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RESEARCH ARTICLE

Prevalence and Associated Risk Factors of Cystic Echinococcosis in Food Animals – A Neglected and Prevailing Zoonosis

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A B S T R A C T

Cystic Echinococcosis (CE) is a neglected tropical disease (NTD) impacting the health and economy of the developing states including Pakistan. It is mainly caused by *Echinococcus granulosus* and renders huge economic losses to the livestock sector. This study investigated the prevalence of CE and associated risk factors. A total of 503 samples from food animals (including 226 cows, 102 buffaloes, 98 sheep, and 77 goats) were inspected for the presence of hydatid cysts by conducting postmortem at various slaughterhouses in district Narowal-Pakistan. Fertility and viability of collected hydatid cysts were assessed by microscopy and confirmatory diagnosis was obtained through conventional Polymerase Chain Reaction (PCR). A pre-structured questionnaire was used to collect baseline data in order to identify the associated risk factors with CE. An overall prevalence of 8.15% was found while in each species it was 12.83% in cattle, followed by buffalo (6.86%), sheep (4.08%) and goat (1.29%), respectively. The order of infection was highest in liver (35.71%) followed by lungs (33.33%) and then both organs at the same time (20%). Such epidemiological findings could help in developing preventive strategies and control programs for CE in Pakistan and other developing countries.

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INTRODUCTION

Echinococcosis is a serious zoonosis and public health problem across the globe. The two most important forms of the disease are cystic echinococcosis (hydatidosis) and alveolar echinococcosis (Romig *et al*., 2017). Cystic Echinococcosis (CE) is caused by a cestode characterized by flat ribbon-like body consisting of anterior scolex, posterior tape and multiple segments (Arbabi *et al*., 2017). Adult worms lack a gut and directly absorb the nutrients (Malik and ul Bari, 2019). Their indirect life cycle involves a variety of hosts in the transmission of infection. Canids (dogs, dingoes, wolves, coyotes) act as definitive/primary hosts for adult worms while humans, domestic animals such as sheep, goat, cattle, buffalo, horse, and wild animals such as deer act as intermediate host for the larval stages (Romig, 2003). Life cycle has three developmental stages i.e. egg, larvae and adult (Romig *et al*., 2017). Adult stages are not usually pathogenic but larval stages are very pathogenic and cause huge socio-economic losses to both human and livestock population (Laurimaa *et al*., 2015). A wide Adult tapeworms reside in mucosa of the small intestine in dogs (definitive host) while the larvae (metacestode) occupy the visceral organs of intermediate hosts involving liver (>65%), kidney (almost 25%), spleen, and rarely in the brain and bones (Moro and Schantz, 2009).

The World Health Organization (WHO) has declared Echinococcosis as a public health issue addressed within its strategic plan to control NTDs. The 2015 WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) estimated CE to be the cause of 19300 annual deaths and around 871000 disability-adjusted life-years (DALYs) globally. Annual costs associated with CE are estimated to be US$ 3 billion for treating cases and losses to the livestock industry (Jain *et al*., 2019). At least 270 million people (58% of the total population) are at risk of acquiring CE in Central Asia, including areas of

Mongolia, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Afghanistan, Iran, Western China and Pakistan.

Pakistan has a large livestock population and rearing of livestock for milk and meat purpose are the only sources of income for rural and peri-urban inhabitants (Usman, 2016). Parasites like CE are prevalent among various geographical areas in Pakistan. The CE is an emerging public health problem that is causing socio-economic losses to the country. There are ten different geographically distributed strains of CE ranging from G1-G10. These genotypically distinct strains exist with unalike host empathies. These include sheep strain (G1), goat strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strains (G7, G9), and deer strains (G8, G10) (Romig *et al.,* 2015; Ehsan *et al.,* 2017; Spotin *et al.,* 2017). The G1 sheep strain is described as the most communal and frequent source of ailment in humans and animals ((Shahzad *et al.,* 2014).

