بِسِلِّمِ الرَّسُولِ الَّذِي يُرَى
GENOTYPING OF *ECHINOCOCCUS GRANULOSUS* IN PUNJAB

A THESIS SUBMITTED TO THE UNIVERSITY OF THE PUNJAB, LAHORE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF

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Oh! Prophet say! If oceans are converted into ink to write the qualities of my Creator, then the whole ocean would be consumed in writing before His qualities come to an end. And even if we produce the like of ink, would also be insufficient.

(Al-Kaf, The Holy Quran)
Dedicated
To
MY
PARENTS,
SISTERS
And
BROTHER
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ABBREVIATIONS

LTL    Large hook total length
LBL    Large hook blade length
STL    Small hook total length
SBL    Small hook blade length
NH     Total number of hooks
$I_{L_{LBL/LTL}}$ Index of LBL/LTL
$I_{S_{SBL/STL}}$ Index of SBL/STL
HCF    Hydatid cyst fluid
log mol.wt. log molecular weights
Rf     Relative flow
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*ASMA ABDUL LATIF*
SUMMARY

Present study revealed molecular analysis of *Echinococcus granulosus* in Punjab, Pakistan. A total of 39738 animals comprising 15857 sheep, 15001 goats, 5300 buffaloes, 2990 cattle (cow/ox) and 590 camels of both sexes were examined for hydatid cyst in different abattoirs of Punjab from 2004-2008. The prevalence (%) of hydatidosis recorded was 7.52 (1193; sheep), 5.48 (822; goats), 5.18 (155; cattle), 7.19 (381; buffaloes) and 17.29 (102) in camels. Camel species showed highest (*P*<0.05) prevalence as compared to sheep, goats, buffaloes and cattle.

The organwise prevalence (%) during present study recorded for lungs and liver was also noted for sheep (32.19, 67.81), goats (32.60, 66.18), buffaloes (48.29, 51.71), cattle (15.48, 84.51) and camels (16.66, 83.33). It was found that existence of cysts were higher in liver as compared to lungs. Chi-square analysis showed occurrence of hydatid cysts were significantly high (*P*<0.05) in lungs and liver of sheep, goats, buffaloes, cattle and camels respectively.

Prevalence (%) of hydatid cysts on the basis of fertile, sterile, calcified and under-developed was noted in sheep 86.40 (108/125), 6.40 (8/125), 4.80 (6/125), 2.40(3/125), goats 79.09 (87/110), 6.36 (7/110), 5.45 (6/110), 9.09 (10/110), cattle 75.24 (76/101), 14.85 (15/101, 3.96 (4/101), 5.94 (6/101), buffaloes 84.31 (86/102), 9.80 (10/102), 4.90 (5/102), 0.98 (1/102) and camels 95 (95/100), 2 (2/100), 1 (1/100), 2 (2/100) respectively. Chi-square revealed that prevalence was significantly (*P*<0.05) higher in fertile, sterile and under developed cysts among all animal species. However, the prevalence of calcified cyst was non-significantly (*P*>0.05) high in camels as compared to sheep, goats, buffaloes and cattle.

Morophometric analysis of hooks of various isolates from sheep, goat, cattle, buffalo, camel and human revealed that large and small hooks were arranged in alternate manner on protoscoleces. The maximum and minimum number of hooks was recorded on protoscoleces of camel (36.2±1.96) and goat origin (27.0±2.00). Values of total number of hooks (NH) were significantly (*P*<0.05) varied among all species examined. It was found that the maximum length of large hooks (LTL)(µm) was recorded in protoscoleces of buffalo (26.83±0.98) and minimum in those of sheep origin (23.35±0.78). However, LTL was non-significant (*P*>0.05) among all species. Large hook blade length (LBL)(µm) was found maximum in sheep origin.
(12.08±0.38) and minimum in buffalo origin (10.20±0.30). LBL and \( I_{\text{LBL/LTL}} \) (%) showed significant \( (P<0.05) \) variation among all experimental species.

Maximum and minimum values of small hook total length (STL)(µm) were observed in buffalo origin (19.72±0.91) and sheep origin (15.65±0.88) respectively. STL was significantly different among all species \( (P<0.05) \). Small hook blade length (SBL)(µm) was measured maximum in camel (8.83±0.42), while minimum in goat origin (8.21 ± 0.33). It was non-significant \( (P>0.05) \) variation among all these species, while \( I_{\text{LBL/LTL}} \) (%) was significant \( (P<0.05) \) in these species.

Fluids from fertile and sterile hydatid cysts of sheep, goat, cattle, buffalo, camel and human isolates were analysed for various biochemical parameters [urea, uric acid, glucose, cholesterol, calcium, creatinine, triglyceride, magnesium (mg/dl), chloride, sodium, potassium, copper (mmol/l) and total protein (g/l)], showed significant \( (p< 0.05) \) difference among all animals and human species.

Fertile and sterile hydatid cyst fluids (HCF) of various animals and human species along with known protein markers segregated for polypeptides using sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis. Number of polypeptides were recorded variable in fertile HCF among various species, in sheep three (209 kDa, 138kDa, 63kDa) and in goat four (46kDa, 29kDa, 22kDa, 18kDa) bands were observed. In camels three (195kDa, 166kDa, 123kDa) polypeptides were examined, where as in cattle three polypeptides (269kDa, 89kDa, 59kDa) were there. Human HCF revealed four polypeptides (195kDa, 138kDa, 21kDa, 6kDa) and in buffalo the number was five (269kDa, 166kDa, 89kDa, 59kDa, 25kDa). In sterile HCF of different species minimum numbers of protein bands were found as compared to fertile isolates. In sheep (123kDa) and goat (78kDa) only one polypeptide band was observed but there was difference in molecular weights. Two polypeptide bands were observed in other species, cattle (27kDa, 18kDa), buffalo (43kDa, 27kDa), camel (195kDa, 141kDa) and human (30kDa, 21kDa).

For genotyping of \( E. \) granulosus after DNA extraction and PCR reaction a 434 base pair fragment of the mitochondrial cytochrome oxidase – 1 gene was obtained from each isolate. Our results showed the presence of two strains G1(sheep strain) and G3 (buffalo strain). However, our findings revealed that G1 and G3 were noted in livestock and G1 strain in humans only. Clear and readable sequences were obtained
for 290 base pairs and phylogenetic analysis was performed for cattle (4 cysts), buffalo (5 cysts), goat (6 cysts), sheep (13 cysts), camel (5 cysts) and human (2 cysts) isolates. Out of 35 isolates of 4 sheep, 4 goat, all camel and cattle and both human were clustered within the G1 genotype of *Echinococcus*, while the rest, including all buffalo isolates clustered within the G3 genotype of *Echinococcus*. A number of microsequence variations within G1 and G3 genotypes were observed varying from reference sequences of G1(EF393619) and G3 (M84663) by 1-6 base pairs. Out of all isolates, only camel isolates were clustered within the G1⁴ haplotype. Both human isolates belonged to the sheep strain or G1 genotype of *Echinococcus granulosus*. Regardless of host species, the isolates belonging to G1 and G3 group have similar morphology for both large and small hooks.

This study demonstrates that hydatid disease is endemic in Pakistan. The presence of the common sheep (G1) and buffalo (G3) strains of *E. granulosus* in livestock in Pakistan coupled with the challenge of controlling illegal and back-yard slaughter practices in rural communities may explains the high incidence. The finding of the common sheep strain (G1) of *E. granulosus* in two humans in Pakistan, reinforces the need for greater attention to be paid on hydatid disease control and public health. However, molecular analysis via PCR can be used as diagnostic tools for hydatidosis.
1. INTRODUCTION

Hydatidosis/Echinococcosis an important zoonosis caused by metacestode of the dog worm *Echinococcus granulosus*, is worldwide in distribution with both sylvatic and pastoral epidemiology (Oku, 2004; Garippa et al., 2004; Torgerson et al., 2005; Roberts and Janovy, 2005; Shafiq, 2005; Torgerson and Budke, 2006; Azlaf and Dakkak, 2006; Haridy et al., 2006; Esatgil and Tuzer, 2007; Zhang and Wang, 2007; Tavakoli et al., 2008; Kebede et al., 2009; Berhe, 2009; Shafiq et al., 2009; Shafiq et al., 2009a; Yang et al., 2009).

The parasite has an indirect life cycle utilizing dogs and other canids as definitive hosts and many herbivorous and omnivorous species, including wildlife and domesticated livestock as intermediate hosts (Fig. 1.1).

![Fig. 1.1: Life Cycle of *Echinococcus granulosus*.](http://www.dpd.cdc.gov/dpdx/HTML/AF/Echinococcosis/body_Echinococcosis_page1.htm)
Mature *Echinococcus* (2-11mm in length) produce eggs that are shed in the faeces. When eggs released from the definitive host, they contain in fully developed larval stage, the oncosphere, which is infective to a susceptible intermediate host. After ingestion of eggs, the oncosphere hatch in the upper gastro-intestinal tract become activated, penetrate the mucosa and migrate to the visceral organs and gradually develop into hydatid cyst. Scoleces are generated within cysts from the inner germinal layer. Life cycle is completed when the definitive host eats an infected intermediate host organ, human exposure is by faecal-oral way, with water or food contaminated by faeces of infected definitive host (Thompson and McManus, 2002).

The appearance of symptoms of hydatidosis as well as the severity of the disease depends on the intensity of infection and the organ involved, it may remain asymptomatic for many years and cysts may found in many organs but most commonly present in the liver, lungs or both (Ammann and Eckert, 1995; Pawłowski, 1997; Khanfar, 2004). Most cystic and alveolar cases of hydatidosis are usually diagnosed by various techniques (Teggi *et al*., 1993; Mohammed *et al*., 1998; Ito *et al*., 1999; Pawlowski *et al*., 2001).

*E. granulosus* has a number of genetically distinct strains, which are known to differ phenotypically and therefore has implications for its control. To date, ten genotypes (*G*1 – *G*10) of *E. granulosus* have been identified using molecular tools and the strain variation closely follow the parasite’s biological and phenotypic characteristics (McManus and Thompson, 2003; Nakao *et al*., 2007). Recently it has been proposed that *E. granulosus* may be a species complex which are likely to be maintained in distinct cycles of transmission comprising of *E. granulosus sensus stricto* (genotypes G1-G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5), G6/G7, *E. canadensis* (genotypes G8 and G10) and *E. felidis* (lion strain) (Nakao *et al*., 2007; Huttner *et al*., 2008). Hydatidosis is also well recognized zoonosis in Pakistan, affecting both human and his livestock, various human cases has been reported by Iqbal *et al*., (2007); Fatimi *et al*., (2007); Ali *et al*., (2008); Shafiq *et al*., (2009) and Amin *et al*., (2009).

In Punjab, Pakistan the prevalence of hydatid disease in individual livestock species has been studied previously (Khan and Haseeb, 1984; Iqbal *et al*., 1989; Shafiq *et al*., 2005) with reported a prevalence ranging from 5 - 46% in sheep, cattle, goats, buffaloes and camels. Shafiq *et al*., (2009) reported that only 4% people were
aware about hydatidosis, level of connection of dogs with livestock and humans. Despite its endemic nature, no studies to genetically characterise the strains of *E. granulosus* has been conducted in Pakistan.

Poor hygienic conditions and unawareness about the parasite life cycle has made the conditions favourable for the perpetuation of the disease. Often no abattoirs are available in rural communities and backyard slaughter of livestock is common, particularly at religious occasions, for example *Eid-ul Adha*. On this occasion no care has been exercised in the disposal of carcasses of offal’s and portions of the carcass are just thrown away carelessly in the streets and roads, which are easily accessible to dogs.

To date, a detailed investigation on the genotypes of *E. granulosus* in livestock and humans of Pakistan has yet to be performed. In view of the medical, veterinary and economic importance of hydatid disease in Pakistan, our study aims to ascertain the molecular epidemiology of hydatid disease by genetically and morphologically characterising hydatid cysts recovered from domestic livestock and humans from the Punjab, Pakistan.
2. LITERATURE REVIEW

Hydatidosis caused by metacestode of the dog worm. *Echinococcus granulosus* is a zoonotic disease and global public health problem. In many areas the disease is being diagnosed in increasing number, whilst in other areas it is re-emerging due to collapse of public health programmes associated with socio-economic changes (Dowling *et al.*, 2000; Taherkhani and Rogan, 2000; Shafiq *et al.*, 2005; Torgerson, 2006; Esatgil and Tuzer, 2007; Tavakoli *et al.*, 2008; Shafiq *et al.*, 2009).

In a study by Dalimi *et al.* (2002), 115 stray dogs, 86 golden jackal, 60 red foxes and 3 female wolves were examined for *E. granulosus* infection, as well as, 32,898 sheep, 15,779 cattle, 10,691 goats, and 659 buffaloes for hydatid infection from five provinces in western Iran during 3 years (1997–2000). Meanwhile fertility rates of different types and forms of cysts isolated from infected animals and the viability of protoscolices were also determined. Results indicated that 19.1% of the dogs, 2.3% of the golden jackals and 5% of the red foxes were revealed with *E. granulosus*. 11.1% of the sheep, 6.3% of the goats, 16.4% of the cattle and 12.4% of the buffaloes were also found to be infected with hydatid cyst. The cysts isolated from liver and lungs of the sheep show higher fertility rate than the cysts of liver and lungs of goats, cattle and buffaloes.

In a cross-sectional study, a total of 5381 slaughtered animals, 928 cattle, 243 buffaloes, 3765 sheep and 445 goats were examined for hydatid cysts in northwest Iran, with prevalence values of 38.3%, 11.9%, 74.4% and 20%, respectively, being recorded. Prevalences were higher in females compared with males, but a major difference (P < 0.001) was only found in sheep and cattle. Most cases which were condemned were seen in the lungs of sheep (13.4%) representing that sheep are the most important intermediate hosts for *Echinococcus granulosus* in this area (Daryani *et al.*, 2007).

Tavakoli *et al.* (2008) reported that out of 359716 animals 37.43% of livers and 51.18% lungs were infected by hydatid cyst. The mean of liver and lung infection in cow was 4.84% and 4.41%, respectively. This rate was 5.05% and 6.84% in sheep. On the basis of season the most infection rates in both animals were observed in turn
in autumn and winter. The estimated economic harm due to exclusion of infected organs, only by hydatidosis in this period was 1667720000 Rails

A study was conducted for a year (2005-2006) in Bahir Dar abattoir to assess the current status of hydatidosis in cattle and sheep. A total 420 cattle and 340 sheep slaughtered in abattoir 143 (34.05%) and 36 (10.6%) animals were found harboring hydatid cysts respectively. Thorough meat inspection in the abattoir revealed that 202 and 54 visceral organs were found harboring one or more hydatid cysts in cattle and sheep respectively. Differences in prevalence rates between the two species of animals were highly significant (P<0.001). The infection of the lung, liver, kidney, spleen and heart were found to be 57.9%, 36.6%, 3%, 1.5%, 1% in cattle and 50%, 48.1% and 1.9% in sheep respectively. A total of 864 in cattle and 138 in sheep hydatid cysts counted 315 (36.4%), 268 (31.0%), 65 (7.5%), 216 (25.0%) in cattle and 92 (66.7%), 20 (14.5%), 1 (0.7%), 25 (18.1%) in sheep were found to be small, medium, large and calcified cysts, respectively and 484 (56.0%), 164 (18.9%), 216 (25%) in cattle and 35 (25.4%), 78 (56.5%), 25 (18.1%) in sheep were sterile, fertile and calcified cysts respectively. Viability rates of 62.2% in cattle and 78.2% in sheep were observed. The rate of cyst calcification was higher in the liver as compared to the lungs while fertility rate was higher among the cysts of the lung for both cattle and sheep (Kebede et al., 2009).

In Ethiopia a cross-sectional epidemiological study was conducted from November 2006 to October 2007 by Berhe (2009) to find out the epidemiology of bovine hydatidosis in cattle. A one-year assessment of 4481 cattle slaughtered at the abattoir showed a prevalence of 32.1% (1,439) for hydatidosis. The percentage of hydatid cysts in different internal organs was observed as 25.62% in the lung, 12.56% in the liver, 0.47% in the heart, 0.17% in the kidney and 0.42% in the spleen, respectively. In this study, the lung was found to be the most predominantly affected organ (63.0%) followed by the liver (36.4%). As regards size of the cyst, the small sized cysts had the highest percentage (80.39%), followed by medium sized cysts (10.90%) and large sized cysts (8.69%). Of the 7,315 cysts examined for fertility and viability, 2349 (32.11%) were sterile, 3979 (54.39%) calcified, 782 (10.66%) fertile and viable while 205 (2.80%) were fertile but nonviable. It was observed that hepatic and pulmonary cysts had fertility rate of 11.75% and 13.83%, respectively. Out of the total cysts examined, the percentage of viable protoscoleces was 10.69%.
In Tunisia hydatidosis causes significant direct and indirect losses in both humans and animals approximately US dollars 10-19 million annually (Majorwski et al., 1990). In Turkey, the minimum total loss for all infected animals was determined to be $583, based on the market prices in the year 2002 (Umur et al., 2003). In Tibetan Plateau annual combined human and animal losses were expected to reach 218,676 U.S. dollars (range 189, 850-247, 871 US dollars), when only liver-related losses in sheep, goats and yaks are taken into account (Budke, 2005). From Pakistan, Shafiq et al. (2005) reported the economic losses of US$ 276.20 per 100 infected sheep and goat and US$ 165.72 per 100 infected buffaloes, cows and camels respectively.

Diagnostic detail of liver hydatid disease and suggestions for its treatment were given by Culafic et al. (2007). Samples were collected from 30 patients with liver hydatidosis. Hepatologic examinations were based on: medical history, physical examination, biochemical and serological tests, ultrasonography and computed tomography scanning. Twenty-five (83.3 %) patients underwent radical cyst resection, while in 5 (16.7 %) cases liver resection with left lobectomy was also performed. Hydatid cyst was histopathologically confirmed in all the patients. Results showed that the patients with multiple cysts had impaired functional liver tests significantly more often than patients with unilocular cyst. In addition, ultrasonography and computed tomography scans are the valid imaging procedures in diagnosis. Radical, surgical resection of hepatic hydatid cysts is the goal of treatment.

A 21-year old pregnant woman case was reported by Ekim and Ekim (2009) with left-sided chest pain, cyanosis and dyspnea. Pulmonary hydatid cyst was a very rare in pregnancy. Chest radiography showed tension pneumothorax, mediastinal shift, and tracheal displacement. Echocardiography revealed perforated hydatid cyst adjacent to pericardium. Histopathological examination confirmed the diagnosis. Approximately 5 months later she had a spontaneous vaginal delivery. Both the patient and her baby were healthy.

In Italy Romano et al. (2008) reported multivesicular, pulmonary and cardiac hydatidosis patient. Clinical, radiological, serological and histological findings described. MR imaging showed the exact anatomic location and the multivesicular nature of the cardiac cyst and was useful in planning surgical treatment. A successful outcome was achieved with a combination of pre and post-operative albendazole
therapy and a three step surgery procedure. MR Imaging was crucial in the pre-
surgical and followup observations.

A 65 year old woman case was reported by Amin et al. (2009) in Attock,
Pakistan. The patient had involvement of liver, diaphragm, pelvic cavity, ovary and
abdominal wall. Hydatid cysts were morphologically different from each other
including calcified, uniloculated and multiloculated cystic lesions. This case was rare
as ovary and abdominal wall involvement was described in less than 1 % cases of
hydatid disease.

