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SEROPREVALENCE AND RISK FACTORS ASSOCIATED WITH TOXOPLASMOSIS IN SHEEP IN MULTAN AND KHANEWAL DISTRICTS OF PUNJAB (PAKISTAN)

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ABSTRACT

During the present study 500 serum samples were examined to determine the seroprevalence of toxoplasmosis and risk factors in sheep by using latex agglutination test (LAT) and enzyme linked immunosorbent assay (ELISA) in Multan and Khanewal districts, Punjab (Pakistan). The aim of the present study was to identify the potential risk factors associated with toxoplasmosis in sheep. The overall seroprevalence was 33.6% by LAT and 27.4% by ELISA. The area wise prevalence of toxoplasmosis was 34.02% and 33.01% by LAT and 28.12% and 26.41% by ELISA in Multan and Khanewal respectively. Difference was statistically non-significant (P>0.05). Out of 449 female sheep 60 were aborted. Relationship between toxoplasmosis and incidence of abortion indicated that seroprevalence was 51.66% and 41.66% in aborted sheep and 31.1% and 26.22% in non-aborted sheep by LAT and ELISA respectively. Differences were statistically significant (P<0.05) by both techniques. The results further revealed the non-significant differences between infection rates in males 31.37% and 19.6% and in females 33.85% and 28.28% via LAT and ELISA respectively. The prevalence was higher in females than males and difference was statistically non-significant (P>0.05). Relationship between age groups showed that toxoplasmosis prevalence was higher in age group 60-73 months (64.5% and 38.7% by LAT and ELISA respectively) and lowest in age group 4-17 months (26.4% and 22.4% by LAT and ELISA respectively) difference was statistically significant (P<0.05) by LAT and non-significant (P>0.05) by ELISA. From the result of present study it is concluded that the large flock size, presence of cats, and the improper disposal of aborted fetuses openly left on ground were the main causes of higher rates of prevalence of toxoplasmosis in sheep which are potential threat of infection for human population of study area.

Key words: *Toxoplasma gondii***,** Sheep, sex, age, abortion, LAT, ELISA.

INTRODUCTION

Livestock rearing is most secure source of earning for the small and landless poor farmers and source of employment generation at the rural level (Ugwu, 2007). It is a sign of prestige for those people related with agriculture sector. It is an essential part of socio-economic activities of the Pakistan rural areas and plays a very vital role in mitigating the effects of poverty by providing necessary items of daily usage, reduced fluctuations in income due to crop failure and improves socioeconomic condition of Pakistan's rural masses (Anonymous, 2012). Its importance in Pakistan's rural economy is evident from the fact that 30-35 million peoples are busy in rearing livestock to earn 30-40 % of their income (Pakistan Livestock Census, 1996). Livestock's have natural ability to convert the shrubs and grasses into quality food such as milk and meat. It is well-known nutritional fact that animal proteins are best source of essential amino acids than vegetable proteins. Sheep and goats are efficient users of Pakistan's rangelands without them it would not be possible to profitably utilize stubble, crop residue, roadsides and scattered vegetation on the rangelands and hilly areas throughout the country. Sheep are well known for their mutton quality and males are especially reared for sale on special occasions as sacrificial animals (Qureshi *et al*., 2010).

Toxoplasmosis is a common zoonotic disease with worldwide distribution from Alaska to Australia and is caused by an obligate intracellular and protozoan parasite, *Toxoplasma gondii* which completes its sexual life cycle only in the intestinal epithelial cells of cats and asexual reproduction in other intermediate hosts such as birds and mammals (Hill and Dubey, 2013). Cats are the definitive hosts and natural reservoirs of infective oocysts and excrete the resistant oocysts to environment with feces (Abu-Dalbou *et al*., 2010; Dubey, 2010). Oocysts of *T. gondii* are environmentally resistant stages which may remain viable in the soil even for years. Sporulation of oocysts takes place outside the body of cat within 1-5 days after defecation, at availability of moderate temperature, aeration and humidity and become infective (Jimenez-Coello *et al*., 2012).

Small ruminants may get infection after use of forage or feedstuffs contaminated with sporulated oocysts or congenitally through placenta (Innes *et al*., 2009). Toxoplasmosis can cause great economic losses to small ruminants by inducing abortions, still birth, early

embryonic or fetal death, or birth of weak kids or lambs and reducing fertility (Edward and Dubey, 2013). In some countries a live vaccine (Toxovax) is commercially used to reduce economic losses to sheep industry which get congenital infection of toxoplasmosis (Abu-Dalbou *et al*., 2010).

