Molecular Characterization of Bubaline Isolate of Cryptosporidium Species from Egypt

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ABSTRACT
Cryptosporidium an apicomplexan parasite has the ability to induce diarrhea in bovines, goats, pigs, dogs and cats worldwide. In this study, buffalo calves fecal samples were examined after staining their smears with Modified Ziehl-Neelsen Stain (MZN). Ileal sections were examined for the detection of pathological changes. Further molecular characterization was done using nested PCR amplification and partial sequence analysis. The detected oocysts were morphologically similar to Cryptosporidium parvum. Light microscopic examination of Cryptosporidium infected ileal Tissue Section (TS) stained with H and E revealed the presence of altered mucosal architecture with congestion of blood vessels, infiltration, sloughing and complete erosion of epithelial cells and shortening, blunting, stunning and atrophy of the intestinal villi. Molecular characterization gave PCR amplicons of 18S SSU rRNA gene products approximately at 823 bp. Sequences proved specified generalized relatedness with 21 species of Cryptosporidium but the nucleotide homogeneity percentage was insufficient to designate species or genotypes. Further bioinformatics analysis showed that resulting Cryptosporidium isolates had the closest match with three isolates. It was implied that the Cryptosporidium isolates is mostly like Cryptosporidium parvum (JX237832.1) previously isolated from buffaloes in Ismailia province.

Key words: Cryptosporidium parvum, buffalo calves, PCR, pathological changes

INTRODUCTION
Cryptosporidiosis is the clinical disease presenting as a gastro enteritis like syndrome in bovines, goats, pigs, dogs and cats (Fayer et al., 2005; Bhat et al., 2013). It causes greenish yellow mucoid or bloody diarrhea, apathy, lack of appetite, mild fever and dehydration in young calves (Abdel Megeed et al., 2015). In Egypt, Cryptosporidium infection was detected in buffalo calves (19.65%) in El Dakahlia Governorate (El-Dessouky and El-Masry, 2005), Middle Egypt (14.19%) (El-Khodery and Osman, 2008), Ismailia (22.5%) (Shoukry et al., 2009), Cairo, Giza, Beni Suef and Qualiobya (52%) (Morsy et al., 2014). Traditionally, the detection of Cryptosporidium oocysts in environmental, water, food, fecal and/or tissue samples had primarily relied on examination by microscopy (O’Donoghue, 1995; Quintero-Betancourt et al., 2003). Oocyst morphology played an important role in Cryptosporidium taxonomy but was inconvenient to clearly differentiate species and genotypes (Fall et al., 2003). Therefore, molecular analyses had been widely used to
characterize the genetic structure of Cryptosporidium parasites and assessment of their zoonotic significance (Xiao, 2010). Various PCR-based techniques employing specific primer pairs for the selective amplification of different genetic loci followed by sequencing had been used to characterize and classify Cryptosporidium species or genotypes (Quintero-Betancourt et al., 2002; Xiao et al., 2004). Some key markers included ribosomal RNA genes and spacers, the Cryptosporidium oocyst wall protein (cowp), the 70 kDa heat shock protein (hsp70), the thrombospondin-related adhesive protein (trap) genes and the 60 kDa glycoprotein (gp60) gene (Jex et al., 2008). Cryptosporidium frequently affected and strong relation between C. parvum infection and diarrhea among Egyptian buffalo calves (Warda et al., 2002). Cryptosporidium parvum, C. ryanae and C. bovis were identified as 65.7, 11.8 and 4.1%, respectively, with combinations of C. parvum plus C. ryanae (11.2%), C. parvum plus C. bovis (5.3%) and of C. parvum plus C. andersoni (1.8%) in Egyptian buffaloes from Ismailia province (Helmy et al., 2013). Also, it was found that C. parvum was the dominant species in buffaloes and cattle in Ismailia province (65.7%) (Helmy et al., 2015). While, Amer et al. (2013a) found that the prevailing occurrence of C. ryanae and the subtype family IId of C. parvum and the absence of C. bovis and C. andersoni represent some features of Cryptosporidium transmission in water buffaloes in Egypt. The PCR analysis of the gp60 gene was successful for seven C. parvum positive specimens as well as two specimens that were negative in SSU rRNA PCR. DNA sequence analyses of microscopy-positive fecal specimens revealed the presence of four major Cryptosporidium species. In pre-weaned calves, C. parvum was most common (30/69 or 43.5%) but C. ryanae (13/69 or 18.8%), C. bovis (7/69 or 10.2%) and C. andersoni (7/69 or 10.2%) were also present. Mixed infections were seen in 12/69 (17.4%) of genotyped specimens. In contrast, C. andersoni was the dominant species (193/195 or 99%) in post-weaned calves and older animals (Amer et al., 2013b). In buffaloes of different farms at Kafr El Sheikh province, Egypt, PCR-RFLP analyses of small-subunit rRNA genes from positive specimens revealed the occurrence of C. parvum and C. ryanae. Genotypes distribution showed that C. ryanae was the dominant species (60%) followed by C. parvum (40%) in Buffalo calves (Mahfouz et al., 2014). This study was aimed to identify Cryptosporidium species isolated from Egyptian buffalo calves and to study the effect of cryptosporidiosis on intestinal tissues referring to its pathological changes.

