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MOLECULAR DIAGNOSIS OF *CRYPTOSPORIDIUM* SPP. VERSUS MICROSCOPY IN DIARRHEIC PATIENTS IN CAIRO

By

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Abstract

Cryptosporidium spp. is considered one of the most common diarrhea-causing protozoa. This cross-sectional study was designed for molecular detection of *Cryptosporidium* spp., comparing results with microscopy using Acid Fast (AF) staining, and to determine predominance of cryptosporidiosis according to patients' age and gender.

Methodology: Stool samples were collected from 150 diarrheic patients attending outpatient clinics of Kasr Al-Ainy School of Medicine, Cairo University. Samples were examined microscopically by direct wet mount before and after sample concentration, and then subjected to copro-PCR assays.

The results showed that out of 150 samples subjected to nPCR, only 9 (6%) samples were positive for *Cryptosporidium* spp. and of those, 2 (1.3%) were positive by microscopy using AF stain. Microscopy showed high specificity but lower sensitivity (22%) compared to nPCR. Six of the 9 molecularly positive cases (66.7%) were children under 9 years and predominantly females with statistical significance.

Key words: Patients, *Cryptosporidium* spp., AF stain, nested PCR.

Introduction

In the developing countries, diarrhea is considered the second most common cause of morbidity & mortality in children (WHO, 2012; WGO, 2013). Cryptosporidiosis is a frequent cause of diarrheal disease in man, particularly children and immunocompromised patients lead to devastating outcomes (Fayer *et al*, 2000). In Egypt, *Cryptosporidium* reports of prevalent & virulent diarrheal agent, mainly in childhood varied between 5.0-31.1% (Abdel-Messih *et al*, 2005; El-Shazly *et al*, 2007; Youssef *et al*, 2008; Mousa *et al*, 2010; Abdel Kader *et al*, 2012; Fathy *et al*, 2014; El-Badry *et al*, 2015; Abdel - razek *et al*, 2016; Ghallab *et al*, 2016).

Microscopic diagnosis alone was neither sensitive nor specific as multiple sampling, concentration and staining techniques are needed to improve its performance (Hamzah *et al*, 2010). More specific and sensitive alternative methods, including PCR and antigen detection tests, were also used for detection of cryptosporidiosis. PCR assays are increasingly available, yielding better sensitivity and specificity in comparison to the microscopy and antigen detection assays (Ghosh *et al*, 2000; Stark *et al*, 2008). Real-time PCR identified *Cryptosporidium* spp. (Haque *et al*, 2007) and determined its burden as enabling parasite quantification (Calderaro *et al*, 2010; Hadfield *et al*, 2011).

This cross-sectional study was designed for molecular detection of *Cryptosporidium* spp., comparing results with microscopy using AF staining, and to determine predominance of cryptosporidiosis according to patients' age and sex.

Materials and Methods

Study design and population: A cross sectional study was performed for 150 diarrheic patients attending Outpatient Clinics of Kasr Al-Ainy Hospitals, Cairo University from January 2015 to October 2015. Patients of

both sexes, aged from 1 to 60 years, old suffering from diarrhea, with or without other GIT symptoms as abdominal pain, vomiting, flatulence and/or fever were included. Those on antidiarrheal treatment were excluded.

Work plan and sample processing: A single fecal sample was obtained from each patient. All stool samples were examined microscopically for fecal *Cryptosporidium* oocysts by acid-fast staining method prior to and after fecal concentration. Part of each fecal specimen was stored at -20^{o}C in Eppendorf tubes for copro-PCR assays. DNA extraction was done using the Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech Corporation Ping-Tung 908, Taiwan) according to the manufacturer's instructions. Extracted copro-DNA was amplified by nested PCR (nPCR) targeting *Cryptosporidium* Oocyst Wall Protien (COWP) Gene, using primers BCOWPF (5́-ACC GCT TCT CAA CAA CCA TCT TGT CCT C-3̀) and BCOWPR (5́-CGC ACC TGT TCC CAC TCA ATG TAA ACC C-3̀) which amplify 796 bp fragment (Pedraza-Díaz *et al*, 2001) and nested primers Cry-15 (5́-GTA GAT AAT GGA AGA GAT TGT G-3̀) and Cry-9 (5́-GGA CTG AAA TAC AGG CAT TAT CTT G-3̀) which amplify 553 bp fragment (Spano *et al*, 1997). The PCR was carried in a volume of 25µl. In the primary reaction, sample was heated to 95°C for 4 min, followed by 35 cycles of 94°C for 1 min (denaturation), 63°C for 1 min (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 10 min. Secondary amplification reagent concentrations were similar to first PCR except that 2µl of primary PCR product were added instead of genomic DNA template and 8.4µl double distilled water. The secondary PCR cycling condition was denaturation at 94°C for 50 sec, annealing at 54°C for 30 sec and extension at 72°C for 50sec. PCR generated amplicons of 553bp, subjected to electrophoresis in 1.5% agarose gels and visualized by a UV transilluminator after staining with ethidium bromide (Spano et al., 1997; Pedraza-Diaz *et al*, 2001).

Coproscopy was done in Diagnostic and Research Unit of Parasitic Diseases (DRUP) and copro-PCR assays were carried out at the Molecular Medical Parasitology Lab (LMMP), Department of Medical Parasitology, Faculty of Medicine, Cairo University.

