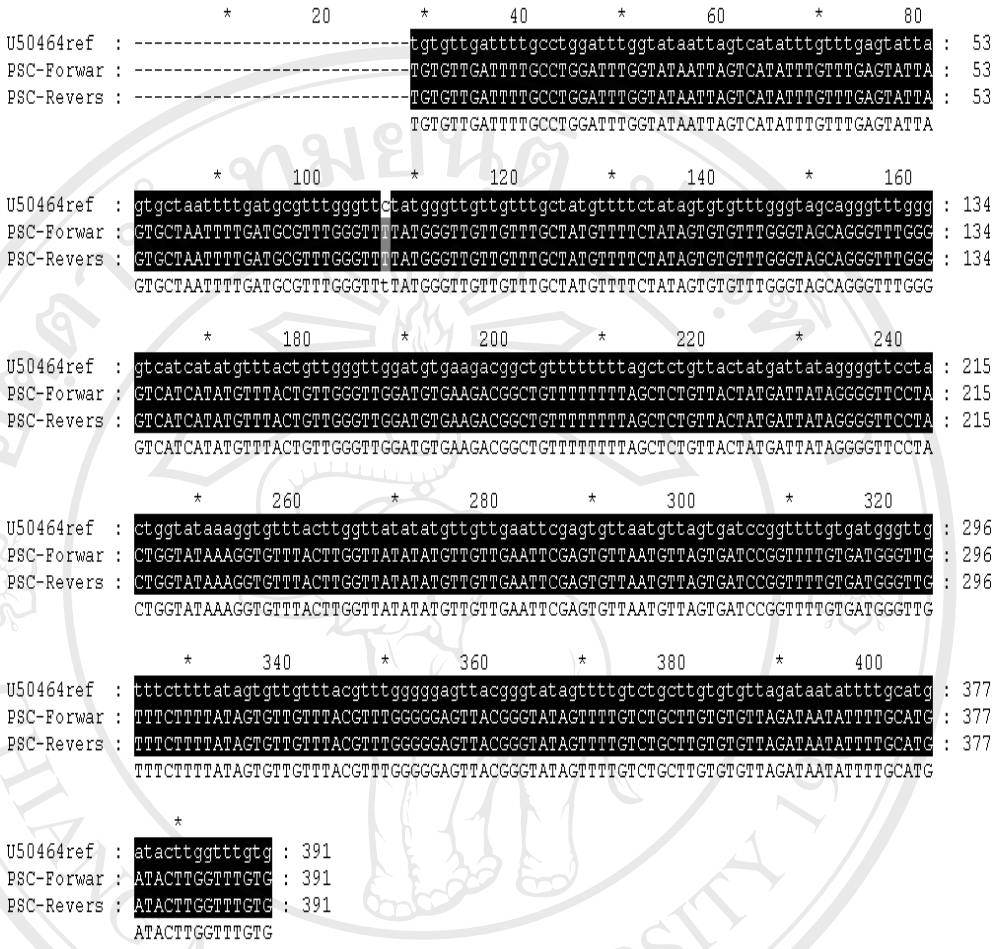

Figure 2. Gene sequence from pooled protoscolices sample (PSC-Forward/ PSC-Reverse) aligned against *E. granulosus* sheep strain G1 (U50464) (M'rad *et al*., 2005)

4.4.2 Strain Identification of *E. granulosus* Eggs from Stray Dogs' Faecal Samples by PCR

The genomic DNA of *Taenia* eggs from 47 microscopically positive dogs' faeces samples were examined by PCR. Out of these samples, 11 (23.4%) were found positive for PCR; the remaining 36 (76.6%) were negative (see Figure 3. for an example). All samples (20) that were negative by concentration technique were also negative by PCR.