MATERIALS AND METHODS
Sample collection: A total number of 571 buffalo calves (age from one day to one year) rectal fecal samples were collected around the year from different Egyptian governorates (Cairo, Giza, Beni Suef, Qualiobya) in a clean labeled container.

Detection of oocysts: Fine fecal smears fixed with methanol spirit and stained with Modified Ziehl-Neelsen Stain (MZN) (Henriksen and Pohlenz, 1981) were examined. The oocysts were measured with the help of stage micrometer conjugated with the light microscope at the eyepiece 10x and the objective 100x. All measurements are in micrometers for about 20-50 oocysts (Fayer and Xiao, 2007).

Histopathological changes: Specimens from different parts of ileum (about 1 cm) were taken from infected buffalo calves for studying histopathological changes. These materials were fixed immediately in 10% formal saline, dehydrated, cleared, embedded in paraffin, sectioned at 4 mm and stained with H and E staining (Drury and Wallington, 1967).
Some infected ileum sections were deparaffinized, hydrated with distilled water and stained with modified-Ziehl-Neelsen (ZN) staining (Sheehan and Hrapchak, 1987). The ileal sections were examined for the detection of pathological changes under microscope.

**Genomic DNA extraction:** Cryptosporidium oocysts were concentrated by flotation using Sheather’s sugar solution (Current et al., 1983). The floated upper third was washed by centrifugation in distilled water for 3 times and suspended in Phosphate Buffered Saline (PBS) (Current and Reese, 1986). The purified oocysts were stored at -20°C in 2.5% potassium dichromate solutions. DNA was extracted from the washed Cryptosporidium oocysts using QIAamp DNA MiniKit (Qiagen Co., USA) with modifications to the manufacturer’s protocols in which, a total of 200 μL of oocysts solution was suspended in 180 μL of ATL buffer and thoroughly mixed by vortexing. Then, subjected to five extra freezing and thawing cycles in liquid nitrogen and a water bath at 65°C as lysis before extraction protocol.

**Nested PCR procedure:** Primers were used as described by Xiao et al. (1999), for the primary PCR (expected amplicon size: 1325 bp): 18 SF: 5’-TTC TAG AGC TAA TAC ATG CG-3’ (forward) and 18 SR: 5’-CCC ATT TCC TTC GAA ACA GGA-3’ (reverse). Each PCR mixture, total volume 100 μL contains 10 μL 10x PCR buffer, 6 mM MgCl2, 200 μM each dNTP, forward and reverse primers at a concentration of 200 nM each, 400 ng μL-1 of non-acetylated BSA, 2.5 U Taq polymerase and 0.5 -3.0 μL DNA template. A total of 35 cycles consisting of; 94°C for 45 sec, 55°C for 45 sec and 72°C for 60 sec make up the PCR program, an initial hot start at 94°C for 3 min and a final extension at 72°C for 7 min were also included. For the nested PCR (expected amplicon size: 819-825 bp, depending on the species) the following primers were used: 18 SNF: 5’-GGA AGG GTT GTA TTT ATT AGA TAA AG-3’ (forward) and 18 SNR 5’-CTC ATA AGG TGC TGA AGG AGTA-3’ (reverse). The reaction mixture was the same as for the primary step with the following exceptions: no BSA was required, increase primer concentration to a 400 nM and the DNA template volume added was 2 μL of primary PCR product. Cycling conditions were identical to the primary PCR except the annealing temperature was increased to 58°C.

