The Journal of Animal & Plant Sciences, 29(4): 2019, Page: 912-918
ISSN: 1018-7081

EPIDEMIOLOGY OF *CYRPTOSPORIDIUM* IN APPARENTLY HEALTHY SHEEP IN SOUTHERN KHYBER PAKHTUNKHWA, PAKISTAN

N. U. Khan[1,8], M. H. Saleem[1], A. Z. Durrani[1], N. Ahmad[2], A. Hassan[1], M. Shafee[3], I. U. Khan[4], M. A. Khan[1], S. Zaman[5], A. U. Khan[6], N. Ullah[1], A. Razzaq[7] and M. L. Sohail[1,9]

[1]Department of Clinical Medicine And Surgery, University of Veterinary and Animal Sciences, Lahore, Pakistan.
[2]Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
[3]Centre For Advanced Studies In Vaccinology And Biotechnology, University Of Balochistan, Quetta, Pakistan.
[4]Department of Livestock Management, Animal Breeding and Genetics, The University Of Agriculture, Peshawar, Pakistan; [5]Department of Microbiology, Quaid-e-Azam University, Islamabad, Pakistan.
[6]Department of Livestock and Dairy Development, The University Of Agriculture, Peshawar, Pakistan.
[7]Department of Veterinary Anatomy And Histology, Sindh Agriculture University, Tandojam, Pakistan.
[8]University College of Veterinary Sciences and Animal Husbandary, Abdual Wali Khan University, Mardan, Pakistan.
[9]Department of Medicine, Cholistan University of Veterinary and Animal Sciences, Bahawalpur, Pakistan.
Corresponding Author e-mail: vet.luqman@gmail.com

ABSTRACT

Cryptosporidium is a prevalent enteric zoonotic parasite of domestic and wild animals, reptiles, birds and fish. This study was conducted to find out the prevalence and risk factors associated with the *Cryptosporidium* in apparently healthy sheep (Ovis aries), in three districts of Khyber Pakhtunkhwa (KPK), Pakistan. From January 2016 to December 2016, 1080 fecal samples were screened for the presence of *Cryptosporidium* oocysts through microscopy of smears stained by modified Ziehl-Neelsen technique. Results showed an overall prevalence of 17.96% (194/1080). The highest prevalence was recorded in Kohat (19.72%), followed by Bannu (18.61%) and Lakki Marwat (15.15%). Season-wise prevalence showed significant difference ($P \leq 0.05$) among different seasons, with highest prevalence during summer (25%), followed by spring (19.44%), autumn (17.72%) and the winter (10.55%). Statistical analysis revealed significant difference ($P \leq 0.05$) among sheep of different age groups with highest prevalence in newborns to ≤1 years of age (22.38%), followed by those of 1-2 years of age (18.03%) and more than 2 years of age (13.46%). Non-significant higher prevalence was recorded in females (18.80%) than males (17.02%). This debut study of *Cryptosporidium* in sheep will help designing disease control measures, as asymptomatic sheep is the key source of infection transmission to humans.

Key words: Asymptomatic, *Cryptosporidium*, Epidemiology, Pakistan, Sheep.

