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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, stunting 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 (*gp*60) 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 *gp*60 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 65ºC 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 $MgCl_2$, 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; 94ºC for 45 sec, 55ºC for 45 sec and 72ºC for 60 sec make up the PCR program, an initial hot start at 94ºC for 3 min and a final extension at 72ºC 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 58ºC.

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).

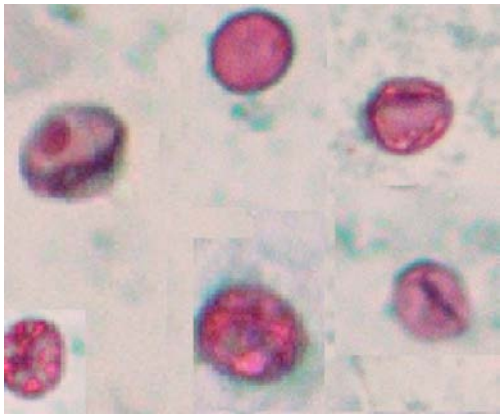

Fig. 1: *Cryptosporidium* oocysts in stained fecal smears of buffalo calves (MZN X1000)

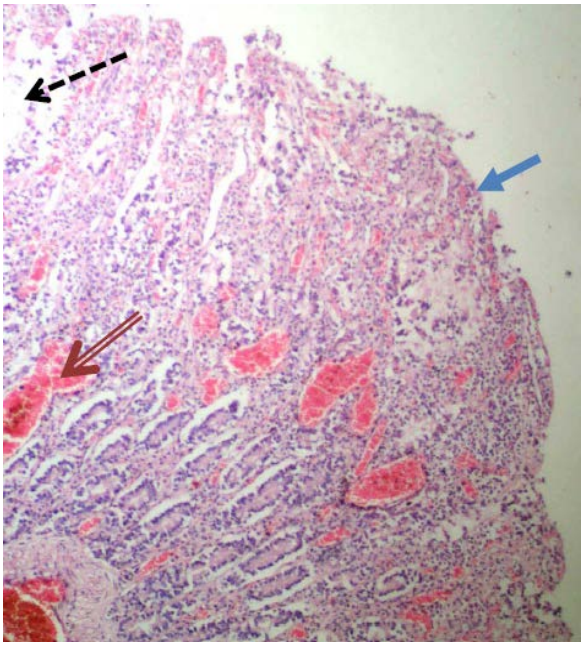

Fig. 2: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing severe hemorrhagic enteritis with congestion of blood vessels (double arrow), blunting of villi (blue arrow) and sloughing and complete erosion of epithelial cells (dashed arrow) (H and E X100)

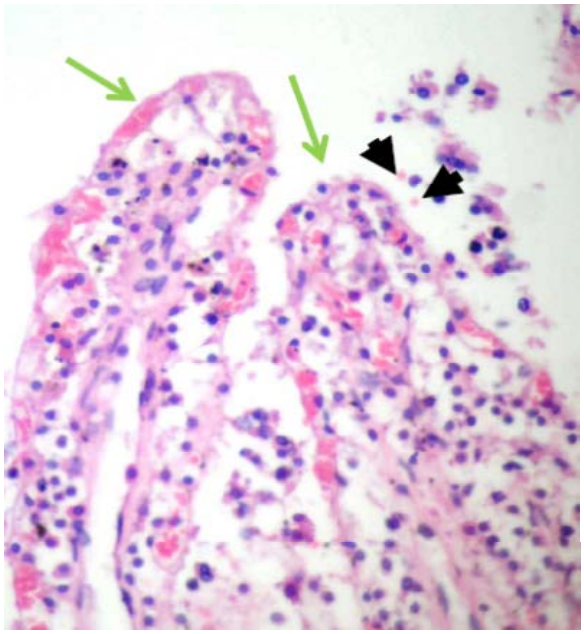

Fig. 3: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing altered mucosal architecture, stunting of villi (green arrows) and *Cryptosporidium* oocysts (arrow heads) (H and E X400)

Phylogenic tree and evolutionary relationships of Egyptian buffalo isolate with *Cryptosporidium* species: The evolutionary relationship by bioinformatics analysis and