**Gel electrophoresis:** Following amplification, PCR products were visualized in a 1% agarose gel stained with ethidium bromide by Molecular Imager (Gel Doc™, BIO RAD).

**Sequencing of PCR amplicons and BLAST of 18S SSU rRNA gene sequences:** All secondary PCR products determined to be Cryptosporidium positive were purified by QIAquick Gel extraction kit (Qiagen Co., USA) and sequenced in both directions in a commercial laboratory (Sigma Scientific Services Co., Egypt). Amplified sequences were compared with reference sequences using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Phylogenetic analysis:** The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length equaling 77.60047543 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown below the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances. Then, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. All
positions containing gaps and missing data eliminated from the dataset (Complete deletion option). There were a total of 560 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

RESULTS

Morphology of the detected Cryptosporidium oocysts: The detected Cryptosporidium oocysts in the examined buffalo calf feces stained with Ziehl-Neelsen technique were morphologically similar to C. parvum oocysts that characterized by spherical to ovoid shape with smooth wall and appeared as acid fast (red-pink) on a green background. The measurements of 50 oocysts were varied from 4.4-5.8×4.3-4.9 μm of mean (5.1×4.6 μm) and the shape index was 1.0-1.2 of mean (1.1) (Fig. 1).

Pathological changes in ileal Tissue Sections (TS): Examination of Cryptosporidium-infected ileal TS stained with H and E revealed the presence of altered mucosal architecture, with congestion of blood vessels, infiltration, sloughing and complete erosion of epithelial cells and shortening, blunting (Fig. 2), stunting and atrophy of the intestinal villi (Fig. 3). Basophilic oval or round organism was found on the surface of villi, free or entering the epithelial cells (Fig. 4 and 5). Oval or round structures oocysts were found also in sections stained with MZN staining technique (Fig. 6).

Identification of Cryptosporidium spp. in buffalo calves

Nested PCR amplicons of 18S SSU rRNA gene products: Molecular characterization was done using nested PCR amplification and partial sequence analysis. The PCR amplicons of 18S SSU rRNA gene products of the Cryptosporidium isolates were about 823 bp. These were used for sequencing process (Fig. 7).

BLAST of Egyptian buffalo isolate 18S SSU rRNA gene sequences with GenBank Database: Sequence compared with reference sequences using BLASTn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was successful to prove specified generalized relatedness with 21 species of Cryptosporidium but the nucleotide homogeneity percentage were insufficient to designate species or genotypes of Cryptosporidium (Fig. 8).

Fig. 1: Cryptosporidium oocysts in stained fecal smears of buffalo calves (MZN X1000)
Fig. 2: Ileal tissue section in Cryptosporidium-infected buffalo calf showing severe hemorrhagic enteritis with congestion of blood vessels (double arrow), blunting of villi (blue arrow) and sloughing and complete erosion of epithelial cells (dashed arrow) (H and E X100)

Fig. 3: Ileal tissue section in Cryptosporidium-infected buffalo calf showing altered mucosal architecture, stunting of villi (green arrows) and Cryptosporidium oocysts (arrow heads) (H and E X400)

Phylogenic tree and evolutionary relationships of Egyptian buffalo isolate with Cryptosporidium species: The evolutionary relationship by bioinformatics analysis and
Fig. 4: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing *Cryptosporidium* oocysts (arrow head) and developmental stages (red arrow) (H and E X1000)

Fig. 5: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing *Cryptosporidium* oocysts (arrow heads) (H and E X1000)

