Bovine Cryptosporidiosis and the Egyptian Diarrheic Buffalo Calves

Dalia A Hamza¹, Soliman M Soliman², Noha Y Salem¹ and Sara M Nader¹*  

¹Department of zoonoses, Faculty of Veterinary Medicine, Cairo University, 12211 Egypt; ²Department of Internal medicine and infectious diseases (Infectious diseases), Faculty of Veterinary Medicine, Cairo University, 12211, Egypt; ³Department of Internal medicine and infectious diseases (Internal medicine), Faculty of Veterinary Medicine, Cairo University, 12211, Egypt  
*Corresponding author: sara.nader@cu.edu.eg

ABSTRACT

The negative impact of diarrhea on livestock health is well known, Cryptosporidium, is one of the protozoan that causes diarrhea in calves especially buffalo calves. Some species of Cryptosporidium represent a zoonotic hazard. This study aimed to distinguish the potential species of Cryptosporidium in affected buffalo calves and evaluate a modified technique to improve the molecular detection and identification of Cryptosporidium. Twenty buffalo calves suffered from diarrhea were enrolled in the study. The enrollment criteria depended on the results of Ziehl–Neelsen stain. Sugar floatation technique was performed followed by oocyst concentration to form a pellet for DNA extraction. Multiplex PCR was performed for identification and differentiation of Cryptosporidium Spp. The results showed a mixed infection in 4 samples; the most common type of Cryptosporidium affecting the examined buffalo calves was C.parvum (10), followed by C.bovis (7 samples) and C.andersoni (6 samples) while no C.reynesi was detected in the examined samples. The zoonotic type, C.parvum was found in 50% of the total affected animals. The current study detects three Cryptosporidium spp, namely C.parvum, C. andersoni, and C.bovis linked to diarrhea in the studied buffalo calves. Mixed infection with more than one species of Cryptosporidium was present. Standard detection of oocYTE in fecal samples using modified Ziehl-Neelsen stain is a simple way for diagnosis of Cryptosporidium. However, a more sophisticated method is recommended to detect and differentiate the zoonotic species in calves, as they symbolize a crucial source of human infection.

Key words: Cryptosporidium; Buffalo calves; Multiplex PCR; C.parvum; Diarrhea

INTRODUCTION

Calf diarrhea is a frequent problem in livestock practices. It can have a negative impact not only on the balance of livestock farms but also extends to state economy (Cho and Yoon, 2014). Both infectious and non-infectious agents were implicated in calf diarrhea (Izzo et al., 2011).

Cryptosporidium, a protozoan parasite, is thought to cause diarrhea in both animal and human (Sakarya et al., 2010); with fatal consequences in young animals (Dubey et al., 1990). Bovine cryptosporidiosis first report dated back to 1971 (Thomson et al., 2017) in an exceedingly report by (Panciera et al., 1971) who described a chronic debilitating diarrhea in eight-month old calf, which had been attributed to Cryptosporidium. Though, approximately 21 types were identified (Sakarya et al., 2011), four Cryptosporidium spp was confirmed in bovine: C.parvum, C.bovis,C. andersoni and C.ryanae; only the latter was linked with zoonotic importance and clinical illness in neonates (Santin et al., 2004; Fayer et al., 2008).

In water buffaloes, few investigations were done to genotype the Cryptosporidium species. Cryptosporidium spp, were isolated from a little number of buffalo calves in Spain, Nepal, and Italy (Gomez-Couso et al., 2005; Caccio et al., 2007; Feng et al., 2012). In Egypt, domestic buffaloes constitute a very important part of the farm animal resources (Wilson, 2012). The country has about 4 million heads, the world water buffalo population and buffaloes are usually kept in close contact with household members raising public health problems. However, detection of Cryptosporidium spp, buffaloes in Egypt so far are done exclusively by microscopy (El-Sherbini and Mohammad, 2006; El-Khodery and Osman, 2008).