There is a dire need of cooperation between developed countries and marginalized Asian countries to control CE. Limited data and epidemiological investigations have been carried out in the remote and border line areas of Pakistan where debilitating parasites are affecting the health and production of animals extensively. The present study has been designed to focus the need of these epidemiological investigations.

MATERIALS AND METHODS

Study Area: This was a cross-sectional study that involved simple random sampling. This study was conducted in district Narowal, Pakistan that has three tehsils named Narowal, Shakargarh, and Zafarwal. Local slaughterhouses are situated within these three tehsils. According to Livestock Census Punjab 2018, total livestock population of the district is 1,213,241 animals. In addition to the huge population of livestock, free roaming dogs are also infected with CE (Dakkak 2010; Ahmed *et al.,* 2017).

Sampling: A total of 503 samples from food animals (226 cattle, 98 sheep, 102 buffalo, and 77 goats) were screened for the presence of hydatid cysts by post-mortem examination at various abattoirs of district Narowal between September 2019 and February 2020. Visceral organs of slaughtered animals were thoroughly examined, palpated, and incised for the detection of hydatid cysts. Samples were collected in sterile plastic containers containing 70% ethanol. The gathered samples were carried to the laboratory and kept at room temperature for further examination (Adwan *et al.,* 2013).

Fertility of Hydatid Cysts: After transportation of samples from abattoirs to the laboratory, cysts were picked up separately from the plastic containers and their surface was washed with alcoholic iodine solution to sterilize these cysts (Oskouei *et al*., 2016). Hydatid cysts were then punctured with a sterile needle to collect inner contents. Fertility of the cysts was examined under light microscope. A cyst having protoscoleces in its inner cavity was referred as fertile cyst while a cyst lacking protoscoleces was termed as infertile cyst (Hidalgo *et al.,* 2019). In case of a fertile cyst, protoscoleces were used for DNA extraction while in the case of an infertile/sterile cyst, the cyst wall (germinal membrane) was used for the same purpose (Ali *et al*., 2015).

Viability of protoscoleces: The viability of the protoscoleces was evaluated by assessing the motility of flame cells and capability of each Protoscolex to be stained with 0.1% eosin. Under a light microscope, live/viable protoscoleces were not stained while the dead ones were stained by the eosin (Mahmoudvand *et al.,* 2016).

DNA extraction: WizPrep™ gDNA Tissue Kit (wizbiosolutions, South Korea, REF # W71060-300) was used to extract DNA from the samples which were declared positive by microscopy for having viable protoscoleces.

Polymerase chain reaction (PCR): PCR was carried out in a DNA thermal cycler (T100 Thermal Cycler, Bio-Rad, USA) using species-specific primers targeting nad1: forward primer Eg1F81 (5’ GTT TTT GGC TGC CGC CAG AAC ‘3) and reverse primer Eg1R83 (5’ AAT TAA TGG AAA TAA TAA CAA ACT TAA TCA ACA AT ‘3) (Boubaker *et al*. 2013).

PCR conditions were adopted and gel was stained with SYBR® Safe dye and examined in a UV trans-illuminator for visualization of PCR products Fig. 1.

DNA sequence and Phylogenetic analysis: PCR products were submitted for sequencing to a commercial company (Advanced Biosystems, Lahore, Pakistan). The accuracy of data was confirmed by bi-directional sequencing. Previously published sequences of *E. granulosus* isolates at National Center for Biotechnology Information (NCBI) were used as reference sequences. The obtained sequences were aligned and compared with reference sequences using ClustalW. Unique nucleotide sequences generated in this study were deposited in GenBank under accession numbers MW846621 and MW846622 for NAD1 sequences.

Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site.

Phylogenetic Analysis: The evolutionary history was implied using the Neighbor-Joining method (Saitou and Nei, 1987).