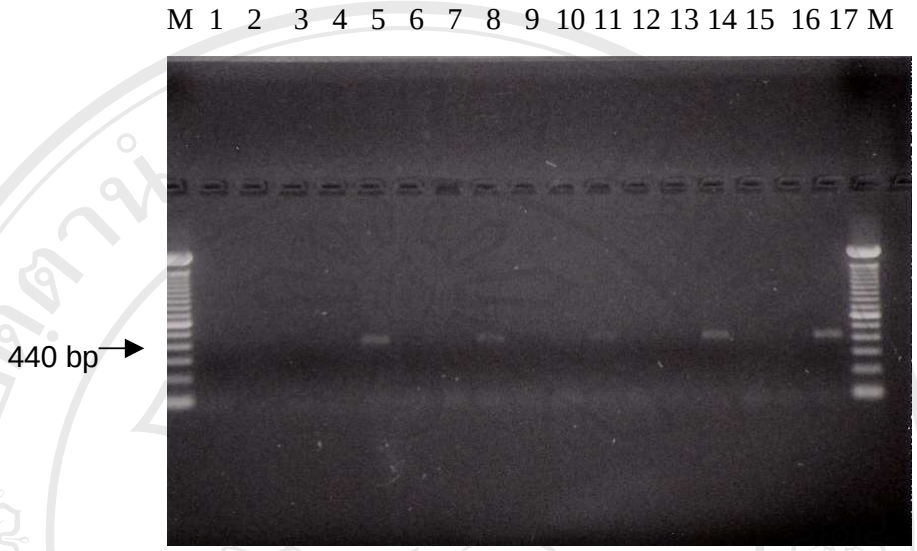

Figure 3. PCR products of genomic DNA from *Taenia* eggs amplified with mitochondrial CO1 primer. M is the molecular size marker of 100 bp. Lanes 1-15 were from faecal samples positive for *Taenia* eggs in microscope. The expected 440 bp PCR products were detected in four of the samples as shown in lanes 5, 8, 11and 14. Lanes 16 and 17 are negative and positive controls, respectively.

Five dog samples chosen from the PCR-positive samples were also sequenced and aligned with the genotypes G1 (sheep strain), G5 (cattle strain) and G6 (camel strain). Because the sequencing data received from four of the faecal samples were unsatisfactory, homology analysis was not performed on these. The one sample (sample number '3') which was analysed, showed 99.3% homology with the genotype G1 (sheep strain), see Figure 4.