Age-related cryptosporidiosis in cattle are proposed (Santin et al., 2004). C. andersani incorporates a great affinity to affect adult cows (Lindsay et al., 2000), C. parvum is more predominant in calves (Thomson et al., 2017). Episode of diarrhea and enteritis with a substantial rate of mortality among young calves, children and immune-compromised patients were described within the association with cryptosporidiosis (Coco et al., 2009; Chalmers and Davies, 2010).
Traditionally, diagnosis of Cryptosporidiosis depends on microscopic examination of Ziehl-Neelsen stained fecal smear and Floatation-concentration technique. The latter technique usually employs salt in preparation, however, sugar usage was also advocated (Shams et al., 2016). Other techniques also are used, for example FAT, ELISA and PCR (Constable, 2014). PCR as a way for detection of Cryptosporidium could be a sensitive, discriminate assay for Cryptosporidium species (Rochelle et al., 1997; Smith 2008). The rationale of using molecular diagnosis for characterization of Cryptosporidium spp. is to estimate the variety of species infecting humans and animals as well as to assess the role of animals within the transmission of human Cryptosporidiosis (Xiao et al., 2004). The identification of Cryptosporidium spp. is vital in drawing the epidemiology of the disease with special respect to the transmission routes to avoid zoonotic hazard. So far, little studies have investigated the prevalence of cryptosporidiosis in Egypt (Iskander et al. 1987; El-Sherif et al. 2000; El- Dessouky and El-Masy 2005; El-Sherbini and Mohammad 2006). The prevalence of cryptosporidiosis was also reported in African buffaloes (Syncerus caffer) in Tanzania (Mbambo et al. 1997). Therefore, the present study aimed to identify Cryptosporidium spp affecting Egyptian buffalo calves and a possible infection with mixed species of this parasite, and to estimate percentage of zoonotic spp in diarrheic Egyptian buffalo calves. Accordingly, the modified sugar concentration floatation technique was used to improve the molecular detection and identification of Cryptosporidium in calves. Using sugar instead of salt for the floatation technique has the advantage of enhancing the extraction of Cryptosporidium spp. Oocyst, as it prevents the oocyst escape within the fecal samples.

**MATERIALS AND METHODS**

**Animals**

Twenty diarrheic native breed buffalo calves were enrolled, clinical signs were recorded and confirmation of Cryptosporidium was done by detecting presence of oocyte in fecal samples using modified Zn stain (Henriksen and Pohlenz, 1981).

**Molecular study**

Multiplex PCR was applied to determine different species of Cryptosporidium that affect bovine species, especially C. parvum which is the zoonotic species for human being.

**Modified sugar floatation technique and DNA extraction**

Fecal samples were examined using sugar floatation technique as modified by Fujino et al., (2006). After that, concentration of the oocyst was performed via centrifugation of pure oocysts at 1000 rpm for 10 min. to form pellet. The resultant pellets were purified prior to DNA extraction. This group was examined via multiplex PCR.

**Purification of Cryptosporidium oocysts and DNA extraction**

The pellets were suspended in 200 µl lysis buffer then centrifugation at 1000 rpm for 10 min followed by addition of Proteinase K with final concentration of 0.15 mg/ml then incubated at 65°C/1 hr. Proteinaceous materials were precipitated by centrifugation at 1000 rpm for 10 min, then transfer the supernatant into a new micro centrifuge tube. DNA were precipitated by adding 100 µl of digested supernatants, which were extracted twice with phenol-chloroform-isomyl alcohol (24:24:1) and pelleted by centrifugation at 2000 rpm/ 10 min. at 4°C. The supernatant was carefully removed and the remaining pellets were washed twice with 75% ethanol, and allowed to dry, finally, pellet was res-suspended in 50 µl TE buffer and stored at -80°C until usage (Balatbat et al., 1996).

**Gene amplification**

Two stages of nested PCR protocol were used to amplify a Cryptosporidium fragment Cryptosporidium. A particular set of primers designed to magnify a portion of the "actin gene" derived from the four common Cryptosporidium spp infecting cows' homogeneous preparations, namely, C. andersoni, C. parvum C. ryanae and C. bovis with expected PCR product of 1095 bp for initial step. (Sulaiman et al., 2002). Nested PCR was performed using three particular primer sets as shown in table 1 (Santin and Zerlenga, 2009). For initial step, the reaction volume is 25 µl from which the primary PCR mixture contains 1X master mix, 10 pmol of each primer and 2.5 µl of the template DNA. This step followed by 35 cycles as follows: 94°C/45 s., 50°C/45 s., and 72°C/1 min. Preliminary preheating step at 94°C/5 min was executed. A final extension step was done at 72°C/10 min.