Statistical analysis: The weighted proportion estimate with 95% Cl (confidence interval) of the overall prevalence was computed by using formula (Thrusfield, 2008) “R” software (version 3.04). The association of risk factors (such as dog keeping and feeding pattern of animals) with disease outcome was determined using Chi-

square and Fisher's exact test Table 4. Data collected through questionnaire was analyzed using Statistical Package for Social Sciences (ver. 20.0).

RESULTS

Postmortem examination: One hundred twenty-five (n=125) hydatid cysts were collected after screening of 503 animals [cattle=81 (64.80%), buffalo=22 (17.60%), sheep=15 (12.00%), goat=7 (5.60%)]. Microscopy found 85 (68%) positive samples (having viable protoscoleces). Other cysts may belong to *Taenia solium* cysticerci (Brunetti and White 2012; Giri and Parija 2012). Out of these samples, 54/85 were fertile, 22/85 were sterile and 9/85 were calcified. DNA extraction was performed on fertile and sterile cysts out of these 85 cysts (n=76). Only 41/76 samples were found having DNA of and amplified a 226-bp fragment in gel electrophoresis. None of the similar amplicons were detected in control negative samples.

Prevalence of *E. granulosus* in district Narowal: An overall prevalence of *E. granulosus* was noted as 8.15% in district Narowal, Pakistan. Highest prevalence was recorded in Shakargarh (11.04%), followed by Zafarwal (7.69%), and Narowal (6.02%) Table 1. Specie wise and organ wise prevalence of *E. granulosus* is given in Table 2 and Table **3** respectively.

Phylogenetic analysis: The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. This analysis involved 18 nucleotide sequences. All uncertain positions were removed for each sequence pair (pairwise deletion option). There were a total of 1213 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018) Fig. 2. Results of present study confirmed the presence of E. granulosis in cattle. These results are in common with other studies conducted in surrounding countries like India (Beigh *et al.*, 2021) and Iran (Shahabi *et al.*, 2021). We can estimate that E. granulosis must be circulating in other livestock species as well and more research should be conducted in this domain.

DISCUSSION

This study showed that out of 503 animals, 38.17% (192/503) were male while 61.82% (311/503) were female. Chi-square analysis revealed that there was a high proportion of female animals brought for slaughtering at various abattoirs. The results corroborated the findings of Iqbal *et al*. (2012) who had conducted a study on small ruminants at Lahore and concluded that 11.2 % male and 12.30% female sheep were infected with *E. granulosus*. Similarly, the rate of infection in female goats was also high with involvement of 7.58% males and 8.25% females (Iqbal *et al*., 2012). Our results differ from that of Khan *et al*. as they recorded that males (9.50%) were more infected with hydatid disease than females (1.96%) in cattle, buffalo, sheep, and goat (Khan *et al*., 2018). Ehsaan *et al*., conducted a gender-wise analysis of Echinococcosis in food animals brought at various abattoirs of Hyderabad and concluded that 9.77% of males and 14.43% of female animals were infected with Cystic Echinococcosis (Ehsan *et al*., 2017).

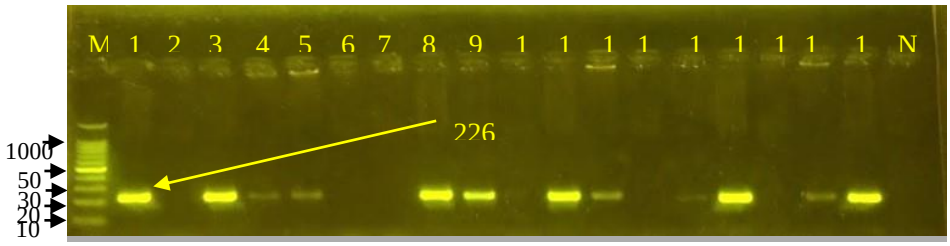

Fig. 1: PCR based detection of *Echinococcus granulosus* species from cattle (Lane 1-7), buffalo (Lane 8-12), sheep (Lanes 13 and 17) and goat (Lane 18). Lane M: Molecular ladder; Lane N: Control Negative; Lanes 1, 3, 4, 5, 8, 9, 11, 12, 14, 15, 17, 18.

