Prevalence and Molecular Discrimination of Cryptosporidium parvum in Calves in Behira Provinces, Egypt

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ABSTRACT

Cryptosporidium parvum is a ubiquitous zoonotic protozoan parasite which is associated with severe acute diarrhoea in humans and animals. Diarrhoeic fecal samples collected from young cattle calves at different localities in Behira Province, Egypt were coproscopically examined concerning the presence of Cryptosporidium spp., oocysts. Small sized Cryptosporidium oocysts were detected of typical shape and measurements of the C. parvum oocysts with a total prevalence of 54.4% (higher prevalence 61.6% in calves less than 1 month of age while lower 38.2% in calves aged 1-2 month). Molecular characterization was done using nested PCR amplification and partial sequence analysis. The nested PCR gave the expected 1st (1325 bp) and the 2nd (825 bp) PCR products from all the examined Egyptian isolates. The DNA sequence alignments and BLAST search analysis of the Egyptian isolates of Cryptosporidium revealed 100% homology between the 825 bp amplified fragment of Egyptian isolates and the counterpart of the 18S rDNA sequences of C. parvum deposited in Gene bank and proved that the 10 positive PCR isolates were Cryptosporidium parvum. The high prevalence of Cryptosporidium parvum among calves obtained by this investigation has pointed to the existence of this zoonotic genotype and suggesting that there is a potential risk of Cryptosporidiosis as a zoonotic transmission between calves and humans in this region.

Key words: Cryptosporidium parvum, calves, coproscopical examination, nested PCR analysis, DNA sequences

INTRODUCTION

The protozoan parasites Cryptosporidium are widespread in the environment have proven potential to cause waterborne and foodborne disease and the major outbreaks of cryptosporidiosis have occurred as a result of contaminated drinking water. Their entire life cycle takes place in the intestinal epithelium and transmission occurs in the form of environmentally resistant oocysts by the fecal-oral route and/or through contaminated food and/or water (Dawson, 2005; Smith and Nichols, 2010).

The genus Cryptosporidium is composed of at least 13 species. Cattle are infected with at least 4 Cryptosporidium parasites: C. parvum, C. bovis, C. andersoni and the C. ryanae. The occurrence of these Cryptosporidium spp., in cattle were shown to be age-related and C. parvum is the only prevalent zoonotic species in cattle, is responsible for about 85% of the Cryptosporidium infections.
in pre-weaned calves but only 1% of the Cryptosporidium infections in postweaned calves and 1-2-year-old heifers (Santin et al., 2004; Fayer et al., 2006).

Cryptosporidium parvum is the important species which infects the gastrointestinal epithelium of most mammalian hosts including cattle and human worldwide, causes severe acute diarrheal enteritis with substantial morbidity and mortality among young calves, children and immunocompromised individuals such as AIDS patients (Chalmers and Davies, 2010). Contact with infected calves has been implicated as the cause of many cryptosporidiosis outbreaks in veterinary students, research technicians and children attending agricultural (Coco et al., 2009).

Extra-intestinal infections by Cryptosporidium parvum have been detected in pigs and sheep. In addition to intestinal infections, cryptosporidial oocysts were found in the gall-bladders of two pigs which were 2 months old and in some organs of sheep aged 5 days, including the gall-bladder, mesenteric lymph nodes, trachea, lung and the uterus of one lamb as reported by Fleta et al. (1995). Moreover, infection of the uterine mucosa was induced after experimental intra-uterine inoculation of adult mice with C. parvum (Liebler et al., 1986).

Lack of distinctive morphologic features of Cryptosporidium oocysts render microscopical examination inconvenient to clearly differentiate species rather genotypes (Fall et al., 2003). Therefore, molecular analyses have been widely used to characterize the genetic structure of Cryptosporidium parasites and assessment of their zoonotic significance (Xiao, 2010). Molecular analysis of C. parvum revealed several families among isolates from humans and/or cattle as well as several subtypes within each family (Wielinga et al., 2008). Consequently, C. parvum can be divided into two genetically distinct subpopulations based on the molecular characterization of oocysts: genotype I (anthropoontic genotype) which is associated exclusively with human infection and is recently proposed as a new species, C. hominis and genotype II (zoonotic or cattle genotype) which is associated with both human and animal infections (Hajdusek et al., 2004; Abe et al., 2006).

