DETECTION AND MOLECULAR STUDY OF *CRYPTOSPORIDIUM* SPP. IN HORSES AT BAGHDAD CITY, IRAQ

Safa Fawzi Jihad* and Mohammed Thabit Salih. Al-Zubaidi

Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Iraq.

**Abstract**

This study was carried out to detection of *Cryptosporidium* spp. oocytes in the feces of horses by using traditional and molecular methods (PCR), also sequencing and phylogenetic analysis. A total of 180 horse fecal samples from equestrian club / Baghdad, from both sex, and different age groups, during the period from October 2019 - March 2020. The total rate of infection was 56.66% (102/180). Both sexes are subjected to the infection with *Cryptosporidium*, 57.40% (62/108) in males and 55.55% (40/72) in females, without significant differences. The infection was highest at age group >3-<6 years 65.51% (57/87%), while the lowest infection rate 35.48% (11/31) at age group 6-20 years with significant differences (P < 0.01). The highest infection rate was recorded in November 21/30 (70%), and December, while the lowest rate was in March 14/30 (46.66%) and January 13/30 (43.3%) with significant differences (P < 0.01). Using 18s rRNA gene, for PCR, the result revealed that 69% of horse fecal samples have *Cryptosporidium* positive. The Iraqi strains of parasite mainly appeared closely related to each other. However, two sequences appeared relatively divergent from the rest (MT476891 and MT476893). Importantly, sequence with accession number (MT476898) looks highly similar to that of Iranian origin. All the sequences appeared far distanced from the sequence of USA. According to the molecular study and phylogenetic tree, *Cryptosporidium parvum* are considered the main species that cause cryptosporidiosis in horses of Baghdad city which recorded for the first time in Iraq by using molecular technique.

**Key words :** Cryptosporidium, 18s RNA, horses, Baghdad.

**Introduction**

*Cryptosporidium* spp. A pathogenic parasite found in the digestive system of many hosts (Cunha et al., 2019). The description of this parasite for the first time in 1907 by Ernest Edward Taser, in the intestinal epithelium of mice. Human infection was first described in 1976, in a child and in an adult in the same year. *Cryptosporidium* spp is the source of public health concern due to reports of disease outbreaks in day care centers, and patients with immunosuppression as well as in reports of transmission of water (Meireles, 2010).

*Cryptosporidium* are common types of food and water borne protozoa that affect a wide range of domestic and wild animals as well as humans. In horses, cryptosporidiosis was first described in immune-deficient Arab foals (Santín, 2013).

**Cryptosporidium** spp is apicomplexan parasites living in the brush border of the intestinal epithelium and respiratory system. At first thought it was to be only a pathogen of young animals such as calves, lambs and foals, and is an important cause of diarrhea, enters colitis in humans and animals (Checkley et al., 2015).

Diarrhea is very common clinical sign in newborn foals and can be a sign of infectious diseases or hypoxic gut injury or dietary changes or disturbances in the intestinal flora, which quickly lead to the emergence of systemic manifestations (Magdesian, 2005; Bernard and Barr, 2011). The most common causes of diarrhea in new-born foals are *Cryptosporidium Rotavirus, Clostridium perfringens* and *Salmonella* spp. (Oliver-Espinosa, 2018).

Due to high economic losses and the delayed growth that determined by diarrhea, more information has been

---

*Author for correspondence : E-mail: safafawzialkazragi@gmail.com*
written about cryptosporidiosis in calves, lambs and kids (De Graaf et al., 1999; Burton et al., 2010).

In these animals, Cryptosporidium parvum is the main cause of cryptosporidiosis, which has been described as a severe diarrheal disease, characterized by yellow faces and unpleasant odor, with a soft to liquid consistency associated with depression, abdominal pain and loss of appetite (Lanci et al., 2018).

In horse, until 2003, only C. parvum was known as main cause of diarrhea (Bjorneby et al., 1991; Veronesi et al., 2010). In 2003, Cryptosporidium horse genotype was described for the first time in Przewalski adult horse and subsequently also isolated in healthy foals, less than 1 month old, in the New York State (Ryan et al., 2003; Burton et al., 2010).

Diagnosis of Cryptosporidium spp. generally occurs by different methods, the common methods used for the identification and detection of oocysts are direct, concentration and staining methods (Henriksen and Pohlenz, 1981; Garcia et al., 1983) these ways gave a good laboratory practice. Cryptosporidium parasites also can be discovered by electron microscopic examination in the intestinal mucosa of the hosts (Juranek, 1995). Also many diagnostic methods were used to diagnosed the parasite, including, immunological tests like IFAT, ELISA (Arrowood and Sterling, 1989; Casemore, 1989) and molecular technique (Polymerase chain reaction) have an active way to identification and genotype of the pathogen (Saramago Peralta et al., 2016). In molecular techniques, the multiple species of Cryptosporidium parasite recognized in faecal samples (Xiao, 2010).

