MOLECULAR DIAGNOSIS OF CRYPTOSPORIDIUM SSP. IN WATER BUFFALOES AT BABYLON PROVINCE, IRAQ
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ABSTRACT
This study was aimed recording the prevalence rates of the Cryptosporidium spp. and identify its species in buffalo at Babylon province by using microscopic (flotation and staining) and molecular technique to examined (100) buffalo fecal samples collected randomly from different sexes, ages, and regions, during the period from the beginning of October 2019, to end of March 2020. The percentage of infection with the Cryptosporidiosis was 40% by using the microscopic method, while, nested polymerase chain reaction (nPCR) technique showed the infection rate was 61%, and according to age groups, recorded higher infection 77.5% at (≤ 6) months age group, followed by age group (> 6 -12) months and (>1 -2) years which showed 60% and 50%, besides, the age group (>2) years recorded 40%, which, the lowest infection rate. Also, females recorded (67.74%) as a higher prevalence rate than males (50%). Sequence analysis for ten samples that were positive by (nPCR) technique showed that 6 sequences belong to Cryptosporidium bovis (MT150692, MT150693, MT150694, MT150698, MT150699, and MT150701), while 3 sequence belongs to C. parvum (MT150696, MT150697, and MT150700), as well as, one of sequence belong to C. andersoni (MT150695), with a genetic difference of (0.010%) Datasets suggest strong genetic distinctiveness amongst species. The first diagnose of the Cryptosporidium and its species in water buffalo in Iraq by using molecular assay (nPCR).

Key words: Cryptosporidiosis, Nested PCR, phylogenetic tree, Iraq.

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INTRODUCTION

Cryptosporidium is a protozoan parasite that completes its life cycle in the intestinal and respiratory surface epithelia of mammals, birds, and reptiles. It's an intracellular extracytoplasmic parasite that acts as a coccidial parasite as a major cause of diarrhea in farm animals and humans worldwide (53). Buffalo cryptosporidiosis is often accompanied by watery diarrhea, which is the most important clinical sign and has contributed to high mortality rates (12). Other signs such as metabolic acidosis and electrolyte loss, anorexia, reduced milk consumption, dehydration, growth disorders, slow walking and depression (18, 30). The severity of the disease depends on various factors, including that of the host such as the immune system and accompanied by another infection like rotavirus (44). Cryptosporidium infection occurs as a result of the ingestion of parasitic oocysts that are excreted in the feces of infected animals. Therefore, autoinfection due to the presence of thin-walled oocysts can occur within the same host (31). Transfer of Cryptosporidium spp. can occur in different ways, through direct or indirect contact from animal to animal, from animal to human (zoonotic transmission). It can also occur due to the ingestion of food or water that is contaminated by infected oocysts, or through vectors such as rodents, arthropods or even birds, they can act as mechanical transmission means (7, 27). The diagnosis of cryptosporidiosis is based on the identification of Cryptosporidium oocysts in the fecal sample using conventional microscopic and immunodiagnostic methods (19), as well as, molecular techniques (more sensitive and specific) such as the polymerase chain reaction (PCR) are often used today for genotyping cryptosporidiosis (40). Several studies agree on the higher sensitivity of PCR targeting the 18S rRNA gene. Nested PCR was introduced to improve detection sensitivity (28). This study aimed to detection of Cryptosporidium spp. in water buffalo by using microscopic examination and molecular method (n PCR) in Iraq.

MATERIALS AND METHODS

One hundred buffalo fecal samples collected randomly from different sexes, ages, and regions of Babylon province (Al-Hilla, Abi-Gharaq, Al-Musayib, Al-Sadda, Al-Mahaweal, Al-Kafel, and Al-Hashimiyah). during the period from the beginning of October 2019, to end of March 2020. The microscope act as the primary diagnosis method by using sheather's flotation, modified Ziehl-Neelsen staining and calibration by ocular micrometer to investigate and measurements of Cryptosporidium oocyst, (29), and finally samples saved in the freeze until DNA extracted to detect Cryptosporidium by Molecular diagnosis (nested PCR), the DNA extracts according to the manufacturer's instructions (AccuPower® PCR Premix Kit) from Bioneer company, based on the 18S ribosomal RNA gene (41). The primers of nPCR are designed according to the instructions of the Korean manufacturer (Macrogen company) table (1), then placed in PCR Thermocycler in a condition that using an n-PCR thermal cycler system according to (41). We constructed a phylogenetic tree for our Cryptosporidium versus NCBI-Blast-GenBank. The positive PCR 18S rRNA gene was analyzed for DNA sequencing (Molecular Evolutionary Genetics Analysis version 6.0) and Multiple sequence alignment analysis (ClustalW). Evolutionary distances were computed by the Maximum Composite Likelihood as described by (48). For statistical analysis, using SPSS version 17 and, Chi-square (X2) test was used for comparison between the results, and the differences were considered statistically significant at (P≤0.05) (37).
Table 1. The primer with their sequence