Table 1: Tehsil wise Prevalence of *E. granulosus* in district Narowal

Tehsil	No. of inspected animals	Positive by PCR	Prevalence (%)	X^2	P - Value
Narowal	166	10	6.02		
Shakargarh	172	19	11.04	6.453	0.040
Zafarwal	156	12	7.69		
Total	503	41	8.15		

While talking about species wise prevalence of *E. granulosus*, highest prevalence was observed in cattle (12.83%) shadowed by buffalo (6.86%), sheep (4.08%) and goat (1.29%). An overall prevalence of *E. granulosus* was recorded as 8.15%.

Table 2: Prevalence of *E. granulosus* in different species of food animals

Specie of animal	No. of inspected animals	Positive by PCR	Prevalence (%)	X^2	P - Value
Cattle	226	29	12.83		
Buffalo	102	7	6.86		
Sheep	98	4	4.08	29.070	0.000
Goat	77	1	1.29		
Total	503	41	8.15		

Table 3: Prevalence of *E. granulosus* in different organs of food animals

Organ	No. of organs having cyst	Positive by PCR	Prevalence (%)	X^2	P - Value
Liver	84	30	35.71		
Lungs	24	08	33.33		
Both (Liver and Lungs)	15	03	20.0	142.106	0.000
No Cyst found	380	00	00.0		
Total	503	41	08.15		

Evaluation of risk factors: Collected data were analyzed through chi-square using Statistical Package for Social Sciences (SPSS).

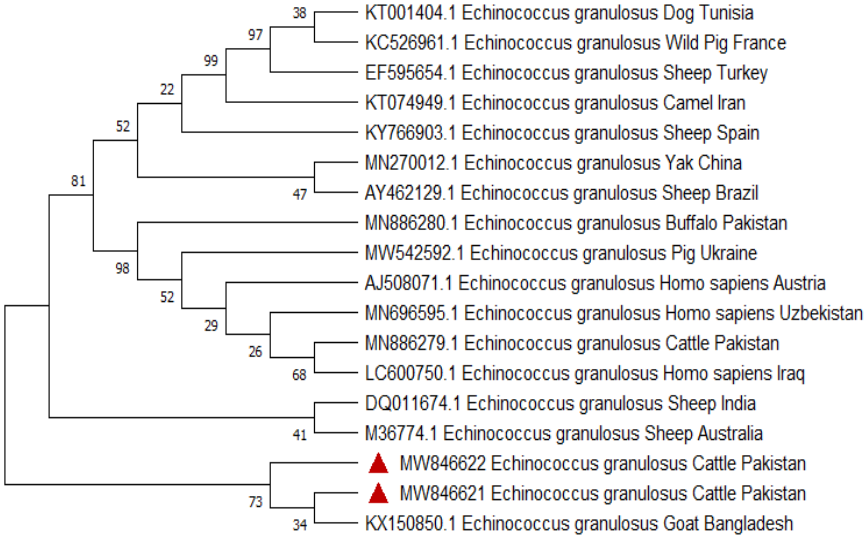

Fig. 2: Phylogenetic relationships of *Echinococcus granulosus* isolates from Pakistan compared to reference sequences of different *Echinococcus* species in NCBI database.