Morphological studies by Hussain et al. (2005) on larval and adult
Echinococcus granulosus of buffalo, cattle, sheep, goats and camel origin showed in-
significant difference as regards the total number of hooks as well as shape and
arrangement of the hooks. The mean total length of large and small hooks did not vary
significantly in protoscoleces from different animal origin but mean total length of
large and small hooks of adult worm differed significantly. No significant variations
were found in segmentation of adult worms, number and distribution of testes, shape
of cirrus sac, position of genital pore and in histological examination hydatid cysts.

Almeida et al. (2007) characterized E. granulosus by the morphometry of the
large and small rostellar hooks in both metacestodes as well as in adult worms. The
rostellar hooks of cattle and sheep hosts varied depending on the geographic
localization, host and organ. Hooks recovered from the lungs were larger than those
collected in the liver. The rostellar hooks presented morphometric polymorphism.

Analysis of hydatid cysts were performed by Frayha and Haddad (1980) for
electrolytes, enzymes, nucleic acids, proteins, nitrogenous waste products,
carbohydrates and lipids. Significant amounts of cations were detected. Potassium,
magnesium and calcium were concentrated in protoscolices as compared to cyst fluid,
but sodium levels were similar in both. Of the anions, chloride constituted the major
volume in the fluid anions, and was not found in protoscolices. In contrast, phosphate
and bicarbonate had more significant values in the protoscolices. RNA and DNA
which were found in considerable amounts in the protoscolices were nearly absent in
the fluid, so was ammonia. The reverse pattern of distribution was observed in urea,
uric acid, creatinine and bilirubin. The proteins of cyst fluid were mainly albumin and
globulin, the latter having always double the concentration of the former. Albumins
and globulins also formed J of protoscollex protein. Among enzymes, LDH, phosphatases, GOT and GPT exhibited high activities. Cholesterol, cholesterol esters, mono-, di- and triglycerides, fatty acids and phospholipids were detected mainly in the protoscolices with phospholipids constituting the major portion. The carbohydrates of the protoscolices were glycogen, trehalose, glucose and alkali stable carbohydrates.

Determination of the chemical and biochemical parameters in hydatid cyst fluids of infected sheep and cattle may help to identify the source of human infection. Refik et al. (2002) reported that mean glucose contents in hydatid cyst fluids of sheep’s lungs were higher than that in livers (44.3 versus 35.8 mg/dl). Mean micrototal proteins were 26.1 and 21.4 mg/dl and mean triglycerides were 2.8 and 2.9 mg/dl in hydatid cyst fluids of sheep’s lung and livers, respectively. Mean glucose and micrototal protein levels in cattle was lower than sheep; 11.1 and 12.9 mg/dl in lungs and 8.1 and 10.4 mg/dl in livers, respectively. Mean triglyceride levels in hydatid cyst fluids of cattle lungs and livers were 2.9 and 2.5 mg/dl; there was not a statistically significant difference from corresponding values of sheep (p>0.05). With regard to electrolytes, mean chloride, potassium and sodium levels in sheep hydatid cyst fluids were lower than those in cattle. In contrast, mean calcium levels were higher in hydatid cyst fluids of sheep than those of cattle (P<0.05).

Radfar and Iranyar (2004) collected hydatid cyst fluids of sheep, goat, camel, cattle and human for comparative study on the biochemical parameters in Iran. Quantitative deviations in the levels of glucose, calcium and creatinine in the cystic fluids of camels were found with hydatid fluids of sheep, goat, cattle and humans. These differences were statistically significant (P<0.05). Similarities in the biochemical composition in hydatid cyst fluids of sheep, goat, cattle and humans proposed the existence of sheep strains of *E. granulosus* and variations in the biochemical composition in hydatid cyst fluids of camel with other domestic animals and humans suggested the existence of camel strains of *E. granulosus* in Iran.

Western-Blot (WB) technique permitted the detection of antibodies in the pre-surgical samples for proteins of 12–14, 16, 20, 24–26, 34, 39 and 42kDa in molecular weight in 15–96% of the patients. The combination involving 2 of the 3 proteins of 20, 39 and 42kDa has made it possible to diagnose 100% of the cases. The antibodies specific to proteins 39 and 42kDa vanished in less than one year in the patients cured
after surgery, while in patients with importunate parasitism the bands present before surgery persisted or other new ones appeared. The WB with purified antigens proved to be highly useful in the finding and post-surgical monitoring of hydatidosis patients. The antigen used was proposed as a standard antigen for the diagnosis (Doiza et al., 2001).

A total of protein antigens from various tissue components of *E. granulosus* and *E. multilocularis* and 4 other cestode species were analyzed by Jiang et al. (1998) by SDS-PAGE. The major protein bands from 4 tissue components (cystic fluid, protoscoleces, the laminated layer and the germinal layer) differed slightly between the 2 *Echinococcus* species. The main polypeptide bands for all antigens were mainly distributed between 14 and 100kDa. The 15 common protein bands for the majority of the antigens were 95, 85, 76, 66, 63, 56-57, 51-52, 46, 41-42, 37-38, 30, 28, 21, 17-19 and 16kDa.

Siles-Lucas and Cuesta-Bandera (1996) reported that purified hydatid fluid patterns, under reducing conditions, acquiesced the most accurate differentiation of Spanish strains of *E. granulosus* into 3 groups (ovine/ bovine/ human, equine and swine). The finding of characteristic 82000 MW band in equine isolates and an unusual arrangement of bands between 50,000 and 6,000 MW in pig samples. In addition, differences were found amongst crude and purified hydatid fluids, especially in bovine and swine isolates. The total protein patterns of protoscoleces were most difficult and, therefore, could not be used for strain demarcation.

A comparison of hydatid fluid antigens revealed that there were differences in the arrangement and MW of developed polypeptide bands: donkey hydatid fluid showed 3 protein bands with MW ranging between 44.6 and 143kDa, whereas camel hydatid fluid showed 7 bands with either low (28.8 to 46.7kDa) or high MW (72.4 to 100kDa). The protein patterns of protoscoleces were complex in both hosts (Derbala, 1998). In sheep the specific protein band for hydatid disease was 116kDa, while the specific protein bands for hydatid disease in human were shown to be 68 and 8kDa. It was demonstrated that the immunodiagnosis of hydatid disease in sheep and humans by western blotting is possible with the use of the purified specific proteins obtained in this study (Burgu et al., 2000).
Crude laminated layer (LL) analysis was performed by Taherkhani and Rogan (2000) by SDS-PAGE showed that this layer contained molecules probably derived from both host (200, 180, 116, 95 and 34kDa) and parasite (31, 27, 20 and 13kDa). The bands at 64, 55, 50, 29, 27 and 25kDa were common in 5 different host species (sheep, horse, mouse, human and gerbil) and bands at 68, 36, 17 and 15kDa were unique in sheep laminated layer (SLL). The important antigenic molecules of LL were confined to two regions (50-66kDa and 25-29kDa). In these regions host IgG molecules were restricted to 50-55kDa and 29kDa regions and parasite molecules were restricted to 27kDa region. It is also likely that both parasite and host molecules of the same molecular weights were present in LL (in the 50-66kDa region and the 25-29 kDa region).

The polypeptides of *E. granulosus* protoscoleces from sheep livers were investigated by SDS-PAGE and western blot; those protein bands specific to hydatid cysts in sheep were then recognized (using 20 positive and 10 negative sheep sera and one non-infected serum) as having molecular weights of 68.24 and 8 kDa (Doganay *et al*., 2004).

In Turkey Simcik and Koroglu (2004) performed SDS-PAGE analysis of hydatid cyst fluids indicated that 6 specific protein bands were detected at molecular weights of 29, 45, 58, 68, 98 and 116 kDa. Enzyme linked immunoelectrotransfer blot (EITB) analysis revealed presence of 29, 38, 42, 58, 62, 68, 98, 116, 120, 150 and 205 kDa bands in positive sheep sera, while 38, 58, 62, 68, 116 and 205kDa bands were detected in negative sheep sera. Therefore, it was concluded that the 116kDa band was specific for diagnosis of sheep hydatid disease by EITB assay.

The laminated layer of hydatid cysts of *E. granulosus* represents a significant amount of parasite material. Its antigenic role, however, is unclear. Extracts of laminated layer taken from sheep cysts were analysed by SDS/PAGE and were found to contain bands at 55 and 25–29kDa, which reacted with an antisheep IgG antibody probe, showing that these were likely to be host-contaminating components within the layer. However, the same bands were also recognised by a significant proportion of human hydatid patients, particularly by IgG4 antibodies, and not by negative control individuals. These individuals did not recognise immunoglobulin heavy and light chains in a sheep serum extract in the same manner. It appears expected that there were either host or parasite antigenic components at similar molecular weights or that
certain parasite antigens may share epitopes with sheep immunoglobulins. The antigens at 25–29kDa were found to be glycoproteins by lectin blot analysis and may be important markers of disease status (Taherkhani et al., 2007).

Hydatid cyst fluid and germinal membrane antigens exhibited respectively 7 and 12 peptides within the range Mr of 12–182kDa. Out of all major peptides of cyst fluid antigen, three low-molecular-weight peptides of 12, 17 and 23kDa were diffuse and fuzzy in nature. Due to polychromatic silver staining these three peptides were become distinctly dark in colour. Additionally, polychromatic stain also detected two dark bands of 58 and 68kDa under reducing conditions. Like cyst fluid, germinal membrane antigen also exhibited a complex profile under non-reducing conditions. Distinct yellow peptides could be seen after polychromatic staining under reducing conditions with Mr of 27, 55, 65 and 182kDa. However, diffuse and fuzzy major bands were absent in germinal membrane antigen that was visible in cyst fluid antigen (Maity et al., 2007).

DNA approaches that have been used for accurate identification of these genetic variants in molecular epidemiological surveys of cystic echinococcosis in different geographical settings and host assemblages (McManus and Thompson, 2004; McManus 2006). To date, 10 distinct genotypes (G1–G10) have been identified for E. granulosus (Bowles et al., 1994; Scott et al., 1997; Dinkel et al., 2004; Busi et al, 2007; Nakao et al., 2007).

Four Echinococcus species are presently recognised namely E. granulosus, E. multilocularis, E. oligarthrus and E. vogeli, which are infective to humans. Evidence for strain diversity within the species E. granulosus, previously mainly based on morphological, biological and biochemical features, has been principally confirmed by recent genetic studies. Epidemiological evidence and molecular studies indicate that the so-called sheep, cattle and cervid strains of E. granulosus are infective to humans, while the camel, horse and pig strains may be less or not infective, but this question demands further studies. Study indicated that E. granulosus infecting patients in Poland shared close molecular affinity with a genotype of pig origin (G7) but exhibited some clear differences. Therefore, it may represent a previously undescribed genotype of E. granulosus, designated as G9. Phylogenetic analysis of molecular data has demonstrated the need to reappraise the taxonomic status of
currently recognised strains. Clear evidence for strain variation in the other species of *Echinococcus* does not exist at present (Eckert and Thompson, 1997).

In Iran molecular characterization of *E.granulosus* strains was previously done based on mitochondrial DNA markers (Zahang *et al.*, 1998) as well as using both molecular (PCR-RFLP of ITS1) and morphological analysis (Faishi-Harandi *et al.*, 2002). In addition to the G1 or common sheep strains, the equine strain (G4) has also been found in Spain, Italy, Lebanon and Syria. The camel strain (G6) has been found in North Africa and the Middle East and the swine strain (G7) in Spain, the Slovak Republic and Poland (Thompson and McManus, 2002).

Genetic variation in *E. granulosus* in Argentina was detected by using mitochondrial cytochrome oxidase subunit 1 (CO1) sequencing, Southern blot of a repetitive DNA element and single strand conformation polymorphism of the 5 non-transcribed region of the cytosolic malate dehydrogenase (MDH) gene. The common sheep, Tasmanian sheep, cattle and camel strains were identified in humans. Unlike the situation found in other countries, where the common sheep strain is the major source of human contamination, the Tasmanian sheep and camel strains produced a significant number of human infections in some regions of Argentina. This is the first report of cattle strain in humans in South America. Goats could be the natural intermediate host of the camel strain, which was not identified in humans from other regions so far. More than one genotype was identified in the same geographic area. These findings may have important consequences for human health and the control of hydatid disease. Within-strain differences were also observed, showing the potential of variation of *E. granulosus* (Kamenetzky *et al.*, 2002).

From different geographical areas of Iran Isolates of *E. granulosus* were collected from humans and other animals and analysed by using both DNA (PCR-RFLP of ITS1) and morphological criteria (metacestode rostellar hook dimensions). The sheep and camel strains) genotypes were shown to occur in Iran. The sheep strain was shown to be the most common genotype of *E. granulosus* affecting sheep, cattle, goats and occasionally camels. The majority of camels were infected with the camel genotype as were 3 of 33 human cases. This was the first time that cases of CHD in humans had been identified in an area where a transmission cycle for the camel genotype exists. In addition, the camel genotype was found to cause infection in both
sheep and cattle. Results also demonstrated that both sheep and camel strains can be readily differentiated on the basis of hook morphology alone (Harandi et al., 2002).

In Algeria, a molecular biology approach was applied to recognize the *E. granulosus* strain. Forty-six samples from a variety of origins were analysed using comparison of PCR-amplified DNA sequences with one genomic (BG 1/3) and two mitochondrial (COI and NDI) targets. Results showed the presence of a “sheep” strain of *E. granulosus* in North Algeria circulating between cattle and ovines and infectious to humans, whereas in South Algeria, a “camel” strain and a “sheep” strain were found to circulate in camels and in sheep, respectively. This study also reported an ambiguous genotype which resembled the “sheep” strain genotype (Gl) on the basis of the partial COI gene sequence, whereas on the basis of the partial NDI gene sequence, it was similar either to the “sheep” strain (Gl) or to the “camel” strain (G6) (Bardonnet et al., 2003).

Ludmila et al. (2003) in Slovak Republic performed sequencing of the mitochondrial ND1 gene, Random Amplified Polymorphic DNA (RAPD) analysis and isoenzyme analyses to differentiate genetically protoscolices of 37 isolates from pigs and cattle and two isolates from humans collected in different regions of the. Solely the G7 genotype (pig strain) was identified by ND1 sequences in all 14 pigs isolate examined by this method. This genotype was also found in two human patients and in two cattle. The restricted finding of the G7 variant has implications for the implementation of control strategies given its unique developmental and biological properties. Despite striking morphological variability, a low degree of the intra-strain variability was found in the Slovak *Echinococcus granulosus* isolates with the genetic methods used. This was particularly associated with the shape and size of hooks, number of testes and shape of ovaries and uterus. GPI and MDH enzymes were found suitable for the discrimination of G7 and G1 (cosmopolitan sheep strain) variant).

In Argentina 114 isolates of *E. granulosus* (Cestoda: Taeniidae) collected from different host species and has been sequenced for 391 bp from the mitochondrial cytochrome *c* oxidase subunit I gene to analyze genetic variability and population structure. Nine different haplotypes were identified, 5 of which correspond to already characterized strains. Analysis of molecular variance and nested clade analysis of the distribution of haplotypes among localities within 3 main geographic regions indicate that geographic differentiation accounts for the overall pattern of genetic variability in
*E. granulosus* populations. Significant geographic differentiation is also present when the sheep strain alone is considered. They suggested that geographic patterns are not due to actual restricted gene flow between regions but are rather a consequence of past history, probably related to the time and origin of livestock introduction in Argentina (Haag et al., 2004).

Eduardo et al. (2004) in Argentina reported that the only known is the domestic cycle that affects dogs and herbivorous, including ovine, swine, cattle and goats. These strains produced a total of 58.6% of primary liver infections, 29.2% primary in lung, 2.4% primary in spleen and 9.8% were multiorgan abdominal infections. The metacestode was classified using the evolutive stages proposed by WHO-IWGE (from CE1 to CE5). We estimated that CE1 cyst has duration of about 22 years, CE2 of 14 years, CE3 of 10 years, CE4 of 19 years and CE5 was not determined. The active types CE1 and CE2 reached 75% of all cases from all strains. In 36 patients with cysts from G1, G5 and G6 strain, there were only two asymptomatic cases. The strains of the *E. granulosus* complex do not present important clinical differences; only G6 seems to have higher growth rate.

Molecular techniques were applied for analysis of cysts in camels and this found that the camels were infected with the G1 sheep strain of *E. granulosus*. Observed data were fitted to a mathematical model by maximum likelihood techniques to define the parameters and their confidence limits and the negative binomial distribution was used to define the error variance in the observed data. The infection pressure to camels was somewhat lower in comparison to sheep reported in an earlier study. However, because camels are much longer-lived animals, the results of the model fit suggested that older camels have a relatively high prevalence rate, reaching a most likely value of 32% at age 15 years. This could represent an important source of transmission to dogs and hence indirectly to man of this zonotic strain. In common with similar studies on other species, there was no evidence of parasite-induced immunity in camels (Lahmar et al., 2004).

In Spain three strains of *E. granulosus* have been previously reported (sheep, ‘horse’ and ‘pig’), but these Spanish strains have not been properly genotyped yet. Cyst isolates were collected from different host species, and the strain to which each belonged was established by analysis of its random amplified polymorphic DNA (RAPD) banding patterns. These results were compared to those obtained with the
analysis of two mitochondrial fragment sequences (cytochrome oxidase 1 (CO1) and NADH dehydrogenase 1 (ND1)) from each isolate. The Spanish ‘sheep’ strain corresponded with the genotype 1 (G1) of the parasite, infecting Spanish sheep, cattle, goat, pig, wild boar and human; the Spanish ‘horse’ strain corresponded with the genotype 4 (G4), only infecting Spanish horses; and the Spanish ‘pig’ strain corresponded with the genotype 7 (G7), infecting Spanish goat, pig and wild boar. Goat, pig and wild boar can be infected by 2 genotypes, G1 and G7 (Mwambete et al., 2004).

In Argentina *E. granulosus* hydatid cysts were examined in 41 patients. Sequencing of mitochondrial cytochrome C oxidase subunit 1 (Co1) revealed in 19 patients G1 (common sheep strain), in 6 patients G2 (Tasmania sheep strain), in 1 patient G5 (cattle strain) and in 36 patients with cysts from G1, G5 and G6 strain, there were only two asymptomatic cases. The strains of *E. granulosus* complex do not present important clinical differences, only G6 seems to have higher growth rate (Guarnera et al., 2004).

Echinococcosis is almost absent from Mexico. A cattle strain of *E. granulosus* was identified by RAPD (Random Amplified Polymorphic DNA), PCR-RFLP (Polymerase Chain Reaction-restriction Fragment Length Polymorphism) and mitochondrial Co1 gene analysis autochthonous case (Maravilla et al., 2004). From PCR analysis amplified and cloned partial Cox I and NADH I genes were analysed, samples of 16 isolates of *E. granulosus* collected from four continents. Genotypes included G1-G3 (sheep, Tasmanian sheep and buffalo), G4 (horse), G5 (cattle), G6 (camel), G7 (pig) (Obwaller et al., 2004).

