MOLECULAR AND MICROSCOPIC DETECTION OF CRYPTOSPORIDIUM SPP IN SHEEP IN AL-TAJI AREA-BAGHDAD/IRAQ

Mohammed T. S. Al-Zubaidi
University of Baghdad/College of Veterinary Medicine
Corresponding author email: mohabood24@gmail.com

ABSTRACT
This study was to detect the Cryptosporidium spp. oocysts and its prevalence among naturally infected sheep. Ninety fecal samples were collected in Al-Taji region aged 1-3 year’s old sheep from the beginning of February to the end of May 2016. Samples examined by lugol's iodine, modified Ziehl-Neelsen stain and PCR. Results revealed that the total prevalence of Cryptosporidium spp infection is 48.88% (44/90) by using PCR technique with highest sensitivity, while the prevalence reach 37.77% (34/90), 27.77% (25/90) by using Ziehl-Neelsen stain and lugol's iodine respectively. Highest prevalence rate of parasite in age group 6 months 70% (14/20) while the lowest prevalence rate in age group 25-36 months 34.78% (8/23). The study found that 58.13% (25/43) of diarrheic sheep shed Cryptosporidium oocysts in feces. The PCR technique was used to determine the presence of Cryptosporidium oocysts, particular gene locus in the extracted DNA which were extracted by modified protocol, proteinase K and seven cycles of freezing-thawing in liquid nitrogen was more efficient than only three cycles by deep freeze in facilitate the subsequent steps of DNA extraction. SSU rRNA–based PCR technique by general tools has been used successfully for the detection of Cryptosporidium oocysts in stool sample with 100% sensitivity.

KEYWORDS: Cryptosporidium, Sheep, Diarrhea, PCR.

INTRODUCTION
Cryptosporidium is an obligate intracellular extra cytoplasmic protozoan parasite belongs to the phylum Apicomplexa. It has an apical compound which helps in the penetration of host cell (Oyibo et al., 2011). Many studies had been proved that Cryptosporidium is one of important zoonotic pathogens which infect digestive epithelium of a variety of vertebrates and cause gastroenteritis which characterized by diarrhea in animals and human, having a great impact of morbidity and mortality (Fayer and Xiao, 2008; Lange et al., 2014). Younger animals appear to be more delicate to cryptosporidiosis and considered as a top cause of diarrhea and death rate in neonatal ruminants (Ozdal et al., 2009; Santín, 2013).

The prevalence of Cryptosporidium infection was reported 4% to 85% in lambs worldwide (Fasihi-Harandi and Fotohi-Ardakani, 2008). Microscopical confirmation of Cryptosporidium differ from 0% in Ethiopia, 2.6% in Australia, 3.7%-47% in Brazil, 13.6%-46.5% in Turkey, 25.7% in Mexico, 29% in Greece, and 42.1% in Serbia (Ozdal et al., 2009; Silva-Fiuza et al., 2011). There are different methods used to detect cryptosporidiosis, such as modified Ziehl–Neelsen stain (modified acid-fast stain) which detects Cryptosporidium oocysts in feces. It is the most commonly used method for the diagnosis of parasite, and Lugol's iodine method which also detect the oocysts of parasite but with less sensitivity (Mehta., 2002; Alles et al., 1995). Molecular method like PCR is the most sensitive and specified (97-100%) assay used for detection of Cryptosporidiosis (Morgan et al., 1998; Bialek et al., 2002). The rRNA (SSU rRNA) gene or the Cryptosporidium oocyst wall protein (COWP) gene is used to determine Cryptosporidium species by PCR technique (Silva-Fiuza et al., 2011). The present study aimed to detect the prevalence of Cryptosporidiosis in sheep in Al-Taji area/Baghdad/ Iraq by using conventional staining methods and PCR.

MATERIALS & METHODS
Collection of Fecal Samples
Ninety fecal samples were collected from sheep with and without diarrhea in Al-Taji area-Baghdad from the beginning of February to the end of May 2016 of both genders (55 males and 35 females), aged 1-3 years old. Each sample was placed in a screw capped plastic container, labeled with the number and the date of collection, and transported in the cooling box, to the Department of Parasitology/ Faculty of Veterinary Medicine / University of Baghdad.

Detection of Cryptosporidium oocysts
1-Direct Microscopic Examination
This method was done according to the procedure of Coles (1986). One drop of 1% lugol's iodine was placed on a glass slide and small quantity of fecal specimen was added and mixed well by a wooden stick. Forceps was used to adjust the cover slip, and the slides were examined under (40X) and (100X) magnifications.