**Materials and Methods**

**Sample collection**

One hundred and eighty horse fecal samples (30-50 g) were collected from both sex and different age groups, during the period from 1st October 2019 to end of March 2020 (30 samples from each month) from equestrian club, this club contain approximately 4000 horses and located in AL-Ameria / Baghdad. Each fecal sample were collected using sterile disposable latex glove, and placed into individual plastic box with ice, which were sealed, labelled, and transported immediately to the Parasitology laboratory, at College of Veterinary Medicine -University of Baghdad.

**Sample preparation**

Small amount from each fecal sample (1/2 tea spoon) of feces placed in Eppendorf tube then labelled and stored at -20°C used later for DNA extraction. Added a sufficient quantity of distilled water (20-30ml) to the remain fecal sample and mixed well, then filtered with four layer of gauze then examined by direct smear, flotation and stain methods, then we added 0.5 ml of potassium dichromate solution 2.5% as preservation for each sample and mixed, and kept in refrigerator at 4°C until used again. Direct smear As in (Anne M. Zajac, 2007), then Floatation Concentration Method by Sheather’s sugar solution technique As in: (Anne Zajac, 2007; Makawi and Al-Zubaidi, 2017), Modified Ziehl-Neelsen staining (Street, 2015).

**DNA extraction**

DNA was extracted from one hundred fecal samples randomly collected and stored previously at -20°C by using addbio DNA extraction kit /Korea. The extraction was performed according to manufacturer’s instruction of the addbio Company. The primer used was according to (Murphy and Arrowood, 2020) the forward 5'-GGAAGGTTGTATTTATTAGATAAG-3’ and reverse 5’-CTCATAAGGTGCTGAAGGAGT-3’ and the size 840bp, the primers were provided as lyophilized form (Macrogen /Korea).

**Results**

**Result of prevalence**

A total of 180 fecal samples from horses were examined for detection Cryptosporidium infection during period from 1st October 2019 to end of March 2020 the total rate of infection was 56.66% (102/180).

**Infection rate of Cryptosporidium according to sex**

The study revealed that both sex were subject to infection with Cryptosporidium 57.40% (62/108) in males, 55.55% (40/72) in females without significant differences between both sex (P<0.05) (Table 1).

**Table 1:** Infection rate of Cryptosporidium in horses according to sex.

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>108</td>
<td>62</td>
<td>57.40%</td>
</tr>
<tr>
<td>Females</td>
<td>72</td>
<td>40</td>
<td>55.55%</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>102</td>
<td>56.66%</td>
</tr>
<tr>
<td>P-value</td>
<td>.....</td>
<td>.....</td>
<td>0.0294 *</td>
</tr>
</tbody>
</table>

With significant difference (P<0.05).

**Infection rate of Cryptosporidium according to age groups**

The study showed that the infection rate with Cryptosporidium was high at age group >3-<6 years 65.51% (57/87) and 54.83% (34/62) at 1-3 years, while at >6-20 group was recorded less infection rate 35.48% (11/31) with significant differences (P<0.01) between
Detection and molecular study of Cryptosporidium spp. in horses at Baghdad city, Iraq

The prevalence of Cryptosporidium were recorded all over the months of the study in horses. The highest infection rate was recorded in November and December 70% (21/30), while the lowest rate was recorded in March 46.66% (14/30) and January 43.3% (13/30) with significant differences (P<0.01) between rate of infection (Table 3-4).

Table 2: Infection rate of Cryptosporidium spp. according to age groups.

<table>
<thead>
<tr>
<th>Month</th>
<th>No. of examined horses</th>
<th>No. of infected horses</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>October</td>
<td>30 sample per month</td>
<td>15</td>
<td>50%</td>
</tr>
<tr>
<td>November</td>
<td>21</td>
<td>13</td>
<td>43.33%</td>
</tr>
<tr>
<td>December</td>
<td>21</td>
<td>14</td>
<td>66.67%</td>
</tr>
<tr>
<td>January</td>
<td>14</td>
<td>13</td>
<td>92.31%</td>
</tr>
<tr>
<td>February</td>
<td>13</td>
<td>12</td>
<td>92.31%</td>
</tr>
<tr>
<td>March</td>
<td>14</td>
<td>13</td>
<td>92.31%</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>102</td>
<td>56.66%</td>
</tr>
</tbody>
</table>

P-value: .....