Molecular analysis by PCR-RFLP of ITS1 sequence showed that all the human, ovine and bovine cysts were due to common sheep strain of *E. granulosus* in Tunisia. The sequencing of the Co1 gene of 37 isolates confirmed the G1 genotype of this strain. For seven of these isolates, found the mutation C56T which is present in the three principal intermediate hosts human (3 cysts), cattle (1 cysts) and sheep (1 cyst). With regard to G1 genotype, they identified three other point mutations. The camel strain G6 is distinctively found (M’rad et al., 2005).

In Iran isolates of *E. granulosus* collected from human, sheep and camel and were distinguished based on rostellar hook morphology of protoscoleces as well as
PCR-RFLP. Morphological study on human and animal isolates revealed the presence of two discrete strains of the parasite, one in sheep and the other one in camels. In this regard, rostellar hook of sheep isolates were significantly different from those of camel origin, meanwhile human isolates were found to be similar to those isolated from sheep. Molecular analysis of the ITS1 region of rDNA derived from human, sheep and camel isolates were in conformity with the morphological findings. Based on the PCR-RFLP method, the sheep and human isolates appeared to relate to the same genotype and the camel isolates were appeared to pertain to a different genotype (Ahmadi and Dalimi, 2006).

Parasite samples (protoscoleces or germinal layers) were obtained from infected intermediate hosts and analysed by comparing the PCR-amplified DNA sequence of three targets one nuclear (B G1/3) and two mitochondrial (Cox 1 and nad 1) in Romania. Three strains were recognized with the mitochondrial sequences: (i) the common sheep strain (G1) which circulates between sheep and cattle and is infective for humans, (ii) the Tasmanian sheep strain (G2) infecting sheep and cattle, and (iii) the pig strain (G7) predominantly found in swine. This is the first report which demonstrate the occurrence of the Tasmanian sheep strain in cattle and sympatric occurrence of these three strains (G1, G2 and G7) in Europe (Bart et al., 2006).

The phylogenetic connections of E. equinus, E. oligarthrus, , E. ortleppi, E. vogeli, E. multilocularis, E. Shiquicus E. granulosus sensu stricto and three genotypes of E. granulosus sensu lato (G6, G7 and G8) was recreated from their complete mitochondrial genomes. Maximum likelihood and separated Bayesian analysis using concatenated data sets of amino acid and nucleotide sequences depicted phylogenetic trees with the same topology. The 3 E. granulosus genotypes communicated to the camel, pig, and cervid strains were monophyletic, and their high level of genetic similarity supported taxonomic species unification of these genotypes into E. canadensis. Sister species relationships were confirmed between E. ortleppi and E. canadensis, and between E. multilocularis and E. shiquicus, regardless of the analytical approach employed. The basal positions of the phylogenetic tree were occupied by the neotropical endemic species, E. oligarthrus and E. vogeli, whose definitive hosts are derived from carnivores that immigrated from North America after the formation of the Panamanian land bridge. Host-parasite co-evolution
assessments suggested that the ancestral homeland of *Echinococcus* was North America or Asia, depending on whether the ancestral definitive hosts were canids or felids (Nakao *et al.*, 2007).

Molecular analysis was used for 12 isolates of *E. granulosus*, collected from domestic animals (cattle, buffalo and sheep) were analysed for DNA nucleotide sequence variation within NADH dehydrogenase subunit I (nadI), mitochondrial cytochrome c oxidase I (coxl) and internal transcribed spacer gene I (ITS1). After analysis of sequence information this was found that the fragment size of ITS1 of buffalo isolate was more in comparison to cattle and sheep isolates. Based on the nadI genotype this was found that Indian cattle, Based buffalo and sheep isolates could be grouped into *E. granulosus* sensu stricto. On coxI genotype two sheep isolates and one buffalo isolate were homologous to G2 genotype. Rests of the isolates were microvariants of G2 genotype. Occurrence of G2 genotype in buffalo is the first report of this genotype from this host (Bhattacharya *et al.*, 2007).

From Mexican livestock isolates of *E. granulosus* were obtained for genotyping. DNA was extracted and observed by polymerase chain reaction (PCR) of rDNA internal transcribed spacer 1 (ITS1-PCR), Eg9-PCR, Eg16-PCR, and PCR-restriction fragment length polymorphism (PCR-RFLP). In addition, fragments of the genes coding for mitochondrial cytochrome c oxidase subunit 1 (CO1) and NADH dehydrogenase 1 (ND1) were sequenced. Two different genotypes of *E. granulosus* were unequivocally identified, the common sheep genotype, G1, and the common pig genotype, G7. In Mexico the G1 genotype of *E. granulosus* has not been previously revealed. Because of its recognized infectivity in humans, G1 genotype is a direct threat to human health and its presence in Mexico is consequently of immediate public health importance and epidemiological relevance (Villabos *et al.*, 2007).

In Italy, sequence analysis of the mitochondrial cox1 and nad1 genes revealed the presence of the G1 genotype (common sheep strain), G3 genotype (buffalo strain) and of one isolate identified as G2 genotype (Tasmanian sheep strain). Sequencing of the rrnS gene exposed significant genetic distinctions between isolates identified as belonging to the G1 and G3 genotypes, with fixed nucleotide substitutions. Theses investigations provided further confirmation of the occurrence of the *E. granulosus* G3 buffalo strain, a strain previously thought to be confined to the Indian region (Busi *et al.*, 2007).
For molecular diagnosis, DNA was extracted from protoscoleces or germinal layers of cysts obtained from sheep and goats and polymerase chain reaction (PCR)/semisested PCR system was utilized. Hence G1, G5, and G6/G7 strains were discriminated by sequencing PCR amplified mitochondrial DNA encoding cytochrome c oxidase subunit I and NADH dehydrogenase I genes. The prevalence of CE was 30.4% in sheep and 14.7% in goats; fertile cysts were found in 16.2 and 7.4%, respectively. Overall, 18 of 20 sheep harbored the G1 genotype (common sheep strain), while the remaining two animals had the G3 (buffalo) strain. All 20 goats were infected with the G7 (pig) strain. These results indicated the prevalence of *E. granulosus* infection in food animals in this geographical area and revealed for the first time the presence of, at least, 3 parasite genotypes (Varcasia *et al.*, 2007).

Cruz-Reyes *et al.* (2007) analysed relationship of the cervid, camel and pig strains and noted question of taxonomic status. Seven pig isolates of *E. granulosus* from Mexico were compared with pig isolates in Poland, envoy strains and species of *Echinococcus*. Isolates in Mexico were found to be genetically identical to *E. granulosus* from Polish pigs and distinct from other main genotypes by sequencing part of the mitochondrial cytochrome c oxidase I (COI) mtDNA locus, restriction fragment length polymorphism (RFLP) of the polymerase chain reaction (PCR) amplified rDNA internal transcribed spacer (ITS) 1 using 5 different enzymes, and random amplified polymorphic DNA (RAPD) analysis. These results reinforce the vision that *Echinococcus* maintained in a cycle involving pigs and dogs is a distinct strain that is conserved genetically in different geographical areas.

The taxonomic and phylogenetic status of *E. granulosus* strains are still controversial and under discussion. In the present study Maillard *et al.* (2007) explored the genetic polymorphism of *E. granulosus* isolates originating from three countries of Africa, including a region of Algeria, where the common G1 sheep and the camel G6 strains coexist sympatrically. Seventy-one hydatid cysts were collected from sheep, cattle, camels, and humans. Two mitochondrial markers (cox1 and nad1) were used for strain identification. Two nuclear markers (actII and hbx2) were used to study the possible occurrence of cross-fertilization. Despite the heterogeneity observed among the G1 isolates, they were all localized within one robust cluster. A second strong cluster was also observed containing all of the G6 isolates. Both strains
emerged as two distinct groups, and no cases of interbreeding were found. Thus, the attribution of a species rank can be suggested.

The molecular study was performed on 30 hydatid cysts from water buffaloes (11) and cattle (19) in the Campania region of southern Italy. Two unlike mitochondrial DNA genes, namely the cytochrome c oxidase subunits 1 and the 12S ribosomal DNA (12S rDNA) were used as genetic markers. Three different genotypes of *E. granulosus* were unequivocally identified, i.e. the G1 (common sheep), G2 (Tasmanian sheep) and G3 (buffalo) genotypes, as well as some G1 and G2 variants. Present study demonstrated for the first time: (i) the presence of the G2 genotype in water buffaloes from a Mediterranean area; and (ii) the fact that the analysed portion of the 12S rDNA gene can not discriminate between the G2 and G3 genotypes of *E. granulosus*. The finding of the G1, G2 and G3 genotypes in large ruminants from southern Italy is of epidemiological significance and immediate public health importance because of their documented infectivity in humans (Rinaldi et al., 2008).

Larval stage of the *E. granulosus* was found by Moke et al. (2008) in wild ungulates in Estonia, genetically characterized *E. granulosus* isolates using mitochondrial gene sequences and used the sequence data, together with those available in a public database, to infer the phylogenetic relationships of *E. granulosus* 'genotypes' G5-G10. While 0.8% of the 2038 moose (*Alces alces*) examined were found to be infected with *E. granulosus*, the parasite was not detected in other wild ungulates, such as roe deer (*Capreolus capreolus*: 1044 specimens examined) and wild boar (*Sus scrofa*: 442 specimens). Genetic analysis of concatenated *atp6*, *nad1* and *cox1* gene (1028 bp) sequences revealed that 2 novel *E. granulosus* haplotypes, namely E8 (11 samples: 69%) and E10 (5 samples: 31%), grouped with *E. granulosus* G8 and G10, respectively, are present in Estonia. first record of an *E. granulosus* G8 in Eurasia. Phylogenetic analyses, using 4 different methods, demonstrated with considerable statistical support that *E. granulosus* G6/7 forms a subgroup together with G10, whereas G8 is a sister taxon to G6/7-G10.

By direct sequencing (G1, G2, G5, G6 and G7) five discrete mitochondrial haplotypes of *E. granulosus sensu lato* isolates were determined. The mdh genotypes were first screened by SSCP: three alleles were identified (Md1–Md3), which were further confirmed by nucleotide sequencing. For EgAgB4, which was analysed by direct sequencing the PCR products, two groups of sequences were found: EgAgB4-1
and EgAgB4-2. No haplotype-specific mdh or EgAgB4 sequences occur. Nevertheless, alleles Md1 and Md2 and type 1 sequences of EgAgB4 showed a higher frequency within the group of haplotypes G1–G2, while allele Md3 and EgAgB4-2 are most frequent in the G5–G7 cluster. 79% of the total genetic changeability was found among haplotype groups. These findings are compatible with two not mutually exclusive evolutionary hypotheses: (a) that haplotypes share an ancestral polymorphism, or (b) that the reproductive isolation between parasites with separate haplotypes is not complete, leading to gene introgression (Badaraco et al., 2008).

A total of 40 Indian animal isolates were displayed by single strand conformation polymorphism (SSCP) collected from sheep, goat, cattle and buffalo by Bhattacharya et al. (2008). The result of the study indicated that nuclear variants of *E. granulosus* were present in both small and large ruminants. Presence of nuclear variants due to mutation of *E. granulosus* has been discussed depending on hypotheses imparted earlier in literature. High polymorphism of AgB demands further investigation because the gene is related with immune evasion and infectivity. This communication reports for the first time the comparative profile of Indian goat, sheep, cattle and buffalo isolates of *E. granulosus* complex.

In Turkey the genetic strains of *Echinococcus granulosus* parasites occurring in sheep and cattle were commenced by Vural et al. (2008). A total of 112 hydatid cysts were collected from sheep (100 isolates) and from cattle (12 isolates) from the Turkish province of Kars. The parasite genotypes were detected by DNA sequencing of part of the mitochondrial Cytochrome C oxidase 1 (cox1) gene. Haplotypes were identified which corresponded clearly to the previously described strain G1 in a total of 107 isolates, including 98 isolates from sheep and 9 isolates from cattle. Five isolates, including 2 sheep and 3 cattle, were determined to belong to the G3 genotype. Parasites of the G3 genotype were identified only in isolates derived from animals in the eastern regions of Turkey. While the majority of the isolates described here had haplotypes corresponding to the G1 genotype, none matched exactly the G1 sequence that was defined in previous studies. Analysis of all GenBank entries for *E. granulosus* cox1 sequences representing G1, G2 and G3 genotypes identified substantial microsequence variability. G1 and G3 could be distinguished as separate strains, however, the existence of G2 as a separate strain could not be supported. Rather, this can be regarded as a microsequence variation of G3.
In Qinghai province of China located on the Tibetan Plateau cystic echinococcosis (CE) is highly endemic. The *Echinococcus granulosus* sheep strain has already been reported in this focal point. To improve the understanding of the role of parasite plays in the high prevalence observed in humans, genetic polymorphism of 55 *E. granulosus* samples (37 from humans) was assessed by using three discriminative mitochondrial markers: coxI, nadI and atp6. A total of 13 discrete genotypes gained were all related to the common sheep G1 strain. Six of these genotypes have already been reported in China and other foci around the world. The remaining seven genotypes were new variations of the strain (Ma *et al.*, 2008).

As an aid to control transmission of the *E. granulosus*, a vaccine EG95 has been created for avoidance of infection in the parasite’s natural animal intermediate hosts. An investigation into the genetic variability of EG95 was undertaken in this study to assess potential antigenic variability in *E. granulosus* with respect to this host-protective protein. Gene-specific PCR conditions were first established to preferentially amplify the EG95 vaccine-encoding gene (designated eg95-1) from the *E. granulosus* genome that also contains several other EG95-related genes. The optimized PCR conditions were used to amplify eg95-1 from several parasite isolates in order to determine the protein-coding sequence of the gene. An identical eg95-1 gene was amplified from parasites showing a G1 or G2 genotype of *E. granulosus*. However, from isolates having a G6 or G7 genotype, a gene was amplified which had substantial nucleotide substitutions (encoding amino acid substitutions) compared with the eg95 gene family members. The amino acid replacements of EG95 in the G6/G7 genotypes may affect the antigenicity/efficacy of the EG95 recombinant antigen against parasites of these genotypes. These investigations reveal the description of eg95 gene family members in other strains/isolates of *E. granulosus* may provide worthless information about the potential for the EG95 hydatid vaccine to be effective against *E. granulosus* strains other than the G1 genotype (Chow *et al.*, 2008).

An etiological agent for hydatidosis is the larval stage of *Echinococcus granulosus*, it is a major zoonotic public health problem and causes great economic losses in many countries, affecting humans and their livestock species. Calreticulin (CRT) is a multifunctional and conserved chaperone calcium-binding protein, present in every cell of higher organisms, except erythrocytes. In other parasites species, CRT
emerges as a key modulator of several immunological aspects of their relationships with their vertebrate hosts. We report herein the cloning of two DNA segments coding for sequences of *E. granulosus* CRT (EgCRT). Collectively with another partial sequence available at the NCBI database, a complete EgCRT cDNA sequence is now proposed. EgCRT mRNA is equally expressed in fertile and infertile hydatid cysts germinal layers, as well as in protoscoleces. In hydatid cysts, EgCRT is a 50 kDa protein, expressed in germinal layer and protoscoleces, mainly in perinuclear and cytoplasmic zones (Cabezon *et al.*, 2008).

In Italy, the livers and the lungs of 2,231 horses from different Italian regions were examined by Varcasia *et al.* (2008) for cystic echinococcosis. Hydatid cysts were found in six horses, namely four from Sardinia, one from Sicily, and one from Tuscany. The location, number, morphology, and fertility of the cysts found were determined. DNA was extracted from the germinal layers and protoscoleces of the fertile cysts and polymerase chain reactions (PCR) were performed in order to strain type DNA isolates for reduced nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1), cytochrome c oxidase subunit 1 (CO1) and 12S partial genes. The PCR products were then purified and sequenced in forward and reverse. Hydatid materials obtained from positive animals were identified as *Echinococcus granulosus* (old G1, sheep strain) and *Echinococcus equinus* (old G4, horse strain) for ND1, CO1, and 12S partial genes.

Galindo *et al.* (2008) studied ultrastructure of the *E. granulosus* protoscolex as observed by scanning electron microscopy, tightly associates with five cellular territories characterized in the interior using light and transmission electron microscopy as well as a histochemical technique. Three of these territories are surrounded by a basal lamina that is also present in the internal side of the tegument, suggesting a complex internal organization. Cellular territories correlated with the expression of specific genes and the regionalization of DNA production in protoscoleces. Additionally, a proposal to explain movements of the body of this form of the parasite in relation to the neck or to the germinal layer of the hydatid cyst is provided.

Echinococcosis was reported in Peru due to genetic deviations in tapeworms. Geographic regions were recognized by the DNA sequencing of genes for mitochondrial cytochrome c oxidase subunit 1 (cox1) and nuclear elongation factor 1
alpha (ef1a). Seventy one larval isolates collected from different intermediate hosts. The G7 genotype (E. canadensis pig strain) was found for the first time in pigs reared in the city of Lima. Echinococcus granulosus sensu stricto (sheep strain or G1) was the most prevalent in human patients, sheep, and cattle and the G6 genotype (E. canadensis camel strain) was found in goats and in one human patient (Moro et al., 2009).

In many Eastern European and Asian countries cystic echinococcosis is an important emerging zoonosis reported by Bruzinskaite et al. (2009). After examinations of slaughtered pig livers (684) in Lithuania showed significantly higher numbers of Echinococcus granulosus infections in animals from family farms (13.2%; 95% CI 10.7-16.2) as compared with those from industrial farms (4.1%; 95% CI 0.8-11.5). The prevalence was also significantly higher in pigs older than 1 year than in younger ones. In addition, in 0.5% of the pigs from the family farms, infertile and calcified E. multilocularis lesions were identified by PCR. Faecal samples from rural dogs (n=240) originating from 177 family farms in 12 villages were investigated for taeniid eggs with two methods. Significantly more dogs excreting taeniid eggs were diagnosed with the flotation/sieving method (n=34) as compared to the modified McMaster method (n=12). Multiplex PCR performed with DNA from taeniid eggs isolated from faeces of 34 dogs revealed 26 infections with Taenia spp., 9 with E. granulosus and 2 with E. multilocularis (4 cases with concurrent Taenia spp. and E. granulosus or E. multilocularis infections). Genotyping of E. granulosus cyst tissues from 7 pigs, 1 head of cattle and from E. granulosus eggs from 8 dog faeces revealed the genotype G6/7 ('pig/camel strain') in all cases.

By using as marker the 12SmtDNA gene, SYBR Green™-based real-time polymerase chain reaction (PCR) technique was utilized for a rapid demarcation of the genotype G1 from the cluster of genotypes G2/G3 of E. Granulosus. Hydatid cysts from buffaloes (11) and cattle (19) were used. Fourteen samples (identified as G1 using sequencing) showed a mean melting temperature (T m) of 76.4°C and 16 samples (identified as G2/G3 using sequencing) showed a mean T m of 77.0°C. The identified mean difference of the T m of 0.6°C between G1 and G2/G3 genotypes might allow a fast and simple discrimination of these genotypes. It was concluded that the real-time PCR developed in the present study offers a powerful tool for molecular
studies on *E. granulosus* with possibilities for extension to other genotypes using different molecular targets (Maurelli *et al.*, 2009).