2-Microscopic Examination by Modified Ziehl–Neelsen
Modified Ziehl–Neelsen stain was done according to the method of Beaver and Jung (1985) and Baxby et al. (1984). Smear of sediment of fecal specimen was left to dry and placed in a slides rack for fixation by methanol for...
Molecular and microscopic detection of *cryptosporidium* spp in sheep

five minutes. Carbol fuchsin stain was applied to the smear for 3 minutes, and the slide was washed with tap water, acid alcohol has been added to the slide for decolorization, the slide was washed with tap water followed by 1% methylene blue for 2 minutes. Then after, the slide was rinsed by tap water and dried in air and examined under (40X) and (100X) magnifications.

3- **PCR Technique for Detection of Cryptosporidium**

**DNA Extraction from Fecal samples**

All fecal samples were used for molecular detection of *Cryptosporidium* using PCR technique according to the Ekanayake *et al.* (2006) and Nichols & Smith, 2004. Tube contains stool specimen with potassium dichromate was placed on a vortex and 200 μl of the stool sample was transferred to 1.5ml eppendorf tube. Eight hundred μl of deionized DW was added to the fecal samples. The specimen was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. One thousand 1 of deionized DW was added and the tube was placed on a vortex to separate the pellet. The specimen was centrifuged at 14.000rpm for 3 minutes and the supernatant was collected. Then, the specimens were transferred to a (55°C) in a shaker water bath, proteinase K was added to the specimens at a final concentration of 200 g/ml.

**DNA extraction**

Fecal oocysts was subjected to DNA extraction after cycles of freezing and thawing using a DNA purification kit (Wizard Genomic DNA purification kit; Promega). Then, DNA concentration was measured by using NanoDrop spectrophotometer at wave lengths of 260 and 280 nm. DNA quality was assessed by agarose gel electrophoresis (Maniatis *et al.*, 1982). Preparations of the Gel electrophoresis: Tris-borate-EDTA (Ethylen Diamine tetra Acetic-acid) (TBE): To prepare a concentration 10X of Tris-borate buffer (TBE) as a diluant, 100ml of TBE (Biotechnology, con. 1X) was dissolved in 900 ml of DW.

- Loading buffer: by DNA purification kit (Wizard Genomic DNA purification kit; Promega). Ethidium bromide dye: Biotechnology (Korea).
- Preparing the gel for electrophoresis: the 1.5% concentration of the agarose gel was prepared for a separation-PCR product, by dissolving (1.5gm) agarose powder in (100 ml) DW, then it was put in the microwave for one minute.

**PCR Technique**

The master mixture was prepared by the kit (promega), by adding SSU-F1 primer: 5′-TTC TAG AGC TAC ATG CG 3′ and SSU-R1 primer: 5′-CCC ATT TCC TTC GAA ACA GGA-3′ for each PCR reaction. DNA bands were visualized by UV transiluminator at a wavelength (302 nm) (Maniatis *et al.*, 1982).

**Statistical analysis**

The Chi-square test was used for the comparison between the results. Differences were considered statistically significant at P<0.05 (Snedecor and Cochran, 1989).

**RESULTS & DISCUSSION**

The results of this study showed significant differences (p<0.05) in prevalence of cryptosporidiosis in sheep in AL-Taji area. Prevalence rates 48.88 recorded (44/90), 37.77% (34/90) and 27.77% (25/90) depending on the result of PCR, ZN and Lugol iodine stain, respectively (Table 1; Figure 1, 2). PCR was considered as more sensitive technique used for detection of Cryptosporidiosis, coincided with Morgan *et al.* (1998), Bialek *et al.* (2002) and Bakheit *et al.* (2008) who argued that PCR-based assays detect one oocyst that could be mis-diagnosed by microscopic investigation.

<table>
<thead>
<tr>
<th>Examination methods</th>
<th>No. of fecal samples</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lugol iodine stain</td>
<td>90</td>
<td>25 (27.77)</td>
</tr>
<tr>
<td>Modified ZN stain</td>
<td>90</td>
<td>34 (37.77)</td>
</tr>
<tr>
<td>PCR</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
</tbody>
</table>

Different superscript refers to significant differences at p<0.05

**FIGURE 1:** Cryptosporidium oocysts (arrows) by (A) Lugol iodine stain and (B) Modified ZN stain 100X
The study showed a significance difference (p 0.05) between age groups and infectivity prevalence of parasite, highest prevalence of cryptosporidiosis was recorded in age group 6 months compared with lowest infection rate in age group 25-36 months which reach 70% (14/20), 39.13% (9/23), respectively (Table 2). This may be due to the low immunity status in the newly born lambs and the increased shedding rhythm of oocysts by the infected dam due to hormonal disturbances (Fayer and Xiao, 2008). This result coincided with AL-Zubaidi (1994), AL Azzawi (2003) in small calves, AL-Zubaidi (2009) in goat and Abd Al-Wahab (2003), Kadhim (2009) in small lambs in Baghdad city who found high infection rate in small age groups. Also, this finding agreed with El-Wahed (1999) in Egypt, Sari et al. (2008) in Turkey who reported high prevalence rate of parasite in small lambs. The highest infection rate in small animal may be due to high shedding rate of Cryptosporidium oocysts from dam which contaminate food and water in farm and give chance to infect lambs (Anderson et al., 1991).