In Egypt, limited studies were devoted to ascertain cryptosporidiosis in human and animals. However, Cryptosporidium was responsible for considerable part of diarrheal illness among American military personnel participated in a military exercise in the northwestern Egyptian desert (Sanders et al., 2005). Human cryptosporidiosis was also reported in different ages (Youssef et al., 2008). Cryptosporidium infection frequently affects domestic animals with strong relation between C. parvum infection and diarrhoea among Egyptian buffalo calves (Warda et al., 2002; El-Khodery and Osman, 2008). Recently, epidemiological and molecular findings on Cryptosporidium spp., from Egyptian dairy calves and stray dogs were performed by Amer et al. (2010) and El-Madawy et al. (2010).

Though cryptosporidiosis is frequent in human and livestock in Egypt, very little information is available about the genetic diversity of this parasite. Therefore, this study was aimed to determine the prevalence and as well as molecular heterogeneity of Cryptosporidium spp., isolated from calves in Behira Province, Egypt. To this end, species identification was based on nested PCR amplification and DNA sequence analysis of these Egyptian isolates and compared with counterparts deposited in Gene bank.

MATERIALS AND METHODS
Coproscopical examination
Fecal samples: A total of 180 diarrhoeic fecal samples were collected from young cattle calves (125 from less than 1 month and 55 from 1-2 months-aged calves) in different localities at Behira Province, Egypt.
Detection of oocysts: Fine feces smears fixated with methanol spirit and stained with Modified Ziehl-Neelsen Stain (MZN) for detection of Cryptosporidium oocysts according to the procedure described by Henriksen and Pohlenz (1981). The preparations were observed and the oocysts were measured with the help of stage micrometer conjugated with a light microscope at the eyepiece 10x and the objective 100x. All measurements were taken in micrometers (µm) for about 20-50 oocysts, with the range in parenthesis following the mean (Fayer and Xiao, 2008).

POLYMERASE CHAIN REACTION (PCR)

PCR-sample preparation: Oocysts of Cryptosporidium were purified from collected calve fecal samples following sucrose and Percoll flotation (Arrowood and Sterling, 1987). The purified oocysts were washed three times with PBS solution and then stored at -20°C in 2.5% potassium dichromate solutions.

DNA extraction: DNA was extracted from the washed Cryptosporidium oocysts using the DNeasy Blood and Tissue Kit (Qiagen Co., Cat. no. 69504) with modifications to the manufacturer’s protocols according to Lalonde and Gajadhar (2009) as follows: (i) oocysts were first suspended in 300 mL of ATL buffer and subjected to 8 cycles of freeze-thaw for 1 min in liquid nitrogen and 1 min in a 95°C water bath; (ii) lysed suspensions were incubated with 20 mL proteinase K (20 mg mL⁻¹, Qiagen) for 3 h at 56°C, followed by incubation at 70°C for 10 min in 300 mL AL buffer with vortexing for 10 sec every 3 min. DNA was purified through the provided columns according to the manufacturer’s protocol and eluted in 50 mL of the supplied AE buffer and stored at -20°C.