It is estimated that one third human's population throughout the world is infected by this parasite (Hill and Dubey, 2013). Although most infections in humans are asymptomatic, some complications may occur as a result of congenital toxoplasmosis such as stillbirth, abortion, mortality and hydrocephalus in newborns or retinochoroidal lesions which cause chronic eye diseases as well as lymphadenopathy, encephalitis and retinitis may be observed in AIDS infected persons due to postnatal acquired infection (Hill and Dubey, 2002; Olivier *et al*., 2007).

MATERIALS AND METHODS

Study Area: Blood samples were collected from various agricultural regions of district Multan (Chak No. 4 KMR, 5 KMR, 11 KMR, 7T, 8T, 9T, 10T) and district Khanewal (2 KMR, Bhai Veer, Jangal Maryala, Chak No. 174/10R, Chak No. 154/10R, Chak No. 155/10R) to determine the seroprevalence of toxoplasmosis. Arid climatic conditions are present in these regions with very hot summers and mild winters.

Collection of Samples: Five hundred blood samples were collected from sheep population (n=500; Lohi breed) of districts Multan and Khanewal through random sampling from April 2012 to June 2013. Briefly the 10 ml blood sample was taken from each animal with the help of disposable syringes by puncturing the jugular vein. Out of this, 3 ml blood was poured in the plastic tubes containing 100 µL of 0.5 M EDTA as anticoagulant for hematological studies and the remaining 7 ml of blood was poured slowly in anticoagulant free plastic tubes to collect the sera samples. The host's sex, age, herd size, presence or absence of cats with herd, methods of disposing aborted fetuses and other related information were recorded on a specially designed questionnaire. The blood samples were taken to Parasitology Research Laboratory at Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan (Pakistan) for further analysis. Antibodies against *T. gondii* in the sera samples were detected with the help of ELISA (ID. Vet, France) and Latex Agglutination (Antec Diagnostic, UK).

Principle of Latex Agglutination Reagent: Toxoplasmosis Latex Kit (Antec Diagnostic, Uk) was used to determine *T. gondii* antibodies in blood samples of sheep which contains the following contents:

a. Latex reagent
b. Positive control
c. Negative control

Reagent contains suspension of polystyrene particles coated with antigen of *T. gondii*. Positive control shows agglutination when added to serum and negative control does not show agglutination. Antigen-antibody reaction could take place when serums which contain antibodies against *T. gondii* were tested and this reaction can be easily visualized because of agglutination.

Both, the reagents and serum were brought at the room temperature prior to use. Sera were diluted 1:16 in 0.9% saline solutions. One drop of diluted serum sample was placed onto the black area of the slide. The latex reagent was mixed well and one drop was added to each serum drop. Both drops were mixed with the help of a stirrer and the slide was tilted. The presence or absence of agglutination was observed within the period no longer than three minutes. The positive sera indicated the milky Latex agglutinates, while in negative sera no agglutination was noted.

Principles of Enzyme-Linked Immunosorbent Assay (ELISA): Multi-species commercial ELISA kit (ID Vet, France) was used to examine *T. gondii* antibodies in sheep according to the instructions of manufacturer. Microwells were covered with the P30 antigen of *T. gondii*. Serum samples, positive and negative controls were added to these microwells. *T. gondii* antibodies if present in serum samples showed agglutination by forming an antibody-antigen complex. After washing with distilled water, a multi-species peroxidase (Po) conjugate was added which fixed and formed antigen-antibody-conjugate-Po complex. Substrate solution (TMB) was added after removing extra conjugate by washing. Resulting coloration depends on the amount of specific antibodies present in the sample to be tested. A blue solution appeared in the presence of antibodies, which turn into yellow color after addition of the stop solution. No coloration in the absence of antibodies. The optical density (O. D) of the microplate was read at 450 nm with microplate reader. Results were written as the % age of the mean absorbance values of the sample (S) to the mean absorbance value of positive (P) control given with the diagnostic kit. According to manufacturer's reference, sera with

$S/P \leq 40\%$	=	Negative
$40\% < S/P < 50\%$	=	Doubtful
$50\% \leq S/P < 200\%$	=	Positive
$S/P \geq 200\%$	=	Strong positive

Statistical Analysis: The collected data/information was analyzed by using SPSS version 23.0. Pearson's Chi-Square test was used to measure the associations between different Variables such as area, aborted, sex, age and risk factors such as herd size, presence of cats and methods of disposing the aborted fetuses. Odds ratio was also calculated to look at the association between various variables. The difference was considered as statistically significant at $P \leq 0.05$ and non-significant at $P > 0.05$.

