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

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Summary


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) parasite 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. Oocysts 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 discarded 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 room temperature 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 x 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 gents: 1× PCR buffer, 1.5 mM MgCl2; 0.1 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.

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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-
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