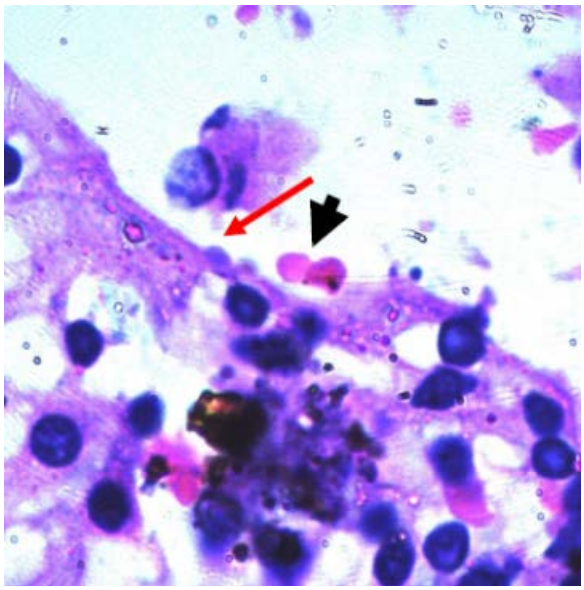

Fig. 4: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing *Cryptosporidium* oocysts (arrow head) and developmental stages (red arrow) (H and E X1000)

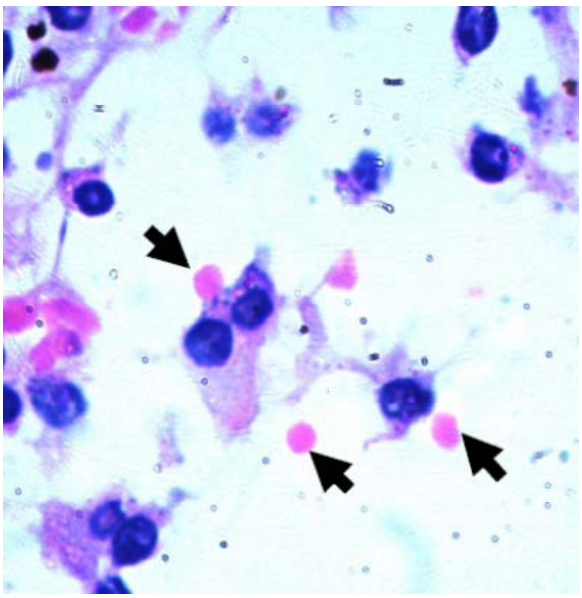

Fig. 5: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing *Cryptosporidium* oocysts (arrow heads) (H and E X1000)

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).

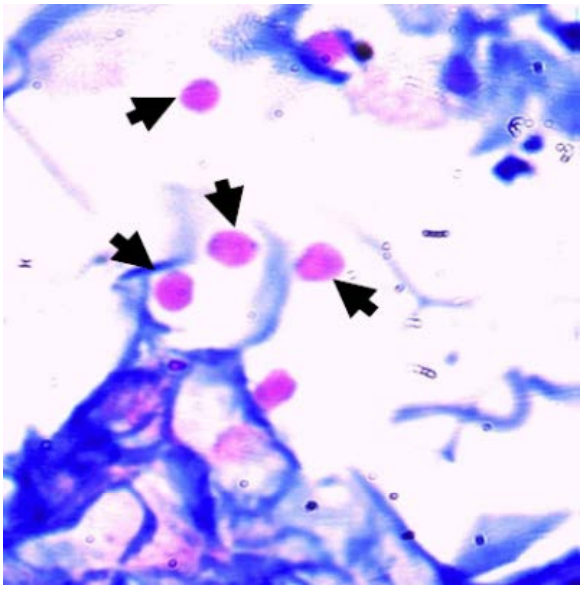

Fig. 6: Ileal tissue section in *Cryptosporidium*-infected buffalo calf showing altered mucosal architecture with *Cryptosporidium* oocysts (arrow heads) (MZN X1000)