Fig. 1: Cryptosporidium oocyst appear (oval to spherical in shape) by using flotation with Sheather’s sugar solution x 100.

Table 3: Infection rate of Cryptosporidium spp. according to months.

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. of examined</th>
<th>No. of infected</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1-3 years</td>
<td>62</td>
<td>34</td>
<td>54.83%</td>
</tr>
<tr>
<td>&gt;3 - &lt;6 years</td>
<td>87</td>
<td>57</td>
<td>65.51%</td>
</tr>
<tr>
<td>&gt;6-20 years</td>
<td>31</td>
<td>11</td>
<td>35.48%</td>
</tr>
<tr>
<td>Total</td>
<td>180</td>
<td>102</td>
<td>56.66%</td>
</tr>
</tbody>
</table>

P-value: .....

Result of molecular study

Genomic DNA estimation

Genomic DNA was extracted from 100 fecal sample using addbio DNA extraction kit /Korea and they done according to company instruction. The extracted genomic DNA was estimated by using Nano-drop spectrophotometer (Thermos, USA), the measured purity of DNA through reading the absorbance at (1.5-1.6) and the concentration of the extracted DNA which ranged from 5 to 50 ng/µl.

Infection rate of Cryptosporidium by using PCR technique

The use of conventional PCR technique to identify Cryptosporidium spp. by using 18s rRNA gene and primer have 840 base pare. The result showed that from 100 horse fecal samples DNA sample of Cryptosporidium 69% (69 out of 100) was identified table.

Detection and genotyping of Cryptosporidium isolates

The molecular detection and genotyping of Cryptosporidium in the faecal samples were achieved through the Polymerase Chain Reaction (PCR) and the sequencing respectively. PCR analysis:

Phylogenetic analysis

Phylogenetic tree of Cryptosporidium 18s rRNA gene generated by maximum likelihood method from a nucleotide sequence alignment in MEGA7, with a bootstrap of 1000 replicates to provide support for individual nodes. The resulted pyelogram depicts splitting the sequences. Importantly, sample number 15 was closely related to the Iranian’s isolate. Both were exhibited divergent from sample number 95. The rest samples were
revealed closely related to each other with exception of samples number 30 and 21. Phylogenetic tree analysis of Cryptosporidium parvum of the currently identified sequences (18S ribosomal RNA) referred as red triangle with their corresponding accession numbers. As can be seen, the Iraqi strains mainly appeared closely related to each other. However, two sequences appeared relatively divergent from the rest (MT476891 and MT476893). Importantly, sequence with accession number (MT476898) looks highly similar to that of Iranian origin. All the sequences appeared far distanced from the sequence of USA (Fig. 4-5).

Fig. 1: Gel electrophoresis image using (1%) agarose shows the amplification of Cryptosporidium gene at 840bp. Amplicon size 800bp. M is molecular marker.

Fig. 2: Phylogenetic tree analysis of Cryptosporidium parvum.

Discussion

Cryptosporidium spp. is a pathogenic protozoan parasite present in the gastrointestinal tract of several hosts (Cunha et al., 2019). The investigation about Cryptosporidium parasite in horses was conducted in Baghdad city during period 6 months. The total infection rate with Cryptosporidium in present study was 56.66%, the result was agree with Moosa, (2019) in Mosul which record infection rate of cryptosporidium (30%) in horses, and Wannas, (2012) in Al Diwaniyah government who record infection rate 20.45% in horses, also agree with Olson et al., (1997) which recorded 17% infection rate in Canadian horses. Also Paper, (2007) record infection rate 15.8% in Urmia area, northwestern Iran, Khan, (2020) in Pakistan, show that the prevalence rate was 11.97%.

This study was disagree with Johnson et al., (1997) in California were the infection rate of Cryptosporidium was zero, and Deng et al., (2017) found the infection rate was 1.8% were detected of Cryptosporidium from Southwestern China by PCR amplification of the partial SSU rRNA gene, Wagnerová et al., (2015) record infection of Cryptosporidium spp. in the Czech Republic and Poland was 3.4%, (Piva et al., 2016) in Italy record 8% infection of Cryptosporidium in horses, also in Italy the prevalence of Cryptosporidium was 8% (Veronesi et al., 2010), the infection rate in western Poland was ranged from 0% to 11.5% in horses (Majewska et al., 2004).

The difference in the infection of Cryptosporidium in various regions may be related to feeding conditions, different local climatic conditions, detection methods, sampling time, sample size, animal husbandry practices as well as the different susceptibility to different strains of horses. The nature of breeding with restricted movement, limited grazing and low density may have played a role in raising the infection rate compared to other parts of the world (Putignani and Menichella, 2010).