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>Amplicon</th>
<th>Company\country</th>
</tr>
</thead>
<tbody>
<tr>
<td>18SrRNAgen Cryptosporidium spp. PCR</td>
<td>F- AGACGGTAGGGTATTGGCCT R- TCCTTGGCAATGCTTTCGC</td>
<td>616bp</td>
<td>Macrogen\Korea</td>
</tr>
<tr>
<td>18SrRNAgen Cryptosporidium spp. Nested PCR</td>
<td>F- AACGGGAATTAGGGTTCGA R- TGCTTTCGCATTAGTTTGTCTT</td>
<td>567bp</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Our results showed the prevalence rate of cryptosporidiosis was 40% by the conventional microscopic examination method (Sheather's flotation, modified Ziehl-Neelsen staining and calibration by using ocular micrometer for oocyst measurements), and the recognized of the oocyst of the Cryptosporidium depend on the morphologically characteristic, the oocysts of Cryptosporidium bovis is similar to C. parvum, in Sheather's (sugar) floatation smear, the oocysts appeared as spherical/rounded in shape with a thin greenish membrane, the four sporozoites looked as black bodies inside the oocysts (Fig. 1), while, in stained with (mZN) stain appeared as spherical/rounded in shape stained red bodies with a clear halo around the oocyst, against a dark blue background of the methylene blue stain, and the measurement was (4.3 x 4.8 ± 0.8) μm by ocular micrometer (Fig.2).

Figure 1. C. parvum oocysts floatation with Sheather's solution, magnification (100X)

And the morphology of C. andersoni oocysts in Sheather's (sugar) floatation, appeared as round or oval in shape with a thin greenish membrane while the four sporozoites looked as black bodies inside the oocysts (Fig. 3), while in mZN stain the oocyst appeared as red ovoid bodies with a clear halo around the oocyst against a dark blue background of the methylene blue stain and the average length and width measurements was (7.0 x 5.6 ± 0.8) μm (Fig. 4).

Figure 2. C. parvum oocysts, stained with Modified Ziehl Neelsen magnification (100X)
While by using the molecular technique (Nested PCR) the results showed that the total infection rate of Cryptosporidium spp. in buffalo was 61% (Table 2), the statistical analysis showed significant differences between these two techniques and its relation with sensitivity and specificity of each diagnostic technique (p<0.05). By nPCR, the fecal samples exhibited a distinct band of 567 bp on agarose gel for Cryptosporidium spp. as depicted (Fig. 5).

Table 2. Total prevalence of Cryptosporidium infection by microscopy and Nested PCR in water buffalo fecal sample

<table>
<thead>
<tr>
<th>Host</th>
<th>No. of samples examined</th>
<th>Conventional microscopy</th>
<th>Molecular-Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffalo</td>
<td>100</td>
<td>40 (40%)</td>
<td>61 (61%)</td>
</tr>
</tbody>
</table>

P<0.05

Fig. 5. Agarose gel electrophoresis image that showed the nPCR product analysis of 18S rRNA gene in Cryptosporidium spp. from buffalo fecal samples. Where M: marker (1500-100bp) and Lane (1-23) some positive Cryptosporidium spp. were showed at (567bp) nested PCR product.

The results showed a significant difference was (p < 0.05) in the prevalence rate among different age groups (Table 3) showed higher infection 77.5% (31/40) observed at (≤ 6) months age group, followed by age group (>6-12) months and (>1-2) years which showed 60% (12/20) and 50% (10/20), in addition, the age group (>2) years recorded the lowest infection rate which was 40% (8/20).
Table 3. Prevalence of Cryptosporidium infection by nested PCR in relation to age groups of water buffaloes

<table>
<thead>
<tr>
<th>Age groups</th>
<th>No. of samples examined</th>
<th>No. of positive</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 6 Months</td>
<td>40</td>
<td>31</td>
<td>77.5</td>
</tr>
<tr>
<td>&gt;6 – 12 Months</td>
<td>20</td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>&gt;1-2 Years</td>
<td>20</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>&gt;2 Years</td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

P<0.05

According to the sex of animal the significant difference was (p < 0.05), the female recorded higher prevalence rate than the male, (67.74%) in females and (50%) in males (Table 4).

