Diagnostic biomarkers in murine *Cryptosporidiosis*: dose- and age-related infection

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Abstract Increasing prevalence of *Cryptosporidium* raises the importance to explore different aspects of its infection. In the absence of reproducible in vitro culturing, animal model is the only experimental method to study *Cryptosporidium*. Our study evaluated *Cryptosporidium* infection using coproscopy, copro-antigen and copro-DNA for early detection of murine cryptosporidiosis. Hundred and forty albino mice (neonates and adult) were divided into two groups, control group received sterile PBS solution, and infected groups were inoculated with molecularly characterized *Cryptosporidium parvum* oocysts and further subdivided into three subgroups for infectious dose response detection. Mice fecal samples were collected every 4 h on the first day and then daily and examined for fecal oocysts, copro-antigen and copro-DNA. Four mice from each subgroup were killed at 12, 24 and 48 h post-infection (P-I), and their intestines were examined for cryptosporidial mucosal DNA. *Cryptosporidium* copro-antigen and copro-DNA were detected 4 and 8 h P-I in infected neonatal and adult mice, respectively, and intestinal mucosal DNA was detected after 12 h in both. Microscopy was able to detect oocysts 48 h P-I. Inoculated *C. parvum* oocysts were recovered in feces of infected mice without genotypic changes. Neonate mice showed higher susceptibility for cryptosporidial infection than adults without statistical differences for the given infectious doses. Both copro-immunoassay and copro-nPCR assays

can early detect *Cryptosporidium* infection; however, nPCR was able to identify *Cryptosporidium* species, making nPCR a reliable biomarker for early detection in murine model.

Keywords *Cryptosporidium* · Diagnostic biomarker · Murine · Copro-DNA · Nested PCR · Fecal immunoassay

Introduction

Cryptosporidium species are water-borne, enteric, zoonotic protozoa that infect mammals including man. They are transmitted through fecal contamination (Oyibo et al. 2011). Infection causes self-limited, mild to severe diarrhea for several days among immunocompetent individuals and life-threatening diarrhea, in immunocompromised ones (Tzipori 1998). Asymptomatic infection is common and constitutes a main source of infection for many hosts (Mirza-Qavami and Sadraei 2011). Increasing prevalence of *Cryptosporidium* with no consistent treatment (Smith and Corcoran 2004) raises the importance to explore different aspects of cryptosporidial infection, understand host-parasite relationship with further innovative therapeutic and preventive studies, taking in consideration common factors affecting the infection pattern in immunocompetent hosts as the dose of infection and age of the host. Being susceptible to *Cryptosporidium* oocysts derived from other hosts, mice appear a convenient experimental model for cryptosporidial infection in humans (Current and Garcia 1991; Tarazona et al. 1998). To monitor experimental cryptosporidial infection in murine model, a diagnostic method for early detection of infection is needed. Our study was designed to evaluate the *Cryptosporidium* oocysts, copro-antigen and copro-DNA shedding as

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diagnostic biomarker(s) of choice for early detection of murine *Cryptosporidium* infection in neonate and adult mice.

Materials and methods

Cryptosporidium isolate

Bovine *Cryptosporidium* isolates were obtained from fecal samples of calves attending veterinary clinics in Faculty of Veterinary Medicine, Cairo University. The stool samples were transferred to the Parasitology Department, TBRI to be screened before and after concentrations for the presence of parasites, stained with modified acid-fast (AF) stain to identify *Cryptosporidium* oocysts. *Cryptosporidium parvum* was obtained after molecular characterization of part of fresh frozen fecal samples, as described below.

Preparation of the inoculum for infection of mice

Each positive stool sample was mixed with 10 ml of distilled water, and then filtered through coarse sterile gauze. The homogenate was then centrifuged at $2500\times g$ for 5 min. The supernatant fluid was discarded, and the sediment was washed twice in 1 ml of phosphate buffer saline (PBS), with centrifugation at $13,000\times g$ for 2 min. After repeated washing followed by centrifugation, fecal debris was totally eliminated (Lumb et al., 1993). Cryptosporidial oocysts were preserved in 2.5% potassium dichromate solution and stored at 4 °C until use for infection (Khalifa et al. 2001).

Just before use, cryptosporidial oocysts were washed three times in distilled water to remove the potassium dichromate, centrifuged at $1500\times g$ for 10 min, and the organisms were counted. Suspension containing the required concentration for the infection (10^4 oocysts/ml) was prepared by dilution of the organism in the appropriate amount of distilled water (Gaafar 2007).