Nested PCR procedure: For the primary PCR (expected amplicon size: 1325 bp), primers were used as described by Xiao et al. (2004): 18 SF: 5'-TTCTAGAGCTATACCTTGCG-3' (forward) and 18 SR: 5'-CCCATTTCC TTGAAAACAGGA-3' (reverse). For the secondary PCR (expected amplicon size: 818-825 bp, depending on the species), the following primers were used: 18 SNF: 5'-GGAATGTGGTGTATTAGATACAG-3' (forward) and 18 SNR 5'-AAGGAGTAAAGGATAATGCAA-3' (reverse). The primary Polymerase Chain Reaction (PCR) contained: 1 µL (50 pmol) of each primer (18SF and 18SR), 28 µL Taq PCR Master Mix (Qiagen Co. Cat. no. 201443) and 3 µL of DNA sample and milliQ water up to a total volume of 50 mL. The conditions for the secondary PCR were the same as for the primary PCR. Both procedures were performed in a thermocycler (PTC-100 Thermal Cycler, MJ Research, Incline Village, USA) starting with initial denaturation of 95°C/2 min with the following cycles: 35 cycles of 94°C/45 sec, 62°C/45 sec, 72°C/1 min², with a final extension of 72°C/10 min. PCR products were separated on 1.2% agarose gels and visualized after ethidium bromide staining.

DNA sequencing: PCR products were sequenced in a commercial sequencing laboratory (Fermentas GMBH, Germany). The sequences obtained were submitted to BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) in order to identify their similarity with sequences of the GenBank. Sequence alignment was performed using CLUSTAL W (Thompson et al., 1994) with manual adjustments.

RESULTS

Morphology and measurements of Cryptosporidium oocysts: The detected Cryptosporidium oocysts in the examined calve feces stained with Ziehl-Nelson technique were morphologically
Fig. 1: *Cryptosporidium parvum* oocysts in calve fecal smears stained with Modified Ziehl-Neelsen stain (MZN) (X100)

Table 1: Dimensions of *Cryptosporidium parvum* oocysts detected in calve fecal samples

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Dimensions of the <em>Cryptosporidium parvum</em> oocysts (µm)</th>
</tr>
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<tbody>
<tr>
<td>Length/width</td>
<td>(4.4-5.8)×(4.3-4.9)</td>
</tr>
<tr>
<td>Mean</td>
<td>5.1×4.6</td>
</tr>
<tr>
<td>Shape Index (SI) 1/w</td>
<td>1.0-1.2 (1.1)</td>
</tr>
</tbody>
</table>

Table 2: Prevalence of *Cryptosporidium parvum* infection among calves

<table>
<thead>
<tr>
<th>Age</th>
<th>Examined No.</th>
<th>Positive No.</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 1 month</td>
<td>125</td>
<td>77</td>
<td>61.6</td>
</tr>
<tr>
<td>1-2 month calves</td>
<td>55</td>
<td>21</td>
<td>38.2</td>
</tr>
<tr>
<td>Total calves</td>
<td>180</td>
<td>98</td>
<td>54.4</td>
</tr>
</tbody>
</table>

Similar to *Cryptosporidium parvum* oocysts which characterized by spherical to ovoid shape with smooth wall and appeared as acid fast (red-pink) on green back. The measurements of 50 oocysts were varied from 4.4-5.8×4.3-4.9 µm of mean (5.1×4.6) and the shape index was 1.0-1.2 of mean (1.1) (Fig. 1 and Table 1).

**Prevalence of Cryptosporidium oocysts:** The extent of prevalence was 54.4% (98 out of 180) of total diarrhoeic fecal calve samples were found to be infected with *Cryptosporidium parvum* oocysts. The older (1-2 month) calves revealed lower prevalence 38.2% (21 out of 55) while the younger (less than 1 month) calves showed higher *Cryptosporidium* infection 61.6% (77 out of 125) (Table 2).

**Nested PCR analysis:** Using DNA extracted directly with simple modification from *Cryptosporidium* oocysts in nested PCR. This technique showed high sensitivity and specificity in the diagnosis of *Cryptosporidium* spp., in ruminants. The nested PCR gave the expected 1st (1325 bp) and the 2nd (825 bp) PCR products from all the examined Egyptian isolates (Fig. 2).
DNA sequencing: The nucleotide sequence alignments and BLAST search analysis of the Egyptian isolates of Cryptosporidium in GenBank revealed 100% homology between the 825 bp amplified fragment of Egyptian isolates and the counterpart of the 18S rDNA sequences of Cryptosporidium parvum. Homology search proved that all the 10 isolates were Cryptosporidium parvum.