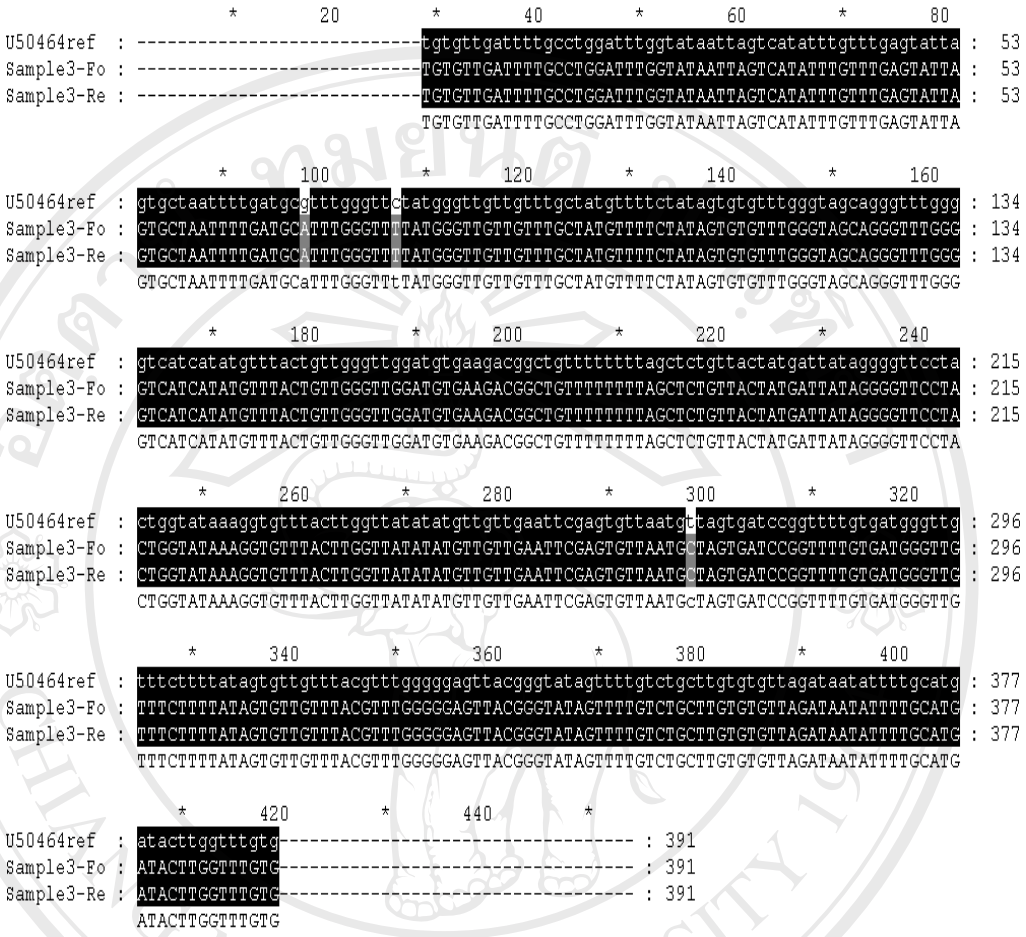

Figure 4. Gene sequence from *Taenia* eggs in a dog faecal sample (sample 3-Forward/sample 3-Reverse-sequence) aligned against *E. granulosus* sheep strain G1 (U50464) (M'rad *et al*., 2005)

4.5 Results from Questionnaire Survey

A small survey was conducted to assess the awareness of butchers regarding slaughtering practices, knowledge of the disease and use and/or disposal of waste materials and disease-infected meat (Table 9). Altogether, 37 butchers responded to the questions.

It is to be noted that only a limited number of buffaloes came from Nepal while the majority (78.4%) were mixed between Nepalese buffaloes and imported buffaloes from India.

It is clear from the response that the disease is a major animal health risk in Nepal. This is proven by the response of the butchers. Among the respondents, 78.4% percent stated that they found the cysts in the slaughtered animals sometimes or rarely, while 10.8% responded that such cysts were found quite often. Only another 10.8% said that such cysts were not found. The response of the majority (89.2%) indicated that such cysts were found sometimes/rarely or quite often, clearly indicating the wide-scale persistence of the problem in Nepal. The most worrisome factor is that a substantial majority did not know about this zoonotic problem. Only one butcher (2.7%) among 37 respondents was found to be knowledgeable about the disease. A lot of misuse of infected meat as shown by subsequent responses may have emanated from lack of knowledge.

The results derived from the survey on the use or disposal of meat are quite horrifying, indicating serious human and animal health risks. A substantial majority (81.1%) said that they sold the infected meat for human consumption after removing the cysts. A small number (8.1%) sold such infested meat for consumption by animals. In either case, the risks to dogs by contamination are aggravated. A majority of butchers (62.2%) gave such infected meat to stray dogs; this enhanced an additional risk for dogs. This response is almost similar to the question, what is the general practice of use/ disposal of infected meat in Kathmandu Valley, where 5.4% were using infected meat for animal feeds, 40.5% were using for human consumption and 54.1% for both purposes.

The waste disposal practice is also far from satisfactory. It may be due to lack of proper waste disposal sites. The 24.3% dispose waste materials at the river sides, polluting and contaminating the water body. However, 56.8% dispose waste material in open sites around the slaughtering places. Also, 18.9% were disposing at both

riversides and open sites. Both these practices are highly undesirable, and increase the possibilities of contamination of environment significantly.

Table 9. Results of questionnaire survey in KTM valley, Nepal.

No.	Question	Number	Percentage
1	Source of buffaloes		
	a. From country	8	21.6
	b. Mixed (Nepal and India)	29	78.4
2	Finding of cyst materials		
	a. Quite often	4	10.8
	b. Sometimes/Rarely	29	78.4
	c. No	4	10.8
3	Awareness about disease		
	Yes	1	2.7
	No	36	97.3
4	Use/disposal of infected organs or meat		
	a. Sell it for consumption by animals	3	8.1
	b. Remove cyst from organ and sell it for human consumption	30	81.1
	c. Cyst not found	4	10.8
5	Feeding of infected raw meat to stray dogs		
	a. Yes	23	62.2
	b. No	13	35.1
	c. No response	1	2.7
6	Disposal of waste materials of slaughtered buffaloes		
	a. Dispose it at riversides	9	24.3
	b. Open site around slaughter	21	56.8
	c. Both (a and b)	7	18.9
7	Use/Disposal of infected meat in Kathmandu Valley		
	a. Use it to feed animals	2	5.4
	b. Use for human consumption	15	40.5
	c. Both (a and b)	20	54.1

Figure 5. Lungs of buffalo infected with hydatid cysts.

Figure 6. Liver of buffalo infected with hydatid cysts.

Figure 7. Protoscolices of hydatid cyst observed under the microscope at 400 magnifications (Ocular 10x and Objective 40x).