McManus (2009) in a review considered aspects of the basic biochemistry, intermediary metabolism and the regulation of respiratory pathways in *E. granulosus* and *E. multilocularis*, and accentuate the occurrence of inter and intraspecies distinction in their general metabolism. The third reports on the comprehensive biochemical analysis of the tegumental surface of the protoscolex of *E. granulosus*, and the final 2 papers describe the genomic cloning of *Echinococcus* DNA fragments and their use, along with other DNA markers, in molecular identification of *E. granulosus* isolates collected worldwide from areas endemic for hydatid disease. A number of years have elapsed since these publications in Parasitology and, in this Centenary Issue article.

In western Turkey Snabel *et al.* (2009) collected various isolates of *E. granulosus* from sheep (12) and humans (10) and examined by DNA sequencing of four mitochondrial genes (cox1, atp6, nad1, rrs). Results demonstrated the presence of two species of *E. granulosus* complex, *E. granulosus* sensu stricto and *E. canadensis*. Of *E. granulosus* sensu stricto, the G1 genotype (including three microvariants) was found in 17 isolates from humans and sheep, the G3 genotype and an intermediate form G1/G3 in one isolate each (both from sheep). Of *E. canadensis*, the pig strain G7 was found in three isolates from sheep and human. Goat and/or wild boar are likely reservoirs for G7 in the region. Based on Gene Bank results G2 (Tasmanian sheep strain) is not considered as a discrete genotypic unit, as its sequences at polymorphic sites conform to microvariants of both G1 and (more often) G3. Various strains of *E. granulosus* were identified by various researchers from different intermediate hosts shown (Table 2.1).
# Table 2.1 Strain identification of *Echinococcus granulosus* from various intermediate hosts.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>Intermediate Host</th>
<th>Geographical location</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>ND1</td>
<td>Human</td>
<td>Iran</td>
<td>Zhang <em>et al.</em>, 1998; Harandi <em>et al.</em>, 2002</td>
</tr>
<tr>
<td></td>
<td>COX1</td>
<td>Camel</td>
<td>Tunisia</td>
<td>Lahmar <em>et al.</em>, 2004</td>
</tr>
<tr>
<td></td>
<td>NAD1</td>
<td>Pigs</td>
<td>Central region of Spain</td>
<td>Gonzalez <em>et al.</em>, 2002</td>
</tr>
<tr>
<td></td>
<td>ND1</td>
<td>Sheep, cattle, goat, pigs, wild boar and humans</td>
<td>Spain</td>
<td>Mwambete <em>et al.</em>, 2004</td>
</tr>
<tr>
<td></td>
<td>COX1</td>
<td>Sheep, cattle, goat, yak, human</td>
<td>West and central China</td>
<td>Yang <em>et al.</em>, 2005</td>
</tr>
<tr>
<td></td>
<td>cob</td>
<td>Human</td>
<td>China</td>
<td>Li <em>et al.</em>, 2008</td>
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<tr>
<td></td>
<td>rrnL</td>
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<td>China</td>
<td>Ma <em>et al.</em>, 2008</td>
</tr>
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<td></td>
<td>atp6</td>
<td>Sheep, cattle, goat, yak, human</td>
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</tr>
<tr>
<td>ITS1</td>
<td></td>
<td>Human, sheep and camel</td>
<td>Iran</td>
<td>Ahmadi and Dalimi, 2006</td>
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<td>Bart <em>et al.</em>, 2006</td>
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<td></td>
<td>nad1</td>
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<td>Varcasia <em>et al.</em>, 2007</td>
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<td>Central Mexico</td>
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<td>Busi <em>et al.</em>, 2007</td>
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<td>rrnS</td>
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<td>Busi <em>et al.</em>, 2007</td>
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<td>Busi <em>et al.</em>, 2007</td>
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</tr>
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<td>Romania</td>
<td>Bart <em>et al.</em>, 2006</td>
</tr>
<tr>
<td></td>
<td>nad1</td>
<td>Sheep</td>
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<td>Busi <em>et al.</em>, 2007</td>
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<td>Turkey</td>
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<td>Ahmadi and Dalimi, 2006</td>
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<td>CO1</td>
<td>Goat</td>
<td>Greece</td>
<td>Varcasia et al., 2007</td>
</tr>
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<td>ND1</td>
<td>Human, Pig, Cattle</td>
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<td>G9</td>
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<td>G10</td>
<td>CO1</td>
<td>Elk, Moose</td>
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<td>Thompson et al., 2006</td>
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</table>
3. MATERIALS AND METHODS

The present study was conducted to note the Prevalence of hydatidosis in animals, Morphometry of protoscoleces, Biochemical and SDS-PAGE analysis of hydatid cyst fluids and Molecular analysis of *Echinococcus granulosus* in livestock animals and human.

3.1 Prevalence (%) of hydatidosis

The present study was conducted from 2004-2008 to find out the prevalence of hydatidosis in sheep (*Ovis aries*), goat (*Capra hircus*), buffalo (*Bubalis bubalis*), cattle, cow/ox (*Bos indicus*) and camel (*Camelus dromedarius*). Cysts and data of animals were collected from different abattoirs and hydatid cyst fluid collected from human beings (*Homo sapiens*) operated for hydatidosis in different hospitals of Punjab, Pakistan.

3.1.1 Survey of different abattoirs

Different abattoirs of Punjab including Faisalabad, Gujranwala, Gujrat, Sheikhupura, Pakpattan and Lahore were visited for cysts collection. In these abattoirs animals were being carried from different areas of Punjab e.g. Sargodha, Bhawalpur, Kasur, Okara, Gujrat, Kamonki, Patoki, Pakpattan, Sheikhupura, Muridkay Rawalpindi, Multan, Sahiwal, Jhang. These abattoirs were visited twice a week from 2004-2008 for collection of data regarding the prevalence of hydatid cysts in the visceral organs (lungs and liver) of slaughtered sheep, goats, buffaloes, cattle and camels.

A total of 39738 adult animals of both the sexes comprising sheep (15857), goats (15001), buffaloes (6300), cattle (2990) and camels (590) were examined for the presence of hydatid disease. Only infected liver and lungs were removed from their bodies. Livers were inspected by cutting and both surfaces were examined by palpation and incisions as well as examined through visual inspection. These cysts were brought to the laboratory for further processing.
Fig. 3.1 Different study areas in Punjab
A: Map of Pakistan  B: Map of Punjab
3.1.2 Sample collection and laboratory investigations

This epidemiological investigation of hydatid cysts for following parameters was
carried out:

1. Prevalence (%) of hydatidosis
2. Organ specificity (lungs and liver)
3. Types of cysts

The prevalence (%) of the disease was recorded by the following modified formula
described by Thrusfield (2005).

\[
\text{Prevalence (\%)} = \frac{\text{No. of infected individuals at particular point in time}}{\text{No. of total individuals at particular point in time}} \times 100
\]

Hepatic and pulmonary hydatid cysts were selected from 125 sheep, 110 goats, 102 buffaloes, 101 cattle and 100 camels for further studies, collected from various abattoirs of Punjab.

3.1.3 Aspiration of hydatid cyst fluid (HCF)

Cyst was washed with distilled water and sterile syringe with hypodermic
needle No.18G was inserted in the cyst and hydatid cyst fluid (HCF) was aspirated.
The fluid was further subjected to centrifuge at 500 rpm for five minutes. The
supernatant was collected for biochemical and SDS-PAGE analysis, preserved in
freezer at -20°C. The sediments were observed under low power objective of
compound microscope for the detection of protoscoleces.

3.1.4 Prostoscoleces collection and preservation

Protoscoleces (PSC) of *Echinococcus granulosus* were collected from fertile
hydatid cysts of liver and lungs from naturally infected animals and humans. Fertility
of cyst was determined by microscopic observation of the germinal layer and viability
of protoscoleces was checked by examination at 10X without staining observing
flame cell activity and also using a vital dye (eosin 0.1%). In cases of non fertile or
sterile cysts, the presence of non viable protoscoleces and degenerative modifications
(calcification) were also noted (Varcasia *et al.*, 2006). Only viable protoscoleces were
used for morphometric and molecular analysis. From each cyst, laminar layers and
protoscoleces were removed, fixed in 70% (v/v) ethanol or stored at -20°C. The
protoscoleces was rinsed several times with sterile distilled water to remove the ethanol prior to DNA extraction (Ahmadi and Dalimi, 2006).

3.2 Morphological analysis

Fresh protoscoleces were randomly chosen for morphometric analysis, three reciprocal groups each containing 10 samples were selected from each animal species isolate and 10 human isolates. Protoscoleces were mounted in polyvinyl lactophenol and sufficient pressure was applied to the cover slip to flatten but not to damage the hooks. All measurements were made by single person using a calibrated eye-piece micrometer under oil immersion. Three large and small hooks were individually measured and all the hooks were counted from each of 6 protoscoleces from each isolate. Morphometric analysis was done as described by Hobbs et al. (1990).

Different variables was considered such as number of hooks per protoscolex (NH), total length of large (LTL) (µm) and small (STL) (µm) hooks, blade length of large (LBL) (µm) and small (SBL) (µm) hooks and the ratio of blade length of total length in large (LBL/LTL)(%) and small (SBL/STL)(%) hooks (Hobbs et al., 1990).The results were statistically analysed using the Analysis of Variance (ANOVA) with Tukeys post hoc test.

3.3 Biochemical analysis of hydatid cyst fluids

All fluid samples were centrifuged at 15000 rpm at 4°C for 30 minutes and supernatants analysed for various biochemical parameters by following Radfar and Iranyar (2004). Three reciprocal groups each containing 10 samples, were selected from each animal species isolate and 10 human isolates (fertile and sterile HCF).

3.3.1 Estimation of urea (BUN)(mg/dl)

Urea was determined by using the RANNOX Kit according to the manufacturer’s instructions followed to the method of Fawcett and Scott (1960), Patton and Crouch (1977).

Principle

The method was based on the following reaction

\[ \text{Urea} + \text{H}_2\text{O} \xrightarrow{\text{urease}} 2\text{NH}_3 + \text{CO}_2 \]
Salicylate and hypochlorite in the reagent react with the ammonium ions to form green complex (2,2 dichroxyindophenol)

**Calculation:**

\[ \text{Urea concentration} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 50 \text{ (mg/dl)} \]

### 3.3.2 Estimation of cholesterol (mg/dl)

Cholesterol was determined by using the RANDOX Kit according to the manufacturer’s instructions by method of Richmond (1973).

**Principle**

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

\[ \text{Cholesterol ester} + H_2O + \text{cholesterol esterase} \rightarrow \text{Cholesterol} + \text{Fatty acids} \]

\[ \text{Cholesterol ester} + O_2 + \text{cholesterol oxidase} \rightarrow \text{Cholesterol} - 3 + H_2O \]

\[ 2H_2O_2 + \text{phenol} + 4 - \text{Aminoantipyrine} \rightarrow \text{quinoneimine} + 4H_2O \]

**Calculation**

\[ \text{Conc. of cholesterol in sample} = \frac{AA_{\text{sample}}}{AA_{\text{standard}}} \times \text{conc. of standard (mg/dl)} \]

### 3.3.3 Estimation of triglyceride (mg/dl)

Triglyceride was determined by using the RANDOX Kit according to the manufacturer’s instructions by method of Tietz (1990), Jacobs and Demark (1960), Koditsheh and Umbreit (1969).

**Principle**

\[ \text{Triglycerides} + H_2O \rightarrow \text{Lipases} \rightarrow \text{glycerol} + \text{fatty acids} \]

\[ \text{Glycerol} + \text{ATP} \rightarrow \text{glycerol-3-phosphate} + \text{ADP} \]

\[ \text{Glycerol-3-phosphate} + O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O_2 \]
2H₂O + 4 - aminoantipyrine + chlorophenol $\xrightarrow{POD}$ quinoneimin + HCl + 4H₂O

**Calculation**

\[
\text{Triglyceride concentration} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 200 \text{ (mg/dl)}
\]

### 3.3.4 Estimation of calcium (mg/dl)

Calcium was determined by using the Randox Kit according to the manufacturer’s instructions by method of Sarkar and Chauhan (1967).

**Principle**

Calcium ions form a violet complex with O-Cresolphthalein complexone in an alkaline medium.

**Calculation**

\[
\text{Concentration} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 10 \text{ (mg/dl)}
\]

### 3.3.5 Estimation of uric acid (mg/dl)

Uric acid was determined by using the Randox Kit according to the manufacturer’s instructions by method of Barham and Trinder (1972), Fossati and Berti (1980).

**Principle**

\[
\text{Uric Acid} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{uricase}} \text{Allantoin} + \text{CO}_2 + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 3,5 \text{ dichloro - 2 - hydroxybenzenesulfonic acid} + 4 \text{ aminophenazone} \rightarrow \text{N - (4 - antipyril) - 3 - chloro - 5 - sulfonate - p - benze - quinoneimine}
\]

**Calculation**

\[
\text{Uric acid concentration} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{conc. of standard (10 mg/dl)}
\]

### 3.3.6 Estimation of glucose (mg/dl)

Glucose was determined by using the Randox Kit according to the manufacturer’s instructions by method of Barham and Trinder (1972).
**Principle**

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed, under catalysis of peroxidase, with phenol and 4- aminophenazone to form a red- violet quinoneimine dye as indicator.

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{gluconic acid} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{- aminophenazone} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4\text{H}_2\text{O}
\]

**Calculation**

\[
\text{Glucose concentration (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times 100
\]

**3.3.7 Estimation of total protein (g/l)**

Total Protein was determined by using the RANDOX Kit according to the manufacturer’s instructions.

**Principle**

Pyrogallol red complexes with proteins in an acid environment containing molybdate ions. The resulting blue -coloured complex absorbs maximally at 600 nm. Therefore the optical density at 600 nm is directly proportional to the protein concentration of the samples.

**Calculation**

\[
\text{Protein concentration (g/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Standard conc.}
\]

**3.3.8 Estimation of copper (mmol/l)**

Copper was determined by using the RANDOX Kit according to the manufacturer’s Instructions.

**Principle**

At pH 4.7 copper, which is bound to Caeruloplasmin, is released by a reducing agent. It then reacts with a specific colour reagent, 3.5-Di-Br-PAESA 4-(3,5-Dibromo-2- pyridylazo)-N-Ethyl-N-(3-sulphopropyl) aniline, to form a stable, coloured chelate. The intensity of the colour is directly proportional to the amount of copper in the sample.
Calculation

\[ \Delta A = \frac{A_2 - A_1}{A_{\text{standard}}} \]

Concentration = \[ \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{conc. of standard} \]

A1 = Initial absorbance of sample
A2 = Absorbance of sample after 5 min of incubation at 37°C.

3.3.9 Estimation of magnesium (mg/dl)

Magnesium was determined by using the Roche Kit according to the manufacturer’s instructions followed to the method of Mann and Yoe (1956).

Principle

In alkaline solution, magnesium forms a purple complex with xylidyl blue, a diazonium salt.

Calculation

Roche automate 912 analyzer was used to calculate the concentration of each sample.

3.3.10 Estimation of creatinine (mg/dl)

Creatinine was determined by using the Roche Kit according to the manufacturer’s instruction by Jaffe’ method.

Principle

\[ \text{creatinine} + \text{picric acid} \xrightarrow{\text{alkaline solution}} \text{creatinine - picric acid complex} \]

In alkaline solution, creatinine forms a yellow – orange complex with picrate. The colour intensity is directly proportional to the creatinine concentration.

Calculation

Roche automate 912 analyzer was used to calculate the concentration of each sample.
3.3.11 Estimation of potassium (mmol/l)

Fluid sample was evaluated for potassium electrolyte in Easy Lyte Plus Na/K/Cl. (MEDICA). Sample size was kept at 100µl and analysed for 60 seconds at 15-32°C and 85% humidity.

3.3.12 Estimation of sodium (mmol/l)

Fluid sample was evaluated for sodium electrolyte in Easy Lyte Plus Na/K/Cl. (MEDICA). Sample size was kept at 100µl and analysed for 60 seconds at 15-32°C and 85% humidity.

3.3.13 Estimation of chloride (mmol/l)

Fluid sample was evaluated for chloride electrolyte in Easy Lyte Plus Na/K/Cl. (MEDICA). Sample size was kept at 100µl and analysed for 60 seconds at 15-32°C and 85% humidity.

3.4 Protein electrophoresis by SDS-PAGE technique

Using Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique protein profiles of hydatid cyst fluids were analyzed (Laemmeli, 1970).

3.4.1 Reagent preparation

3.4.1.1 Monomer solution (acrylamide-bisacrylamide)(30%)

To prepare 30% solution of acrylamide-bisacrylamide 29.2g of acrylamide and 0.8 g of bis-acrylamide were dissolved in 50ml of distilled water using magnetic stirrer. Once the clear solution was obtained the total volume was made up to 100ml with distilled water using volumetric cylinder and stored at 4°C in a refrigerator.

3.4.1.2 Resolving gel buffer (Tris-HCl)(pH 8.8, 3.0 M)

To prepare 3M solution of Tris-HCl, 36.3 g of Trizma base [Tris (Hydroxymethyl) amino methane] was dissolved in distilled water using magnetic stirrer. The pH of the solution was adjusted to 8.8 using concentrated HCl. Finally the volume was made volume up to 100ml with distilled water using a volumetric cylinder and stored at 4°C in a refrigerator.
3.4.1.3 Stacking gel buffer (Tris-HCL)(pH 6.8, 1.0 M)

1M Tris-HCl was prepared by dissolving 12.11g Trizma base [Tris (Hydroxymethyl) amino methane] in 100ml of distilled water (final volume) with the help of a magnetic stirrer. The pH of the solution was adjusted to 6.8 using concentrated HCl and stored at 4°C.

3.4.1.4 SDS 10%

Freshly prepared 10% SDS was used and prepared by dissolving 10g of SDS in 80ml of distilled water on a magnetic stirrer. Once the SDS got dissolve completely the final volume was made up to 100ml and the solution was stored at room temperature.

3.4.1.5 Electrophoresis buffer (tank or running buffer)

To prepare 1M of the running buffer 3g of Tris, 14.4g of glycine and 1g of SDS were dissolved in distilled water in a beaker. Final volume of the solution was made up to 1 liter with distilled water in a volumetric cylinder and stored at 4°C.

3.4.1.6 Loading dye

154mg of Dithiothreitol (DTT) and 200mg of SDS was dissolved in 8ml of 1M Tris (pH 6.8). 10ml of glycerol was added to the mixture and when the glycerol was dissolved 20mg of bromophenol blue dye was added in the solution and mixed completely. The loading dye was aliquot in 1.5ml eppendorf tubes covered with aluminium foils and stored at 4°C.

3.4.1.7 Staining solution

125mg of Coomassie Blue R-250 was taken in a stopper flask and 112.5ml methanol, 22.5ml of acetic acid and 112.5ml of distilled water was added in the flask. The solution was dissolved completely using magnetic stirrer and stored in a dark bottle at room temperature.

3.4.1.8 Destaining solution

50ml of methanol and 70ml of glacial acetic acid were mixed and the final volume was made up to 1 liter in a graduated cylinder using distilled water and stored at room temperature.
3.4.1.9 Preparation of working dilutions

Hydatid cyst fluids (fertile and sterile) from various intermediate hosts were centrifuged at 10,000 rpm for 30 mins and supernatants were separated for analysis. Samples were mixed with loading dye to prepare the working dilutions for gel electrophoresis. For this purpose, 100µl of each of fluid samples, 50 µl of loading dye was added in new eppendorf tubes. The samples were gently but thoroughly mixed using vortex and heated in a boiling water bath for 5 mins to denature the proteins. 40µl of each of the sample was loaded in to the well of the gel.

