Identification of *Cryptosporidium species* infecting camels *(Camelus dromedarius)* in Egypt.

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Abstract: Cryptosporidium sp was investigated among 145 camels (5-8 years old) from Egypt. The prevalence of infection was 19.3%. The detected oocysts were ellipsoidal in shape with a mean length and width 7.5 × 5.6 um. Ten Cryptosporidium free mice were orally inoculated each with 350.000 oocysts (camel isolate). The prepatent period in mice was 2 days and the patent period could not be determined since they were still shedding oocysts until day 100 post- infection. The camel isolate of Cryptosporidium and the same isolate propagated in mice was non infective for lambs during an examination period of 3 months. Molecular characterization of the camel isolate indicated that the target gene (18SrRNA) gave positive result for C. muris at 435bp.
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Introduction

The genus *Cryptosporidium* includes a group of protozoan parasites that infect the gastrointestinal tract and other organs of mammals including human, birds, reptiles and fish (Xiao et al., 1999).

The emergency of *Cryptosporidium* as important cause of diarrheal illness and its increasing role in both localized and widespread outbreaks of disease initiated a public and animal health problems of global proportion for both developed and developing countries (Fayer and Xiao,2007).

Identification of *Cryptosporidium* species based on morphology and dimensions of oocysts is difficult since oocysts of many species lack unique features and are indistinguishable from each other. The life cycle of cryptosporidia and its variations can provide species specific information but this is also impractical. More recently gene sequence information has become the most widely applicable factor for defining *Cryptosporidium* species**.** Such genetic data were based primarily on slight differences in the sequence base pair within the gene referred to 18s or small subunit ribosomal (ssr) RNA. Accordingly, 16 species of *Cryptosporidium* were defined as valid species (Fayer and Xiao, 2007).

Previous studies on camel cryptosporidia were scanty. Molecular characterization of camel *(Camelus bactrianus) Cryptosporidium* sp**.** revealed *C. muris* in one study (Xiao et al., 1999) and *C. andersoni* in another one **(**cited by Santin et al., 2007**).** Cryptosporidia were reported from camels *(Camelus dromedarius)* in Egypt without identification of the species (El-Kelesh et al., 2009).

Therefore, this investigation was initiated to study the prevalence and molecular identification of *Cryptosporidium* spp. infecting camels *(Camelus dromedarius)* from Egypt. The susceptibility of mice and lambs for infection with the isolated *Cryptosporidium* sp. was also investigated

Materials and Methods

1-Collection of fecal samples:

Fecal samples were collected from 145 camels of various ages (5-8 years old) and sexes at El-Basatein (Cairo) and El- Warak (Giza) abattoirs (Egypt).Fecal samples were examined after preparation of smears stained with modified Zeihl Nelseen stain. Positive fecal samples were preserved in 2.5% potassium dichromate at 4ºC until used for isolation of *Cryptosporidium* sp.

2-Oocysts concentration and experimental inoculation of mice with *Cryptosporidium* spp:

The oocysts were concentrated in order to be used for animal inoculation and molecular study. Ten, one week old *Cryptosporidium* free mice (*Mus musculus*) (10 gm weight) were purchased from private farm and each was inoculated orally with 350.000 oocysts (camel isolate). The faeces of the inoculated mice were collected and examined daily for the presence of *Cryptosporidium* oocysts for a period of 100 days post inoculation.

3-Inoculation of lambs with *Cryptosporidium* sp. oocysts:

Three, four months old lambs were reared individually and conventionally in isolated pens during the period of the experiment (100 days) and fed sterilized and balanced ration. Two lambs were used for experimental inoculation with *Cryptosporidium* sp. while the 3rd lamb was used as non-infected control. One of the experimental lambs was inoculated orally with 10^6 oocyst (camel isolate) while the 2nd lamb received the same dose of *Cryptosporidium* sp. oocyst

of camel isolate propagated in mice. Faeces of the experimentally inoculated lambs were collected and examined daily for the presence of *Cryptosporidium* oocyst and for studying the prepatent and patent periods of infection.

4-Preparation of oocyst lysates as PCR templates:

For DNA extraction of *Cryptosporidium* sp. (camel isolate) was concentrated from faecal material of positive cases. They were washed four times by successive pelleting (10.000 × g for 10 min at 4°C) and resuspension in distilled water and finally suspended in 10 mM Tris (pH 8.3)-50 mM KCl. Purified oocysts were suspended at a density of 250 oocysts/µl in 100-µl aliquots of 10 mM Tris (pH 8.3)-50 mM KCl containing 0.5% (wt/vol) Tween 20. After freeze-thawing (15 cycles), samples were heated for 15 min at 100°C and then centrifuged for 2 min at 16.000 × g to remove particulate matter. Supernatants were recovered and stored at -20°C until used for PCR amplification (Gobet et al., 1997).

5-PCR amplification and gel analysis of PCR products:

One-microliter volumes of the oocyst lysates were used as amplification templates in 50-µl reaction mixtures containing 75 mM Tris (pH 9); 20 mM $(NH_4)_2SO_4$; 0.01% (wt/vol) Tween 20; 0.2 mM each dGTP, dATP, dCTP, and dTTP; 2 to 4 mM $MgCl_2$ (Table 1); 50 pM each primer; and 1 to 2 U of Gold Star *Taq* DNA polymerase (Eurogentec). Reaction mixtures were overlaid with 50 µl of sterile mineral oil and subjected to denaturation, thermal cycling (Minicycler; MJ Research), and then a final elongation at 72°C. The conditions of denaturation, annealing, and elongation varied depending on the primers of the target genes (18 SrRNA gene and Hsp70 gene). PCR products were analyzed on horizontal agarose gels in TAE buffer (40 mM Tris acetate, 2 mM $Na_2EDTA\ 2H_2O$). Each amplification run included a negative control (PCR water).