### TABLE 2: Prevalence of Cryptosporidium spp according to the age groups

<table>
<thead>
<tr>
<th>Age group (months)</th>
<th>No. of fecal Samples examined</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>20</td>
<td>14 (70)</td>
</tr>
<tr>
<td>6-12</td>
<td>23</td>
<td>12* (52.17)</td>
</tr>
<tr>
<td>13-24</td>
<td>24</td>
<td>10* (41.66)</td>
</tr>
<tr>
<td>25-36</td>
<td>23</td>
<td>9* (39.13)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
</tbody>
</table>

Different superscript refers to significant differences at p<0.05

There was no significance difference between male and female infection rates, 49.09% (27/55) and 48.57% (17/35), respectively (Table 3). This result agreed with Abd Al-Wahab (2003), Kadhim (2009) in small lambs in Baghdad city and Rasheed (1997) in goat kids in Iraq, who found no significance differences in the infection rate between male and female due to equal possibility of exposure to the contaminated environment (Fayer and Xiao, 2008).

### TABLE 3: Prevalence of Cryptosporidium spp according to gender

<table>
<thead>
<tr>
<th>Animal gender</th>
<th>No. of fecal Samples examined</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>55</td>
<td>27* (49.09)</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>17* (48.57)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
</tbody>
</table>

Different superscript refers to significant differences at p<0.05

FIGURE 2: An agarose gel electrophoresis stained with ethidium bromide, revealed Cryptosporidium SSU rRNA gene sequence (~550 bp) PCR amplification products for the 90 sheep fecal samples.
Significance difference (p 0.05) was observed in infection rate of cryptosporidiosis in sheep according to the months of study. The highest infection rate (68.16%; 15/22) was recorded in April, while the lowest infection rate (17.39%; 4/23) in February. This result agrees with Abd Al-Wahab (2003) and Kadhim (2009) who recorded high infection rate of cryptosporidiosis among lambs in March and April. This result may be due to good environmental condition (temperature and humidity) for the parasite and large number of Cryptosporidium oocysts, that shed from pregnant and lactating ewes in the farm which considered as a source of infection to the lambs (Smith et al., 1993; Bulent and Huseyin, 2004; Sari et al., 2008; Fayer and Xiao., 2008).

The result of this study showed significance difference (p 0.05) between infection rate of cryptosporidiosis among diarrheic and non diarrheic sheep, which reach 58.13% (25/43), 40.42% (19/47), respectively (Table 5). This result explains the role of Cryptosporidium as an important cause of diarrhea in sheep and agrees with several researchers, Abd Al-Wahab (2003), Kadhim (2009) in Baghdad, Zorana et al. (2006) in Serbia and Sari et al. (2008) in Turkey who recorded shedding of Cryptosporidium oocysts in 13.7% , 18.61%, 69% and 38.8%, respectively. The result of this study showed significance difference (p<0.05) between infection rate of cryptosporidiosis among diarrheic and non diarrheic sheep, which reach 58.13% (25/43), 40.42% (19/47), respectively (Table 5).

### TABLE 4: Prevalence of Cryptosporidium spp according to the Months

<table>
<thead>
<tr>
<th>Months of study</th>
<th>No. of fecal Samples Examined</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>February</td>
<td>23</td>
<td>4* (17.39)</td>
</tr>
<tr>
<td>March</td>
<td>24</td>
<td>16* (66.66)</td>
</tr>
<tr>
<td>April</td>
<td>22</td>
<td>15* (68.16)</td>
</tr>
<tr>
<td>May</td>
<td>21</td>
<td>9* (42.85)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
</tbody>
</table>

Different superscript refers to significant differences at p<0.05

### TABLE 5: Prevalence of Cryptosporidium spp according Type of feces

<table>
<thead>
<tr>
<th>Types of feces</th>
<th>No. of fecal Samples Examined</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrheic</td>
<td>43</td>
<td>25* (58.13)</td>
</tr>
<tr>
<td>Non diarrheic</td>
<td>47</td>
<td>19* (40.42)</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>44 (48.88)</td>
</tr>
</tbody>
</table>

Different superscript refers to significant differences at p<0.05

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