INTRODUCTION

Cryptosporidium is the most common enteric zoonotic parasite, infecting humans and a wide range of domestic and wild animals, including reptiles, birds and fish (Bamaiyi *et al*., 2016). It is ranked 5th among the 24 most important food-borne parasites globally (Aniesona *et al*., 2014). *Cryptosporidium* belongs to the phylum Apicomplexa and the family Cryptosporidiidae (Kvac *et al*., 2016). It is mostly prevalent in hot and humid weather during the year, with global distribution. (Jafari *et al*., 2013). It was first discovered by Tyzzer as "sporozoan found in the peptic glands of the common mouse" in 1907 (Tyzzer 1907). In sheep, *Cryptosporidium* infection was first described in a diarrheic lamb (Barker and Carbonel, 1974). A single *Cryptosporidium* oocyst is sufficient to cause infection in any susceptible host (Ryan *et al*., 2014). The direct transmission occurs through feco-oral route, while indirect transmission occurs through contaminated food and water, moreover, aerosol transmission has also been reported (Bamaiyi *et al*., 2016). *Cryptosporidium* infected animals shed a large number of oocysts (10^8–10^9 OPG of fecal material) (Romero-Salas *et al*., 2016). Epidemiological studies confirmed the zoonotic potential of the *Cryptosporidium* infection when veterinary professionals caught infection through pets and small and large ruminants (Gharekhani *et al*., 2014). Young animals are highly sensitive to the infection whereas infection in adults is mostly asymptomatic (Ozdal *et al*., 2009). In sheep clinical cryptosporidiosis is characterized by foul smelling semi liquid to watery yellowish diarrhea, abdominal pain, loss of weight, severe depression, dehydration and high mortality at the age of one month (Jacobson *et al*., 2016). Heavy economic losses have been reported due to cryptosporidiosis in different animal husbandry practices (Ramo *et al*., 2016). In addition, infected animals are a source of infection for humans, highlighting its zoonotic potential which imparts serious and life threatening intractable diarrhea in patients of autoimmune diseases (Ryan *et al*., 2016). The most commonly practiced modified Ziehl Neelsen (ZN) acid fast staining is a key player in the detection of

Cryptosporidium oocysts in fecal smears (Rekha *et al*., 2016). Recent advancements in molecular biology research techniques (PCR, RT-PCR, nested PCR) aided in the detection of oocysts in fecal samples of apparently healthy animals (Silva-Fiuza *et al*., 2011). To design effective disease control and prevention strategies, a comprehensive understanding of various risk factors contributing to the spread of disease among human and animal population is unavoidable (Collinet-Adle *et al*. 2015).

In Pakistan, a country with 30 million of sheep population, experiencing an annual increase of more than 1% (Pakistan Economic Survey 2015-2016), no study has been conducted ever to address *Cryptosporidium* in southern Khyber Pakhtunkhwa, a province where a major portion of population rely on sheep and other livestock to make the ends meet (Jan *et al*., 2015). This made us plan and design this much needed study, to prevent humans and their livestock assets from this devastating infection, as sheep reportedly is the major reservoir for transmission of infection to human beings (Romero-Salas *et al*., 2016). Limited access of human population to health care facilities will further aggravate the issue if left, unreported.

MATERIALS AND METHODS

Study Area: The present study was conducted in three selected districts of southern Khyber Pakhtunkhwa *viz;* Bannu, Lakki Marwat and Kohat.

Global positioning system was used to determine the coordinates of the selected districts in KPK, Pakistan. Bannu is located at 32.99° N latitude, 70.61° E longitude and have an elevation of 371 meters from the sea level. Lakki Marwat is the neighbor district of Bannu, having coordinates 32° 36' 27" N, 70° 54' 45" E. Kohat district is among the southern districts of KPK Pakistan and lies between latitude 32° 47' and 33° 53' N and longitude 70° 34' and 72° 17 E.

All the samples were processed at Diagnostic Laboratory, Department of Clinical Medicine and Surgery and in the Parasitology Laboratory, Department of Parasitology, Faculty of Veterinary Science, University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan.

Sampling Strategy and Sample Size: A total of 1080 fecal samples (30 samples per month from each district) were collected through convenient sampling from sheep reared under different managemental conditions from January, 2016 to December, 2016. For each sampled animal, an interview was conducted by trained local research assistants, conversant in respective local languages, using a predesigned questionnaire composed of open and closed ended questions along with the complete details of individual and managemental profile of animal

Ethics Statements: All the fecal samples were collected after the formal written consent of sheep owners. Sufficient necessary veterinary care and use of personal protective equipment were assured at the time of sampling and free veterinary services were provided to every animal, if needed.

Sample collection and handling: Each fecal sample (about 3 g) was collected directly from the rectum of sheep. All the collected samples were preserved in 10% formalin (1:3) into sterile, clean, moisture resistant, labeled and disposable wide-mouthed plastic bottles avoiding any contamination from urine. All the samples were transported and refrigerated at 4°C till further processing (shafiq *et al*., 2015).