The study revealed that both sex were subject to infection with Cryptosporidium. The total rate of infection in males was 62 (57.40%) out of 108 males examined while in females was 40 (55.55%) out of 72 females examined with significant differences between both sex (P<0.05).

The result was agree with (Wagnerová et al., 2015)
in Czech Republic and Poland which Statistical analyses
did not show any association between sex in horses,
(Paper, 2007) in Iran also record no significant differences
between the rate of infection in sexes (17.1%) males
and (13.6%) females horses. This study was disagree
with Khan, (2020) in Pakistan were the highest prevalence
was recorded in male horses (13.76%) followed by female
horses (10.97%) statistically significant (p<0.132).
(Burton et al., 2010) in New York State showed that the
infection was higher in female than male.

The study showed that the infection rate with
Cryptosporidium was high at age group >3-<6 years 57
(65.51%) out of 87 examined and 34(54.83%) at <1-3
years out of 62 examined, while at >6-20 group was
recorded less infection rate 11 (35.48%) out of 31 horses
examined with significant differences (Pd<0.01) between
three age group.

This result was agree with Khan, (2020) in Pakistan
where the highest prevalence (16.96%) was determined
in young equines at the age of (<1-5) years while lowest
infection (9.92%) was observed in adult equines at the
age of (>6- 10) years as presented by and statistically
significant (p<0.001), (Li et al., 2019) in China identified
in all three age groups: <6 (1.4%), 6-12 (3.7%) and >12
(12.5%).

(Moosa, 2019) in Mosul showed an increase in
the percentage of infection of foals compared to adult horses
(26%, 4%) respectively, (Veronesi et al., 2010) in Poland
show that the higher infection rate (26.66%) was observed
in foals younger than <8 weeks of age. ( Olson et al.,
1997) in Canada showed that the infection was (21%) in
>6 months of age and (10%) in <6 months. The results
of this study confirmed that Cryptosporidium infection
is common in foals (Xiao and Herd, 1994), (Burton
et al., 2010) / USA show that the infection in foals
was higher than adult.

Wagnerová et al., (2015) there is no significant
differences between the rate of infection in different ages
(Paper, 2007) also show o significant differences between
the rate of infection in different ages.

The prevalence of Cryptosporidium were recorded
all over the months of the study in horses. The highest
infection rate was recorded in November 21/30 (70%)
, December 21/30 (70%) February 18/30 (60%), October
15/30 (50%), while the lowest rate was recorded in March
14/30 (46.66%) and January 13/30 (43.3%) with
significant differences (P<0.01).

This result was disagree with (Khan, 2020) highest
prevalence was record in the month of June (23.07%) followed by April (16.12%), July (15.62%), September
(14.70%), October (13.79%), August (10.71%), January
(10.71%), March (8.82%), February (8.69%) while the
lowest in the month of December (6.06%) and statistically
significant association (p<0.05).

The study showed according to the molecular
technique and phylogenetic tree, Cryptosporidium
parvum was considered the main species that cause
cryptosporidiosis in horses of Baghdad city, which
recorded for the first time in Iraq by using molecular
technique.

This study was disagreement with Wagnerová et al.,
(2015) in Czech Republic and Poland which found more
than one spp. of Cryptosporidium by Analysis of partial
sequences of the SSU gene showed the presence of C.
parvum, Cryptosporidium horse genotype and C. muris,
also this study was disagree with Li et al., (2019) in China
which identified Four Cryptosporidium species/
genotypes in horses, including C. parvum,
Cryptosporidium horse genotype. Hijjawi et al., (2016)
detect six species; C. xiao, C. andersoni, C. ryanae,
C. parvum, C. baileyi from horses.

References
of conventional staining methods and monoclonal antibody-
based methods for Cryptosporidium oocyst detection. J.
cryptosporidiosis in horses with severe combined
Bowman and L. Xiao (2010). “The prevalence of
Cryptosporidium, and identification of the
Cryptosporidium horse genotype in foals in New York State”.
Checkley, W., A.C. White, D. Jaganath, M.J. Arrowood, R.M.
Chalmers, X.M. Chen, R. Fayer, J.K. Griffiths, R.L.
Guerrant, L. Hedstrom, C.D. Huston, K.L. Kotloff, G Kang, J.R.
Mead, M. Miller, W.A. Petri, J.W. Priest, D.S. Roos, B. Striepen,
R.C.A. Thompson, H.D. Ward, W.A. Van Voorhis, L. Xiao,
burden, novel diagnostics, therapeutics, and vaccine
85–94.
into the detection and molecular characterization of
Cryptosporidium with emphasis in Brazilian studies: A