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Original article

MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIOSIS IN PRE-WEANED CATTLE CALVES IN EGYPT

N. M. T. ABU EL EZZ[1], F. A. M. KHALIL[1] & K. A. ABD EL-RAZIK[2]

[1]Department of Parasitology and Animal Diseases, [2]Department of Animal Reproduction; National Research Centre, Dokki, Cairo, Egypt

Summary

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The aim of this study was to throw more light on the genetic diversity of *Cryptosporidium parvum* isolates originating from pre-weaned cattle calves in Egypt using multilocus gene analysis. *Cryptosporidium parvum* (*C. parvum*) is a global zoonotic protozoan causing severe acute diarrhoea in humans and different animals. In this study, 172 diarrhoeic faecal samples collected from pre-weaned cattle calves at Giza and Sharkia governorates of Egypt were screened by modified Ziehl-Neelsen acid-fast microscopy for detection of *Cryptosporidium* oocysts. From them, 79 (45.9%) samples were positive for this test. Molecular characterisation using nested PCR showed a high sensitivity and accuracy in the verification of all *C. parvum* isolates. Sequence and phylogenetic analysis of five isolates confirmed three buffalo and two cattle variants of *C. parvum*. Moreover, there was a high homology between present isolates with others from different governorates of Egypt and also with that of Latin America that may be due to the introduction of live animals from these countries to Egypt. In conclusion, this study demonstrates some features of *Cryptosporidium* transmission in cattle in Egypt and addresses the probable role of cattle calves in zoonotic cryptosporidiosis. More consideration should be focused on the role of the imported livestock in the transmission of the disease.

Key words: cattle calves, *Cryptosporidium*, Egypt, genotyping, molecular epidemiology

INTRODUCTION

Genus *Cryptosporidium* members are intracellular protozoa which target the epithelial cells of the digestive and respiratory tract of a broad spectrum of vertebrate hosts producing significant economic damages in livestock (Budu-Amoako *et al.,* 2012). They have received much attention in the last decade as clinically significant zoonotic pathogens (Chalmers & Davies, 2012). Cryptosporidiosis is similarly a vital reason of diarrhoea in young livestock such as calves, lambs, goat kids and piglets (Santin & Trout, 2008). It is also an emerging protozoan disease connected to great waterborne outbreaks (Omoruyi *et al.,* 2011).

Latest studies in many countries reported that cattle were infected at least with four *Cryptosporidium* species or genotypes which displayd a host age-associated susceptibility: *C. parvum* dominated in pre-weaned calves, *C. bovis* and the *Cryptosporidium* deer identical genotype in post-weaned calves and *C. andersoni* in old calves and mature cattle (Fayer *et al.,* 2006), though *C. bovis* and the *Cryptosporidium* deer-identical genotype have been described in all ages (Feng *et al.,* 2007). Moreover, *C. parvum* is the main species involved in diarrhoea in young calves with zoonotic value in cattle (Amer *et al.,* 2010a; Hassanain *et al.,* 2011).

The diagnosis of cryptosporidiosis relies upon clinical signs and recognition of the organism in faeces using direct microscopic investigation of faeces or after acid-fast staining, use of fluorescent-labelled antibodies and ELISA on faecal samples (Cacciò & Pozio, 2006). All these methods are suitable to detect oocysts in clinical samples, but cannot differentiate *Cryptosporidium* species (Smith *et al.,* 2006). Because of this, molecular techniques are implemented to define the genotype and evaluation of pathogen's zoonotic impact (Fayer *et al.,* 2006; Xiao, 2010).

In Egypt, little studies have been devoted to investigating cryptosporidiosis in animals. Shoukry *et al.* (2009) reported the following prevalence rates of *C. parvum* in farm animals in Egypt: 25.9% in goats, 23.7% in cattle, 22.5% in buffaloes and 20.9% in sheep.

Therefore, this study was performed to clarify more detailes associated with the genetic diversity of *Cryptosporidium* isolates originating from pre-weaned cattle calves at Giza and Sharkia governorates, Egypt. Multilocus gene analysis is reported as an outstanding approach for molecular epidemiology of *C. parvum* (Caccio *et al.,* 2005); therefore, it was used in this study.