Results

1-prevalence of the *Cryptosporidium* sp. in camels**:**

Examination of stained fecal smears from 145 camels revealed that 28 camels (19.3%) were positive for *Cryptosporidium* sp. The oocysts were ellipsoidal in shape with a mean dimensions 7.5 × 5.6 um.

2-Results of experimental inoculation of mice with *Cryptosporidium sp.* (camel strain):

Daily examination of stained fecal smears from experimentally inoculated 10 mice with *Cryptosporidium* sp. (camel isolate) revealed that the prepatent period was 2 DPI. The mice excreted *Cryptosporidium* oocysts daily until 100 days post-infection (termination of the experiment). The mean dimensions of 50 *Cryptosporidium* sp. oocysts excreted by mice were 6.9 × 5 µm.

3-Results of experimental inoculation of lambs with *Cryptosporidium* sp. (camel isolate)

Daily examination of stained fecal smears from experimentally inoculated lambs with *Cryptosporidium* sp. (camel isolate) and the same isolate propagated in mice revealed negative finding during the examination period (100 days).

4-Results of PCR:

PCR gave the same-sized fragment of *C. muris* (435 bp). Primer pairs targeting the 18S rRNA gene gave positive result for *C. muris*. While the PCR showed negative result to *C. parvum* using primer pairs targeting Hsp70 gene (Fig.1).

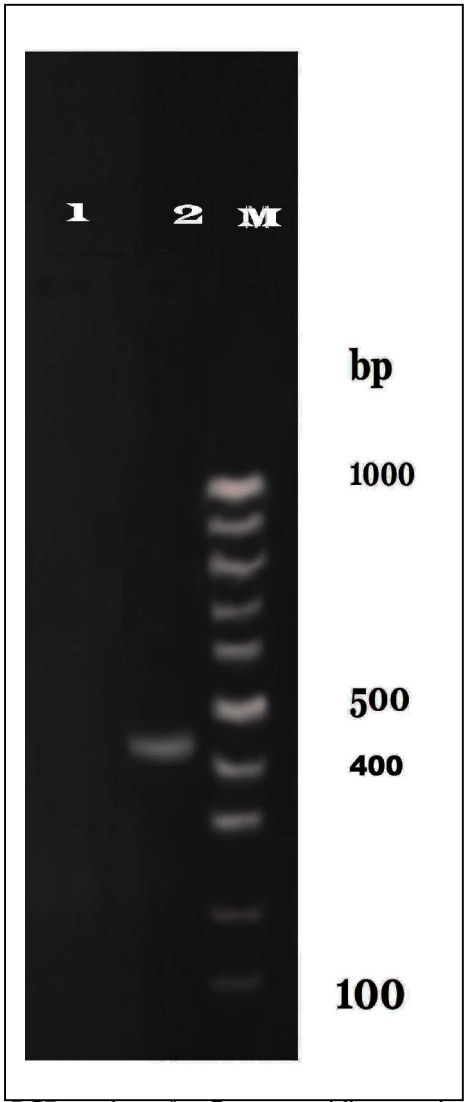

Fig. (1): PCR products for *Cryptosporidium muris*. Lane1: negative control sample. Lane2: positive camel sample for *C. muris*. (435bp) and lane M: 100 bp marker

Discussion

Examinations of stained faecal smears from 145 camels revealed 19.3% infection rate with *Cryptosporidium* sp. El Kelesh et al. 2009 reported 17.5% out of 80 camels were positive to *Cryptosporidium* spp. Alves et al. (2005) in Portugal found that, no positive cases were detected in examined camels.

In our study the camel isolate of *Cryptosporidium* sp. was infective to mice. Xiao, et al., 1999 reported that *C. muris* isolated from rodents, bactrian camel (*Camelus bactrianus*) and rock hyrax *(Procavia capensis*) were infectious to mice and *Cryptosporidium* oocysts from cattle were not infective to mice.

The dimensions of our camel isolate of *Cryptosporidium* were comparable with *C. muris* described by Upton and Current (1985) (7.4x 5.6 um). Also, Fayer and Xiao, 2007 reported that both *C. muris* and *C. parvum* were established based on differences in oocysts morphology and infection sites.

In this study PCR gave the same-sized fragment of *C. muris* (435 bp) and primer pairs targeting the 18S rRNA gene gave positive results to *C. muris*. While the PCR showed negative results for *C. parvum* using primer pairs targeting Hsp70 gene. *C. muris* was detected from camel (*Camelus bactrianus*) and rock hyrax (*Procavia capensis)* at 448 bp and from cattle at 485 bp using nested PCR (RFLP) (Xiao et al., 1999). Also, Bornary-Llinares et al., 1999 detected that the *C. felis* specific fragment was 455 bp when compared with *C. muris* specific diagnostic band (431pb). Finally Champliaud et al., 1998 declared that the group which included *C. muris* and *C. baileyi* gave positive results with the two primer pairs targeting the 18S rRNA gene **(**Johnson, 1995 (435 bp)).

We could conclude from our morphological, biological and molecular study that the detected *Cryptosporidium* sp. oocysts from camels (*Camelus dromedarius*) were *C. muris.*

Table 1: Target genes and primers for detection of *Cryptosporidium* DNA

Cryptosporidium Spp.	Target genes	Primer pair	Fragment size (bp)
1-*C. muris* (Johnson et al., 1995).	1-18 SrRNA gene	5'-AAGCTCGTAGTTGGATTTCTG	435
2-C.parvum (Rochelle et al., 1997).	2-Hsp70 gene	5'-AAATGGTGAGCAATCCTCTG	361

bp: base pair

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