Laboratory Analysis of Fecal Samples: All the collected fecal samples were processed using a Faust modified centrifuge flotation technique (Romero-salas *et al*., 2016). Briefly, 3 g of fecal material was dissolved in distilled water to make homogenized solution. After homogenization the solution was centrifuged for 1 min at 1500 rpm and supernatant was discarded, while sediment was suspended in floatation solution (44% $ZnSO_4$). The solution was then again centrifuged at 1500 rpm for 1 min. Finally, sediment was examined under microscope. *Cryptosporidium* oocysts were stained by modified Ziehl-Neelsen (MZN) staining technique (Shafieyan *et al*., 2016). Briefly, smears were fixed using absolute methanol and were stained by carbol fuchsin solution (4 g of basic fuchsin crystals, dissolved in 25 ml of 99% ethyl alcohol) for 15 minutes. Non-*Cryptosporidium* materials were decolorized by acid alcohol solution for 2 minutes. Then, the slides were treated with 2% malachite green solution for one minute, washed, dried and examined for presence of the oocysts under light microscopy.

Identification of Cryptosporidium oocysts: Cryptosporidium oocysts appeared as bright red granules on a blue-green background in MZN stained fecal smears and were identified on the basis of morphology, size and other key features as described by Wantanabe *et al*., 2005.

A fecal sample was considered positive if at least one, clearly identifiable oocyst was recognized. The total number of oocysts per gram (OPG) of feces was calculated by multiplying the total number of oocysts on the slide by 50 (Tzanidakis *et al*., 2014).

Statistical analysis: The data thus collected were subjected to statistical analysis through Statistical Product and Service Solutions (SPSS) version 20.0 program (2016) to establish associations between prevalence of *Cryptosporidium* and the risk factors at 95% level of confidence. Statistical differences in prevalence were determined using Chi-square test (X^2) for all the studied

variables (age, sex and season). All values at $P \leq 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Cryptosporidium infects a wide range of livestock animals and humans, causing substantial economic losses and serious public health concern. It causes gastrointestinal disorders specially diarrhea in newborns (Li *et al*., 2016). Various molecular techniques are used for oocysts detection, which are costly, time consuming, laborious and require a range of equipment rendering them not the best candidate for screening studies, so for large scale investigations, microscopy of stained fecal smears is more useful. Taking these aspects of diagnosis in consideration modified ZN method is arguably a useful tool for *Cryptosporidium* diagnosis (Shafieyan *et al*., 2016). To the best of our knowledge, this communication is the first documented study on *Cryptosporidium* in small ruminants of Southern KPK, Pakistan. An overall prevalence of *Cryptosporidium* oocysts in sheep was 17.96% (194/1080) during the study period, from January, 2016 to December, 2016. Statistical significant difference (P≤0.05) was found among the prevalence in three study areas. The highest prevalence of *Cryptosporidium* oocysts was recorded in District Kohat (19.72%) followed by District Bannu where the prevalence was 18.61% and the lowest prevalence was observed in District Lakki Marwat (15.15%). Previous literature reports prevalence of *Cryptosporidium* oocysts was 11.7% in Nigeria and 12.3% in china (Danladi *et al*., 2015; Li *et al*., 2016) which is in agreement with the findings of this study. When compared with previous reports, results of this study do not contradict significantly from already published data. According to two different studies in Iran the prevalence of *Cryptosporidium* oocysts was 12% and 11.3% in small ruminants (Shafieyan *et al*., 2016). Another report from India recorded prevalence of 26.25% in small ruminants (Maurya *et al*., 2013; Khursheed *et al*., 2018). Coherence of findings of present study with the results of studies conducted in neighboring countries may be because this region shares a similar kind of climate and socio-economic status of sheep rearing farmers. On the contrary, studies in Kuwait (3.6%), China (4.7%) and Australia (6.5%) showed lower prevalence rates (Koinari *et al*., 2014; Majeed *et al*., 2018; Zhong *et al*., 2018). This varied prevalence indicates geographical incongruity in the frequency of infection that may be attributed to differences in animal age, breed, or the management and husbandry practices involved (Kauke *et al*., 2017) Some reports showed considerably higher prevalence of *Cryptosporidium* oocysts as in Spain (31-59%), which warns that all these comparisons should be translated with care because matching for characteristics of animals and their raising conditions is challengingly variant (Romero-Salas *et al*., 2016).