3.4.1.10 Gel preparation

Polyacrylamide gel was prepared by following Laemmli (1970). Proteins were resolved on 12% gel. Perfect protein marker™ 10-225 KDa, catalogue number 69079-3 manufactured by Novagen was used.

3.4.1.11 Preparation of 12% gel

Before casting the gel for polymerization the glass plates of gel assembly was placed together using 1 mm thick spacers.

3.4.1.11a Resolving gel preparation

Resolving gel was prepared by following

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-Bisacrylamide (30%)</td>
<td>8.0ml</td>
</tr>
<tr>
<td>Tris-HCl (pH8.8) 3.0M</td>
<td>3.4ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>0.2ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>8.4ml</td>
</tr>
</tbody>
</table>

Mixed well and then add

TEMED (N-N’-N’-Tetramethyl ethylene diamine) 14µl
Ammonium per sulphate (APS) 250µl

(APS was always freshly prepared by dissolving 0.1g of ammonium per sulphate in 1 ml of distilled water).

This resolving gel solution was poured in between the glass plates assembled in the gel assembly leaving 0.5 inch vacant at the top for stacking gel. Almost 100 µl of distilled water was layered at the top of the gel to avoid dryness, to give a flat surface and to remove the oxygen from the surface of the gel as it inhibits
polymerization. Gel was then left undisturbed at room temperature for about 20 minutes for polymerization.

**3.4.1.11b Stacking gel preparation**

The time resolving gel takes for polymerization it is good to prepare the stacking gel using following:

- Acrylamide-Bisacrylamide (30%) 0.94ml
- Tris-HCl (pH 6.8) 1.0M 0.35ml
- SDS (10%) 55µl
- Distilled water 4.9ml
- TEMED 5µl
- Ammonium per sulphate (APS) 230µl

Mixed well and then added. Once the resolving gel is polymerized the overlying water was removed by tilting the gel assembly upside down. Then the stacking gel was pipetted at the top of resolving gel. A comb was inserted in the stacking gel to obtain required number of wells for sample loading. The gel was kept at room temperature for 15 minutes for polymerization.

**3.4.1.12 Gel electrophoresis**

The gel plates were fixed in the electrophoresis chamber and were placed in the electrophoresis tank filled with electrophoresis buffer to a volume so as the bottom of the gel was dipped in it. The central area of the electrophoresis chamber was filled completely with the same buffer. 10µl protein marker was loaded in the first well and 40µl of each of the samples was loaded in consecutive wells with the help of micropipette. The electrophoresis was performed for about 1-1.5 hour or until the time (15 minutes after) the dye seems to diffuse in the buffer of lower chamber at a current supply of 170 V in a cooling chamber maintained at 4°C.

**3.4.1.13 Staining**

After the electrophoresis, the gel was removed from the plates very carefully. The two glass plates were separated from each other using a spatula and the gels were transferred to the staining solution. The boxes containing the gel and stain were placed on a shaker with constant agitation for 30 minutes.
3.4.1.14 Destaining

After staining gel, it was destained with constant agitation until background became transparent and protein bands became visible in the form of blue coloured light and dark bands. After destaining, the Image was captured on Gel Documentation System and by Olympus camera.

3.5 Molecular techniques

Molecular analysis of protoscoleces was done in Parasitology Laboratory, School of Veterinary Science, University of Queensland, St. Lucia Campus, Australia.

Prior to DNA extraction, protoscoleces were washed in distilled water by taking 1ml aliquot from the base of each vial and added to labelled 2ml microtube, spin for 1 minute at 14,000 rpm to separate protoscoleces. Supernatant was removed and the pellet was resuspended in 1.5ml distilled water and then stored in refrigerator till ready to use. After one day this microtube was spun for one minute at 14,000 rpm, supernatant was removed from each only pellet was left.

3.5.1 DNA extraction

Total *E. granulosus* genomic DNA was prepared from samples of protoscolices or germinal membrane preserved in 70% ethanol. The DNA was isolated using the DNeasy Tissue Kit (Qiagen) according to the manufacturer’s protocol for DNA isolation from tissues with the following exceptions: Approximately 250mg of tissue (protoscoleces/ germinal membrane) was suspended in

1. 180µl ATL (tissue lysis buffer) Buffer to clean DNA from Ethanol.
2. 40µl Proteinase K to remove excess proteins.
3. Vortex these tubes for a second to mix DNA pellets in the solution.
4. These tubes were left overnight at 56°C in a water bath.
5. For freeze and thaw put them in a Nitrogenous liquid gas containing in a iron cylinder, slightly covered with the lid, after 1-2 minutes samples became solidified and put these tubes in water bath at 96°C even whole samples became liquified, this step was repeated for 3-6 times.
6. Samples were centrifuged for 30 seconds at 14,000 rpm.
7. 200µl AL (lysis buffer) Buffer were added to the samples and mixed thoroughly by vortexing. Then 200µl ethanol (100% molecular) and vortexed for thoroughly mixing.

It is essential that the sample, Buffer AL and ethanol are mixed immediately and thoroughly by vortexing to yield a homogeneous solution,

8. Pipetted the mixture and shifted into the DNeasy Mini spin column placed in 2 ml collection tube. Centrifuged at 8000 rpm for 1 min.

9. Placed DNeasy Mini spin column in a new 2ml collection tube, added 500µl Buffer AW1(wash buffer 1) and centrifuged for 1 min at 8000 rpm. The filtrate was discarded.

10. Placed DNeasy Mini spin column in a new 2ml collection tube, added 500µl Buffer AW2 (wash buffer 2) and centrifuged for 1 min at 14,000 rpm.

11. Again spin column tube was kept in a new 1.5 ml or 2 ml microcentrifuge tube and 100µl AE (elution buffer) buffer added directly on the DNeasy membrane. These tubes were incubated at room temperature for 10 minutes.

12. After this column was centrifuged for one minute at 8000 rpm to elute DNA in a new micro tube.

13. Extracted DNA was preserved at -20°C.

3.5.2 PCR amplification

A 434 base pair fragment of the mitochondrial cytochrome oxidase – 1 gene was amplified from each isolate using the following primer pairs:

Forward primer RT_1_E.g.Cox1_F (5'-GCCATCCTGAGGTTTATGTGTT-3'),
Reverse primer RT_1_E.g.Cox1_R (5'-CGACATAACATAATGAAAATGAGC-3').

3.5.2.1 Reaction mixture for PCR

The PCR was carried out in a 20 µl reaction mixture containing

1. 10x Buffer (fermentas) 2.0µl
2. dNTPS (10 mM)(Bioline) 0.4µl
3. MgCl₂(25mM) 1.6µl
4. COX 1-F(12.5 pmol) 0.5µl
5. COX 1-R(12.5 pmol) 0.5µl
6. Taq polymerase(Bioline) 0.2µl
7. Distilled water 13.8µl
8. Template DNA 1.0µl

**Total volume** 20µl

Whole process was performed on ice containing box.

### 3.5.2.2 Polymerase chain reaction (PCR) cycling conditions

Thermocycler conditions were as follows:

- **Step 1:**
  - Denaturing step at 94°C for 2 minutes
  - Annealing step at 50°C for 1 minute
  - Extension step at 72°C for 2 minutes

- **Step 2:**
  - 94°C 30 sec.
  - 50°C 30 sec. x 35 times
  - 72°C 30 sec.

- **Step 3:** Final extension at 72°C for 7 minutes

- **Step 4:** Incubated at 12°C for infinity

### 3.5.3 PCR product analysis

After completion of PCR, amplified products were confirmed and analysed by 1.5% Agarose gel electrophoresis. Any nonspecific reaction or difference in size of band was observed by running the 100bp DNA ladder (Fermentas) along with PCR product.

### 3.5.3.1 Gel electrophoresis for PCR product

TAE (Tris/acetate/EDTA) solution 50x was diluted up to 1x by taking 20ml TAE in 980ml distilled water.

1. Place a clean gel caster on a horizontal smooth surface, sealed its open ends with a paper tape and fixed the appropriate comb into gel caster.
2. 1.5% Agarose gel was prepared by dissolving 1.50gm agarose (Promega) in 100ml 1x TAE in a glass flask and mixed this carefully in a microwave set at 170°C for one minute and then again repeated until clear solution was formed.

3. 5µl Ethidium bromide (concentration of 0.5µg/ml) was also added at the base of the flask and allowed to cool before solidification this gel was poured on a plastic tray fixed on a stand containing a plastic comb for wells formation.

4. After solidification of the gel, the combs were removed. Gel was shifted into plastic tank containing TAE (1x), comb was removed carefully.

5. Loading sample was prepared by adding 1µl (6x) loading dye (# RO611, Fermentas). 5µl PCR product mixed and loaded in a gel from 2nd well. In a first well 6µl DNA ladder (100 bp) was loaded.

6. The electrophoresis apparatus was setted at 100 Volts, run for approximately 45 minutes.

7. After required time carefully gel was removed and shifted on U.V Transilluminator of gel documentation system (Bio-Rad Gel Doc.) and photographed

3.5.4 Post PCR product purification

For PCR product purification QIAGEN Kit was used under the following instructions

1. 5 volumes of buffer PBI (binding buffer) was added to 1 volume of the PCR sample and mixed for example PCR product was 15µl then 75µl buffer PBI was added. These samples were shifted into QIAquick spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column and centrifuged for 30-60sec. at 13,000 rpm.

2. Discarded flow-through and placed the QIAquick column into the same tube.

3. Then 0.75ml (750µl) buffer PE (wash buffer) was added, centrifuged for 30-60sec. at 13,000 rpm.

4. Discarded flow-through and placed the QIAquick column back in the same tube, centrifuged the column for an additional 1 minute.
One of the important things is residual ethanol from buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

5. Placed column in a clean 1.5ml microcentrifuge tube.

6. To elute DNA 30µl Buffer EB (elution buffer, 10mM Tris. Cl, pH 8.5 to the center of the QIAquick membrane and let the column stand for 30 minutes, centrifuged the column for 1 minute.

Another important thing is ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average elute volume was 28µl from 30µl elution buffer. Elution efficiency is dependent on pH. The maximum elution buffer efficiency is achieved between 7.0 and 8.5.

3.5.4.1 Gel electrophoresis for post PCR purified product

TAE (Tris/acetate/EDTA) solution 50x was diluted up to 1x by taking 20ml TAE in 980ml distilled water.

1. Place a clean gel caster on a horizontal smooth surface, sealed its open ends with a paper tape and fixed the appropriate comb into gel caster.

2. 1.5% Agarose gel was prepared by dissolving 1.50gm agarose (Promega) in 100ml 1x TAE in a glass flask and mixed this carefully in a microwave set at 170°C for one minute and then again repeated until clear solution was formed.

3. 5µl Ethidium bromide (concentration of 0.5µg/ml) was also added at the base of the flask and allowed to cool. Before solidification this gel was poured on a plastic tray fixed on a stand containing a plastic comb for wells formation.

4. After solidification of the gel, the comb was removed. Gel was shifted into plastic tank containing TAE (1x), comb was removed carefully.

5. Loading sample was prepared by adding 1µl (6x) loading dye, 5µl PCR product, mixed and loaded in a gel from 2nd well. In a first well 6µl DNA ladder (100 bp) was loaded.

6. The electrophoresis apparatus was set at 100 Volts, run for approximately 45 minutes.
7. After required time carefully gel was removed and shifted on U.V Transilluminator of gel documentation system (Bio-Rad Gel Doc.) and photographed

3.5.5 DNA sequencing and phylogenetic analysis

PCR amplification products were cut from agarose gels and purified using QIAEX II Gel Extraction Kit (Qiagen) according to manufacturer’s recommendations and sequenced in both directions using the Big Dye® Terminator system, 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

The sequencing reaction was carried out in a 10µl reaction mixture containing 1.0µl Big Dye Terminator, 3 pmol of primer, 1.25µl of 5x Big Dye Sequencing Buffer, 7.25µl purified PCR product (approximately 20-40ng DNA), made up with water. The sequencing reaction was as follows:

Step 1 95°C for 1 min,

Step 2 96°C for 1 min, 54°C for 1 min, 60°C for 4 min, repeated for 35 thermal cycles,

Step 3 72°C for 7 min

Step 4 Final hold at 4°C.

DNA was precipitated and pelleted using ethanol/EDTA method

For single reaction

1. 100 mM EDTA 2µl
2. 3 M NaOAc 2µl
3. Absolute Ethanol 50µl
   Total volume 54µl

1. After sequencing PCR samples were shifted into 0.5ml PCR tubes, then 54µl master mix added in each tube

2. These tubes were left at room temperature about for 1 hour, centrifuged them for 30 minutes at 13,000 rpm.

3. Supernatants were removed with the help of micropipette
4. 250µl 70% ethanol was added in each sample, centrifuged these for 10 minutes at 13,000 rpm

5. Supernatants were removed with the help of micropipette

6. After this these tubes were put under a hood for evaporation for about 15 minutes

These processed samples were sequenced using an Applied Biosystems/Hitachi 3130xl Genetic Analyzer, 50cm capillary array and POP7 polymer (Applied Biosystems). Sequences chromatograms were read and analysed using the software program Finch TV v 1.4.0 (Geospira Inc. ©). Clear sequences were obtained for a 290 base pair fragment. These were aligned and compared with previously published sequences of E. granulosus (GenBank accession numbers AF297617, M84662, M84663, M84664, M84665, M84666, M84667, DQ144021, AF525457), E. vogeli (M84670), E. oligarthus (M84671), E. multilocularis (M84668, M84669) and T. solium (AB086256) using Clustal W (GenomeNet, Japan) and Bioedit (Hall, 1999). Distance-based analyses were conducted using Kimura 2-parameter distance estimates and trees were constructed using the Neighbour Joining algorithm using Mega 3.1 software.

3.6 Morphology of protoscoleces

Protoscoleces preserved in 70% ethanol were mounted in polyvinyl lactophenol on clean glass slide and sufficient pressure was applied to the cover slip with the help of a thumb to flatten but not to damage the hooks. The hook components were measured according to Hobbs et al. (1990). Measurements of the total length and blade length were made on 3 large and 3 small hooks per rostellum from each of 6 protoscoleces for each isolate.
4. RESULTS

Prevalence (%) of hydatidosis in sheep, goats, cattle (cow/ox), buffaloes and camels were studied. The results provided data on prevalence, about organ specificity and types of hydatid cysts in test organisms during year 2004 to 2008.

4.1 Prevalence (%)

A total of 39738 animals comprising 15857 sheep, 15001 goats, 5300 buffaloes, 2990 cattle and 590 camels of both sexes were examined for hydatid cyst in different abattoirs of Punjab. The prevalence of hydatidosis recorded was 1193 (7.52%) in sheep, 822 (5.48%) in goats 155 (5.18%) in cattle 381 (7.19%), in buffaloes and 102 (17.29%) in camels (Table 4.1, Fig. 4.1).

Chi-Square analysis revealed the prevalence ($p = 0.00$) was significantly higher ($P<0.05$) among all animal species. However, camel species showed high ($P<0.05$) prevalence as compared to sheep, goat, buffalo and cattle.

4.1.1 Organ specificity (%)

The organ specificity for hydatid cyst in infected animals was studied for year 2004 to 2008. The prevalence (%) documented during study was 32.19 in lungs and 67.81 in liver for sheep, for goats 32.60 in lungs and 66.18 in liver, for buffaloes 48.29 in lungs and 51.71 in liver, in cattle 15.48 in lungs and 84.51 in liver, for camels 16.66 in lungs and 83.33 in liver (Table 4.1, Fig. 4.1 and Plate 4.1).

Chi-Square analysis revealed significantly higher distribution of hydatid cysts ($P<0.05$) in lungs and liver of sheep, goats, buffaloes, cattle and camels respectively.

4.1.2 Types of cysts

Prevalence of cysts was noted on the basis of fertile, sterile, calcified and under-developed. In sheep, for total of 125 hydatid cysts examined, 108 (86.40%) fertile, 8 (6.40%) sterile, 6 (4.80%) calcified, 3 (2.40%) under developed were found. In goats total of 110 hydatid cysts found to comprise 87 (79.09%) fertile, 7 (6.36%) sterile, 6 (5.45%) calcified, 10 (9.09%) under developed. In cattle total of 101 hydatid cysts contained 76 (75.24%) fertile, 15 (14.85%) sterile, 4 (3.96%) calcified, 6 (5.94%) under developed. In buffaloes total of 102 hydatid cysts, 86 (84.31%) fertile, 10 (9.80%) sterile, 5 (4.90%) calcified, 1(0.98%) under developed were recorded. In camels total of 100 hydatid cysts, 95 (95.00%) fertile, 2 (2.00%) sterile, 1 (1.00%) calcified, 2 (2.00%) under developed were found (Table 4.2, Fig. 4.2).
Table 4.1: Prevalence (%) of hydatidosis in various organs of animals slaughtered in different abattoirs of Punjab during 2004-2008.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of animals</th>
<th>Infected Animals</th>
<th>Infected Liver</th>
<th>Infected Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Sheep</td>
<td>15857</td>
<td>1193</td>
<td>7.52</td>
<td>809</td>
</tr>
<tr>
<td>Goat</td>
<td>15001</td>
<td>822</td>
<td>5.48</td>
<td>554</td>
</tr>
<tr>
<td>Cattle</td>
<td>2990</td>
<td>155</td>
<td>5.18</td>
<td>131</td>
</tr>
<tr>
<td>Buffalo</td>
<td>5300</td>
<td>381</td>
<td>7.19</td>
<td>197</td>
</tr>
<tr>
<td>Camel</td>
<td>590</td>
<td>102</td>
<td>17.29</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>39738</td>
<td>2653</td>
<td>6.67</td>
<td>1776</td>
</tr>
</tbody>
</table>

Chisquare analysis revealed highly significant differences \( p<0.05 \) in the prevalence (%) of hydatid disease between various animals and also highly significant differences in \( p<0.05 \) in the prevalence (%) of hydatidosis in the liver and lungs among different animals.

Table 4.2: Prevalence (%) of various types of hydatid cysts in different animals slaughtered in different abattoirs of Punjab 2004-2008.

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of Cysts</th>
<th>Types of Cysts</th>
<th>Under Developed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fertile</td>
<td>Sterile</td>
</tr>
<tr>
<td>Sheep</td>
<td>125</td>
<td>108</td>
<td>86.40</td>
</tr>
<tr>
<td>Goat</td>
<td>110</td>
<td>87</td>
<td>79.09</td>
</tr>
<tr>
<td>Cattle</td>
<td>101</td>
<td>76</td>
<td>75.24</td>
</tr>
<tr>
<td>Buffalo</td>
<td>102</td>
<td>86</td>
<td>84.31</td>
</tr>
<tr>
<td>Camel</td>
<td>100</td>
<td>95</td>
<td>95.00</td>
</tr>
<tr>
<td>Total</td>
<td>538</td>
<td>452</td>
<td>84.01</td>
</tr>
</tbody>
</table>

Chisquare analysis revealed prevalence of various hydatid cysts were highly significant \( p<0.05 \) for fertile, sterile and under developed cysts and non-significant differences \( p>0.05 \) was found in calcified cysts of different experimental animal species.
Fig. 4.1: Bar graph represents prevalence (%) of hydatidosis in various species of animal slaughtered in different abattoirs (Gujrat, Gujranwala, Sheikhupura, Faisalabad, Lahore, Pakpattan) of Punjab during 2004-2008.
(A) Total number of animals examined
(B) Comparative prevalence (%) of hydatidosis in various animals
(C) Prevalence (%) of hydatidosis in various organs (lungs & liver) of infected animals
Among all animal species various numbers of cysts selected for study then sheep showed comparatively maximum number of fertile cysts i.e. 108 and minimum number was observed in cattle i.e. 76. Maximum number of sterile cysts (15) was present in cattle and minimum (2) was documented in camels. Maximum numbers of calcified cysts (6) were present in sheep and goats, and minimum number was reported in camels (1). Cattle showed relatively maximum number of under-developed cysts i.e. 6 while goats and buffaloes showed only 1 under-developed cyst each.

