EPIDEMIOLOGY OF CYRTOSPORIDIUM IN APPARENTLY HEALTHY SHEEP IN SOUTHERN KHYBER PAKHTUNKHWA, PAKISTAN


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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<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<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 (109–1010 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 managerial 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% ZnSO4). 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 (Shafeeyan 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 obtained was gathered in the Microsoft Excel version 2010 and was exported to the 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. The prevalence was the proportion of positive animals out of the total number of
animals analyzed and was presented in percentage (%). 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 < 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 (Shafieyean 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 (Shafieyean et al., 2016). Another report from India recorded prevalence of 26.25% in small ruminants (Maurya et al., 2013; Khurshed 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 curtail 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.

<table>
<thead>
<tr>
<th>Months</th>
<th>Bannu</th>
<th>Lakki Marwat</th>
<th>Kohat</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve/Total</td>
<td>Prevalence (%)</td>
<td>+ve/Total</td>
<td>Prevalence (%)</td>
</tr>
<tr>
<td>January</td>
<td>3/30</td>
<td>10</td>
<td>2/30</td>
<td>6.66</td>
</tr>
<tr>
<td>February</td>
<td>2/30</td>
<td>6.66</td>
<td>1/30</td>
<td>3.33</td>
</tr>
<tr>
<td>March</td>
<td>6/30</td>
<td>20</td>
<td>5/30</td>
<td>16.66</td>
</tr>
<tr>
<td>April</td>
<td>8/30</td>
<td>26.66</td>
<td>5/30</td>
<td>16.66</td>
</tr>
<tr>
<td>May</td>
<td>7/30</td>
<td>23.33</td>
<td>6/30</td>
<td>20</td>
</tr>
<tr>
<td>June</td>
<td>8/30</td>
<td>26.66</td>
<td>7/30</td>
<td>23.33</td>
</tr>
<tr>
<td>July</td>
<td>7/30</td>
<td>23.33</td>
<td>6/30</td>
<td>20</td>
</tr>
<tr>
<td>August</td>
<td>11/30</td>
<td>36.66</td>
<td>7/30</td>
<td>23.33</td>
</tr>
<tr>
<td>September</td>
<td>5/30</td>
<td>16.66</td>
<td>7/30</td>
<td>23.33</td>
</tr>
<tr>
<td>November</td>
<td>3/30</td>
<td>10</td>
<td>4/30</td>
<td>13.33</td>
</tr>
<tr>
<td>December</td>
<td>3/30</td>
<td>10</td>
<td>2/30</td>
<td>6.66</td>
</tr>
<tr>
<td>Total</td>
<td>67/360</td>
<td>18.61</td>
<td>56/360</td>
<td>15.15</td>
</tr>
<tr>
<td><strong>Season wise Prevalence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>11/120</td>
<td>9.16</td>
<td>9/120</td>
<td>7.5</td>
</tr>
<tr>
<td>Spring</td>
<td>14/60</td>
<td>23.33</td>
<td>10/60</td>
<td>16.66</td>
</tr>
<tr>
<td>Summer</td>
<td>33/120</td>
<td>27.5</td>
<td>26/120</td>
<td>21.66</td>
</tr>
<tr>
<td>Autumn</td>
<td>9/60</td>
<td>15</td>
<td>11/60</td>
<td>18.33</td>
</tr>
<tr>
<td>Total</td>
<td>67/360</td>
<td>18.61</td>
<td>56/360</td>
<td>15.55</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Age</th>
<th>Bannu</th>
<th>Lakki Marwat</th>
<th>Kohat</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve/Total</td>
<td>Prevalence (%)</td>
<td>+ve/Total</td>
<td>Prevalence (%)</td>
</tr>
<tr>
<td>&lt; 1 year</td>
<td>30/132</td>
<td>22.72</td>
<td>24/114</td>
<td>21.05</td>
</tr>
<tr>
<td>1-2 years</td>
<td>24/132</td>
<td>18.18</td>
<td>18/126</td>
<td>14.28</td>
</tr>
<tr>
<td>&gt; 2 years</td>
<td>12/96</td>
<td>12.5</td>
<td>12/120</td>
<td>10</td>
</tr>
<tr>
<td><strong>Gender wise Prevalence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>18/108</td>
<td>18.75</td>
<td>12/84</td>
<td>14.28</td>
</tr>
<tr>
<td>Female</td>
<td>48/252</td>
<td>19.04</td>
<td>42/276</td>
<td>15.21</td>
</tr>
</tbody>
</table>

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 (Fashti-Haranidi et al., 2008). Prevalence of Cryptosporidium 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.

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