DISCUSSION

The Cryptosporidium oocysts detected in calve fecal smears in this study are morphologically similar to those of Cryptosporidium parvum described in calves in many previous studies (Fall et al., 2003; Fayer et al., 2003; Quiles et al., 2008). Identification of the Cryptosporidium species was depending upon the conventional criteria, such as oocyst morphology and measurements, this opinion was agreed with Fayer et al. (2000) and Morgan-Ryan et al. (2002), who cited that morphometric measurement of oocysts represents the cornerstone of cryptosporidium taxonomy and is one of the requirements for establishing a new species, however, is not adequate by itself and should using multiple parameters as electron microscopy, developmental biology, host specificity, histopathology and/or molecular biology.

In the present study, 98 out of 180 (54.4%) of the total investigated calves were confirmed by acid fast stain to be infected with Cryptosporidium. These finding was higher than (21.7 and 30.2%) detected by Amer et al. (2010) and El-Khodery and Osman (2008) in Egyptian buffalo calves and dairy calves, respectively; but lower than (95%) estimated by Ramirez et al. (2004) in US dairy farms; whereas, these results are consistent with (52%) had been reported by Silverlas et al. (2009) in European young calves. Several factors may be responsible for the differences in the prevalence from the present study to those previously surveyed, such as the breed of the calves, husbandry and management system, exac age of the host, nursing conditions of the calves, season of the sample collection as well as the sanitary conditions inside and around the
farms. Some of these factors may act individually or collectively to increase the risk factor associated with transmission and prevalence of Cryptosporidium between calves (Kvac et al., 2006; Duranti et al., 2009).

Concerning age, higher prevalence of cryptosporidiosis in our study (61.6%) was detected in calves less than 1 month however lower prevalence (38.2%) in 1-2 month aged calves. These findings agreed with Fayer et al. (2006) in the United States who demonstrated that only preweaned calves are important sources of zoonotic cryptosporidiosis in humans and also Kvac et al. (2006) and Xiao (2010) reported that, calves less than 2 months of age are the major contributors of zoonotic C. parvum.

It is crucial to differentiate among Cryptosporidium species infecting cattle because only C. parvum is zoonotic type of C. parvum and is known to be infectious to many mammalian hosts worldwide, where C. bovis, C. andersoni and C. ryanae are cattle adapted (Fayer et al., 2006; Santin and Zarlenga, 2009). Using nested PCR and sequences of the rDNA, this study revealed that calves, in Behira Province, Egypt, are only infected with the zoonotic C. parvum assumed to be of the cattle genotype. Such genotype is detected among animals worldwide (Fayer et al., 2000; Hajdusek et al., 2004; Abe et al., 2006). In Egypt, DNA amplification of C. parvum has been identified only in calves (Warda et al., 2002). However, little information on gene sequence of isolates from Cryptosporidium species associated with animal hosts in Egypt has been reported so, extensive studies are extremely important including biological aspects associated with molecular techniques.

Regarding the epidemiology of C. parvum bovine genotype, water-, food-, or air-borne transmission and person-to-person or animal-human contacts are reported routes of infection, with a median dose of 130 oocysts (Fayer et al., 2000). In developing countries, the risk of infection may be higher and the disease probably exerts most of its impact on neonates and infants and raditionally, cattle have been considered the primary non-human species impacted by cryptosporidiosis and the major reservoir of Cryptosporidium for human infections (Hunter et al., 2009). The animal-human contact is considered as the most important transmission route in Egypt, with cattle probably representing the most important source of human and animal infections (Warda et al., 2002).

CONCLUSION

Calves in Egypt carrying the zoonotic C. parvum could represent the important farm animal source of infection to human. The high Cryptosporidium prevalence rates among calves obtained by this study indicate the potential risk of its transmission to humans through contact with infected cattle feces or contaminated milk or food with these oocysts. Further molecular analyses are needed to elucidate the nature of the other unknown genotypes of Cryptosporidium sp., to better understand the source of infection and routes of transmission and ultimately, to improve risk assessment and measures for prevention and control of cryptosporidiosis

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