Bulgarian Journal of Veterinary Medicine (2010), **13**, No 2, 104–110

DETECTION OF CRYPTOSPORIDIAL INFECTION AMONG EGYPTIAN STRAY DOGS BY USING *CRYPTOSPORIDIUM PARVUM* OUTER WALL PROTEIN GENE

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Summary

El-Madawy, R. S., N. O. Khalifa & H. F. Khater, 2010. Detection of cryptosporidial infection among Egyptian stray dogs by using *Cryptosporidium parvum* outer wall protein gene. *Bulg. J. Vet. Med.*, **13**, No 2, 104–110.

Cryptosporidium parvum is a common intestinal parasite which is associated with severe acute diarrhoea in humans and animals. This work aimed to determine *Cryptosporidium* sp. in positive faecal samples by using polymerase chain reaction (PCR). *Cryptosporidium* oocysts were isolated from 10 out of 20 Egyptian stray dogs with diarrhoea. The zoonotic *C. parvum* was found to be present in two isolates which showed successful amplification of a specific DNA fragment at 550 bp with *C. parvum* outer wall protein (COWP) gene amplicon using two specific primers CRY-15 and CRY-9. This is the first investigation on the presence of *C. parvum* among Egyptian stray dogs and it has pointed to the existence of a genotype that may play an important role as a source of human and farm animal cryptosporidiosis.

Key words: *Cryptosporidium parvum*, Egypt, outer wall protein gene, PCR, stray dogs

INTRODUCTION

The representatives of the genus *Cryptosporidium* (Apicomplexa) parasitize the intestinal tract of a great range of vertebrate hosts. In humans and many other mammals, *Cryptosporidium parvum* is recognized as a significant pathogen, primarily as a causative agent of acute and severe diarrhoeal illness (Fayer *et al.*, 2000; Hajdusek *et al.*, 2004; Abe *et al.*, 2006). The lack of effective treatment and the resistance of the organism to routine disinfection procedures are further reasons for the interest in cryptosporidial infections.

Based on the molecular characterization of oocysts, *C. parvum* can be divided into two genetically distinct subpopulations: genotype I (anthroponotic 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 (Patel *et al.*, 1998; Hajdusek *et al.*, 2004; Abe *et al.*, 2006). Although outbreaks of cryptosporidiosis are associated with indirect transmission via contaminated food or public water supplies, the source of infections is thought to be either infected animal faeces or human sewage. Cryptosporidiosis is common among the Egyptian children and may be associated with severe diarrhoea (Youssef

et al., 2008). In addition, there is a strong relation between *C. parvum* infection and diarrhoea among Egyptian buffalo calves (Warda *et al.*, 2002; El-Khodery & Osman, 2008).

In Egypt, the dog population was estimated to be more than 3 million and the ratio of stray to owned dogs is about 15:1 (Samaha, 2008). Several public health problems are raised by stray dogs such as transmission of rabies (Samaha, 2008), leishmaniasis (Morsy *et al.*, 1994) and several intestinal trematodes (El-Gayar, 2007). *Cryptosporidium* oocysts excreted by dogs are morphologically similar to those of *C. parvum* and have therefore been assumed to be zoonotic (Morgan *et al.*, 2000). In Egypt, 47.3% of stray dogs were found to be infected with *Cryptosporidium* spp. (El-Madawy, 2006). In general, all *Cryptosporidium* species are morphologically very similar and have low host specificity. Ooocysts in direct smears could be also easily confused with yeasts and moulds by less experienced investigators. Therefore, identification techniques with higher sensitivity and specificity are needed. That is why molecular techniques have become essential for the correct identification of the species involved in outbreak and epidemiological studies. Genotyping of *Cryptosporidium* isolates allows for the detailed identification of isolates present in various hosts, and helps to elucidate the epidemiology of cryptosporidiosis (Hajdusek *et al.*, 2004; Abe *et al.*, 2006).

The application of polymerase chain reaction (PCR) is particularly useful for *Cryptosporidium* detection since routine culture techniques for the amplification of the organism are lacking. Outer wall protein (COWP) gene is a most useful tool for identification of *Cryptosporidium* isolates (Patel *et al.*, 1998; Giangaspero *et al.*, 2006).

There is no report in the available literature about any *Cryptosporidium* species infecting Egyptian dogs except that of El-Madawy (2006) and genotyping studies are still rare.

The aim of this study was to use PCR to detect the incidence of the most zoonotic *Cryptosporidium* species – *C. parvum*, among the Egyptian population of stray dogs through DNA amplification of C. parvum (COWP) gene.

MATERIALS AND METHODS

All chemicals used for purification of DNA were purchased from Sigma Aldrich Co., Germany. Oligonucleotide primers were synthesized by MWG-Biotech AG, Germany. Deoxyribonucleotide triphosphates (DNTPs) and RNase were obtained from Boehringer Mannheim, Germany. Taq DNA polymerase was obtained from Qiagen, USA. The DNA marker was ΦX174-Hae III digest from MWG-Biotech AG, Germany.