RESULTS AND DISCUSSION

Seroprevalence of toxoplasmosis across the world shows the variation from 0 to 100 % (Tenter *et al.*, 2000; Olivier *et al*., 2007) depending upon traditions, customs, climatic conditions, age and husbandry practice of the animals (Tasawar *et al*., 2012). Out of 500 sheep examined 168 and 137 were found positive with overall prevalence of 33.6% by LAT and 27.4% by ELISA. Infection rate was 34.02% and 28.12% in Multan and 33.01% and 26.41% in Khanewal by LAT and ELISA respectively (Table 1 and 2) difference was statistically non-significant (P>0.05). Result of toxoplasmosis in the present study are very close to some studies conducted by Mendonca *et al*. (2013) in Brazil, Liu *et al.* (2010) in China, Gebremedhin *et al*. (2013[a]) in Ethiopia, Sechi *et al*. (2013) in Italy who reported 28.22%, 29.8%, 31.59%, 33.9% prevalence in sheep respectively. However, certain studies in the past and near past have reported infection rates of toxoplasmosis in sheep higher than the present results such as 37.31% in Pakistan (Ahmad and Tasawar, 2015)**,** 57.5% in Sudan (Khalil and Elrayah, 2011), 61.4% in Egypt (Hassnaein *et al.,* 2011), 78% in Italy (Gaffuri *et al.,* 2006), 85% in New Zealand (Dempster, 2011) and 94.8% in Texas (Edwards and Dubey, 2013) and lower rates 1.26% in Urmia (Tavassoli *et al*., 2013), 4.4% in China (Yang *et al.*, 2013), 5.73% in Iran (Dehkordi *et al.*, 2013), and 19.88% in Pakistan (Lashari and Tasawar, 2010). These variations in prevalence of toxoplasmosis in different countries might be due to difference in contact of animal to oocysts contaminated feed and water (Khezri *et al.,* 2012), difference in the relative cat densities, difference in serological techniques, cut off values, species of animals, gender, number of individuals and sampling procedures (Ramzan *et al.*, 2009; Andrade *et al.,* 2013; Halova *et al.*, 2013; Zewdu *et al.*, 2013). Age, breed, flock size, management systems, water source, stage of the pregnancy, presence of rodent's feces near the food sources may be associated with toxoplasmosis prevalence in sheep (Romanelli *et al.,* 2007). The insufficiency of basic health facilities for the livestock animals also promotes the prevalence of toxoplasmosis (Ahmad and Tasawar, 2016[a]). Furthermore differences in various regions may be due to difference in climatic conditions such as variations in temperature and annual rainfalls. Humidity and moderate temperatures may be favorable for oocysts sporulation and dry climatic conditions reduce the chance of oocysts survival (Andrade *et al.*, 2013; Gebremedhin *et al*., 2013[a]).

In the current study we evaluated the relationship between toxoplasmosis and incidence of abortion in sheep. The results indicated that prevalence was 51.66% and 41.66% in aborted sheep and 31.1% and 26.22% in non-aborted sheep by LAT and ELISA respectively (Table 1 and 2) difference was statistically significant (P<0.05) by both techniques. Prevalence was higher in aborted sheep than non-aborted sheep. In aborted sheep 17.35%, 32.3% and 97.4% prevalence was reported by Khadi *et al.* (2009) in Iraq; Abu-Dalbouh *et al.* (2012) in Jordon and Issa and Omer (2011) in Iraq. It is considered that toxoplasmosis may be the most important factor causing worldwide abortions in sheep and goats (Dubey, 2010) therefore prevalence was higher in aborted sheep (Khadi *et al.* 2009; Edwards and Dubey, 2013). The important relationship between *T. gondii* infection and animal level abortion suggested that there might be excessive contamination of environment with oocysts due to cat feces because farmers kept cats to protect their crops from rodent's attack and moderate climate may increase chance of oocysts survival for long period in feeds, water and moist soil. One gram of infected cat's feces may contain up to 13 million oocysts which is sufficient to infect hundreds of thousands of animals, causing abortion or stillbirth in most of them (Schares *et al*., 2008). Congenital toxoplasmosis and recrudescence of internal infection may be main cause of transmission (Buxton *et al*., 2007). Mostly sheep aborted due to toxoplasmosis may develop protection against infection but this immunity is not absolute (Edwards and Dubey, 2013). The Higher abortion rates caused by *T. gondii* infection in large herds as compared to those found in the small herds may be related due to low management standards, environmental stress (Ahmad and Tasawar, 2016[b]) and improper feeding regime (Gebremedhin *et al*., 2013[b]).