MATERIALS AND METHODS

Faecal samples collection

A total of 172 diarrhoeic faecal samples were collected from pre-weaned cattle calves at Giza and Sharkia Governorates, Egypt. Each faecal sample was gathered in a labelled clean container, transferred in ice boxes to the laboratory for further processing.

Detection of oocysts

Faecal smears were fixed in methanol and stained with Modified Ziehl-Neelsen stain (MZN) for recognition of *Cryptosporidium* oocysts (Henriksen & Pohlens, 1981). The preparations were observed and the oocysts were measured according to Fayer & Xiao (2008).

Purification of Cryptosporidium oocysts

Cryptosporidium oocysts were refined by Sheather's sucrose flotation method and oocysts were kept at –4 °C in 2.5% potassium dichromate till DNA extraction. Oocysts were washed with 0.025M phosphate-buffered saline (PBS, pH 7.2) and counted in a haemocytometer by means of a phase-contrast microscope.

PCR analysis and DNA sequencing

DNA was extracted from oocysts using the QIAamp DNA Mini Kit (Qiagen, Cat.No.51304) according to the manufacturer's description. This nested PCR used amplifies a 435 bp region of the *Small subunit (SSU) ribosomal RNA* gene that is specific for *Cryptosporidium* species. PCR was performed in 25 µL volume containing 0.5 µL of each primer (10

µM), 0.5 µL dNTP (10 mM) (Finnzymes, Espoo, Finland), 2.5 µL 10× PCR buffer, 1.5 µL $MgCL_2$ (Qiagen GmbH, Hilden, Germany), 0.25 µL HotStart Taq DNA polymerase (5U/µL) and 1 µL BSA (Promega, Madison, WI) according to Nichols *et al.* (2003). The used primers were: P1 5-AAG CTC GTAGTT GGA TTT CTG-3 and P2 5-TAAGGT GCT GAA GGA GTA AGG-3, P3 5-CAA TTG GAG GCC AAG TCT GGT GCC AGC-3, P4 5-CCT TCC TAT GTC TGG ACC TCG TGA GT-3. Primary PCR consisted of initial denaturation at 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 68 °C for 60 s, 72 °C for 30 s, and final extension at 72 °C for 10 min while secondary PCR comprised the same conditions but with annealing at 60 °C for 60 s.

The positive PCR products were then sequenced on an ABI Prism 3100 Genetic Analyzer by using a Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems). The correctness of data was defined by two-directional sequencing with the forward and reverse primers that was used in secondary PCR.

The nucleotide sequences obtained in this study were analysed using the BioEdit 7.25 and ClustalW2 (http://www.clustal.org) programmes. The resultant sequences were aligned with SSU rRNA reference sequences of *Cryptosporidium* spp. using a neighbour-joining analysis of the aligned sequences implemented in the program CLC Sequence Viewer 6.

Nucleotide sequence accession numbers

Five sequences PCR samples used in this study have been placed in the GenBank database three from Giza Governorate under accession numbers KP899812, KP941593, KP941594, and two from Sharkia governorate under accession numbers KP941595 and KP941596.

RESULTS

In the current study, 79 out of 172 (45.9%) investigated pre-weaned diarrhoeic calves were confirmed to be infected with *Cryptosporidium parvum* by acid fast stain (Fig. 1). The *C. parvum* oocysts appeared with length × width 4.4–5.8 × 4.3–4.9 (mean 5.1 × 4.6) and shape index (SI=l/w) of 1.0–1.2 (1:1) with 50–100 oocysts/50 examined fields.

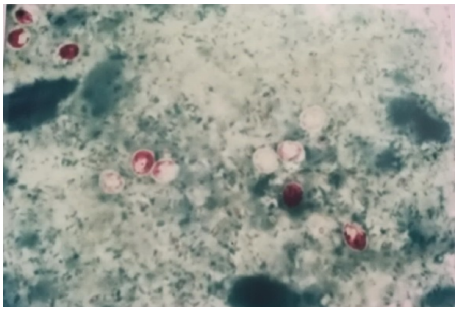

Fig. 1. *Cryptosporidium parvum* oocysts in calves' fecal smears stained with Modified Ziehl-Neelsen stain (×100).