Sampling

Faecal samples were collected in January, 2008, from 20 stray dogs, suffering from diarrhoea aged 2 to 5 months. The dogs were captured to an animal centre at the Faculty of Veterinary Medicine, Moshtohor, Benha University, Egypt. The collected samples were labeled and directed to the laboratory for coprological examination.

Coproparasitological examination

Faecal samples containing *Cryptosporidium* were purified using the centrifugation-floatation technique (Huber *et al.,* 2007) and stained with safranin methylene blue (Baxby *et al.,* 1984) to avoid the misdiagnosis with other *Cryptosporidium*-

like parasites. Positive samples were stored in 2.5% potassium dichromate at 4°C for DNA extraction and subsequent PCR detection.

Oocyst disruption and DNA extraction

The collected materials from dog faeces were washed with saline. Oocyst disruption, extraction, and purification of DNA were performed as previously described by Patel *et al.* (1998). For each sample, 500 µL total volumes was added to 900 µL of 10 M guanidinium thiocyanate in 0.1 M Tris HC1, pH 6.4, plus 35 mM EDTA, (w/v) pH 8.2, Triton X-100 together with 0.3 g Zirconia beads of diameter 0.1 mm. The tube was shaken for 2 min at maximum speed then left at room temperature for 5 min, and centrifuged after that. Size fractionated silica suspension (120 µL) was added to the supernatant (Boom *et al.,*, 1990) followed by incubation at room temperature for 10 min with gentle agitation. The supernatant was discarded and the pellet was washed several times – twice with 300 µL of 10 M guanidinium thiocyanate in 0.1 M Tris-HCl, pH 6.4; twice with 300 µL ice cold 80% ethanol; and once with 300 µL acetone. The pellet was then dried at 56 °C for 5 min. The DNA was eluted into 100 µL of water after vortex mixing and incubated at 56 °C for 5 min. The supernatant (DNA samples) was recovered by centrifugation. RNA content was digested by RNase and used directly for PCR amplification or stored at –20°C before analysis.

PCR Detection

Polymerase chain reaction amplification for COWP genes was performed in 50 µL total volume reaction. Polymerase chain reaction of the COWP gene was performed using the primer pair CRY-15 and CRY-9 with application of various reagents: 1× PCR buffer, 1.5 mM $MgC1_2$; 0.l mM DNTP mix, 100 pmol µM primer CRY-15; 100 pmol µM primer CRY-9, and 1.25 units Taq polymerase. The tubes were subjected to the first denaturation step at 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 2 min, 72°C for 2 min, and final extension for 10 min at 72 °C.

Amplification of 550 bp fragment of COWP had been done by the forward primer CRY-15 (5'- GTA GAT AAT GGA AGA GAT TGT G -3') and the reverse primer Cry-9 (5'- GGA CTG AAA TAC AGG CAT TAT CTT G -3'), according to Morgan *et al.* (1998).

Identification of PCR product by agarose gel electrophoresis

A 10 µL aliquot of the PCR products for COWP gene fragments was examined following electrophoresis (Horizon, Gibco BRL, USA) in 1% agarose/ethidium bromide gels. The gels were visualized using UV transilluminator. The size of DNA fragments was determined from the DNA marker using Gel Pro 3.1 Software computing programme (Media Co., USA).

RESULTS

Direct examination revealed that cryptosporidial oocysts were found in 50% dogs (10/20) and had spherical or slightly ovoid bodies with smooth, thick colorless walls, containing four elongated, naked sporozoites and a cytoplasmic residual body. The tiny oocysts were 4.5–5 µm × 4.7–5.5 µm in diameter. The oocysts stained with safranin methylene blue appeared as rounded or slightly ovoid orange bodies against a blue background (Fig. 1).

PCR amplification of DNA of ten positive faecal samples showed that two of

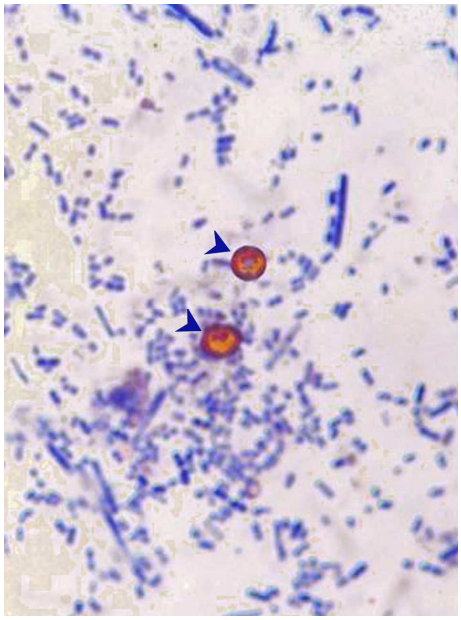

Fig. 1. *Cryptosporidium* oocysts found in the faecal samples of Egyptian stray dogs. Oocysts (arrowheads) are rounded or slightly ovoid orange bodies against a blue background containing four elongated, naked sporozoites and a cytoplasmic residual body. Safranin methylene blue staining.

the tested samples had a successful amplification of the target sequence. The other samples failed to give any amplicon compatible with the positive control, which is identical to *C. parvum* sample and to show a specific DNA fragment at 550 bp with COWP gene amplicon using the CRY-15/CRY-9 primer pair (Fig. 2).