The results revealed the toxoplasmosis infection rates 31.37% and 19.6% in males and 33.85% and 28.28% in female sheep through LAT and ELISA respectively (Table 1 and 2). Prevalence was higher in females than males however difference was statistically non-significant (P>0.05) as has been reported by Wu *et al.* (2011) in China, Khezri *et al.* (2012) in Iran, Gebremedhin *et al.* (2013[a]) in Ethiopia and Halova *et al*. (2013) in Ireland. The results disagreed with (Ahmad and Tasawar, 2016[b]) who have recently reported non-significant seroprevalence of anti-*Toxoplasma* antibodies in small ruminants, nevertheless, with higher rates of infections in females as compared with males. The higher seroprevalence rates in females than males may be due to difference in management system because ewes are retained for longer periods for breeding and milk production purposes while few rams are retained in the herd for mating while the most of them may be sold or culled for cash purpose during special occasions (Gebremedhin *et al.*, 2013[a]) such as Eid ul Azha festival, as has been reported by Ahmad and Tasawar (2016[a]). Variations in prevalence rate at the gender level might be influenced by the physiological and hormonal conditions of the animals which directly affects the immune system. Generally there is more immunity in females than males, due to the secretion of estrogen, which normally increase

the immunity by antibody production, while testosterone in males decrease the immunity by suppressing T-cell and B-cell immune responses (Roberts *et al*., 2001; Romanelli *et al*., 2007). However, female animals are more susceptible to protozoan infection than males because there are various factors which may breakdown the immunity in females such as changes in hormone secretions, pregnancy, lactation, nutrition, environmental factors and stress (Martin, 2000; Craig *et al*., 2001; Kelly *et al*., 2001). During the present study higher prevalence in females may be due to examination of large numbers of female animals as compared to males (Tasawar *et al*., 2011).

Relationship between age of sheep and toxoplasmosis showed that prevalence was higher in age group 60-73 months 64.5% and 38.7% by LAT and ELISA respectively and lowest in age group 4-17 months 26.4% and 22.4% by LAT and ELISA respectively (Table 1 and 2) difference was statistically significant (P<0.05) by LAT and non-significant (P>0.05) by ELISA. Prevalence increases as age of animals increase. These results are supported by Khanmohammadi (2011) in Iran, Wang *et al.* (2011) in China, Andrade *et al.* (2013) in Brazil, Gebremedhin *et al.* (2013[a]) in Ethiopia and Ahmad and Tasawar (2016[b]) in Pakistan, suggesting that most animals acquire infection post-nataly. The increase in infection rate along the age of sheep might be due to high opportunity of contact to numerous predisposing factors or ingestion of infective oocysts from the environment with the increase of animal age. Therefore these differences in infection rates could be linked with cumulative effect of age (Hall *et al*., 2001; Dubey, 2010) because as animal get old their chance of exposure to *T. gondii* oocysts increase and furthermore older animals are less resistant to infection than younger due to low immunity (Roberts *et al.,* 2001). The climatic changes, environmental potential for sporulation of oocysts, breeding management systems, suitable pastures and health also have an important effect on the occurrence of blood borne infection (Ramzan *et al*., 2009; Khanmohammadi, 2011; Khezri *et al*., 2012).

Data related to risk factors of toxoplasmosis in sheep was collected on a specially designed questionnaire (Table 3 and 4) and cats association with herd, size of the herd and the method of discarding the aborted fetuses were all significant (P≤0.05) risk factors that were related with toxoplasmosis prevalence. Romanelli *et al.* (2007) in Brazil, Abu-Dalbouh *et al.* (2012) in Jordon, Sechi *et al.* (2013) in Italy, Andrade *et al.* (2013) in Brazil, and Gebremedhin *et al.* (2013[a]) in Ethiopia reported the various risk factors which may be significantly associated with toxoplasmosis prevalence in sheep such as herd size, water source, access of cats and rodents to food and water source, grazing land, farm management system and mineral salt supplementation. Cats are the final host and only animal that can pass oocysts of *T. gondii* in their feces and great source of environmental contamination (Buxton *et al*., 2007; Gazzonis *et al*., 2015).