Cryptosporidial infection in mice

Two batches of mice were used in this study. A batch of 70 adult male Swiss albino mice of CDI strain about 5 weeks old (a) and another 70 neonate male Swiss albino mice of CDI strain about 3 weeks old (n). Mice were housed in well-ventilated cages with clean wood-chip bedding and provided with pelleted food and water ad libitum. The mice were allowed to adapt to the laboratory environment for 1 week before the experiment, and their stools were examined by direct wet saline smear, and iodine before and after concentrations then stained with AF stain to exclude the presence of parasites. Each batch of mice was divided

into two groups; control, non-infected group (Group Ia & In), received only sterile PBS, and experimental group, *Cryptosporidium*-infected group (Group IIa & IIIn), which were further subdivided into three subgroups according to the dose of inoculated oocysts; GIIa-A & GIIIn-A: infected by intragastric inoculation of 2500 *C. parvum* oocysts per mouse, GIIa-B & GIIIn-B: infected by intragastric inoculation of 5000 *C. parvum* oocysts per mouse and GIIa-C & GIIIn-C: 10^4 infected by intragastric inoculation of *C. parvum* oocysts per mouse using esophageal tube. Experimental infection was done in the laboratory of Parasitology Department, TBRI. The animal study has been conducted with permission of the Ethics Committee of TBRI.

Detection of *Cryptosporidium* infection

Coproscopy and immunoassays were carried out in the Diagnostic and Research Unit of Parasitic Diseases (DRUP), and copro-nPCR assay was held in the Laboratory of Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Faculty of Medicine, Cairo University (Cairo, Egypt).

Samples collection and processing

Fecal samples were obtained from each group/subgroup 4, 8, 12 and 24 h in first day and then daily till the end of the first week post-infection (P-I). Part of each fecal specimen was preserved in formalin saline for parasitological examination, and the rest of the specimen was divided into two aliquots and stored at -20 °C for immunoassays and molecular studies. Four mice from each group/subgroup were killed 12, 24 and 48 h and 1 week P-I, and their intestine were dissected, opened and washed two times in PBS and then their mucosa were scraped, collected and stored at -20 °C for molecular studies.

Parasitological Coproscopy and AF staining

Fecal samples were examined directly by wet mount and after using modified Ritchie's biphasic concentration method (Garcia 2007); the samples were permanently stained using cold Kinyoun's AF stain to detect *Cryptosporidium* oocysts.

Copro-immunoassay

The frozen fecal aliquots were processed for detection of *Cryptosporidium* copro-antigen using RIDASCREEN® *Cryptosporidium* ELISA Kit (R-Biopharm AG, Landwehrstr. 54, D-64293 Darmstadt, Germany) according to manufacturer's instructions.

DNA extraction and nested PCR (nPCR) conditions

Genomic DNA was extracted from frozen stool aliquots using Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan) after thermal shock (five cycles of deep freezing in liquid nitrogen and immediately transferred into water bath 95 °C each for 5 min.), according to manufacturer's instructions with modification in the form of prolongation of incubation in 95 °C for 1 h after 56 °C at 10 min. Also, Genomic DNA was extracted from scraped mucosa using GeneJET Genomic DNA Purification Kit (Fisher Scientific–UK) according to manufacturer's instructions.

Extracted copro and mucosal DNA was amplified by nPCR targeting COWP gene, a fragment of 830 bp was amplified using primer pair; BCOWPF and BCOWPR for the primary PCR and a fragment of 530 bp using primer pair; Cry-15 and Cry-9 for the secondary PCR. The reaction mixture contained 12.5 µl Master Mix (Fisher Scientific–UK), 200 nM from each primer, 0.2 units of Taq polymerase (as an activator) and 3 µl of the template DNA for the primary reaction and 1 µl for the secondary reaction and double distilled water to get a total volume of 25 µl. The cycling conditions were carried out as previously described (Spano et al. 1997; Pedraza-Díaz et al. 2001) with modification in the form of 63 °C annealing temperature for the primary and 54 °C for the secondary PCR. The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining. nRFLP-PCR was done on nPCR products by RsaI enzyme kit (# ER1121–Fermentas UAB, V. Graiciuno 8, LT-02241 Vilnius, Lithuania) according to manufacturer's instructions and the restriction fragments were analyzed by gel electrophoresis in 2% agarose gel, the fragments were visualized by UV light to determine *Cryptosporidium* genotype.