Table 4: Detailed analysis of the respondent responses collected through Questionnaire

Variable	Response	No. of Positive/ Total	X^2	P - Value
Animal gender	Male	14/192	0.306	0.619
	Female	27/311		
Age (years)	≤ 1	2/69	8.029	0.045
	2	11/167		
	3	12/151		
	≥ 4	16/116		
Hydatid Cyst	Yes	41/125	134.987	0.000
	No	0/378		
Cyst Type	Fertile	31/54	237.861	0.000
	Sterile	8/22		
	Calcified	2/9		
Herd Type	Mixed	36/415	0.869	0.519
	Single	5/88		
Dewormer usage in animals	Yes	4/154	20.713	0.000
	No	30/202		
	Do not Know	7/147		
Feeding pattern	Grazing	22/118	22.983	0.000
	Confined	5/130		
	Mix	14/255		
Dog keeping	Yes	33/217	25.382	0.000
	No	8/286		
Reason for keeping dog	Hunting	5/36	37.463	0.000
	Watch dog	26/133		
	Companion/pet	3/45		
Dogs accompany the herd while grazing	Yes	31/154	42.981	0.000
	No	3/60		
Deworming of dog	Yes	8/87	38.549	0.000
	No	26/127		
Dog feeding with viscera	Yes	33/166	45.530	0.000
	No	1/48		
Free roaming of dog	Yes	30/179	30.891	0.000
	No	4/35		
Proper disposal of dog feces	Yes	5/61	36.520	0.000
	No	29/153		
Presence of stray dogs in community	Yes	41/485	1.657	0.385
	No	0/18		
Presence of other dogs at grazing site	Yes	37/359	7.781	0.004
	No	4/144		
Home slaughtering	Yes	7/130	1.792	0.199
	No	34/373		
Disposal of offal	Buried/burn	2/61	2.278	0.320
	Leftover/scavenge	5/63		
Meat inspection	Yes	0/1	1.554	0.460
	No	7/125		
Distance from the nearest abattoir	≤3 km	5/42	15.971	0.003
	4-5 km	16/97		
	6-10 km	10/110		
	≥11 km	7/174		
	Do not know	3/80		

Iqbal *et al*. (2012) conducted a study in Lahore, Pakistan on the estimation of hydatidosis prevalence and found 8.85% prevalence in sheep and 6.21% in goats. In Quetta, Ahmad *et al*. (2006) has calculated 46.74% prevalence of hydatidosis in sheep. Cattle prevalence was (56.6%) as compared to another species of cattle called *E. multilocularis* (43.3%) (Ali *et al*., 2015). The highest prevalence of *Echinococcus granulosus* in cattle were (12.83%), followed by buffalo (6.86%), sheep (4.08%), and goat (1.29%). During 2004-2008, Latif *et al*. (2010) have also reported 15.18% prevalence in cattle, 11.19% in buffalo, 7.52% in sheep, and 5.48% in goat. Our study differs with that of Latif (2009) as they have estimated the highest prevalence in camels (17.29%), followed by sheep (7.52%), buffalo (7.19%), cattle (5.18%), and goat (5.48%). In another study, the prevalence of hydatidosis was found to be 3.24% in sheep and 2.44% both in goat, and cattle (Mustafa *et al*. 2015). Surhio *et al*. (2011) found 10.6% prevalence of hydatidosis in sheep and 10.2% in goat. Shahzad *et al*. (2014) conducted an epidemiological study in three districts of Punjab including Lahore, Jhang and Okara and reported highest prevalence in buffalo (60.46%), followed by cattle (45.45%), sheep (20%) and goat (20%). Highest prevalence of Echinococcosis was estimated and reported in buffalo (5.25%) followed by cattle (3.43%), sheep (2.20%) and goat (1.76%) (Khan *et al*., 2020). Similarly, Khan *et al*. (2021) have conducted a prevalence study in the province of Khyber Pakhtunkhwa (KPK) and recorded highest prevalence of hydatid cysts in buffaloes (15.88%) shadowed by cows (15.79%), sheep (15.38%), and goats (3.25%). These differences could be related to geographical locations, age of studied animals and sample sizes.