Fig. 4.2: Bar graphs showing epidemiological investigation of various types of hydatid cysts in animal species infected with hydatidosis in Punjab (Gujrat, Gujranwala, Sheikhupura, Faisalabad, Lahore, Pakpattan) during 2004-2008. (A) Comparison of prevalence (%) of fertile cysts among various animals. (B) Comparison of prevalence (%) of sterile cysts among different animals. (C) Comparison of prevalence (%) of calcified cysts among various animals. (D) Comparison of prevalence (%) of under developed cysts among different animals.
Chi-Square analysis revealed that prevalence was significantly ($P<0.05$) higher in fertile, sterile and under developed cysts among all animal species. However, in livestock suffering from hydatidosis the prevalence of calcified cyst ($p=0.51$) was nonsignificantly ($P>0.05$) high in camels as compared to sheep, goats, buffaloes and cattle.

4.2 Protoscoleces

4.2.1 Rostellar hooks morphology

Measuring factors were total number of hooks (NH), their total length (TL)(µm) and blade length (BL)(µm) showed in (Plate 4.2, Table 4.3).

4.2.1.1 Total number of hooks (NH)

The total number of hooks on protoscoleces was 27.0±2.00 in goat origin 31.4±1.27 in sheep origin, 35.8±1.82 in cattle origin, 35.0±2.31 in buffalo origin, 36.2±1.96 in camel origin and human origin 35.00±1.00 (Table 4.3, Fig. 4.3A). It is evident from these values that the maximum number of hooks was recorded on protoscoleces of camel origin and minimum on those of goat origin.

Values of NH significantly varied among all species examined ($P<0.05$). Total number of hooks in goat isolates found significantly varied from other species but showed least variance from NH of sheep isolates. The maximum number of hooks was recorded on protoscoleces of camel origin (36.2±1.96) and minimum on those of goat origin (27.0±2.00). The one way ANOVA applied to the data of total number of hooks on protoscoleces showed that significant difference among all animals species ($F_5, s_d= 3.99, P =0.00$). The Tukeys post hoc test gave two homogeneous subsets (Table 4.4).

4.2.1.2 Large hook total length (LTL)(µm)

The total length of large hooks on protoscoleces was measured in micrometers (µm) as 23.94±0.96 in goat origin, 23.35±0.78 in sheep origin, 26.55±1.239 in cattle origin, 26.83±1.00 in buffalo origin, 24.6±1.23 in camel origin and 24.4±0.07 in human origin (Table 4.3, Fig. 4.3B). It is evident from these values that the maximum length of large hooks was recorded in protoscoleces of buffalo origin (26.83±0.98) and minimum in those of sheep origin (23.35±0.78). Total length of large hook (LTL) was non-significant among all these species ($P>0.05$).

ANOVA showed non-significant difference ($F_5, s_d= 2.18, P =0.07$) and Tukeys post hoc test gave one homogeneous subset (Table 4.5).
Table 4.3: Rostellar hooks morphology of protoscoleces of *Echinococcus granulosus* from various animal species and human isolates in Punjab. Values given are (mean±S.E) of 10 samples of three reciprocal groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Isolates (mean ± S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheep origin</td>
</tr>
<tr>
<td>Total number of hooks* (NH)</td>
<td>31.40±1.27</td>
</tr>
<tr>
<td>Large hooks total length (LTL) (µm)</td>
<td>23.35±0.78</td>
</tr>
<tr>
<td>Large hooks blade length (LBL) (µm)</td>
<td>12.08±0.38</td>
</tr>
<tr>
<td>LBL/LTL (%)</td>
<td>51.95±1.70</td>
</tr>
<tr>
<td>Small hooks total length (STL) (µm)</td>
<td>15.65±0.89</td>
</tr>
<tr>
<td>Small hooks blade length (SBL) (µm)</td>
<td>8.27±0.47</td>
</tr>
<tr>
<td>SBL/STL (%)</td>
<td>52.37±0.98</td>
</tr>
</tbody>
</table>

* Arrangement of Hooks on Protoscoleces: All hooks were arranged in alternate manner

The statistical analysis was done by applying one-way analysis of variance (ANOVA) with the Tukeys post hoc test was applied to give homogenous subsets for all groups, table showing NH, LBL and STL have significantly different (P<0.05) values, while LTL and SBL were non-significant different (p>0.05) among all isolates.
4.2.1.3 Large hook blade length (LBL)(µm)

The blade length of large hooks on protoscoleces was measured as 10.54±0.45 in goat origin, 12.08±0.38 in sheep origin, 11.89±0.32 in cattle origin, 10.2±0.30 in buffalo origin, 11.26±0.47 in camel origin, and 11.3±0.27 in human origin (Table 4.3, Fig.4.3C). It is evident from these values that the LBL was found maximum in sheep origin (12.08±0.37) and minimum in buffalo origin (10.20±0.30). Blade length showed significant variation in large hooks (LBL) ($P$=0.004).

Analysis of variance showed significant variation (F 5, 54= 4.00, $P$=0.00) and Tukeys post hoc test gave two homogeneous subsets (Table 4.6).

4.2.1.4 Index of LBL/LTL (I\_LBL/LTL)(%)

Index of LBL/LTL (%) data had been given in Fig. 4.3 and Table 4.3. The index of large hook was measured as 44.01±1.00 in goat origin, 51.95±1.70 in sheep origin, 45.33±1.85 in cattle origin, 38.11±0.82 buffalo origin, 46.74±2.98 in camel origin and 46.42±0.42 in human origin (Table 4.3, Fig. 4.3F). It is evident from these values that the $I\_LBL/LTL$ was noted maximum in sheep (51.95±1.70), while minimum in buffalo origin (38.11±0.82). $I\_LBL/LTL$ was significantly different ($P$=0.00) among all these species ($P$<0.05).

Analysis of variance showed significant variation (F 5, 54= 6.50, $P$=0.00) and Tukeys post hoc test gave two homogeneous subsets, which also showed no significant variation in data (Table 4.9).

4.2.1.5 Small hook total length (STL)(µm)

The total length of small hooks on protoscoleces was measured as 17.21±0.81 in goat origin, 15.65±0.89 in sheep, 17.67±1.00 in cattle, origin 19.72±0.91 in buffalo origin, 17.6±0.98 in camel origin and 19.4±0.07 in human origin (Table 4.3, Fig.4.3D). It is evident from these values that the maximum and minimum values of STL were observed in buffalo (19.72±0.91) and sheep origin (15.65±0.88) respectively. Total length in small hooks (STL) was significantly different among all species ($P$<0.05).

One way ANOVA showed that significant results (F 5, 54= 3.17, $P$=0.01). Tukeys post hoc test gave two homogeneous subsets (Table 4.7).
Fig. 4.3: Bar graph shows morphological analysis of rostellar hooks of protoscoleces from various animals (sheep, goat, buffalo, cattle, camel) and human isolates (mean±S.E).

(A) Total number of hooks
(B) Large hook total length (LTL) (µm)
(C) Large hook blade length (LBL) (µm)
(D) Small hook total length (STL) (µm)
(E) Small hook blade length (SBL) (µm)
(F) Index of LBL/LTL and SBL/STL (%)
4.2.1.6 Small hook blade length (SBL)(\(\mu m\))

The blade length of small hooks on protoscoleces was measured as 8.21\(\pm\)0.332 in goat origin, 8.27\(\pm\)0.406 in sheep origin, 8.8\(\pm\)0.434 in cattle origin, 8.42\(\pm\)0.524 buffalo origin, 8.83\(\pm\)0.422 in camel origin and 8.68\(\pm\)0.11 in human origin (Table 4.3, Fig. 4.3E). It is evident from these values that the SBL was measured maximum in camel (8.83\(\pm\)0.42), while minimum in goat origin (8.21 \(\pm\) 0.33). It was non-significant (\(P=\)0.794) variation for small hook blade (SBL) among all these species (\(P>\)0.05).

Analysis of variance showed non-significant variation (\(F_{5, 54}= 0.47, P =0.79\)) and Tukeys post hoc test gave one homogeneous subset (Table 4.8).

4.2.1.7 Index of SBL/STL (\(I_{SBL/STL}\)) (%)

Index of SBL/STL (%) data has been given in Fig. 4.3 (F) and Table 4.3. The index of small hook was calculated as 48.02\(\pm\)1.57 in goat origin, 52.37\(\pm\)0.98 in sheep origin, 50.05\(\pm\)1.38 in cattle origin, 42.99\(\pm\)2.64 buffalo origin, 50.01\(\pm\)1.87 in camel origin and 44.7\(\pm\)0.70 in human origin. It is evident from these values that \(I_{SBL/STL}\) was maximal in sheep (52.37\(\pm\)0.98) and lowest in buffalo origin (42.99\(\pm\)2.64). \(I_{LBL/LTL}\) was significant (\(P=\)0.00) among all these species (\(P<\)0.05).

Analysis of variance showed significant variation (\(F_{5, 54}= 5.02, P =0.00\)) and Tukeys post hoc test gave three homogeneous subsets (Table 4.10).

4.2.1.8 Arrangement of hooks on protoscoleces

Morophometric analysis of hooks of various isolates from sheep, goat, cattle, buffalo, camel and human revealed that all large and small hooks were arranged in alternate manner on protoscoleces of all isolates.

4.3 Biochemical analysis of fertile & sterile isolates of animals & humans

Fluids from fertile and sterile hydatid cysts of sheep, goat, cattle, buffalo, camel and human isolates were analyzed for various biochemical parameters i.e. urea (mg/dl), uric acid (mg/dl), glucose (mg/dl), cholesterol (mg/dl), total protein (g/l), chloride (mmol/l), calcium (mg/dl), sodium (mmol/l), creatinine (mg/dl), triglyceride (mg/dl), potassium (mmol/l), magnesium (mg/dl) and copper (mmol/l) (Table 4.11).

4.3.1 Sheep isolates

In Sheep fertile isolates maximum level of following parameters were recorded, glucose \((100.91\pm6.63)\), cholesterol \((201.21\pm1.26)\), triglyceride
(192.4±1.83), potassium (119.85±0.49), and minimum values were sodium (4.52±0.30), chloride (7.33 ±0.43), Urea (47.34±0.79), while in sterile groups maximum values were observed for total protein (118.39±8.78) and magnesium (7.75±0.35).

T-test was applied among fertile and sterile isolates of sheep revealed significant differences \( p < 0.05 \) in urea, total protein, triglyceride, calcium, cholesterol, uric acid, creatinine, sodium, potassium, chloride and non-significant differences \( p > 0.05 \) in the values of glucose, magnesium, and copper (Table 4.26).

### 4.3.2 Goat isolates

Maximum values of chloride (104.48±0.51), copper (37.45±1.42) and minimum values of uric acid (9.56±0.13) were recorded in goat sterile groups.

T-test was applied among fertile and sterile isolates of goat revealed significant differences \( p < 0.05 \) in urea, triglyceride, calcium, cholesterol, uric acid, magnesium, potassium, copper and non-significant differences \( p > 0.05 \) in the values of glucose, total protein, creatinine, sodium, chloride (Table 4.26).

### 4.3.3 Cattle isolates

In cattle maximum fertile values were recorded for calcium (10.18±0.24), chloride (107.51±0.37), urea (67.58±0.44) and in sterile isolates maximum values were observed for calcium (10.2±0.26), potassium (197.23±0.73) and minimum for glucose (60.19±0.45), cholesterol (148.05±6.55).

T-test was applied among fertile and sterile isolates of cattle revealed significant differences \( p < 0.05 \) in urea, total protein, triglyceride, cholesterol, magnesium and insignificant differences \( p > 0.05 \) in the values of glucose, uric acid, creatinine, sodium, potassium, calcium, chloride and copper (Table: 4.26).

### 4.3.4 Buffalo isolates

In fertile isolates of buffalo maximum value recorded was creatinine (0.042±0.01) and minimum were glucose(63.92±0.80), calcium (7.56±0.10), potassium (5.20±0.51), copper (21.17±1.51) and in sterile isolates maximum observed values were calcium (7.91±0.23), triglyceride (155.01±8.76), creatinine (0.03±0.01), magnesium (0.08±0.01), potassium (1.40±0.05), sodium (19.05±1.22) and chloride (24.51±0.87) was recorded minimum.

T-test was applied among fertile and sterile isolates of buffalo revealed significant differences \( p < 0.05 \) in urea, triglyceride, calcium, cholesterol, uric acid,
potassium, magnesium, copper and insignificant differences $p>0.05$ in the values of chloride, creatinine, sodium, glucose, total protein (Table 4.26).

**4.3.5 Camel isolates**

In camel fertile isolates, values for total protein (111.3±5.32), copper (41.24±1.38), urea (73.08±3.68) were found maximum while minimum values were recorded for parameters like uric acid (9.45±0.15), cholesterol (103.23±0.75), triglyceride (105.50±3.29), and magnesium (0.30±0.02).

In sterile isolates, values for uric acid (10.69±0.25), glucose (118.97±0.31), urea (372.93±8.92), cholesterol (201.73±2.11), triglyceride (183.39±1.93) were recorded maximum and minimum was recorded for copper (15.47±0.92).

T-test was applied among fertile and sterile isolates of camel revealed significant differences $p<0.05$ in glucose, total protein, triglyceride, calcium, magnesium and insignificant differences $p>0.05$ among the values of urea, cholesterol, uric acid, creatinine, sodium, potassium, chloride and copper (Table 4.26).

**4.3.6 Human isolates**

In human fertile isolates maximum values were recorded for uric acid (13.01±0.36), creatinine (0.20±0.01), magnesium (9.2±0.24) and sodium (122.3±0.64), and in sterile isolates maximum value was creatinine (0.10±0.01) and minimum was urea (43.72±0.29).

T-test among fertile and sterile isolates of human revealed significant differences $p<0.05$ in glucose, triglyceride, uric acid and insignificant differences $p>0.05$ in the values of urea, total protein, calcium, cholesterol, creatinine, sodium, potassium, chloride magnesium, and copper (Table 4.26).

Multivariant analysis (MNOVA) was done by using SPSS 13.0 showed in between all species of fertile isolates and sterile isolates all values were significantly different ($p<0.05$) (Table 4.25).

**4.3.7 Fertile and sterile isolates of animals and humans**

**4.3.7.1 Calcium (mg/dl)**

Calcium level was higher in fertile isolates of cattle (10.18±0.24) and lower in buffalo (7.56±0.10), while also higher in sterile isolates of cattle (10.2±0.26) and lower in buffalo (7.91±0.23) (Fig. 4.4E).
The General Linear Model (GLM) was applied on the data for fertile and sterile isolates. The test showed significant difference among all animals and human species \((P =0.00)\) \(p< 0.05\) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and two for sterile (Table 4.12).

4.3.7.2 Cholesterol (mg/dl)

Cholesterol level was higher in fertile isolates of sheep \((201.21±1.26)\) and lower in camel \((103.23±0.75)\), while higher in sterile isolates of camel \((201.73±2.11)\), and lower in cattle \((148.05±6.55)\) (Fig. 4.4C).

The General Linear Model (GLM) applied to the data of fertile and sterile isolates showed that significant difference among all animals and human species \((P =0.00)\) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and two for sterile (Table 4.13).

4.3.7.3 Total protein (g/l)

Experimental study revealed, total protein was higher in fertile isolates of camel \((111.3±5.32)\) and less in buffalo \((90.92±0.65)\), while maximum in sterile isolates of sheep \((118.39±8.78)\) and lower in camel \((5.04±0.65)\) (Fig. 4.5G).

The General Linear Model (GLM) applied to the data for fertile and sterile isolates showed that significant difference among all animals and human species \((P =0.00)\) \(p<0.05\) (Table 4.25). The Tukeys post hoc test gave two homogeneous subsets for fertile and three for sterile isolates (Table 4.14).

4.3.7.4 Triglyceride (mg/dl)

Observed values showed that triglycerides was higher in fertile isolates of sheep \((192.41±1.83)\) and less in camel \((105.50±3.29)\), while maximum in sterile isolates of camel \((183.39±1.93)\) and lower in buffalo \((155.01±8.76)\) (Fig. 4.4D).

The General Linear Model (GLM) applied to the data fertile and sterile isolates provided that significant difference among all animals and human species \((P =0.00)\) \(p<0.05\) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and two for sterile isolates (Table 4.15).

4.3.7.5 Magnesium (mg/dl)

Level of magnesium was higher in fertile isolates of human \((9.20±0.24)\) and less in camel \((0.30±0.02)\), while maximum in sterile isolates of sheep \((7.76±0.35)\) and lower in buffalo \((0.08±0.01)\) (Fig. 4.5I).
The General Linear Model (GLM) was applied on the data for fertile and sterile isolates. The results showed significant differences among all animals and human species \((P = 0.00) \ p < 0.05\) (Table 4.25). The Tukeys post hoc test gave four homogeneous subsets for fertile and four for sterile isolates (Table 4.16).

**4.3.7.6 Potassium (mmol/l)**

Estimated value of potassium was higher in fertile isolates of sheep (119.85±) and less in buffalo (5.21±0.51), while maximum in sterile isolates of cattle (7.83±0.36) and lower in buffalo (1.40±0.05) (Fig. 4.5M).

The General Linear Model (GLM) applied to the data of fertile and sterile isolates, showed significant difference among all animals and human species \((P = 0.00) \ p < 0.05\) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and four for sterile (Table 4.17).

**4.3.7.7 Uric acid (mg/dl)**

Uric acid level in fertile groups was more in human (13.01±0.0.36) and less in camel (9.45±0.15), while in sterile groups the value was higher in camel (10.69±0.25) and lower in goat (9.56±0.13) (Fig. 4.4A).

The General Linear Model (GLM) applied to the data fertile and sterile isolates showed that significant difference among all animals and human species \((P = 0.00) \ p < 0.05\) (Table 4.25). The Tukeys post hoc test gave two homogeneous subsets for fertile and three for sterile isolates (Table 4.18).

**4.3.7.8 Copper (mmol/l)**

Copper level was higher in fertile isolates of camel (41.24±1.38) and less in buffalo (21.17±1.51), while maximum in sterile isolates of goat (37.46±1.42) and lower in camel (15.47±0.92) (Fig. 4.5L).

The General Linear Model (GLM) applied to the data fertile and sterile isolates showed that significant difference among all animals and human species \((P = 0.00) \ p < 0.05\) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and three for sterile (Table 4.19).