Statistical analysis

Data were coded and entered using the statistical package SPSS version 17 (Chicago, IL, USA) for statistical analysis. Comparisons between groups were done using Chi-square test for qualitative variables, and data were considered statistically significant if *P* values was <0.05.

Results

Cryptosporidium infection in mice

Cryptosporidium oocyst infectious dose response

Regarding the infectious dose response of the three given doses (2500, 5000 and 10⁴ oocysts/mouse), all were

effective in producing cryptosporidial infection in mice with no detectable statistical differences (*P* value >0.05) for the given infectious doses in either the oocyst or antigen and DNA shedding.

Mice age susceptibility to infection

Mice susceptibility to infection with *C. parvum* oocysts was higher in neonatal (3 weeks of age) than adult mice (5 weeks of age). Copro-DNA and copro-antigen detection were as short as 4 h P-I in infected neonatal mice [IIn-A, IIn-B, IIn-C] in comparison with their detection in infected adult mice [IIa-A, IIa-B, IIa-C] being 8 h P-I (Table 1).

Detection of *Cryptosporidium* infection (Table 1)

Microscopy was able to detect *Cryptosporidium* oocysts (the transmissive stage & sexual life cycle form of parasite) after 48 h P-I. Whereas, antigen detection reveals positive results within 4 and 8 h P-I and was overlapped with PCR-based assays which gave the same result in infected neonatal and adult mice, respectively.

Moreover, intestinal mucosal DNA was able to prove cryptosporidial infection from shedded *Cryptosporidium* copro-DNA of asexual life cycle forms in all infected subgroups within 12 h P-I.

Detection of genotypic changes

Inoculated molecularly characterized *C. parvum* oocysts were recovered in feces of infected mice without any genotypic changes (Fig. 1).

Discussion

Mice were selected as model in experimental studies of *Cryptosporidium* infection (Hijawi 2010); they were susceptible to inoculums derived from other hosts with limitations (Tzipori et al. 1980; Moon et al. 1988; Harp et al. 1990; Current and Garcia 1991; Enriquez and Sterling 1991; Harp et al. 1992; Tarazona et al. 1998). However, variations in infection biomarkers shedding profiles are less well established.

The infection outcome in our study was not dose related. However, the relation between the extent of pre-patent period and infection is dose related or not is still questionable (Current and Snyder 1988; Tarazona et al. 1998).

Regarding age-related susceptibility in this study, we revealed higher susceptibility of infection in neonatal than adult mice that may be explained by the age-related maturation of the local secretory IgA immunity (Van Der Heijden et al. 1988), as evidenced by the severe clinical

Table 1 Showing comparison between diagnostic methods used for early detection of cryptosporidial infection in neonatal and adult mice groups

		Neonate mice groups				Adult mice groups			
		In	IIn-A	IIn-B	IIn-C	In	IIn-A	IIn-B	IIn-C
4 h P-I	A-F Stain	–	–	–	–	–	–	–	–
	Copro-Ag	–	+	+	+	–	–	–	–
	Copro-DNA	–	+	+	+	–	–	–	–
8 h P-I	A-F Stain	–	–	–	–	–	–	–	–
	Copro-Ag	–	+	+	+	–	+	+	+
	Copro-DNA	–	+	+	+	–	+	+	+
12 h P-I	A-F Stain	–	–	–	–	–	–	–	–
	Copro-Ag	–	+	+	+	–	+	+	+
	Copro-DNA	–	+	+	+	–	+	+	+
	Mucosal DNA	–	+	+	+	–	+	+	+
24 h P-I	A-F Stain	–	–	–	–	–	–	–	–
	Copro-Ag	–	+	+	+	–	+	+	+
	Copro-DNA	–	+	+	+	–	+	+	+
	Mucosal DNA	–	+	+	+	–	+	+	+
48 h P-I	A-F Stain	–	+	+	+	–	+	+	+
	Copro-Ag	–	+	+	+	–	+	+	+
	Copro-DNA	–	+	+	+	–	+	+	+
	Mucosal DNA	–	+	+	+	–	+	+	+
1 week P-I	A-F Stain	–	+	+	+	–	+	+	+
	Copro-Ag	–	+	+	+	–	+	+	+
	Copro-DNA	–	+	+	+	–	+	+	+
	Mucosal DNA	–	+	+	+	–	+	+	+