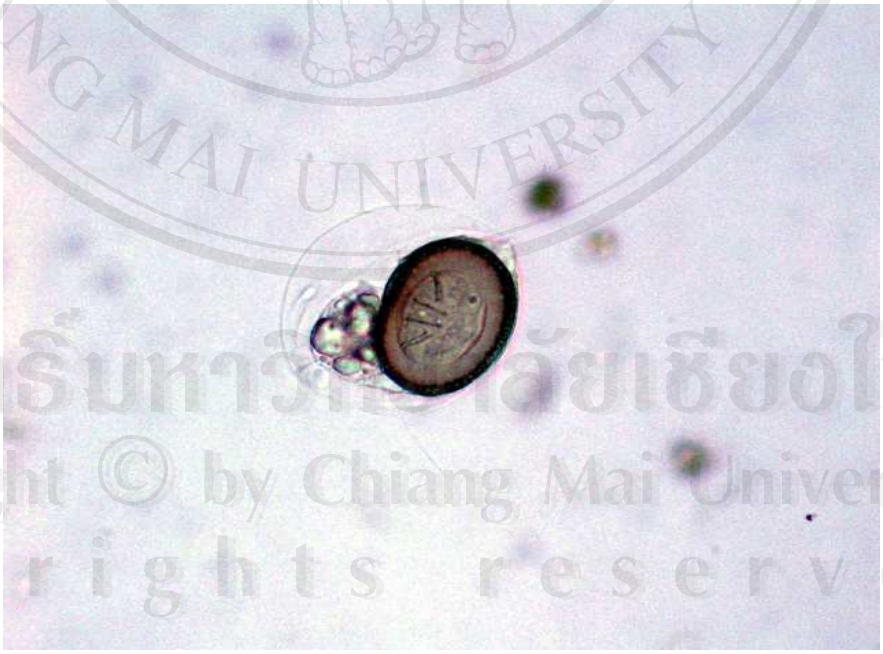

Figure 8. *Taenia* egg observed under the microscope at 400 magnifications (Ocular 10x and Objective 40x).

5. DISCUSSIONS

Meat is one of the most delicious and nutritious foods. In Nepal, the most commonly used meat is buff, mutton and chicken. Among those, buffalo meat is most popular in Kathmandu Valley, particularly among Newars, the majority of the population and other ethnic hill groups. Due to their socio-economic conditions and culture, the buffalo meat consumption is very high. These days, the demand of buffalo meat is increasing day by day in restaurants and hotels.

Echinococcosis/hydatidosis is one of the main zoonotic problems in Nepal. The present study on this thesis was conducted in the period from November, 2004 to April, 2005 at different slaughter places in Kathmandu Valley. From a total of 500 randomly selected slaughtered buffaloes 10.6% were infected with hydatid cysts. It is different from the findings of a previous study, which showed the prevalence of 18% by Joshi *et al*. (1996 a) and higher than another study, which showed the prevalence of 5% by Joshi *et al*. (1995). Khatri (2003) has reported the prevalence of 22.9% hydatid cysts among buffaloes slaughtered in three adjoining towns of Kathmandu Valley. However, it indicated the higher prevalence of hydatidosis outside Kathmandu Valley. Another study by Bajagai (2004) showed the prevalence of 12.4% positive cases conducted at ward numbers 19 and 20 of Kathmandu municipality. This finding is quite similar to the findings of the present study. It is also comparable to the study in Southern India by Hafeez (1997), which reported a prevalence rate of 9.4%. It should be taken into consideration that the majority of slaughtered buffaloes in Kathmandu Valley are imported from India. Such hydatidosis occurrence was also reported by Mondal (1997), in Bangladesh ranging from 2.4% to 56.0% among livestock animals including water buffaloes.

The presence of buffalo hydatidosis in the slaughterhouse was 6.7%, in riversides it was 10.0%, and in individual butchers' places it was 12.7%. The percentage is somewhat variable in different sites and may be due to the age factor

and different origins of buffaloes. But statistically these occurrences were not different as shown by the higher computed p value ($p>0.05$).