For second set, in a 25 µl reaction volume, multiplex PCR was done via PCR mixture contained 1X master mix, 10 pmol of each primer and 2 µl of the primary PCR reaction. PCR reaction was entailed 40 cycles as follows: 95°C/30 s., 60°C/30 s. and 72°C/2 min. Initial preheating step at 95°C/2 min was done. The final extension was a done at 72°C/7 minutes. PCR products were analyzed using electrophoresis on a 1.5% agarose gel and stained with ethidium bromide (Santin et al., 2004; Santin and Zerlenga, 2009).

**RESULTS AND DISCUSSION**

The sensitivity of immune-detection over coproscopical method are scrutinized repeatedly. However, contradicting verdicts are reported (Doganci et al., 2002). Nevertheless, PCR steadily gained a wide popularity in Cryptosporidium detection (Minarovicova et al., 2007). This study aimed to identify Cryptosporidium spp affecting Egyptian buffalo calves, possibility of mixed infection and crude estimation of percentage of zoonotic spp in diarrheic Egyptian buffalo calves.

In order to detect Cryptosporidium spp, 20 positive diarrheic native breed buffalo calves with confirmed cryptosporidium affection was enrolled during this study. Though, detecting presence of oocyte in fecal samples using modified Zn stain (Henriksen and Pohlenz, 1981) is feasible to spot the infection but lack ability to differentiate sub spp. Diagnosis of Cryptosporidium from stool samples by the direct microscopy using acid-fast stains remains the gold standard in many laboratories worldwide (Morgan et al., 1998; Ridley and Olsen, 1991; Webster et al., 1996). However, this method is insensitive, time consuming, and requires skillful personal, as compared to PCR, which, is more sensitive and particularly allows identification and subtyping of Cryptosporidium spp. (Xiao, 2010).
In the current study, the DNA was extracted from oocyst pellet retrieved from a modified concentration floatation technique by using sugar instead of salt to enhance the extraction technique of Cryptosporidium spp. oocyst by avoidance of oocyst escape within the fecal samples (Fujino et al., 2006; Koompapong et al., 2009). Multiplex PCR using DNA extracted from oocyst pellet is advocated over conventional PCR, as a result of PCR inhibitors in feces together with resistance of oocysts lysis, false-negative PCR results were expected (Lantz et al., 1997). The increased sensitivity of sugar floatation method is believed to linked with the power of sugar to concentrate oocysts and provides high yield whether or not the quantity of oocysts are low (Rekha et al., 2016). Moreover, the hypertonicity of solution was presupposed to cause oocyst collapse if it left for more than 15 minutes (Bowman, 2014).

Results of molecular detection is shown in figure 1. C. bovis was detected at 300 bp, C. parvum at 400 bp, C. andersoni at 350 bp while C. reyne was not detected in all the studied group. Although four types of Cryptosporidium are known to be mostly affect bovine, however, only C. parvum is the only one of zoonotic importance (Fayer et al., 2006; Santin and Zarlenaga, 2009).

In this study, C. parvum was identified in 10 samples (50%) followed by C. bovis in seven samples (35%) and C. andersoni in six samples (30) while C. reyne was not identified in all 20 samples. Mixed infection was detected in four samples (20%). in mixed infection, three samples have mixed infection with two spp (70%) and one sample with three spp as shown in Table 2.

In a study performed in Egypt (Mahfouz et al, 2014) who isolated Cryptosporidium spp. From buffaloes in different farms at Kafir El Sheikh Province. The overall prevalence was 1.29% in buffalo (4.17% in calves versus 0.48% in adults) followed by PCR-RFLP analyses of small-subunit rRNA genes from positive specimens revealed C. ryanae was the dominant species (60.0%) followed by C. parvum (40.0%) in buffalo calves. This is different from our results, which indicated the highest infection rate with C. parvum (50%) in buffaloes while C. reyne was not identified. However, it is similar to a study conducted by (Amer et al., 2013) from Al Monofia, Kafir El Sheikh and Alexandria Province from which the distribution of C. ryanae is 59% and C. parvum is 41%. Whereas Gomez- Couso et al. (2005) and Caccio et al. (2007) reported only C. parvum in water buffalo calves in Spain and Italy, respectively.