Fig. 1 Showing agarose gel electrophoresis for the DNA products. Lane 1: 100 bp DNA molecular weight marker. Lanes 1–3: Products of the nPCR targeting COWP gene of *Cryptosporidium* at 553 bp. Lane 4: Negative control. Lanes 5–7: RFLP products after digestion with *RsaI* endonuclease with *C. parvum* genotype 2 digestion products at 34, 106 and 410 bp (34 band is very small, faint and difficult to see)

course of cryptosporidial infection observed in different hosts with selective IgA depletion including mice (Heyworth 1990; Jacyna et al. 1990; Tarazona et al. 1997).

In the current work, we were able to detect *Cryptosporidium* oocysts 48 h P-I by microscopy. A 2-day pre-patent period in mice (*Mus musculus*) inoculated with cryptosporidial camel isolate was reported (Yu and Lee 2007). On the contrary, *C. parvum* (KKU isolate) oocysts were shed through faeces in neonatal female mice (C57BL/6 J) from day 5 P-I (Abdel-Wahab and Abdel-Maogood 2011). This discrepancy may be attributed to the presence of many factors such as; host-specific reaction against each cryptosporidial isolate, the sensitivity of stool examination by acid-fast staining which requires high number of oocysts up to 50–500,000 per g in formed stools (Weber 1991), in addition to nutritional status and genetic background which may affect pre-patent period of this disease.

Immunoassay and PCR assays in our study detected infection 4 and 8 h P-I in neonate and adult mice, respectively. This can be mostly resulting from *Cryptosporidium* antigen and DNA of asexual life cycle forms (Smith 2008). Mauzy and his team observed appearance of excysted sporozoites at 2 h P-I, followed by immature (12 h) and mature (24 h) meront development in culture.

However, morphological detection by light microscopy started after a full cycle of asexual replication has been completed (between 24 and 36 h P-I), with no distinguishing between sexual or asexually derived meronts (Mauzy et al. 2012).

Although the results of copro-antigen detection assay proved the same detecting accuracy as PCR, besides, ELISA prevailed as being quick, economical, requiring the least expertise and practically useful in batch testing situations (Ungar 1990), but false-positive results are common (Chapman et al. 1990). Moreover, a variety of purified oocysts and copro-antigen detection kits is commercially available, but up till now, there are no international standards for the preparation of purified oocysts, antigens, or monoclonal antibodies, especially with the well-known antigenic diversity among *Cryptosporidium* species (OIE Terrestrial Manual 2008).

Contrariwise, PCR-based detection assays have become a favorable option in most of the situations, especially in clinically asymptomatic cases with very low oocysts excretion rate (Connelly et al. 2008; Weber et al. 1991). Taking in consideration, some mentioned limitations in PCR assays (Vohra et al. 2012), it was proven that they offer both improved sensitivity and specificity and most important, species/genotype/subtype determination (Morgan et al. 1998; Bialek et al. 2002). Also, nPCR, offers higher diagnostic index and more sensitivity than direct PCR (Uppal et al. 2014).

Employing mucosal DNA detection, in our study plan, as an index of true established infection rather than relying on detected oocysts in fecal smears, confirmed the sensitivity of copro-DNA detection method by giving positive results within 12 h P-I. The question is whether these asexual life cycle forms represent true threat of producing infectious oocysts. Answering this question will help other challenge studies to evaluate further innovative therapeutic and preventive measures, since only infectious oocysts shed from the risk of humans and are thus of epidemiological relevance.

To conclude, infection outcome was not infectious dose related but there was higher susceptibility of infection in neonatal than adult mice. In spite that both microscopy- and copro-antigen-based immunoassays can detect *Cryptosporidium* infection in clinically ill animal but neither morphological- nor copro-antigen-detection can do in clinically normal animal. Nested PCR sensitivity surpass them, and can determine the species of incriminated *Cryptosporidium*, making it a reliable, higher diagnostic biomarker for *Cryptosporidium* infection in murine model, allowing early diagnosis and important prerequisite for further innovative therapeutic and preventive studies.

Compliance with ethical standards

Author contribution All manuscript authors contributed to every activity of it; idea of paper, study design, collection of materials, methodology, writing the paper and revising it.

Conflict of interest The authors declare that they have no competing interests.

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