In our study, DNA extracted from *Cryptosporidium* oocysts was used in PCR targeting the SSU ribosomal RNA gene. This technique showed combined great sensitivity and accuracy in the identification of *Cryptosporidium* spp. from cattle calves. The PCR provided the expected product (435 bp) specific for *Cryptosporidium* spp from all samples as shown on Fig. 2.

From our results, clear sequences of the SSU rRNA were obtained from five selected isolates (three from Giza and two from Sharkia governorates). DNA sequencing of current samples approved the identity of *C. parvum* (Fig. 3 and 4). Blasting the gained sequences with those in GenBank database and the phylogenetic analysis (Fig. 3) demonstrated that all the five isolates showed high homology (98–

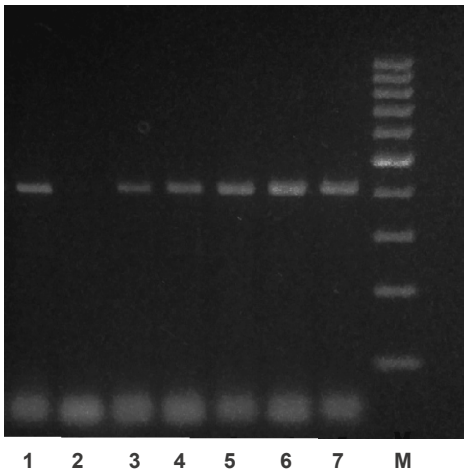

Fig. 2. PCR of *Cryptosporidium* DNA: Lane 1=positive control; Lane 2=negative control; Lanes 3–7, positive PCR products (435 bp) from *Cryptosporidium* spp. oocyst DNA; M=100 bp molecular DNA size markers.

99%) with reference sequence of *C. parvum*. The Neighbor-Joining (NJ) phylogenetic analysis relied on the SSU rRNA (Fig. 3 and 4) and showed that the three sequences of Giza *C. parvum* isolates clustered with relevant sequences of *C. parvum* isolates (JX237832 & JX298601) isolated from buffalo faeces at Ismailia province of Egypt.

Regarding the two Sharkia *C. parvum* isolates, they were clustered with relevant sequences of *C. parvum* Al Karada 1 isolate (AB922118) isolated from cattle faeces at Kafr El-Sheikh governorate of Egypt (Fig. 3 and 4).

In this study, the *Cryptosporidium* isolates originating from pre-weaned cattle calves faeces were recognised as *C. parvum* 'buffalo' genotype for the three Giza isolates and *C. parvum* 'cattle' genotype for the two Sharkia isolates by the sequence analysis of a portion of the SSU rRNA (Fig. 4).

Concerning the geographical distribution (Fig. 5), SSU rRNA gene analysis of our isolates sequenced PCR product with that of the other *C. parvum* isolates from Egypt, Middle East, Europe and Asia showed that all Giza isolates had a high

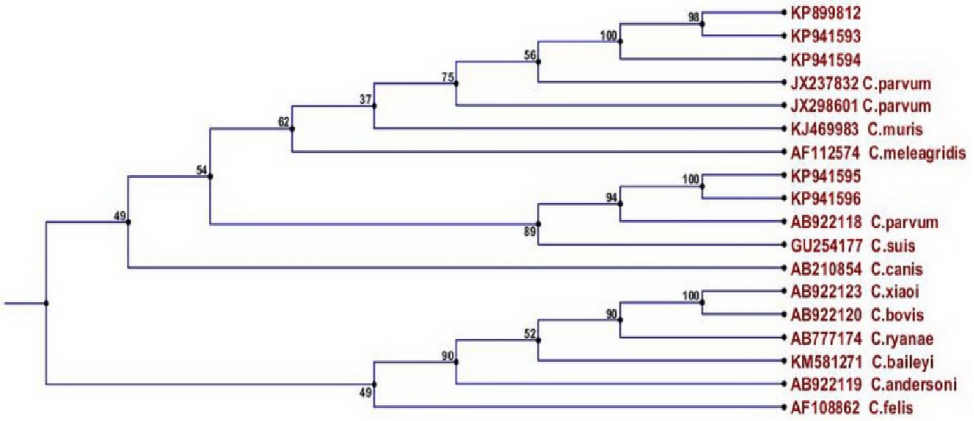

Fig. 3. Phylogenetic tree for partial SSU rRNA sequence of the genus *Cryptosporidium*. *C. parvum* (AB922118), *C. bovis* (AB922120), *C. meleagridis* (AF112574), *C. felis* (AF108862), *C. canis* (AB210854), *C. suis* (GU254177), *C. baileyi* (KM581271), *C. andersoni* (AB922119), *C. xiaoi* (AB922123), and *C. muris* (KJ469983) were obtained from the GenBank database. KP941593, KP941594, KP941595, KP941596 and KP899812 are the *Cryptosporidium* isolates from cattle calves in Egypt.