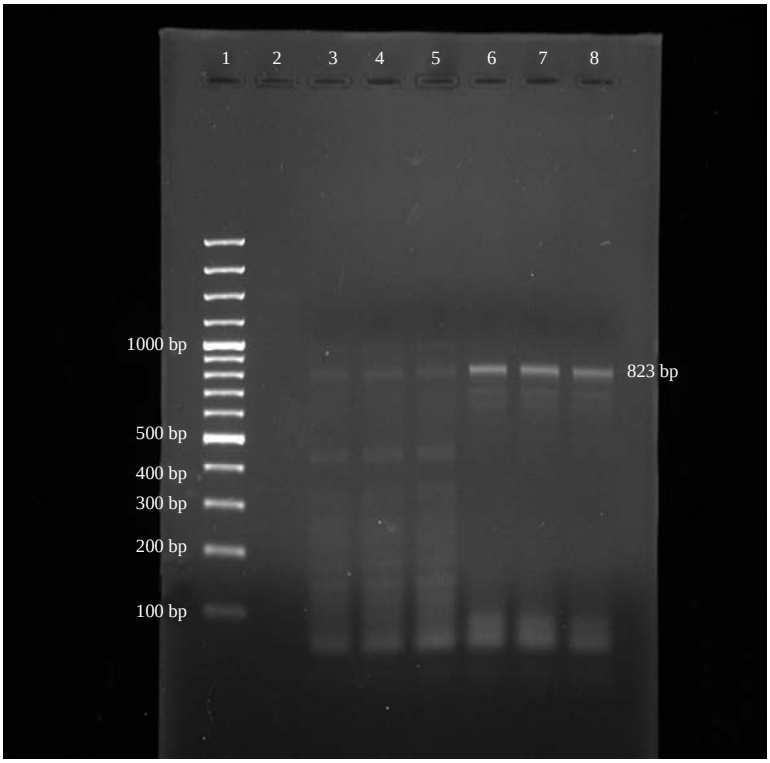

Fig. 7: PCR amplification products of respective *Cryptosporidium* isolates: Lane 1: 100 bp DNA ladder, lane 2: Negative control, lanes 3-8: 18S rRNA amplification products. PCR amplicons of 18S SSU rRNA gene products of the *Cryptosporidium* isolates were about 823 bp