Table 4. Prevalence of Cryptosporidium infection by n PCR in relation to sex of water buffaloes

<table>
<thead>
<tr>
<th>Sex of buffalo</th>
<th>No. of samples examined</th>
<th>No. of positive</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>38</td>
<td>19</td>
<td>50</td>
</tr>
<tr>
<td>Female</td>
<td>62</td>
<td>42</td>
<td>67.74</td>
</tr>
</tbody>
</table>

P<0.05

Ten samples randomly selected from water buffalo were positive by nested PCR, the obtained sequences of nested PCR products were analyzed by using the NCBI BLAST tool, and the results showed the presence of three Cryptosporidium species in the buffaloes, namely C. bovis C. parvum, and C. andersoni. and C. bovis had the highest incidence in the analysis samples (6/10), followed by C.parvum (3/10), and C. andersoni (1/10) (Table 5).

Table 5. Cryptosporidium strains in water buffalo and NCBI BLAST homology sequences

<table>
<thead>
<tr>
<th>Cryptosporidium spp. isolate No.</th>
<th>Genbank Accession number</th>
<th>Identical NCBI BLAST Cryptosporidium species</th>
<th>Genbank Accession number</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.1</td>
<td>MT150692</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.42%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.2</td>
<td>MT150693</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.62%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.3</td>
<td>MT150694</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.61%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.4</td>
<td>MT150695</td>
<td>Cryptosporidium andersoni</td>
<td>KF271468.1</td>
<td>99.43%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.5</td>
<td>MT150696</td>
<td>Cryptosporidium parvum</td>
<td>AY458612.1</td>
<td>99.63%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.6</td>
<td>MT150697</td>
<td>Cryptosporidium parvum</td>
<td>AY458612.1</td>
<td>99.26%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.7</td>
<td>MT150698</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.81%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.8</td>
<td>MT150699</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.65%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.9</td>
<td>MT150700</td>
<td>Cryptosporidium parvum</td>
<td>AY458612.1</td>
<td>99.26%</td>
</tr>
<tr>
<td>Cryptosporidium spp. IQ- buffalo isolate No.10</td>
<td>MT150701</td>
<td>Cryptosporidium bovis</td>
<td>MK982466.1</td>
<td>99.22%</td>
</tr>
</tbody>
</table>