**4.3.7.9 Glucose (mg/dl)**

Glucose (mg/dl) level was maximum in fertile isolates of sheep (100.91±6.63) and less in buffalo (63.92±0.80), while in sterile isolates was higher in camel (118.97±0.31) and lower in cattle (60.19±0.45) (Fig. 4.4F).
The General Linear Model (GLM) applied to the data fertile and sterile isolates showed significant difference among all animals and human species \( (P =0.00) \ p<0.05 \) (Table 4.25). The Tukeys post hoc test gave two homogeneous subsets for fertile and three for sterile isolates (Table 4.20).

### 4.3.7.10 Creatinine (mg/dl)

Creatinine was higher in fertile isolates of human \((0.20\pm0.01)\) and less in buffalo \((0.04\pm0.01)\), while maximum in sterile isolates of human \((0.10\pm0.01)\) and lower in buffalo \((0.03\pm0.01)\) (Fig. 4.5H).

The General Linear Model (GLM) for fertile and sterile isolates showed that significant difference among all animal and human species \( (P =0.00) \ p<0.05 \) (Table 4.25). The Tukeys post hoc test gave four homogeneous subsets for fertile and two for sterile isolates (Table 4.21).

### 4.3.7.11 Urea (mg/dl)

Urea level was higher in fertile groups of camel \((73.08\pm3.68)\) and less in sheep \((47.34\pm0.79)\), while in sterile groups values were higher in camel \((372.9\pm8.92)\) and lower in human \((43.72\pm0.29)\) (Fig. 4.4B).

The General Linear Model (GLM) applied to the data of fertile and sterile isolates showed significant differences among all animals and human species \( (P =0.00) \ p<0.05 \) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and four for sterile isolates (Table 4.22).

### 4.3.7.12 Chloride (mmol/l)

Chloride contents were higher in fertile isolates of cattle \((107.51\pm0.37)\) and less in sheep \((7.33\pm0.43)\), while maximum in sterile isolates of goat \((104.48\pm0.51)\) and lower in buffalo \((24.51\pm0.86)\) (Fig. 4.5K).

The General Linear Model (GLM) applied to the data of fertile and sterile isolates showed significant difference among all animals and species \( (P =0.00) \ p<0.05 \) (Table 4.25). The Tukeys post hoc test gave three homogeneous subsets for fertile and six for sterile (Table 4.23).

### 4.3.7.13 Sodium (mmol/l)

Sodium level were found higher in fertile isolates of human \((122.30\pm0.64)\) and less in sheep \((4.52\pm0.30)\), while maximum in sterile isolates of cattle \((197.23\pm0.73)\) and lower in buffalo \((19.05\pm1.22)\) (Fig. 4.5J).
Table 4.11: Biochemical profiles (mean±SE) of fertile and sterile hydatid cyst fluid from various animals and human isolates, values given are of 10 samples of three reciprocals collected from various Punjab areas during 2004-2008.

<table>
<thead>
<tr>
<th>Biochemical Profiles</th>
<th>Units</th>
<th>Sheep</th>
<th>Goat</th>
<th>Cattle</th>
<th>Buffalo</th>
<th>Camel</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fertile</td>
<td>Sterile</td>
<td>Fertile</td>
<td>Sterile</td>
<td>Fertile</td>
<td>Sterile</td>
</tr>
<tr>
<td>Total protein</td>
<td>g/l</td>
<td>96.37 ± 1.99</td>
<td>118.39 ± 8.78</td>
<td>96.27 ± 1.95</td>
<td>92.02 ± 0.63</td>
<td>91.56 ± 0.27</td>
<td>85.85 ± 1.82</td>
</tr>
<tr>
<td>Glucose</td>
<td>mg/dl</td>
<td>100.91 ± 6.63</td>
<td>77.2 ± 5.52</td>
<td>77.11 ± 4.29</td>
<td>85.77 ± 1.82</td>
<td>66.07 ± 0.34</td>
<td>60.19 ± 0.45</td>
</tr>
<tr>
<td>Urea</td>
<td>mg/dl</td>
<td>47.34 ± 0.79</td>
<td>62.77 ± 3.56</td>
<td>51.44 ± 1.49</td>
<td>45.95 ± 0.67</td>
<td>67.58 ± 0.44</td>
<td>76.62 ± 2.37</td>
</tr>
<tr>
<td>Uric acid</td>
<td>mg/dl</td>
<td>9.97 ± 0.15</td>
<td>9.94 ± 0.06</td>
<td>10.12 ± 0.33</td>
<td>9.56 ± 0.13</td>
<td>10.11 ± 0.11</td>
<td>10.26 ± 0.22</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mg/dl</td>
<td>201.21 ± 1.26</td>
<td>191.48 ± 4.03</td>
<td>163.57 ± 14.86</td>
<td>192.17 ± 0.46</td>
<td>164.44 ± 0.90</td>
<td>148.05 ± 6.55</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>mg/dl</td>
<td>192.4 ± 1.83</td>
<td>160.51 ± 7.97</td>
<td>169.09 ± 11.61</td>
<td>175.34 ± 3.86</td>
<td>162.42 ± 0.26</td>
<td>158.67 ± 6.27</td>
</tr>
<tr>
<td>Calcium</td>
<td>mg/dl</td>
<td>9.92 ± 0.10</td>
<td>10.05 ± 0.22</td>
<td>9.72 ± 0.12</td>
<td>8.54 ± 0.32</td>
<td>10.18 ± 0.24</td>
<td>10.2 ± 0.26</td>
</tr>
<tr>
<td>Creatinine</td>
<td>mg/dl</td>
<td>0.16 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.104 ± 0.02</td>
<td>0.053 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mg/dl</td>
<td>9.106 ± 0.31</td>
<td>7.76 ± 0.35</td>
<td>5.712 ± 0.65</td>
<td>1.59 ± 0.13</td>
<td>1.44 ± 0.09</td>
<td>0.57 ± 0.015</td>
</tr>
<tr>
<td>Potassium</td>
<td>mmol/l</td>
<td>119.85 ± 0.49</td>
<td>5.17 ± 0.36</td>
<td>7.09 ± 0.85</td>
<td>5.82 ± 0.15</td>
<td>13.47 ± 0.45</td>
<td>7.83 ± 0.36</td>
</tr>
<tr>
<td>Sodium</td>
<td>mmol/l</td>
<td>4.52 ± 0.30</td>
<td>100.6 ± 0.92</td>
<td>119.07 ± 1.00</td>
<td>119.33 ± 0.80</td>
<td>120.61 ± 0.67</td>
<td>197.23 ± 0.73</td>
</tr>
<tr>
<td>Chloride</td>
<td>mmol/l</td>
<td>7.33 ± 0.43</td>
<td>91.56 ± 1.24</td>
<td>99.64 ± 0.51</td>
<td>104.48 ± 0.57</td>
<td>107.51 ± 0.37</td>
<td>95.75 ± 0.46</td>
</tr>
<tr>
<td>Copper</td>
<td>mmol/l</td>
<td>36.6 ± 1.13</td>
<td>35.02 ± 1.33</td>
<td>31.29 ± 2.44</td>
<td>37.46 ± 1.42</td>
<td>37.5 ± 1.04</td>
<td>34.53 ± 0.86</td>
</tr>
</tbody>
</table>

The statistical analysis has been determined by applying multivariate analysis of variance (MNOVA) and for the comparison of mean, the Tukeys post hoc test was applied to give homogenous subsets for all groups. T-test applied to compare the means of fertile and sterile isolates of same species, all have significant difference (P<0.05).
The General Linear Model (GLM) applied to the data of fertile and sterile isolates showed significant differences among all animals and human species ($P = 0.00$) $p < 0.05$ (Table 4.25). The Tukeys post hoc test gave four homogeneous subsets for fertile and five for sterile isolates (Table 4.24).

### 4.4 Polypeptide analysis of hydatid cyst fluids by SDS-PAGE

Results of hydatid cyst fluids along with known protein markers segregated for polypeptides using sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) electrophoresis on 12% resolving gel following the protocol as described by Laemmli (1970) are presented at plate 4.3. Protein markers of catalog number 69079-3 manufactured by Novagen depicted nine polypeptide bands having molecular weights 225, 150, 100, 75, 50, 35, 25, 15 and 10kDa respectively. The log molecular weights (log mol.wt.) and relative flow (Rf) values of above mentioned protein markers are shown in Table 4.27. Standard curve plotted between log mol.wt. of protein markers and their Rf values is displayed in Fig. 4.6. The mol.wt. of unknown polypeptides of hydatid cyst fluids (HCF) from different species were calculated using standard curve of known protein markers shown in Table 4.28.

Both sterile and fertile HCF collected from sheep were electrophoriesed on 12% resolving gel by SDS-PAGE. Electrophoretic pattern of proteins of sterile as well as fertile HCF collected of sheep are presented at plate 4.3 (2, lane A,B) and plate 4.3 (4, lane H-K) and plate 4.4 (5, lane E) respectively. Sterile HCF of sheep revealed only one polypeptide band of 123kDa (log mol.wt.2.09, Rf 0.1927). Fertile HCF from sheep exhibited different polypeptide patterns. In one case, there was only one peptide band of 63kDa (log mol.wt. 1.8, Rf 0.289) are shown in plate 4.4 (5E) whereas in other cases two bands were observed of having mol.wt. 209kDa (log mol.wt. 2.32, Rf 0.1084) and 138kDa (log mol.wt. 2.14, Rf 0.1686) as shown in plate 4.3(4 H-K).

Electrophoretic pattern of proteins of sterile as well as fertile HCF collected from goat is presented at plate 4.4 (5, lane A) and plate 4.4 (6, lane B) respectively. Sterile HCF of goat revealed only one polypeptide band of 78kDa (log mol.wt.1.89 and Rf 0.265). Fertile HCF from goat exhibited four polypeptide bands, molecular weights of polypeptides calculated were 46kDa (log mol.wt. 1.66, Rf 0.3373), 29kDa (log mol.wt. 1.46, Rf 0.4096), 22kDa (log mol.wt. 1.34, Rf 0.4457) and 18kDa (log mol.wt.1.25, Rf 0.4819) shown in plate 4.4(6, lane A).
Plate 4.3: Polypeptide analysis of hydatid cyst fluid by sodium dodecyl sulphate polyacrylamide gel electrophoresis using 12% resolving gel.
1: Protein markers of known molecular weights used as standard  
2: Lanes A,B: Hydatid cyst fluid (fertile) of sheep  
Lanes C,D: Hydatid cyst fluid (fertile) of camel  
3: Lanes A,B: Hydatid cyst fluid (sterile) of human isolate  
4: Lanes: A,B,C,D,E,F,G, H: Hydatid cyst fluid (fertile) camel  
Plate 4.4: Polypeptide analysis of hydatid cyst fluid on 12% resolving gel by sodium dodecyl polyacrylamide gel electrophoresis.

5: Electrophoretogram of proteins of hydatid cyst fluid (fertile) from various intermediate hosts Lane A: Sterile hydatid cyst fluid of goat Lane B: Hydatid cyst fluid (fertile) of cattle Lanes C,D: Hydatid cyst fluid (fertile) of buffalo Lane E: Hydatid cyst fluid (fertile) of sheep

6: Lane A: Hydatid cyst fluid (fertile) of goat B: Hydatid cyst fluid (sterile) of cattle C: Hydatid cyst fluid (sterile) of buffalo

7: Lanes A,B: Hydatid cyst fluid (fertile) of human.
Electrophoretic pattern of proteins of sterile as well as fertile HCF collected from cattle is presented at plate 4.4 (6, lane B) and plate 4.4 (5, lane B) respectively. Sterile HCF of cattle revealed two polypeptide bands having molecular weights 27kDa (log mol.wt.1.43, Rf 0.4216) and 18kDa (log mol.wt.1.25, Rf 0.4819). Fertile HCF from cattle showed three polypeptide bands were found of 269kDa (log mol.wt. 2.43, Rf 0.0722), 89kDa (log mol.wt. 1.95, Rf 0.2409) and 59kDa (log mol.wt. 1.77, Rf 0.3012) as visible in plate 4.4 (5, lane B).

Polypeptides of buffalo hydatid cyst fluids (sterile and fertile) are presented at plate 4.4 (6, lane C) and gel 5, lanes C,D respectively. Sterile HCF of buffalo revealed two polypeptide bands of 43kDa (log mol.wt.1.63 and Rf 0.3493) and 27kDa (log mol.wt.1.43 and Rf 0.4216). Fertile HCF from buffalo showed five protein bands with mol.wt. of 269kDa (log mol.wt. 2.43, Rf 0.0722), 166kDa (log mol.wt. 2.22, Rf 0.1445), 89kDa (log mol.wt. 1.95 and Rf 0.2409), 59kDa (log mol.wt. 1.77 and Rf 0.3012) and 25kDa (log mol.wt. 1.4, Rf 0.4337).

Electrophoretic pattern of proteins of sterile as well as fertile HCF obtained from camel is presented at plate 4.3 (2, lane C,D) and plate 4.3 (4, lane A and B-G) respectively. Sterile HCF of camel showed two polypeptide of 195kDa (log mol.wt.2.29, Rf 0.1204) and 141kDa (log mol.wt.2.15 and Rf 0.1686). Number of polypeptides observed in fertile HCF of camel was two, however there was difference in molecular weights of polypeptides. In one case mol.weights were 195kDa (log mol.wt.2.29, Rf 0.1204) and 166kDa (log mol.wt.2.22, Rf 0.1445), where as in other case mol.wt. of 195kDa (log mol.wt.2.29, Rf 0.204) and 123kDa (log mol.wt. 2.09 and Rf 0.1927) were recorded.

Electrophoretic pattern of proteins of sterile as well as fertile HCF collected from human is presented at plate 4.3 (3, lane A,B) and plate 4.4 (7, lane A,B) respectively. Sterile HCF of human revealed two polypeptide bands of 30kDa (log mol.wt. 1.47 and Rf 0.4096) and 21kDa (log mol.wt.1.32, Rf 0.4578). Fertile HCF from human having four polypeptides were segregated mol.wt. of 195kDa (log mol.wt. 2.29, Rf 0.1204), 138kDa (log mol.wt.2.14 and Rf 0.1686), 21kDa (log mol.wt.1.32, Rf 0.4578) and 6kDa (log mol.wt.0.76, Rf 0.6506).

4.5 Molecular analysis
4.5.1 DNA extraction and amplification

For genotyping of *Echinococcus* first DNA from cysts were extracted and then DNA were confirmed on agarose gel electrophoresis. After confirmation of genomic
DNA, PCR analysis using primers for mitochondrial cytochrome oxidase – 1 gene was done. After completion of PCR reaction the product size and quality were confirmed by 1.5% agarose gel electrophoresis.

A 434 base pair fragment of the mitochondrial cytochrome oxidase – 1 gene was obtained from each isolate (Plate 4.5).

### 4.5.2 Phylogenetic analysis

Phylogenetic tree based on the aligned sequences of partial cytochrome oxidase-1 is displayed in Figure 4.7. Clear and readable sequences were obtained for 290 base pairs and phylogenetic analysis was performed for cattle (4 cysts), buffalo (5 cysts), goat (6 cysts), sheep (13 cysts), camel (5 cysts) and human (2 cysts) isolates. Out of 35 samples 4 sheep isolates, 4 goat isolates, all camel and cattle isolates and both human isolates clustered within the G1 genotype of *Echinococcus*, while the rest, including all buffalo isolates clustered within the G3 genotype of *Echinococcus*. A number of microsequence variations within G1 and G3 genotypes were observed varying from reference sequences of G1(EF393619) and G3 (M84663) by 1-6 base pairs. All camel isolates clustered within the G14 haplotype previously described by Vural *et al*. (2008). Both human isolates belonged to the sheep strain or G1 genotype of *Echinococcus granulosus*.

### 4.5.3 Morphology of protoscoleces

Figure 4.8 is a scatterplot of blade length and total length of (A) large rostellar hooks, and (B) small rostellar hooks, measured in micrometers. The means for all isolates from each host species within G1 and G3 are displayed along with data from previous studies according to Thompson *et al*. (2006) and Pednekar *et al*. (2009). Regardless of host species, the isolates belonging to G1 and G3 group together for both large and small hook morphology (Table 4.29).
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>No. of hooks</th>
<th>Small hook total length (µm)</th>
<th>Small hook blade length (µm)</th>
<th>Large hook total length (µm)</th>
<th>Large hook blade length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>G3</td>
<td>34.66</td>
<td>19.23</td>
<td>8.61</td>
<td>23.47</td>
<td>10.73</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>34.88</td>
<td>19.44</td>
<td>8.72</td>
<td>24.55</td>
<td>11.27</td>
</tr>
<tr>
<td>Goat</td>
<td>G3</td>
<td>35.33</td>
<td>17.67</td>
<td>7.83</td>
<td>25.55</td>
<td>11.77</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>31</td>
<td>19.41</td>
<td>8.70</td>
<td>23.83</td>
<td>10.81</td>
</tr>
<tr>
<td>Cattle</td>
<td>G1</td>
<td>32.5</td>
<td>19.9</td>
<td>8.95</td>
<td>24.8</td>
<td>11.4</td>
</tr>
<tr>
<td>Buffalo</td>
<td>G3</td>
<td>36.5</td>
<td>18.16</td>
<td>8.08</td>
<td>22.66</td>
<td>10.33</td>
</tr>
<tr>
<td>Camel</td>
<td>G1</td>
<td>34</td>
<td>18.90</td>
<td>8.45</td>
<td>25.06</td>
<td>11.53</td>
</tr>
<tr>
<td>Human</td>
<td>G1</td>
<td>35</td>
<td>19.4</td>
<td>8.68</td>
<td>24.4</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Table showing G1 is most common strain in all intermediate hosts, while G3 restricted to buffalo and also found in sheep and goat isolates. The isolates belonging to G1 and G3 group together for both large and small hook morphology.
Plate 4.5: Gel electrophoresis (1.5% agarose gel) of polymerase chain reaction products (DNA) produced from protoscoleces of *Echinococcus granulosus* showing bands of 434 base pair of different intermediate host isolates.

Fig. 4.7: Phenogram construction of the cytochrome oxidase -1 gene of *Echinococcus* isolates from livestock and humans sourced in this study (each number represents one isolate, a letter represents a different cyst isolated from the same animal; “S” refers to a sterile cyst) together with GenBank reference strains, using the neighbour-joining algorithm and maximum parsimony.
Fig. 4.8: Displays a scatterplot of blade length and total length of: (A) large rostellar hooks, and (B) small rostellar hooks, measured in micrometers from isolates of *Echinococcus* characterized in this study as well as previously published and unpublished data.
5. DISCUSSION

The results of the present study provides the preliminary information on the prevalence (%) of hydatidosis from year 2004-2008 in various animals, sheep 7.52 (n:1193), goats 5.48 (n:822), cattle 5.18 (n:155), buffaloes 7.19 (n:381) and camels 17.29 (n:102).

Prevalence (%) of hydatidosis was reported in different animals, cattle (3), sheep (7) and camels (45) in Sudan (Elmahdi et al., 2004), goats (4.5), sheep (3.6), cattle (19.4) and camels (61.4) in Kenya (Njoroge et al., 2002) respectively. Hydatidosis occurrence in India was also recorded in sheep (2.30–93.2%), cattle (7.1–68.9%), buffaloes (8.90–69.0%), goats (1.1–72.7%) and pigs (11.5%) by Mathur & Khanna (1977); Prasad & Mandal (1978); Kosalaraman & Ranganathan (1980); Prabhakaran et al., (1980); Deka et al. (1983); Hafeez (1997). The results of present study are different from the various studies carried out globally in recent years due to the variations in geographical conditions.