Comparing the distribution of cysts in the organs like spleens, kidneys, heart, only lungs and livers were infested with hydatid cysts. Among the 500 carcasses, the cysts found in the lungs of animals were 6.4%, in livers were 2.4% and in both, livers and lungs were 1.8%. It showed that the occurrence of cysts was significantly ($p=0.0001$) different among the organs with a higher percentage of cysts present in the lungs. Similar results had been reported by Maharjan (1999) and Bajagai (2004) that lungs were found more infected in buffaloes in Nepal. In Ethopia Bekete *et al.* (1988), Mersie (1993) and Jobre *et al*. (1996) have reported similar data that lungs were found to be the most infected organ in cattle and sheep similar to Pandey *et al*. (1988) reported in cattle from Morocco. The findings in the study were thus in conformity with previous studies made in Nepal and abroad. The organ predilection may be due to the strains of *E. granulosus* involved as certain strains have been shown to have high predilection to specific organs (Thompson and Lymbery, 1988; Eckert and Thompson, 1997). Also, this organ predilection could be due to the age of the animals at the time of infection. Hubbert *et al.* (1975) reported that the higher degree at which the lungs are affected in older animals is attributed to the greater diameter acquired with the age of the capillaries of the portal vein.

Out of the 500 slaughtered buffaloes, 53 were infected with hydatid cysts. Among these total infected buffaloes, 58.5% were fertile and 41.5% were sterile cysts. So the percentage of fertile cysts was found to be higher than sterile cysts. But these two proportions were not significantly ($p>0.05$) different. Examinations of the fertility status of the cysts in previous studies on buffaloes in Nepal showed that 80-83% cysts were fertile (Khatri, 2003; Bajagai, 2004). However, all this data revealed the extensive occurrence of fertile hydatid cysts among water buffaloes in Nepal. It could be hypothesized that high occurrence of fertile cysts in the present study could be due to old aged buffaloes slaughtered in Kathmandu Valley. This fact is further supported by Hahn (1987) that fertile cysts were found in older age (>4 years) cattle and sterile

ones were found in young age (< 3years). The study showed that hydatidosis prevalence is a high risk for dogs becoming infected with echinococcosis.

By concentration method, the prevalence of *Taenia* eggs in stray dogs' faecal samples in Kathmandu Valley was 12.8%. Around the slaughterhouse and individual butchers' places *Taenia* eggs were found 17.6%, in riversides 14.1%, in garbage sites 9.6%, and in temple sites 7.6%. This showed that a higher percentage of positive samples were found in buffalo slaughtering areas. The most positive cases of *Taenia* eggs were also found around garbage sites. This showed that the people are throwing the infected meat around these sites also. People also leave leftovers around temple sites making ritual feasts or other religious ceremonies, going there for worshipping or other purposes. Also, some people are still sacrificing the animals (buffaloes, goats, etc.) in some temples. People leave bits of food around and stray dogs come to eat this food. These findings are not directly comparable with the prevalence rates (10-39%) of echinococcosis found in dogs in different countries of Africa, because they represent *Taenia* eggs occurring in general (Mersie, 1993; Jobre *et al*., 1996; Macpherson *et al*., 1985). Also, the present reports on the prevalence of *Taenia* eggs in dogs are not directly comparable with the higher prevalence rates (6%) of *E. granulosus* by the ELISA Coproantigen test found around the slaughtering area in Kathmandu Valley (Baronet *et al*., 1994).

The differentiation of *Taenia* eggs and hydatid materials was performed by PCR. Eleven dog faecal samples were PCR positive using mitochondrial CO1 primers. The rest of the *Taenia* egg-positive samples were PCR negative, indicating that the test lacks sensitivity for *Taenia* eggs. Abbasi *et al*. (2003) have reported that this test enabled species-specific identification of *E. granulosus* isolated ova, but the lower sensitivity limit was at least 100 eggs per gram of faeces. Also, Cabrera *et al*. (2002) reported a sensitivity equivalent of 200 eggs for *E. granulosus*. All negative (20) randomly selected samples and the negative control (DNA free) were negative with PCR, and the positive control was PCR positive. This shows that there was no cross-contamination in this test, and the test had high specificity.

Both the results of the pooled protoscolices and egg DNA analysis were highly homologous (99.7% and 99.3% respectively) with the genotype G1 (sheep strain) of *E. granulosus*. This implied that in the study area, buffaloes may harbor the sheep strain of *E. granulosus*. However, these results are preliminary and are based only on one pooled protoscolices sample and one DNA extract from *Taenia* eggs in a dog fecal sample, and we cannot rule out the presence of other strains of *E. granulosus* in the dog and buffalo populations. Further investigation is needed to comprehensively identify the strains present and their prevalence. Thompson *et al*. (1995) mentioned that buffaloes may harbor three strains, namely sheep, cattle and buffalo strains, suggesting there may be other strains present which we did not find, due to the small sample size. Thompson also reported that the buffalo strain is genetically very similar to the sheep strain.