Phylogenetic tree construction showed that resulting *Cryptosporidium* isolates had the closest match with three isolates; *Cryptosporidium* spp. pig genotype II (isolated from pigs in China, GenBank accession number: HQ844733.1), *C. parvum* (Egyptian isolate from buffalo in Ismailia province, GenBank accession number: JX237832.1) and *C. baileyi* (isolated from quails in China, GenBank accession number: EU717805.1) (Fig. 9).
Fig. 6: Ileal tissue section in Cryptosporidium-infected buffalo calf showing altered mucosal architecture with Cryptosporidium oocysts (arrow heads) (MZN X1000)

Fig. 7: PCR amplification products of respective Cryptosporidium isolates: Lane 1: 100 bp DNA ladder, lane 2: Negative control, lanes 3-8: 18S rRNA amplification products. PCR amplicons of 18S SSU rRNA gene products of the Cryptosporidium isolates were about 823 bp
DISCUSSION

Modified Ziehl Neelsen was used in this study for the detection of *Cryptosporidium* oocysts microscopically. This method has been used in many previous studies; Henriksen and Pohlenz (1981), Fathia (1993), Kvac and Vitovec (2003), El-Sherbini and Mohammed (2006), Diaz-Lee et al. (2011) and Bhat et al. (2012). The MZN staining was the most efficient method in the detection of *Cryptosporidium* oocysts, so it was recommended as rapid, easy and less costly method for diagnosis of cryptosPORIDIOsis (Abdel-Rady and Sayed, 2008).

In the present study, light microscopic examination of H and E stained *Cryptosporidium* infected intestinal sections revealed the presence of altered mucosal architecture, with congestion...
of blood vessels, infiltration, sloughing and complete erosion of epithelial cells and shortening, blunting, stunting and atrophy of the intestinal villi. Basophilic oval or round organism was found on the surface of villi, free or entering the epithelial cells. These findings agreed with Fathia (1993), Abu El Ezz et al. (2011), Gaafar (2012), Toulah et al. (2012) and Jin et al. (2015).

The Cryptosporidium oocysts detected in buffalo calf fecal smears in this study were morphologically similar to those of C. parvum described in calves in many previous studies (Fall et al., 2003; Fayer et al., 2006; Hassanain et al., 2011; Randhawa et al., 2012b). Identification of the Cryptosporidium species depended upon the oocyst morphology and measurements (Fayer et al., 2000). Fayer et al. (2000) cited that morphometric measurements of oocysts represented the cornerstone of Cryptosporidium taxonomy and was one of the requirements for establishing a new species, however, it was not adequate by itself and multiple parameters as electron microscopy, developmental biology, host specificity, histopathology and/or molecular biology should be used. However, morphological identification only was insufficient to identify species or genotypes of Cryptosporidium (Egyed et al., 2003; Monis and Thompson, 2003).

In the present study, Cryptosporidium identification based on morphology of oocysts had provided generalized prevalence data for the infection but was insufficient alone to identify species or genotypes of Cryptosporidium. Therefore, molecular analysis had been used to characterize the genetic structure of Cryptosporidium oocysts. The PCR could detect up to single oocyst per sample and could ensure specific diagnosis up to species level coupled with 100% diagnostic sensitivity and specificity (Coupe et al., 2005; Shields et al., 2013).
In accordance, during the present study, 823 bp fragments amplified from the 18S SSU rRNA gene could be noted after nested PCR reaction from buffalo’s feces. Previous studies indicated the usefulness of the small subunit (SSU) ribosomal RNA genes as genetic markers for the specific identification of Cryptosporidium having relatively low intraspecific and relatively high interspecific sequence variation (Fayer et al., 2000; Xiao et al., 2004; Jex et al., 2007). Thus, they had been utilized in systematic (phylogenetic) investigations of Cryptosporidium providing the basis for the current classification of members within the genus (Morgan et al., 1999a; Xiao et al., 2004).