The prevalence was calculated on monthly basis and season wise to find out the time of the year with highest prevalence of *Cryptosporidium* oocysts in sheep. Results showed that August (31.11%) had the highest prevalence, followed by June, July (23.33% each), May (22.22%), April (20%), September (20%) and March (18%). Likewise, summer (25%) and spring (19.44%) had the highest seasonal prevalence. The lowest prevalence (7.77%) was recorded in the month of January and understandably during winter season (10.55%) (Table 1). Statistical analysis revealed significant difference (P≤0.05) among the months of the year and seasons during the year. The *Cryptosporidium* infection occurs globally and is prevalent in hot and humid months of the year where higher temperature and humidity were recorded (Brankston *et al*., 2018). In the present study, higher prevalence rates were recorded from June to August, as these months experience higher rainfall in the study area because of monsoon (Singh *et al*., 2018). *Cryptosporidium* has been reported to be waterborne infection (Efstratiou *et al*., 2017), hence higher prevalence in wetter months of the year is no surprise. Higher prevalence of cryptosporidium oocysts during monsoon and post monsoon months may be attributed to the overcrowding of animals in shelters curtailing the drying of floors and walls of shed, causing rapid spread of etiological agent due to optimum temperature and high humidity (Maurya *et al*., 2013). Mirhashemi *et al*. (2016) also reported higher peak prevalence during the month of June in sheep. Similar results were also reported in other ruminant species (Essa *et al*., 2014). Infection trends in human beings have no difference than animals when compared in terms of seasonality. Agrawal *et al*., 2018 reported higher prevalence of *Cryptosporidium* in months with higher rainfall and humidity facilitating its spread. Some reports suggest higher prevalence in winter and autumn (Morsy *et al*., 2014) which is in contrast to the findings of this study. These differences can be interpreted as, overcrowding due to larger production of newborns as a result of synchronized breeding plans aids in infection spread (Maurya *et al*., 2013)

During current investigation, prevalence recorded in lambs (less than one year of age) (≤ 1 year) was highest (22.38 %), followed by sheep of 1-2 years of age (18.03%) while lowest prevalence was recorded in sheep of older than two years. On the basis of statistical analysis, prevalence rate was significantly different (P≤0.05) among animals of different age in all the study areas (Table 2). On the other hand, non-significant (P>0.05) higher prevalence was recorded in females (18.80%) as compared to male animals (17.02%). A similar trend in results were observed in all the three districts of Bannu, Kohat and Lakki Marwat, yielding no significant difference (P>0.05) (Table 2).

Table 1. Month and season wise percent (%) prevalence of Cryptosporidiosis in Sheep in three districts of South KPK.

Months	Bannu		Lakki Marwat		Kohat		Overall	
	+ve/ Total	Prevalence (%)	+ve/Total	Prevalence (%)	+ve/ Total	Prevalence (%)	+ve/ Total	Prevalence (%)
January	3/30	10	2/30	6.66	2/30	6.66	7/90	7.75[a]
February	2/30	6.66	1/30	3.33	5/30	16.66	8/90	8.80[b]
March	6/30	20	5/30	16.66	6/30	20	17/90	18.68[c]
April	8/30	26.66	5/30	16.66	5/30	16.66	18/90	20[d]
May	7/30	23.33	6/30	20	7/30	23.33	20/90	22.12[e]
June	8/30	26.66	7/30	23.33	6/30	20	21/90	23.23[v]
July	7/30	23.33	6/30	20	8/30	26.66	21/90	23.32[v]
August	11/30	36.66	7/30	23.33	10/30	33.33	28/90	31.11[n]
September	5/30	16.66	7/30	23.33	6/30	20	18/90	20[d]
October	4/30	13.33	4/30	13.33	5/30	16.66	13/90	14.43[u]
November	3/30	10	4/30	13.33	6/30	20	13/90	14.43[u]
December	3/30	10	2/30	6.66	5/30	16.66	10/90	11.11[k]
Total	**67/360**	**18.61**	**56/360**	**15.15**	**71/360**	**19.72**	**194/1080**	**17.95**
Season wise Prevalence								
Winter	11/120	9.16	9/120	7.5	18/120	15	38/360	10.45[a]
Spring	14/60	23.33	10/60	16.66	11/60	18.33	35/180	19.54[b]
Summer	33/120	27.5	26/120	21.66	31/120	25.83	90/360	25[c]
Autumn	9/60	15	11/60	18.33	11/60	18.33	31/180	17.22[d]
Total	**67/360**	**18.61**	**56/360**	**15.55**	**71/360**	**19.72**	**194/1080**	**17.26**