A Phylogenetic tree of C. bovis, C. parvum, and C. andersoni referenced against those of Gen-Bank which highlight differences by DNA STAR (48). Cryptosporidium spp. (1, 2, 3, 7, 8 and 10) were closely related to NCBI-Blast Cryptosporidium bovis (MK982466), (5, 6 and 9) were closely related to NCBI-Blast Cryptosporidium parvum (MK982466), and No.(4) to NCBI-Blast Cryptosporidium andersoni isolates (KF271468) with a genetic difference of (0.010%) Datasets suggest strong genetic distinctiveness amongst species (Fig.6).
The morphology of Cryptosporidium species oocysts were isolated from the feces of the naturally infected animal that were completely sporulated, spherical, ovoid-shaped to ellipsoidal with a thin greenish membrane. It contains four sporozoites that resemble black bodies in oocysts on a wet smear (floatation) and red ovoid-shaped bodies with a transparent halo around the oocyst on a dark blue background of methylene blue in (mZN), but with slightly different dimensions. The oocysts of the two species: C. parvum and C. bovis had almost the same morphological properties and measured (4.4 x 4.8 ± 0.8) μm by ocular micrometer, and the average length and width of C. andersoni oocysts were (7.0 x 5.6 ± 0.7) μm. These results were in agreement with the results of (34) reported the C. parvum oocyst measurements were (3.2-5.8 x 4.3-4.9) μm in size in Egypt, in the province of Babylon (Iraq), the average length of the C. andersoni oocysts was (7.1 ± 0.92) μm and width (5.6 ± 0.61) μm (5), In the United States, reported the mean dimensions of C. andersoni was (7.30 x 5.19) μm (50), and in the city of Baghdad recorded the dimensions of C. parvum were (4.9 x 4.3 ± 0.9) (5). The overall prevalence of cryptosporidiosis in buffalo in this study was 40% by conventional microscopic examination and 61% by molecular technique (nested PCR), and the agreement or disagreement with the results of other studies depends on the different factors that affected on the result between countries such as the sampling plan, study season, level of hygiene measures applied and the diagnostic methods used, besides, attributed to the system of rearing, water supply, management, and sanitary conditions in and around the farms (21). Microscopically, in Baghdad city, recorded the infection rate was 53.93% in buffalo (3), in Salah al-Din Province, the prevalence rate was 31.5% reported by (2), and reported the prevalence rate of cryptosporidiosis in buffalo was 10% in Babylon Province (39). Also, the prevalence of infection in water buffalo in different regions of the world, such as in Egypt, recorded the prevalence rate of C. parvum was 22.5% in water buffalo (45). In the calves, reported the infection rate was 21.8% in diarrheal calves in Turkey (26), in Iran, recorded the infection rate of diarrheal buffalo calves were 45.5%, and 2.5%, respectively by (10,49), and in India, Sudan and Thailand, recorded the infection rate were (21%, 85%, 58.3%, and 51%) respectively, in diarrheic bovin calves (14,42,47). By molecular (nPCR) examination, we recorded a higher prevalence rate of Cryptosporidiosis (61%) in this study. The widespread use of the SSU rRNA gene in the detection/genotyping of Cryptosporidium is mainly due to the multiple copies of the gene and the presence of semi-conserved and hypervariable regions that facilitate the design of genus-specific primers as well as the 18S rRNA-based PCR protocol was shown to be more sensitive than the modified Ziehl-Neelsen (mZN) staining technique (17,52,55). The agreement or disagreement with the other study depends on several factors mentioned previously that make it expected that there will be a difference in the results of studies for different countries and regions such as in Pakistan, recorded infection rate was 24% in buffaloes (36), and the prevalence rate was 37.5% in water buffaloes in Nepal recorded by (20). However, the prevalence rate of Cryptosporidium infection was 73.3% of the herds and 32.2% of the individual buffalo in the positive herds in Egypt (23). In Australia, the prevalence rate of Cryptosporidium in water buffaloes was 30%
recorded by (53), and reported the prevalence rate of Cryptosporidium infection was 48.2% in Brazil (9). According to age groups the results showed the highest rate in the age group (≤ 6) and the lowest rate in the age group (>2) years. The higher prevalence of infection in newborns is due to lower tolerance levels in young newborns due to the poor development of acquired immunity (46). Our results were consistent with other studies showing that younger age groups have the highest infection rate, which decrease with age of the animal increase, this corresponds to the studies have shown in the, in Egypt by (15) for buffalo calves, in Australia by (1) in water buffalos, and in India by (46) in buffaloes. According to the sex, our study showed the female was higher proportion than male. It has been agreed with other studies that indicate that animals raised in the same place and exposed to the same conditions have close prevalence rates where male or female have no factors that facilitate infection, but the highest proportion found in adult females could be due to the stress during pregnancy, parturition and milking times, which make the females more prone to the infection (6,35,51). In addition to the PCR technique the phylogenic tree analysis act as one of the good ways to detect and identify Genetic variants.(16). The DNA Sequence of the ten samples showed the presence of three species of Cryptosporidium in water buffalo, they are C. bovis, C. parvum, and C. andersoni and the most prevalent species was: C. bovis. Our results were consistent with other results that recorded in many studies such as in Egypt reported Cryptosporidium parvum, C. ryanae, C. bovis and combinations of C. parvum plus C. andersoni in buffalos (23). In China, Cryptosporidium parvum, C. bovis, C. andersoni and C. ryanae were recorded by (32,38,54). In Egypt, the identification of Cryptosporidium species / genotypes were C. ryanae (~ 59%) and C. parvum (~ 41%) in buffalo calves was reported by (33), while, the results were C. parvum (88.2%) and C. ryanae (11.8%) in buffaloes in Thailand (27). Two species of Cryptosporidium, C. parvum and C. bovis, have been identified in buffalo fecal samples, Cryptosporidium parvum is the most frequently classified as positive(~80%) in farmed buffaloes in Australia (1,53). Other studies from several countries have reported that C. parvum is alone or the predominant species in buffalo calves, such as in Egypt, Spain, Italy, and India (8,11,22,46), also in adult buffalo such as the Italian, South Africa, and Egypt (13,25, 43). This study described the diversity of Cryptosporidium in buffalo in Babylon province/Iraq. Where, three species of Cryptosporidium were detected in this study, C. bovis, C. parvum and C. andersoni, and the absence of the other Cryptosporidium species that infected buffalo in this study can be attributed to the small number of samples collected for sequence analysis that did not fully cover the study area and therefore would not represent the total number of buffaloes present in them or possibly due to the low distribution of these species in our study area. 

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