From Pakistan prevalence (%) was reported in ruminants by Munir (1980,1982); Khan & Haseeb (1984); Pal & Jamil (1986) and Iqbal et al. (1989) in sheep (8.3), cattle (5.5–9.6), goats (7.5) and buffaloes (12.3–4). In Pakistan, prevalence of hydatidosis was also reported by various investigators i.e. Iqbal et al. (1989) studied occurrence of cyst in different animals slaughtered at Faisalabad abattoir and found 49 % buffaloes, 33 % cattle, 14.8 % sheep, and 5.9 % goats infected with disease. Pal and Jamil (1986) reported prevalence (%) of hydatidosis in cattle (31.5), goats (1.79) and sheep (5.3) at Rawalpindi abattoir respectively. Our investigations are different from previous reported results because they studied before 20 to 25 years and they collected information only from one abattoir from each selected city. From our present results only prevalence (%) in goats was matched with findings of Iqbal et al. (1989).

Our present results showed that the location of hydatid cysts in various organs (lungs and liver) differed significantly among sheep, goats, buffaloes cattle and camels as well as between different organs of the same species of animal. In 2006 Ahmed et al. reported prevalence (%) of hydatidosis in livers of sheep (46.74) and goats (23.28). The liver was the predominant site of infection in both animals found by Baswaid (2007). In Egypt Haridy et al. (2006) studied prevalence between camels,
goats, sheep, pigs, cows and buffaloes, reported significant differences between animals regarding liver and lungs infection. Tavakoli et al. (2008) also reported prevalence of hydatidosis, they found the rate of infection of liver and lung in cows 4.84 % and 4.41 %, in sheep 5.05 % and 6.84 % respectively.

In Pakistan liver infection in sheep has been reported by Iqbal et al. (1989) 14.8 %, Hayat et al. (1986) 8 %, Anwar et al. (1993) 2.83 % and Iqbal et al. (1995) 15.9 %. In present study liver was more infected organ with hydatidosis instead of lungs among all animals, sheep (67.81%), goats (66.18%), buffaloes (51.71%), cattle (84.51%) and camels (83.33%). In our results more hepatic (51.71) infections were found as compared to pulmonary (48.29) in buffaloes, which were similar to the results of Khanmohammadi et al. (2008). The present study revealed significantly higher occurrence of hydatid cysts (P<0.05) in lungs and liver of sheep, goats, buffaloes, cattle and camel respectively. These present results were similar to the investigations of Haridy et al. (2006).

Different hydatid cysts were investigated under developed cysts might be due to immunological response of the host which might prevent extension of cyst (Torgerson et al., 1998; Lahmar et al., 1999; Larrieu et al., 2001; Torgerson, 2002). The calcified cysts in liver might be due to the numerous connective tissue reaction of the organ (Kebede et al., 2009). Different strains of E. granulosus might cause the variation in fertility rate in various environmental regions (McManus, 2006). Two types of cysts were examined in horses by Gordo and Bandera (1998) found that one similar to that of sheep, caused by small, non-fertile cysts with a thin wall, and an other type caused by medium to large, always fertile cysts with a thick wall.

Various types of hydatid cysts of cattle were examined in Tigray Region of Ethiopia and found 32.11% sterile, 54.39% calcified, 10.66% fertile and viable, while 2.80% were fertile but nonviable by Berhe (2009). Our results revealed that the prevalence of various types of cysts in Punjab was different from the findings of Rinaldi et al. (2008), they investigated that the cysts were either sterile (42.7%) or calcified/caseous (57.3%), no fertile cysts were present. Scala et al. (2006) studied hydatidosis in sheep and found out of 4072 collected cysts, 1023 were sterile, 178 purulent/caseous, 2339 calcified and 532 fertile in Sardinia. Result deviations might be associated with many factors of intermediate host like age, seasonal diversity in different regions (Macpherson, 1984; Arbabi and Hooshyar, 2006).
These characteristics findings have never been reported in Pakistan earlier. Our investigations revealed that prevalence of type of cysts was significantly higher in fertile, sterile and under developed cysts among all animal species. However the prevalence of calcified cyst was non-significantly high in camels as compared to sheep, goats, buffaloes and cattle. These findings showed variations from previous researchers work might be due to geographical distributions. Our results revealed that prevalence was significantly higher among all animal species. However, camels showed high prevalence as compared to sheep, goats, buffaloes and cattle. Keeping in view, it is concluded that in Pakistan hydatidosis is highly out of control due to stray dogs and their easy approach to the abattoirs, improper disposal of hydatid organs and unhygienic conditions of abattoirs. Thus it is, therefore, suggested that more effort should be done for the prevention of hydatidosis.

From the inner germinal layer of hydatid cysts protoscoleces were created. Hooks were first discriminated structures formed at the apical part of the developing protoscoleces (Galindo et al., 2002). It was observed by Antoniou and Tselentis (1993) there were two types of hooks, larger hooks of upper row had a projecting rounded stout guard and smaller hooks of lower row had a flattened guard.

Echinococcus granulosus showed phenotypic variations in different intermediate hosts isolates. Ten genotypes of E. granulosus have been discovered by using various molecular techniques. Each genetically identified strain has specific phenotypic distinctions. Due to this variation, taxonomy of E. granulosus was revised by previous researchers (Thompson et al., 1995; Thompson and McManus, 2002). Different techniques have been used to identify the diverse strains. First of all morphological study of E. granulosus was reported by Thompson et al. (1984) then followed by many other investigators (Kumaratilake et al., 1986; Worbes et al., 1989).

It was reported by Thompson and Lymbery (1988) that morphological study could be less informative for strain identification. Three to four variables were used to analyse the larval hooks (Hosseini and Eslami, 1998). This was established as more convenient method for identification of various sub species of E. granulosus. Five parameters were selected for this study. From this data it was found that all values of total number of hooks and total length in small hooks (STL) of protoscoleces were found significant among all species but non-significant variations in large hooks.
length (LTL). Blade length showed significant variation in large hooks (LBL) but non-significant variations in small hooks (SBL).

This study revealed that total numbers of hooks were maximum in the protoscoleces of camel (36.2 ± 1.96) and minimum in the goat origin (27.0 ± 2.00). Total number of hooks of camel origin in this study was similar to camel isolates reported by Karimi and Dianatpour (2008). Large hook total length (23.35 ± 0.78) and blade length (12.08 ± 0.38) of sheep isolate was similar to sheep strain studied by Karimi and Dianatpour (2008); Ahmadi and Dalimi (2006); Gordo and Badera (1997); Kumaratilake and Thompson (1984).

Small hook total length (19.4 ± 0.07) and blade length (8.68 ± 0.11) of protoscoleces from human isolates in the present study was similar to the human isolates identified by Gordo and Badera (1997); Sweatman and Williams (1963). Both molecular and morphological techniques collectively or separately have been applied by various scientists for parasite identification (Kumaratilake and Thompson, 1984; Kumaratilake et al., 1986; Hope et al., 1992; Bowles and McManus, 1993; Washira et al., 1993; Bowles et al., 1995; Hosseini and Eslami, 1998; Zahang et al., 1998a; Fasihi-Harandi et al., 2002). Cattle origin investigations were also different from results of Worbes et al. (1989). NH (35.8 ± 1.82), SBL (11.89 ± 0.32) values of cattle origin were similar to the findings of Gordo and Badera (1997).

Our present investigations on morphometric analysis of protoscoleces, total numbers of hooks, large hook total length, large hook blade length, small hook total and blade length in cattle, buffalo and goat origin varied from the findings of Hussain et al., 2005. Buffalo and goat species are endemic to India and Pakistan, thus detailed literature on the traits of this particular species was not available.

Morphological study and statistical analyses showed that hooks morphology is not sufficient for strains identification of *E. granulosus* in Punjab, Pakistan. Advanced study is required for describing better criteria for strain identification among different species. From our molecular study we found various sub species of *E. granulosus* in various intermediate hosts.

Hydatid cyst fluid is not a simple substance; it is composed of many inorganic and organic materials (Frayha and Haddad, 1980; Sultan et al., 1984; Richards et al., 1987; Chowdhury and Singh, 1993; Thompson and Lymbery, 1995; Shaafie et al.,
In the present work biochemical analysis of fertile and sterile hydatid cyst fluid (HCF) was conducted to detect various components. The samples were collected from different intermediate hosts (sheep, goats, cattle, buffaloes, camels and human).

Biochemical analysis of hydatid cyst fluid was done by many investigators (McManus and Smyth, 1978; McManus 1981; Çelik, 1989). By quantitative analysis of HCF of liver it was found that values of alanine aminotransferase, glucose, urea, and sodium were not statistically significant (Macpherson and McManus, 1982). Sharif et al. (2004) were found significant differences in the following parametric values potassium, creatinine, calcium, triglyceride, cholesterol, uric acid, albumin, phosphokinase gamma glutamyl transferase, aspartate, aminotransferase and creatinine, variations in levels of chemical components of different HCF isolates revealed that there could be more than one sub species of *E. granulosus*.

It was found by various investigators (McManus and Macpherson, 1984; Thompson and Lymbery, 1995; Shaafie et al., 1999) that due to intermediate host specificity there was quantitative difference of biochemical parameters in the hydatid cyst fluids. There was possibility if same strain develops in different intermediate hosts, it may acquire changes in metabolism for parasite endurance (Thompson and Lymbery, 1995). Rahdar et al. (2008) investigated for Ca, P, Mg, Na and K, there were significant variation between serum and fluid of cyst, also in sheep. In our present study elevated levels of uric acid, creatinine, magnesium and sodium were found in human fertile isolates and also creatinine in sterile isolates, similar elevated level of uric acid was also reported by Shaafie et al. (1999) and Radfar and Iranyar (2004)

This present data for fertile and sterile isolates for biochemical profiles showed significant (*p*<0.05) differences among all animal species and human isolates. The focal strain which causes infection in human is sheep strain (Thompson and Lymbery, 1995). In our findings it was found that potassium (mmol/l) values for sheep (sterile), camel (fertile) and goat (fertile) were nearly similar to the findings of Izadi and Ajami (2006), similar findings have also been reported by Radfar and Iranyar (2004). In our results it was found that sodium (mmol/l) values for goat (sterile), human (fertile) and cattle (fertile) were comparable as reported by Izadi and Ajami (2006). Glucose, cholesterol, triglyceride and urea values found in present investigations were lower and creatinine were higher in cattle, sheep, goat, camel and
human isolates as compared to the results of Izadi and Ajami (2006). Values of calcium for sheep (sterile), camel (fertile) and cattle (fertile, sterile) found in our results were nearly similar to those of Izadi and Ajami (2006).

In sheep (fertile) isolates, our results for Ca, Mg, Na, K were different as compared to the Rahdar et al. (2008). Total protein (g/l), copper (mmol/l), triglyceride levels for fertile and sterile isolates showed significant difference among human and animal species \( p<0.05 \), our values were different from those reported by Radfar and Iranyar (2004) and Izadi and Ajami (2006). Values of copper for fertile and sterile groups of sheep were lower than the values reported by Kojouri and Moshtaghi (2008). Values of Refik et al. (2002) for glucose, triglycerides, calcium varied from our work but sodium, potassium, chloride were nearly similar in all sterile samples of sheep and cattle. Buffalo species is endemic to India and Pakistan, so detailed report on this study could not be found from the literature available. Present analysis showed fertile and sterile isolates of buffalo revealed significant differences \( p<0.05 \) in urea, triglyceride, calcium, cholesterol, uric acid, potassium, magnesium, copper and insignificant differences \( p>0.05 \) in the values of chloride, creatinine, sodium, glucose and total protein.

Present study revealed significant variation of biochemical parameters in different hydatid cyst fluids of various intermediate hosts (animals and human) isolates. Variation in composition of hydatid cyst fluids in different species of animals and human may be due to multiple strains of \( E. \) granulosus in Punjab, Pakistan. This analysis will be further useful for immunological studies, diagnostic tests and may be helpful to find out various strains of \( E. \) granulosus in Punjab.

Polypeptide analysis of purified hydatid cyst fluids of different species (Sheep, goat, cattle, buffalo, camel and human) was conducted under reduced conditions to evaluate number of polypeptides and their molecular weights in each species. Both number of polypeptides and molecular weight as observed in our study were not similar. Variable segregation pattern of proteins was observed as mentioned in results. Fractionation of fertile purified, the fluids of sheep (209kDa, 138kDa, 63 kDa), cattle (269kDa, 89kDa, 59kDa) and camel (195kDa, 166kDa, 123kDa) revealed 3 protein in each case but their molecular weights varied alot. According to our analysis proteins of HCF are variable as have been reported by Sabry (2007), while conducting research on diagnosis of hydatidosis in human and living animals. Similar findings on
variation of total protein patterns of protoscoleces of *E. granulosus* were reported by Jiang *et al.* (1998). In addition difference among crude are purified hydatid cyst fluid of Bovine and Swine isolates were most complex in the findings of Siles-Lucas Cuesta-Bandera (1996). Derbala (1998) performed polypeptide analysis of camel HCF and observed seven polypeptide bands with variable molecular weights. The findings of Derbala vary with reference to number of molecular weight polypeptides, as only three proteins were present in electrophotograms of camel HCF in present study.

Polypeptides separated by SDS-PAGE differed in cyst fluid samples of cattle and sheep in the findings at Itagaki *et al.* (1994), which supports present results, they further reported 5 polypeptide bands. Segregation patterns of protein were also variable in goat, human and buffalo isolates. Number of polypeptide bands ranged between 3 to 5. However, a common protein having molecular weight of 166kDa was also found in isolates of buffalo and camel. Similarly a protein with molecular weight of 138kDa was common in sheep and human isolates. Common proteins have been reported by Burgu *et al.* (2000), while working on immunodiagnosis of hydatidosis in sheep and humans by western blotting. The molecular weight of this protein was 116 kDa, a little lower than the proteins observed in preset findings.

The protein of *E. granulosus* from sheep liver’s as investigated by Doganay *et al.* (2004) ranged between 68.24 and 8kDa, quite different from our results of SDS-PAGE conducted on HCF of sheep. Simsik and Koroglou (2004) analyzed HCF and detected 6 bands from sheep. Reports on the suggested work that the profile of proteins of HCF from different species differs from one geographical region to another. Similar comments have been indicated by Shambesh *et al.* (1995); Siles-Lucas and Cuesta-Bandera (1996), in their reports on partial characterization of cyst fluid of buffaloes.

The protein patterns of protoscoleces (PSC) by SDS-PAGE performed on HCF exhibited variable results in number and molecular weight of polypeptides, both in protoscoleces and hosts. Therefore it was concluded that diagnosis of hydatidosis in human and animals can not be relied only on detection of polypeptides. For exact diagnosis serological as well as molecular techniques may be used for assistance.
The prevalence of hydatid disease in livestock present in the present study reveals that the prevalence of hydatidosis in Pakistan has been declining over the past few decades (Khan and Haseeb, 1984; Iqbal et al., 1989; Shafiq et al., 2005), but that the disease is still present at a moderately endemic level. In spite of the rules and regulations of the provincial and local governments about the slaughter of animals, a vast majority of animals are slaughtered and their meat sold or disposed off without any veterinary supervision throughout the countryside and in towns and where proper disposal of offal is not practiced (Khan and Purohit, 2006). Nevertheless, there is a trend of declining prevalence observed among livestock likely due to the presence of over 180 local council or municipal owned abattoirs in peri-urban regions. However, in stating this, it is likely that in the present study, the government-run abattoirs that were sampled are more likely to attract livestock from large-scale livestock production facilities that are intensively managed rather than the poorly resourced rural farmer. This study is therefore unlikely to represent the prevalence of hydatid disease of food producing animals in poorly resourced rural communities, which is expected to be significantly higher.

Camels and buffalo were found to have the highest prevalence of hydatid cysts. This is most likely due to the older age at which the animals are slaughtered. Most cattle bought for slaughter are young male calves, a vast majority younger than three months, which may account for the lower prevalence in this host.

This is the first study to genetically characterise the strains of *E. granulosus* from livestock and humans in Pakistan. The common sheep strain (G1) and buffalo strain (G3) were the only genotypes found to cycle among livestock in Punjab and appear to be highly host adapted to producing fertile cysts in all domestic livestock, including camels. Although the camel strain of *E. granulosus* (G6) has been shown to occur in the Middle East (Ahmadi and Dalimi, 2006), China (Zhang et al., 1998) and Africa (Mrad et al., 2005), it is surprising that this strain is not present in the Punjab region of Pakistan. Camels provide an important source of subsistence and income with the major portion of the camel population found in Balochistan (mountainous south west) (36%), followed by Punjab (34 %). Camels frequently migrate across the Afghan border, especially when raised by nomadic herders (Aujla, 1998).
Moro et al. (2009) found that *Echinococcus granulosus* sensu stricto (sheep strain or G1) was the most prevalent in human patients, sheep, and cattle and the G6 genotype (*E. canadensis* camel strain) was found in goats and in one human patient.

The morphological and molecular results support earlier studies suggesting that *Echinococcus* of buffalo origin is phenotypically and genetically similar to the sheep (G1) and Tasmanian Sheep (G2) strains of *Echinococcus*. Both sheep and buffalo strains occur sympatrically, which adds further evidence to support its recognition as one species *viz*., *Echinococcus granulosus* sensu stricto. However, despite the high prevalence of the buffalo strain, both isolates of hydatid cysts recovered from humans in Punjab were found to belong to the G1 genotype of *E. granulosus*, in agreement with previous studies showing it to be the most common genotype to infect humans throughout the world (Li et al., 2008; Snabel et al., 2009).

This study demonstrates that hydatid disease is still highly endemic in Pakistan. The presence of the common sheep (G1) and buffalo (G3) strains of *E. granulosus* in livestock in Pakistan coupled with the challenge of controlling illegal and back-yard slaughter practices in rural communities may explain why the incidence of human echinococcosis is so high in the area. The finding of the common sheep strain (G1) of *E. granulosus* in two humans in Pakistan, reinforces the need for greater attention to be placed on hydatid disease control and public health.
Conclusion

The outcome of present investigation demonstrates that hydatidosis is endemic in Pakistan. Two strains, G1 (sheep strain) and G3 (buffalo strain) were found in livestock and G1 strain in human only. However, camel isolates was found clustered within G1^4 haplotype. Regardless of host species the isolates belonging to G1 and G3 group have similar morphology for both large and small hooks of protoscoleces, that clearly showed this may not have any significance with species identification. On the basis of morphological, biochemical, and SDS-PAGE analysis conducted during present investigation it is suggested that PCR based analysis can play major role for strain identification.

Recommendations

- Regular deworming of dogs.
- Killing of stray dogs.
- Safe disposal of infected viscera.
- Practicing personal hygiene, especially the washing of hands after handling dogs and before eating by following teaching of Islam regarding personal hygiene.
- Avoiding unnecessary handling of dogs.
- Not feeding dogs with uncooked meat / beef or offal.
- Strict legislation against butchering in houses especially at the time of religious festival Eid-ul-Adha.
- Serological survey of people, which are at risk butchers and herdsman.
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