There are mainly two organs infected with hydatid cysts, which are the liver and lungs. This study found liver infectivity (35.71%) and lungs (33.33%) while both liver and lungs involvement were found in 20% cases. Shahzad *et al*. (2014) found 37.5% prevalence in liver and 57.14% in lung samples. Latif (2009) recorded the organ wise prevalence (%) of *Echinococcus granulosus* for cattle (84.51, 15.48), buffaloes (51.71, 48.29), goats (66.18, 32.60), sheep (67.81, 32.19) and camels (83.33, 16.66) in liver and lungs respectively. The prevalence of hydatid cysts was higher in liver as compared to lungs. In 2006,

Ahmed and co-workers stated the prevalence of hydatidosis in sheep liver as 46.74% and goat liver as 23.28% (Ahmed *et al*. 2006). Mehmood *et al*. (2020) conducted a comprehensive study on prevalence of Echinococcosis in Punjab and observed liver as the most preferred site for cyst localization followed mainly by lungs. Microscopy is an excellent screening test for Echinococcosis. Various parameters like fertility and viability of hydatid cysts can be detected through this method. However, in order to confirm the species, one must go for PCR (Moro and Schantz, 2009; Ergin *et al*., 2010).

Another study revealed the percentage prevalence of hydatid cysts based on the type of cyst (fertile, sterile, calcified) in cattle 52.5 (42/80), 11.25 (9/80), 7.5 (6/80), buffalo 26.08 (6/23), 39.13 (9/23), 8.69 (2/23), sheep 33.33 (5/15), 20 (3/15), 0 (0/15), and goat 14.28 (1/7), 14.28 (1/7), 14.28 (1/7) respectively. Latif (2009) conducted a similar study and noted the prevalence (%) of hydatid cysts on the basis of fertile, sterile, calcified, and under-developed in sheep 86.40, 6.40, 4.80, 2.40, goats 79.09, 6.36, 5.45, 9.09, cattle 75.24, 14.85, 3.96, 5.94, buffaloes 84.31, 9.80, 4.90, 0.98 and camels 95, 2, 1, 2 respectively. Manterola *et al*. (2006) found fertility rates of hydatid cyst in sheep as 37% for liver and 26% for lung, while in water buffalo, it was 46% for liver and 44% for lung.

In the present study, animals were divided into four age groups. ≤1, 2, 3 and ≥4 years. First group (≤1) was comprised of 13.71% (69/503) animals while highest number of animals, 33.20% (167/503), were found in second age group. Animals with 3 years of age were 30.01% (151/503) while 31.21% (157/503) were having age ≥4 years. Statistical analysis showed that animals older than 4 years of age are significantly associated (p-value < 0.05) with the positivity of disease. Surhio *et al*. (2011) estimated that sheep older than 2 years of age are greatly infected (16%) with *E. granulosus*, followed by sheep having age between 1 to 2 years (7.24%). Sheep having age less than 1 year showed least potential of disease (2.05%). Ali *et al*. (2015) found <1-year animals as 11.29 %, 1-5 years as 12.32% and >5 years as 28.26% having CE.

Conclusions: It is concluded that owing to the presence of socio-economic conditions favorable for hydatidosis and maintenance of high level of infection in cattle and sheep, hydatidosis is one of the most important diseases in the study area. The findings of the present study reflect the economic and zoonotic impact of hydatidosis which deserves serious attention by the various stakeholders in order to reduce losses to livestock sector and safeguard the public health. Establishment of policy on dog keeping and handling including registration, treatment and elimination of stray dogs, promoting construction of abattoirs with their appropriate disposal pits with obligatory meat inspection services are highly essential. Furthermore, detailed investigation into the basic local epidemiological factors governing the spread of hydatidosis in the region is recommended.

Authors contribution: QM, URZ, MY and AI conceived the study, drafted the manuscript, prepared the questionnaire, performed the statistical analysis and revised manuscript. QM and SK performed experiments in laboratory. SK and AI entered data and filled questionnaires, SS and SI provided intellectual inputs. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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