Apart from *Taenia* eggs, different other helminths eggs were found in dogs' faecal samples. Particularly *Toxocara* and hookworm eggs contaminate the environment leading to zoonotic infections primarily in children. From this it can be concluded that there is high parasitic infection in the stray dogs. Lohani *et al*. (1996) also reported that there was a higher prevalence of helminths infestations in dogs, representing 41.6% of total.

Appropriate meat inspection procedures are rarely practiced throughout the country. Indeed, dogs have free access to condemned organs even in slaughterhouses and slaughter sites located in urban centers including Kathmandu, Nepal. Dogs were observed roaming freely within the slaughtering places. Discussions with the butchers revealed that no meat inspection is done to the slaughtered animals. These observations lead to the conclusion that dogs are unhindered in gaining access to infected organs. This further confirms enhanced risk to humans being as observed by Bhatia and Pathak (1990). Besides, there is very little public awareness on the importance of this disease. These findings are similar to what Dar and Alkarmi (1997) and Koskei (1998) described in the Arab and African countries. They mentioned that dogs kept as guards or shepherds are often 'rewarded' with the discarded offals of privately slaughtered livestock. The large stray dog population around poorly or non-

supervised abattoirs constitutes the principle definitive host reservoir of *E. granulosus*.

In Nepal, people involved in slaughtering and the meat business are generally unaware of the problems of echinococcosis/hydatidosis. This has led to use of infected meat feeding stray dogs. This enhances the risk of infection for humans and animals health by environmental contamination. Considering the DNA results that the sheep strain is predominantly in Kathmandu Valley, the risk of infection for human beings with cystic echinococcosis is increasing. It shows the need for generating awareness among all those involved in slaughtering and the meat trading business as well as consumers. It also justifies the need for strengthening the monitoring by government or local government (municipalities) agencies to ensure selling of healthy meat only, and the safe control of condemned offals.

The waste disposal practices are also highly undesirable, which enhances the possibilities of contamination significantly. Schantz (1996) has reported that such careless disposal increases risk. Improper disposal and open slaughtering increases the occurrence of hydatidosis (Gemmell, 1987; Dar and Alkarmi, 1997). The present disposal practice adds pollution to water bodies and surroundings that create further health hazards. Overall, the buffalo meat, one of the staple meats of Kathmanduites, is full of risk and a health hazard. The findings of the study clearly justify that there is a need to improve disposal practice, create proper disposal facilities and a need for safe meat inspection. The responsibilities of the government or local bodies (municipalities) thus are significantly increased.