DISCUSSION

Cryptosporidium oocyst is an essential stage of the parasite's life cycle and of primary importance for its dispersal, survival, and infectivity. Oocysts excreted by stray dogs are morphologically similar to those of *C. parvum* and *C. canis* described by Fall *et al.* (2003). No oocysts with less than 4.5 µm in length or more than 5.5 µm in diameter were detected in this study.

The cattle genotype of *Cryptosporidium* is commonly recognized as a zoonotic type of *C. parvum* and is known to be infectious to many mammalian hosts worldwide (Patel *et al.,* 1998; Fayer *et al.,* 2000; Hajdusek *et al.,* 2004).

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). 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).

Using PCR, this study revealed that stray dogs, which are common in Egypt, are infected with the zoonotic *C. parvum* assumed to be of the cattle genotype. Such genotype is detected among dogs worldwide (Fayer *et al.,* 2001; Hajdusek *et al.,* 2004; Abe *et al.,* 2006).

Dogs are infected with *Cryptosporidium canis,* formerly *C. parvum* dog genotype (Fayer *et al.,* 2001; Giangaspero *et al.,* 2006; Huber *et al.,* 2007; Xiao *et al.,* 2007) which have been found in human beings (Xiao *et al.,* 2007). *C. canis* oocysts from both dogs and humans are infectious for calves (Fayer *et al.,* 2001).

C. canis oocysts differed markedly at the molecular level from all known *Cryptosporidium* species based on sequence data from the 18S rDNA and HSP 70 gene. The differences in genetics and host specificity clearly differentiate *C. canis* as new species (Fayer *et al.,* 2001).

In Egypt, DNA amplification of *C. parvum* has been identified only in calves (Warda *et al.,* 2002). However, no infor-

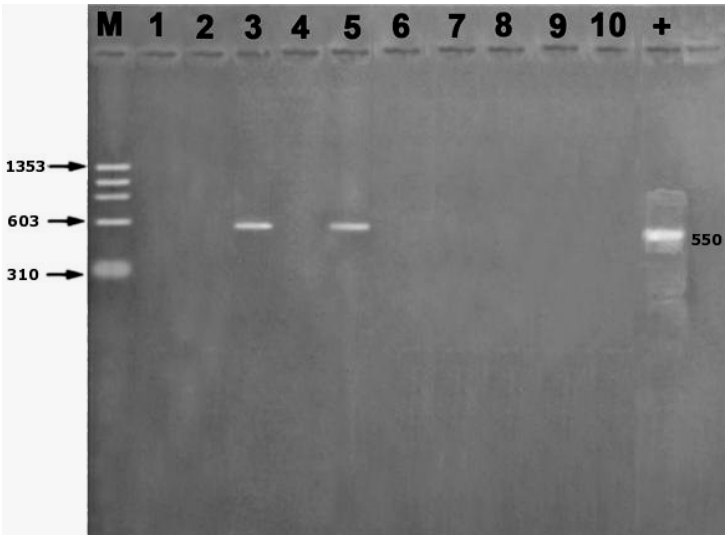

Fig. 2. PCR amplification product from *Cryptosporidium* species isolated from faecal matter of stray dogs tested with CRY-15 and CRY-9 primers. Lane M: molecular DNA size markers; lanes 1, 2, 4, 6–10: negative *Cryptosporidium* sp. isolates; lanes 3, 5: positive *C. parvum* isolates, lane +: specific 550 bp positive control *C. parvum* sample.

mation on gene sequence of isolates from *Cryptosporidium* species associated with animal hosts in Egypt has been reported. Extensive studies are extremely important including biological aspects associated with molecular techniques.

In addition to infection with *C. parvum* cattle genotype and *C. canis*, dogs are infected with *Cryptosporidium meleagridis*, the primary parasite of turkeys, which has been isolated from humans (Hajdusek *et al.,* 2004). Consequently, the negative samples in this study are probably for *C. canis* or *C. meleagridis* but this assumption needs further investigation.

CONCLUSION

Stray dogs in both urban and rural environments in Egypt carrying the zoonotic *C. parvum* could represent the missing link between human and farm animal cryptosporidiosis. The utilization of modern techniques as polymerase chain reaction is preferred because of their speed, specificity and sensitivity for detecting a number of pathogens including *Cryptosporidium*.

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.

ACKNOWLEDGMENT

The authors would like to thank Prof. Dr. Magdy Ghoneem, The Biotechnology Center, Faculty of Veterinary Medicine, Cairo University, Egypt; Prof Dr. Mohamed Hafez, Plant Pathology Department, Faculty of Agriculture, Benha University, Egypt, for their

support, and BJVM referees for helpful comments on the manuscript.

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Paper received 06.11.2009; accepted for publication 26.03.2010

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