The results showed a positive correlation between herd size of sheep and seroprevalence of *T. gondii* in agreement with (Ahmad and Tasawar, 2016[b]) who reported significantly positive correlation (r=0.9275) between herd size and occurrence of toxoplasmosis, leading to the suggestion that a large herd size can increases the chance of infection because animals have greater chance to come in contact with each other and with infectious materials such as cat's feces in the environment, especially during night because floor space of the pen per animal is less (Gebremedhin *et al.,* 2013[b]). In addition, animals in large herds might have received insufficient care from managers due to more work and effort as compared to small herds where nutrition could be better and easier to monitor the animals. The improper dumping of aborted fetuses was also positive risk factor because infection was higher on farms that left them on bare ground as compared to those farms that give them to dogs and properly buried them in ground which decrease the chance of infection in animals (Abu-Dalbouh *et al.,* 2012).

Table 1. Relationship between different parameters and prevalence of toxoplasmosis in sheep (LAT).

Parameters	Animals Examined	Animals positive	Prevalence (%)	Chi-Square	P-Value	OR	95 % CI
Overall Seroprevalence							
	500	168	33.60	4.534	0.033*	1.153	1.015,1.310
Area							
Multan	288	98	34.02	0.056	0.813	1.031	0.802,1.324
Khanewal	212	70	33.01				
Aborted and non-aborted sheep							
Aborted	60	31	51.66	9.814	0.002*	1.661	1.248,2.211
Non-aborted	389	121	31.10				
Sex							
Male	51	16	31.37	0.126	0.722	0.927	0.605,1.419
Female	449	152	33.85				

Age					
4-17 months	125	33	26.40	18.163	0.001*
18-31 months	152	49	32.23		
32-45 months	120	37	30.83		
46-59 months	72	29	40.27		
60-73 months	31	20	64.50		

* P-Value < 0.05 (Statistically significant)

Table 2. Relationship between different parameters and prevalence of toxoplasmosis in sheep (ELISA).

Parameters	Animals Examined	Animals positive	Prevalence (%)	Chi-Square	P-Value	OR	95 % CI
Overall Seroprevalence							
	500	137	27.40	4.534	0.033*	0.860	0.745,0.992
Area							
Multan	288	81	28.12	0.179	0.672	1.065	0.796,1.424
Khanewal	212	56	26.41				
Aborted and non-aborted sheep							
Aborted	60	25	41.66	6.114	0.013*	1.589	1.128,2.238
Non-aborted	389	102	26.22				
Sex							
Male	51	10	19.60	1.733	0.188	0.693	0.390,1.232
Female	449	127	28.28				
Age							
4-17 months	125	28	22.40	4.018	0.404		
18-31 months	152	43	28.28				
32-45 months	120	32	26.66				
46-59 months	72	22	30.55				
60-73 months	31	12	38.70				

* P-Value < 0.05 (Statistically significant)

Table 3. Risk factors associated with prevalence of toxoplasmosis in sheep in Multan and Khanewal (LAT).

Parameters	Animals examined	Animals positive	Prevalence (%)	Chi-Square	P-Value	OR	95 % CI
Herd size							
1-30	158	35	22.15	13.569	0.000*	0.570	0.413,0.785
31-60	342	133	38.88				
Cat's association with herd							
Present	311	121	38.90	10.385	0.001*	1.565	1.177,2.079
Absent	189	47	24.86				
Methods of disposing the aborted fetuses							
Left on ground	301	115	38.20	7.192	0.007*	1.435	1.093,1.882
Given to dogs	199	53	26.63				

* P-Value < 0.05 (Statistically significant)

Table 4. Risk factors associated with prevalence of toxoplasmosis in sheep in Multan and Khanewal (ELISA)

Parameters	Animals examined	Animals positive	Prevalence (%)	Chi-Square	P-Value	OR	95 % CI
Herd size							
1-30	158	28	17.72	10.877	0.001*	0.556	0.384,0.805
31-60	342	109	31.87				
Cat's association with herd							
Present	311	104	33.44	15.091	0.000*	1.915	1.353,2.711F
Absent	189	33	17.46				

Methods of disposing the aborted fetuses							
Left on ground	301	95	31.56	6.584	0.010*	1.495	1.090,2.051
Given to dogs	199	42	21.15				

* P-Value < 0.05 (Statistically significant)

Conclusion: The large flock size, presence of cats, and the improper disposal of aborted fetuses openly left on ground were the main causes of higher rates of prevalence of toxoplasmosis in sheep which are potential threat of infection for human population of study area.

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