The blast of the sequenced PCR products, amplified from the morphologically characterized oocytes in the present study, on GenBank database was successful to prove specified generalized relatedness with 21 species of Cryptosporidium. But the nucleotide homogeneity percentages were insufficient to designate species or genotypes of Cryptosporidium. However, the evolutionary relationship by bioinformatics analysis and phylogenetic tree construction showed that resulting Cryptosporidium isolates had the closest match with three isolates; Cryptosporidium sp. pig genotype II (isolated from pigs in China), C. parvum (Egyptian isolate from buffalo in Ismailia province) and C. baileyi (isolated from quails in China). Despite that these results disagree with McLauchlin et al. (2000) and others (Insulander et al., 2013; Friesema et al., 2012; Wang et al., 2011) who could identify C. parvum genotypes by 18S rRNA gene sequence, they confirmed the previous conclusion of many researchers who found the use of multi-loci analysis had better results with regards to Cryptosporidium genotyping (Abe and Teramato, 2012; Amer et al., 2010). Because their sequences had higher intraspecific variation than the ribosomal RNA gene regions (Morgan et al., 1999b), it was suggested that other Cryptosporidium genes targets should be used for amplification including the Cryptosporidium oocyst wall protein (COWP), 16S rRNA, Hsp70, Actin, β-Tubulin, gp60, microsatellites, minisatellites and extrachromosomal double-stranded RNA (Xiao et al., 2004; Caccio et al., 2005; Coklin et al., 2007). As well as the Internal Transcribed Spacers (ITS) of ribosomal DNA were useful for the detection of genetic variability within species (Chalmers et al., 2005; Schindler et al., 2005). Synchronized analysis of the obtained morphological simultaneous with molecular criteria of Cryptosporidium buffalo’s oocytes in the present study could prove that the isolates were C. parvum. Theoretically, it was known that C. parvum is infectious to many mammalian hosts worldwide (Fayer et al., 2006; Santin and Zarlenga, 2009). Calves were the major recognized reservoirs for C. parvum (Caccio et al., 2000; Warda et al., 2002; Condoleo et al., 2007; Paul et al., 2008; Helmy et al., 2013; Mahfouz et al., 2014) with strong relation between C. parvum infection and diarrhea among Egyptian buffalo calves (Warda et al., 2002). Cryptosporidium parvum was mostly dominant in preweaned calves (El-Dessouky and El-Masry, 2005; Santin et al., 2008; Keshavarz et al., 2009; Karanis et al., 2010; Randhawa et al., 2012a). Also, it’s known that C. baileyi infected a broad range of birds and found in the small and large intestine, bursa, respiratory tissues such as the conjunctiva, sinus and trachea. Viable C. baileyi oocysts measured 6.2 by 4.6 μm (5.6-6.3 by 4.5-4.8 μm). Oocysts of C. baileyi were inoculated orally into several animals to determine its host specificity. Mice and goats inoculated with C. baileyi oocysts did not become infected (Current et al., 1986; Xiao et al., 2004). Based on these theories and hence the detected Cryptosporidium oocysts in the examined young buffalo calf feces stained with Ziehl-Neelsen technique were morphologically similar to C. parvum oocysts. It was implied that the Cryptosporidium isolates resulted in this study was mostly approaching to the C. parvum (JX237832.1) isolated previously from buffaloes in Ismailia province. In this study, the Cryptosporidium species with unclear identity were observed. Additional studies, using more
genes, with a larger number of isolates from various geographic areas, different husbandry and management systems, covering the item of seasonality should be conducted to identify the species of Cryptosporidium. Little information on gene sequence of isolates from Cryptosporidium species associated with animal hosts in Egypt had been reported so, extensive studies were extremely important including biological aspects associated with molecular techniques (Hassanain et al., 2011). Also, Amer et al. (2010) stated that very little was known about genetic structure of Cryptosporidium spp., in Egypt. Important research gaps remained including lack of subtyping tools for many Cryptosporidium species of public and veterinary health importance, poor understanding of host specificity of Cryptosporidium species and impact of climate change on their transmission (Ryan et al., 2014).

CONCLUSION
In this endeavor the parasitic and pathological studies identified the Cryptosporidium spp. as a causative agent of diarrhea in buffalo calves. While, molecular studies revealed that the similar Cryptosporidium species and C. parvum genotype that was previously isolated in outbreak-associated buffalo calves in Egypt were identified. Sequencing the PCR products obtained from the Egyptian buffalo calf samples may assist in elucidating the Cryptosporidium species C. parvum genotype signature of the amplifiable Cryptosporidium DNA isolated.

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