Fig. 4. Phylogenetic tree for partial SSU rRNA sequence of *Cryptosporidium parvum. C. parvum* cattle (AB922118, AB777179), *C. parvum* buffalo (JX237832, JX298601), *C. parvum* goat (KM199757), *C. parvum* horse (KJ469985), *C. parvum* human (AJ493548), *C. parvum* mouse (AF112571) were obtained from the GenBank database. KP941593, KP941594, KP941595, KP941596 and KP899812 are the *Cryptosporidium* isolates from cattle calves in Egypt.

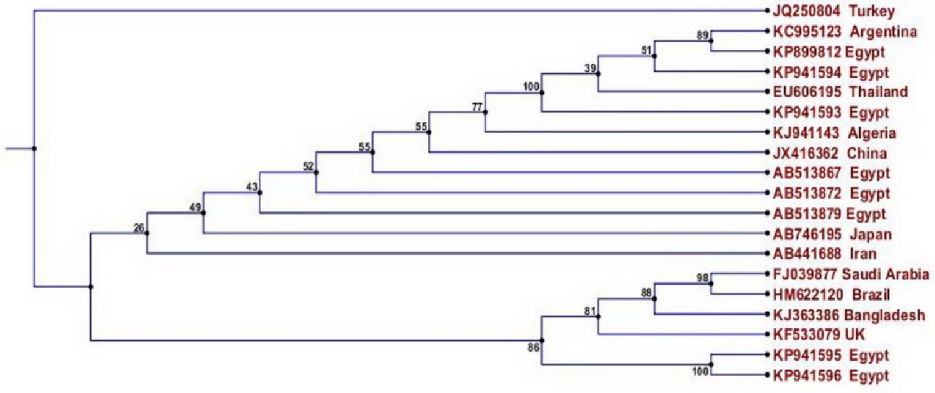

Fig. 5. Phylogram illustrating the genetic relationships among *Cryptosporidium* genotypes. Dendrogram of *C. parvum* genotype clusters (neighbour-joining analysis) corresponding to the three *C.parvum* Egyptian isolates and their groups (Middle East, Africa, Europe, and Asia). KP941593, KP941594, KP941595, KP941596 and KP899812 are the *Cryptosporidium* isolates from cattle calves in Egypt.

homology with cattle calf isolates from Thailand and Argentina while all Sharkia isolates had a high homology with cattle calf isolates from UK, Bangladesh, Saudi Arabia and Brazil.

DISCUSSION

Restricted knowledge on *Cryptosporidium* is available in all developing countries as well as Egypt, where only a few investigations are known (Amer *et al.,* 2013) and data are mostly uneven with concentration on humans cases only. Moreover, there is scarce information about the genomic variation of this protozoan parasite in Egypt (Amer *et al.,* 2010b).

In Egypt, a solid connection between farmers and their animals infected with *C. parvum* was reported, demonstrating a

zoonotic prospective for infection with cryptosporidiosis (El-Khodery & Osman, 2008). Therefore, this study was devoted to clarify more details on genetic diversity of *Cryptosporidium* isolates from pre-weaned cattle calves at Giza and Sharkia governorates, Egypt. Multilocus gene analysis was employed as an outstanding approach for molecular epidemiology of *Cryptosporidium*.