Statistically mean values that are carrying same superscript are differ from each other non-significantly (P>0.05) while those statistical mean values having different superscripts are differ significantly (P≤ 0.05).

Table 2. Age and gender wise percent (%) prevalence of Cryptosporidiosis in Sheep in three districts of South KPK.

Age	Bannu		Lakki Marwat		Kohat		Overall	
	+ve/ Total	Prevalence (%)	+ve/ Total	Prevalence (%)	+ve/ Total	Prevalence (%)	+ve/ Total	Prevalence (%)
≤ 1 year	30/132	22.72	24/114	21.05	36/156	23.07	90/402	22.38[a]
1-2 years	24/132	18.18	18/126	14.28	24/108	22.22	66/366	18.03[b]
> 2 years	12/96	12.5	12/120	10	18/96	18.75	42/312	13.46[c]
Gender wise Prevalence								
Male	18/108	18.75	12/84	14.28	18/90	20	48/282	17.32[m]
Female	48/252	19.04	42/276	15.21	60/270	22.22	150/798	18.89[n]

Means with different superscripts differ at (P ≤ 0.05)

Age is the key risk factor in occurrence of *Cryptosporidium* among different species and maximum morbidity has been reported in younger animals (Khan *et al*., 2017). Li *et al*. (2016) and Gharekhani *et al*. (2014) reported highest prevalence in sheep under one year of age and lowest in sheep of more than two years of age, which is in agreement with the findings of this study. Highest prevalence rate in neonatal animals may be due to immature immune system and their highest sensitivity against *Cryptosporidium* (Fasihi-Harandi *et al*., 2008). Prevalence of *Cryptospordium* is reported to be as high as 86% in lambs in Pakistan (Shafiq *et al*., 2015). Similar results were reported in neonates of other ruminant species and camel, showing higher prevalence in younger animals which decreases as the age progresses (Yakhchali and Moradi, 2012; Morsy *et al*., 2014; Romero-Salas *et al*., 2016). Interestingly, similar inverse relation between age and infection was reported in human beings where infection was found to be highest in children less than 5 years of age (Ghenghesh *et al*., 2012). Literature also reports non significant relation between age and *Cryptosporidium* (Shafieyan *et al*., 2016). The difference can be the result of variation in presence of oocysts in the environment, infectivity of *Cryptosporidium*, zoohygienic conditions of animal husbandry and grazing practices (Majewska *et al*., 2000).

Non-significant higher prevalence of *Cryptosporidium* was found in female animals as compared to male ones in Iran (Jafri *et al*., 2013), whereas significant higher prevalence was reported by (Maurya *et al*., 2013). Various other reports mentioned higher prevalence in female sheep as compared to male

sheep (Gharikhani *et al*.2014). Similar non-significant relation between age and *Cryptosporidium* prevalence was reported in camels by (Yakhchali and Moradi, 2012). This independent relation between gender and *Cryptosporidium* prevalence may attribute to the differences in sensitivity of individual animal to etiological agent (Kaupke *et al*., 2017).

Conclusion: Findings of this study show the need of devising proper control and preventive strategies to reduce the rate of infection among humans and animals. Improved management, hygienic measures and farmer education can abate the risk of infection to a considerable level.

Acknowledgements: We humbly thank the skilled staff of Department of Clinical Medicine and Surgery (CMS) Department of Parasitology of University of Veterinary and Animal Sciences Lahore, College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University Mardan, Veterinary Research Institute Peshawar, Government Post Graduate College Bannu and Department of Livestock and Dairy Development KPK to provide technical support for processing and collection of fecal samples from study areas.

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