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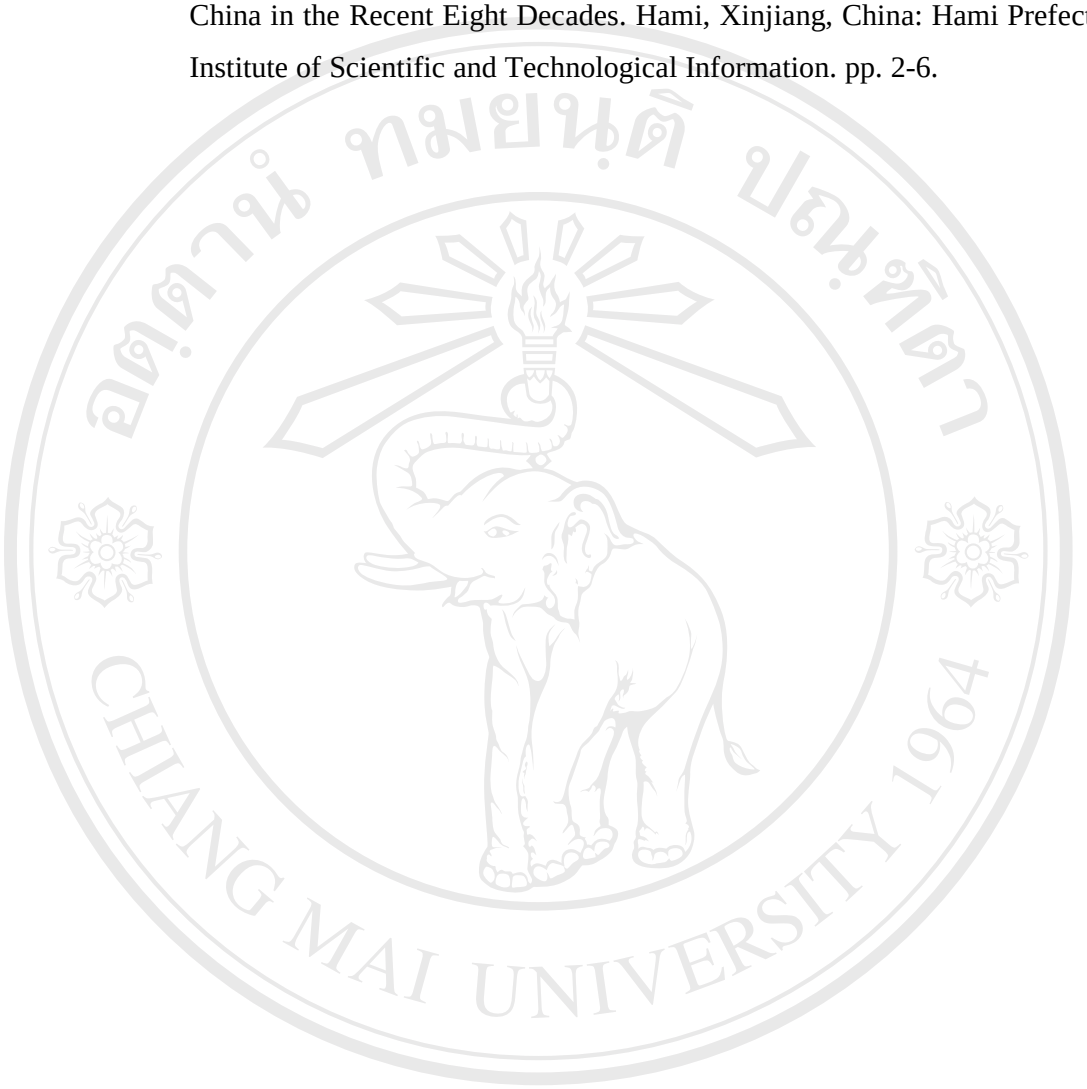

8. ANNEXES

Annex I. Questionnaire survey for butchers

1) Where do buffaloes come from?

a) From the countryside (Nepal)
b) Outside the country (Neighboring country)
c) Mixed (Nepal and India)
d) Own farm

2) How often do you get cyst-type materials in slaughtered buffaloes?

a) Quite often
b) Sometimes/Rarely
c) No

3) Are you have aware of this disease?

a) Yes
b) No

4) What do you do to infected organs or meat?

a) Throw it away
b) Sell it for consumption by animals
c) Remove cyst from organ and sell it for consumption purpose
d) Any other ------

5) Do you give raw or infected raw meat to feed stray dogs?

a) Yes
b) No

6) How do you dispose waste materials from slaughtered buffaloes?

a) Dispose it properly at waste disposal sites
b) Dispose it at riverside
c) Burn it
d) Bury
e) Any other ------

7) What is done in general to infected meat in Kathmandu?

a) Throw it away
b) Use it to feed animals
c) Use for human consumption
d) Any other ----------

Annex II. Reagents, chemicals and buffers

Agarose	INVITROGEN™ Life Technologies, Karlsruhe, Deutschlan
Ampli Taq Gold® DNA Polymerase	Applied Biosystems, GmbH, Darmstadt, Germany
Chelex 100® Resin	Sigma Chemie GmbH, Deisenhofen, Germany
DNA Marker (50, 100 bp)	Gibco, Life Technologies, Karlsruhe, Deutschlan
DNase and RNase free water	Sigma Chemie GmbH, Deisenhofen, Germany
dNTP's	Amersham, Bioscience Europe, GmbH, Freiburg, Germany
DTT (Dithiothreitol)	Sigma Chemie GmbH, Deisenhofen, Germany
Ethidium bromide	Sigma Chemie GmbH, Deisenhofen, Germany
Ether	
Formalin (10%)	
$MgCl_2$	Applied Biosystems, GmbH, Darmstadt, Germany
Oil (mineral oil)	Sigma Chemie GmbH, Deisenhofen, Germany
Tris	Sigma Chemie GmbH, Deisenhofen, Germany

Agarose Gel 2%

Agarose	2 g
1 × Electrophoresis buffer	100ml

Sample Buffer (for 10ml)

Bromophenol (0.05%)	5.0mg
Glycerin (50%)	5.0ml
EDTA (100 mM)	0.37g
1 ×Electrophoresis buffer	5.0ml