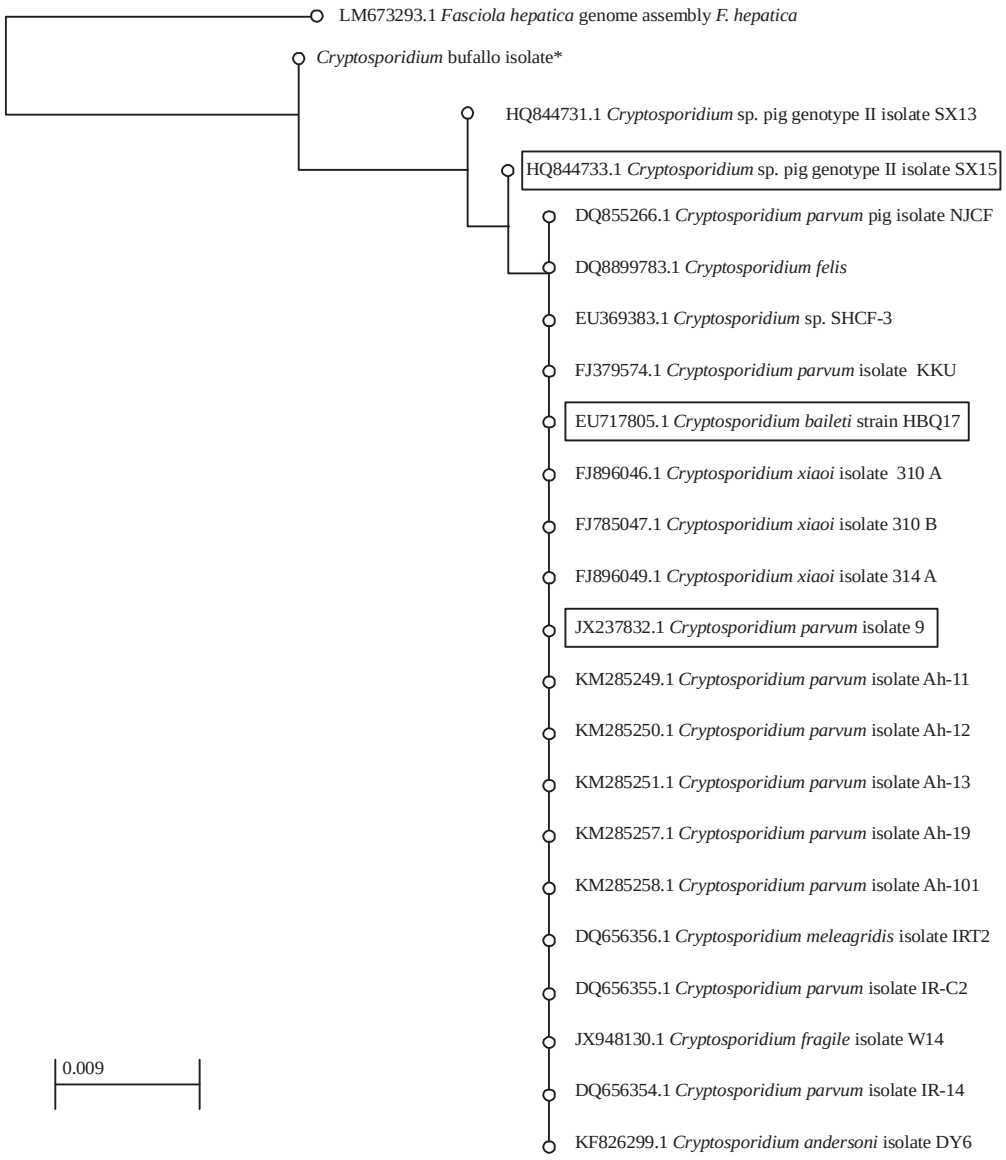

Fig. 8: Phylogenetic tree of sequence with GenBank database using BLASTn program utilizing forward strand sequence, *18S rRNA sequences from the respective *Cryptosporidium* isolates identified

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

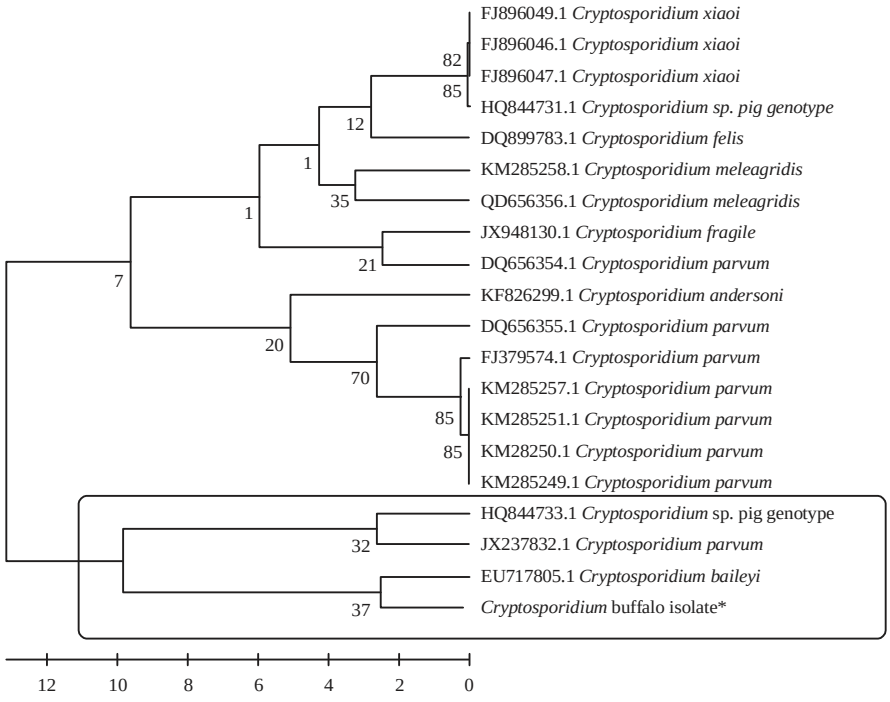

Fig. 9: Phylogenetic tree illustrating the evolutionary relationships of Egyptian buffalo isolate obtained during the present study with 21 *Cryptosporidium* species recorded in the GenBank database, *18S rRNA sequences from the respective *Cryptosporidium* isolates identified

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, *gp*60, 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). *Cryptospordium 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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