In this study, C. parvum accounted for 50% of affections. C. parvum infected many animal species including cattle which act as a reservoir for human infection (Xiao et al., 2004). During this study, multiplex PCR was able not only to detect cryptosporidium, but also, to detect the mixed infection and to signal those with potential hazard for human being (C.parvum). The C.parvum in this study was assumed to be of cattle genotype (Abe et al., 2006). The lack of microscopic ability to differentiate between oocysts, make the necessity to perform an affirmative test is necessitated to rule out or signal presence of the zoonotic type in animal feces (Santin et al., 2004). Hence, multiplex PCR was designed to distinguish the zoonotic type (C.parvum) from other three types (Santin and Zarlenaga, 2009). Although use of PCR was hindered by cost, required time to process samples and false negative results, recent revolution in molecular detection and differentiation made its usage surpluses the hurdles (Khurana and Chaudhary, 2018).

![Fig. 1: Multiplex PCR amplification of C. parvum, C. bovis, and C. andersoni observed on 2% agarose gels stained with ethidium bromide. Lane M, 100-bp DNA ladder; lane 1, C. parvum (400 bp); lane 2, C. bovis (300 bp) and C. andersoni (350 bp); lane 3, C. andersoni (350 bp); lane 4, negative; lane 5, C. andersoni; lane 6, C. bovis and C. andersoni; lane 7, C. parvum, C. andersoni and C. bovis; lane 8, control negative.](image-url)

Table 1: Primer sets sequences

<table>
<thead>
<tr>
<th>Step</th>
<th>Primer set 5'-3'</th>
<th>Amplicon length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R:</td>
<td>AGAARCAYTTCCTGTGKACAT</td>
<td></td>
</tr>
<tr>
<td>Second set 5'-AATCCTGAGAAGATGACTCAAATA</td>
<td>400 bp fragment “C. parvum”</td>
<td>Santin and Zarlenga, (2009)</td>
<td></td>
</tr>
<tr>
<td>5'-CTGCTAGTCTATATGTTCTCTCAA</td>
<td>350 bp fragment “C. andersoni”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-GCTGTGTCTTCCCATCAATTTGACGA</td>
<td>300 bp fragment of both C. bovis and C. ryanae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-GGATCCTCAGGAATCTCTCAGAATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'-TAATCTTCTAGTGAAAGGGTCTTAT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Results of multiplex PCR in affected calves

<table>
<thead>
<tr>
<th>Sample</th>
<th>C. Parvum</th>
<th>C. bovis</th>
<th>C. andersoni</th>
<th>C. reyne</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 1: Multiplex PCR amplification of C. parvum, C. bovis, and C. andersoni observed on 2% agarose gels stained with ethidium bromide. Lane M, 100-bp DNA ladder; lane 1, C. parvum (400 bp); lane 2, C. bovis (300 bp) and C. andersoni (350 bp); lane 3, C. andersoni (350 bp); lane 4, negative; lane 5, C. andersoni; lane 6, C. bovis and C. andersoni; lane 7, C. parvum, C. andersoni and C. bovis; lane 8, control negative.
Moreover, *Cryptosporidium* spp. has been identified from buffalo heifers without diarrhea by using the molecular characterization (Gómez-Couso et al., 2005).

**Conclusions**

The current study identified the presence of three *Cryptosporidium* spp., namely *C. parvum*, *C. andersoni* and *C. bovis*, which are linked to diarrhea in the studied buffalo calves. Mixed infection with more than one species was detected. Standard detection of oocyte in fecal samples using modified Ziehl-Neelsen stain is an easy way for diagnosis of *Cryptosporidium*. However, a more sophisticated method is needed to detect the zoonotic species like *C. parvum*, which constitutes 50% of the detected *Cryptosporidium* in the studied group, implicating calves as zoonotic hazard to their handlers.

**REFERENCES**