In the current study, 79 out of 172 (45.9%) of the total investigated pre-weaned diarrhoeic calves were confirmed by acid fast stain to be infected with *C. parvum*. This rate was higher than that detected by Mahfouz *et al.* (2014) and Helmy *et al.* (2013) in Egyptian buffalo and cattle calves (6.9 and 36% respectively), but lower than rates (54.4% and 69.4%) reported by Hassanain *et al.* (2011) and Joutes *et al.* (2016) in pre-weaned diarrhoeic calves respectively. Our results are in line with findings in pre-weaned young calves reported by Helmy *et al.* (2015) – 43%. Several issues may be respondible for the variability in the prevalence between the current trial and mentioned previous reports: the calves' breed, age and nursing situations, farming and organisation scheme, period of sample collection, the sanitary situation of the environment inside and outside the farms. These factors may act independently or cooperatively to raise the hazard aspect connected with incidence and transmission of *C. parvum* between calves (Duranti *et al.,* 2009).

Regarding age, currently high prevalence of cryptosporidiosis (45.9%) was detected in pre-weaned calves. This agreed with data of Fayer *et al.* (2006) and Kvac *et al.* (2006) who confirmed that only pre-weaned calves are the key contributors of zoonotic *C. parvum* in humans.

Molecular techniques are implemented to define the pathogenic genotype and to evaluate their zoonotic impact (Xiao, 2010). They use predominantly sequence study of the 18S rRNA gene and *Cryptosporidium* oocyst wall protein (COWP) (Geurden *et al.,* 2006; Swai *et al.,* 2007; Adamu *et al.,* 2010; Amer *et al.,* 2010b), 60-kDa glycoprotein (GP60) gene (Adamu *et al.,* 2010), 70 kDa heat shock protein (HSP-70) , small subunit of ribosomal RNA (SSU rRNA) gene and restriction fragment length polymorphism of the SSU rRNA gene (Amer *et al.,* 2010a; Maikai *et al.,* 2011) .

In our study, DNA extracted from *Cryptosporidium* oocysts was used in PCR targeting the SSU ribosomal RNA gene. This technique showed combined great sensitivity and accuracy in the identification of *Cryptosporidium* spp. from cattle calves and demonstrated the expected product (435 bp) specific for *Cryptosporidium* spp. from all positive samples.

In the present study, the two *C. parvum* isolates from Sharkia were clustered with relevant sequences of *C. parvum* Al Karada 1 isolate (AB922118) isolated from cattle faeces at Kafr El-Sheikh governorate of Egypt (Fig. 3 and 4). This was in agreement with Amer *et al.* (2013) and Mahfouz *et al.* (2014) who reported the predominance of *C. parvum* from cattle calves. On the contrary, Amer *et al.* (2010a) approved the predominance of *C. andersoni.*

It was recently demonstrated that the sequence exploration of the variable region of the SSU rRNA gene was a reliable typing technique for distinguishing species or genotypes of *Cryptosporidium* by exploration of the phylogenetic relations and genetic distances (Xiao *et al.,* 1999; Margaret *et al.,* 2001). In this study, the

Cryptosporidium isolates from pre-weaned cattle calves' faeces were recognised as *C. parvum* 'buffalo' genotype for the three Giza isolates and *C. parvum* 'cattle' genotype for the two Sharkia isolates. Surprisingly, the mouse genotype forms another cluster. This was in contrary to Chen & Huang (2007) who reported the isolation of C. *parvum* 'mouse' genotype as the source of infection to cattle.

Our data were in line with Inpankaew *et al.* (2014) who affirmed that the water buffaloes in Thailand can behave as a significant natural reservoir for transmission to human and other livestock either by direct or indirect way via pollution of food and water.

Concerning the geographical distribution (Fig. 5), all Giza isolates had a high homology with cattle calf isolates from Thailand and Argentina while all Sharkia isolates – with cattle calf isolates from UK, Bangladesh, Saudi Arabia and Brazil. The high homology of the present Egyptian isolates with that those countries may be due to the location of all these countries on the world trade movement i. e. the import of live cattle especially from Latin America (Argentina & Brazil) to Egypt.

In conclusion, additional studies including more extensive sampling from humans and the other livestock from different governorates of Egypt are crucial to determine the prevalence of cryptosporidiosis, relationship of loss of calves with *Cryptosporidium* species and genotypes, and zoonotic spread extent. More attention should be paid to the significance of live farm animals' import as a way of transmission of new species and genotypes to Egypt.

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Correspondence:

Khaled A. Abd EL-Razik, PhD
Department of Animal Reproduction,
National Research Center (NRC),
El-Behouth St., Dokki, Giza,
Cairo, Egypt
e-mail: khaled707@hotmail.com