10 × Electrophoresis buffer (pH 8)

Tris (0.36 M)	43.6g
NaH_2PO_4 (0.30.M)	41.1g
EDTA (0.01 M)	3.72g
Distilled water	1000.0ml

Autoclave

Store at 4^0 C

Ethidium Bromide Stock Solution

Ethidium Bromide	10.0mg
DNase and RNase free water	1.0ml

Store at room temperature

DNA Marker

DNA Marker 50 or 100 bp	50.0 µl
1 × Electrophoresis buffer	400.0 µl
Sample buffer	50.0 µl

Aliquot and store at -20^0C

Annex III. Equipments

Adjustable Pipettes (10, 100, 1000 µl)	Eppendorf, Hamburg, Germany
Applicator sticks, wooden	
Balance	Sartorius, GmbH, Göttingen, Germany
Bottles, dispensing or plastic "squeeze" 250ml or 500ml	
Centrifuge	Heraeus Sepatech GmbH, Osterode, Germany
Cotton swabs	
Coverslips	
Electrophoreses chamber	Whatman Biometra, Göttingen, Germany
Funnel	
Freezer	Bosch, Germany
Glass slides	
Graduated 15ml centrifuge tubes	
Minicentrifuge	Roth GmbH, Karlsruhe
Pipettes Tips (10, 100, 1000)	Biozyme Hessisch Oldendorf, Germany
Pipettes, Pasteur, with rubber bulbs	
QIAmp® DNA Mini Kit	QIAGEN, GmbH, Hilden, Germany
Rack or support for tubes	
Surgical gauze	
Trio-Thermoblock™	Whatman Biometra, Göttingen, Germany
Vortex	Heidolph Instruments GmbH, Schwabach, Germany

CURRICULUM-VITAE

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Qualification

Level	Institution
B.V.Sc. and A.H.(1995)	Tribhuvan University, Nepal.
I.Sc.(1987)	Tribhuvan University, Nepal.
S.L.C. (1983)	S.L.C. Board, HMG/Nepal.

Computer Knowledge: Basic, Word Perfect and Microsoft Window.

Work Experience: Veterinary Officer, Department of Livestock Services, Kathmandu, Nepal, 1996 to date.

Research Associate, involved in laboratory investigation of biochemical changes in animals in a DFID (UK) funded collaborative research on "the interaction of fasciolosis and nutrition in growing ruminants" jointly undertaken by Centre for Tropical Veterinary Medicine (CTVM) University of Edinburgh, Midlothian, EH259RG, Scotland, UK, and Pakhribas Agriculture Centre, Dhankuta, Nepal, 1997-1999.

Health Programmer, (Partly involved) Women for Environment Protection, 1991-1995.

Consulting Doctor, Petclinic 'n'Veterinary Consulting, Ekantkuna, Jawalakhel.

Research Experience : Co- researcher, Gender Analysis in Livestock

Farming Among Small Farmers in Shripur Village of Chitwan, Nepal 1993.
Research Assistant, in Monitoring and Performance Evaluation of Kathmandu Muncipality Activities 1992-1993.

Training Obtained

"Project Planning and Management" organised by Livestock Services Training & Extension Directorate, o8th-19th April 2002, Nepal.

"Diagnosis and Control of Rabies and Other Viral Zoonoses" from Hokkaido University, Japan, 10th Jan-19th March, 2000.

"Animal Tissue Culture" organised by Animal Health Research Division in collaboration with JICA/Nepal From 27th Dec-19th Jan, 1999, Nepal.

"Assertiveness and Management" organised by Nepal Administrative Staff College, 5th Nov-21st Dec 1998, Nepal.

"Training of Trainers" Programme jointly organised by Livestock Services Training Division and Strengthening of Veterinary Services for Livestock Disease Control (SVSLDC, EU Project), 15th-29th March 1998, Nepal.

"Decentralised Agriculture Livestock Services Programme Planning Monitoring and Evaluation (PME) Model" organised By Agriculture Projects Services Centre (APROSC), 22nd June- 2nd July 1997, Nepal.

"Orientation Training", HMGN, Livestock Services Training Division, 29th Sept-13th Oct 1996, Nepal.

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DECLARATION

I, the under signed, declare that the thesis is my original work and has not been presented for a degree in any University.

Name Salina Manandhar

Signature Salina

Date of Submission 20th September 2005