Results

Of 150 samples examined by microscopy using AF staining, *Cryptosporidium* spp. oocysts were detected in 2 samples (1.3%), while 148 (98.7%) samples were free from *Cryptosporidium* spp., suggesting other etiological diarrheic causes. Using nPCR, *Cryptosporidium* spp. copro-DNA was detected in 9 samples (6%) six molecularly positive cases (66.7%) were children under 9 years and predominantly females with statistical significance. Microscopy showed sensitivity of 22% and specificity of 100% compared to nPCR and Kappa showed fair agreement between both methods. Details are in tables (1, 2 & 3) and figures (1 &2).

Table 1: Microscopic examination using AF stain compared to nPCR (n=150) to detect *Cryptosporidium* spp.

Item		nPCR positive	nPCR negative	Total
Microscopic examination	+ve	2	0	2
	-ve	7	141	148
	Total	9	141	150

Table 2: Age and sex of positive *Cryptosporidium* samples by microscopy of AF stained fecal smears and nPCR.

Number	Sex	Age	Microscopy	nPCR
1	Female	3 years	Negative	positive
2	Female	8 years	Positive	positive
3	Female	3 years	Positive	positive
4	Male	8 months	Negative	positive
5	Male	9 years	Negative	positive
6	Female	7 years	Negative	positive
7	Female	35 years	Negative	positive
8	Female	45 years	Negative	positive
9	Female	55 years	Negative	positive

Table 3: Diagnostic yield and Kappa agreement of microscopy compared to nPCR for detection of *Cryptosporidium* spp. among group

Variants	Microscopy
Sensitivity	22%
Specificity	100%
PPV	100%
NPV	95%
Kappa*	0.349

* Kappa: <0 Poor agreement, 0.01-0.20 Slight agreement, 0.21-0.40 Fair agreement, 0.41-0.60 Moderate agreement, 0.61-0.80 Substantial agreement, 0.81-1.00 almost perfect agreement

Discussion

Microscopy is the most commonly used method for routine diagnosis of parasites in developing countries; however, it lacks both sensitivity and specificity (Utzinger *et al*, 2010). In developing countries, replacing microscopy with more sensitive and specific molecular methods is usually hindered by the cost (Taniuchi *et al*, 2011).

In the present study, the low *Cryptosporidium* oocysts copro-prevalence (1.3%) found by microscopy using AF stain agreed with El- Helaly *et al*. (2012) who detected *Cryptosporidium* oocysts in 2.6% of diarrheic stools of patients attending the Stanley Medical College Hospital, Alexandria by using microscopy with AF stain. In Greater Cairo, Nazeer *et al.* (2013) reported *Cryptosporidium* oocysts in 1% of cases and Banisch *et al*. (2015) reported 7 cases of cryptosporidiosis (6.7%) using immunochromatographic assay (ICA) not detected by microscopy. Perch et al. (2001) in West Africa reported (7.7%) in diarrheic patients. Stark *et al*. (2011) in Australia detected *Cryptosporidium* oocysts in 1.1% of samples submitted to St. Vincent's Hospital, Sydney and Acquah *et al*. (2012) in Ghana reported (7.3%) oocysts of *Cryptosporidium*. However, higher prevalence of cryptosporidiosis was reported by Al Braiken *et al*. (2003) in Saudi Arabia, using microscopy detected *Cryptosporidium* oocysts in stools of 32% of children with diarrhea with AF stain. El-Hamshary *et al*. (2008) and El-Settawy and Fathy (2012) in Egypt reported 21% and 18.6% respectively using microscopy with AF stain.

In the present study, due to incompliance of the patients and inability to examine three successive specimens, the reported microscopic prevalence of *Cryptosporidium* spp. might be considered lower than expected.

In general, decreased sensitivity of AF stain for detection of *Cryptosporidium* spp. was explained by the high threshold necessary (a concentration of 10,000 oocysts/g of watery stool) for oocyst detection in stool specimens (Weber *et al*, 1991). Also, there might be some variability in stain uptake, related to the stain itself or the age of the oocyst after prolonged storage (Current and Garcia, 1991).

In the present study, more cases were detected using nPCR targeting *Cryptosporidium* COWP gene of *Cryptosporidium* copro-DNA. Many studies reported using nPCR for detection of *Cryptosporidium* spp and stated that PCR proved more sensitive compared to microscopy with conventional staining (Kaushik *et al*, 2008; Stark *et al*, 2011; Fathy *et al*, 2014; El-Badry *et al*, 2015; Abdelrazek *et al*, 2016; Ghallab *et al*, 2016). In the present study, there was a significant predominance of *Cryptosporidium* copro-DNA detection in children as compared with adults. While *Cryptosporidium* affected both sexes, there was a significant predominance in females. Predominance of cryptosporidiosis in children and contradictory sex predominance was reported in many studies from different countries (ANOFEL, 2010; Abd El-Kader *et al*, 2012; El-Badry *et al*, 2015). Predominance of cryptosporidiosis in children may be due to the absence of pre-existing immunity as well as more exposure to recreational water, increasing the risk of getting infected. Also, previous studies have reported a more frequent attendance of diarrheic children than adults in outpatient clinics (ANOFEL, 2010; Abd El-Kader *et al*, 2012; El-Badry *et al*, 2015).

The low prevalence of *Cryptosporidium* detection (6%) agreed with Abd El-Kader *et al.* (2012) who reported cryptosporidiosis in

children patients suffering from diarrhea (4.6%) using nPCR, and Banisch *et al*. (2015) who reported 7 cases (6.7%) using ICA majority were adults. On the other hand, higher prevalence rates ranged from 16-25% were recorded in stool of diarrheic Egyptian children agreed with studies carried out in Cairo using nPCR (Fathy *et al*, 2014; El-Badry *et al*, 2015; Abdelrazek *et al*, 2016; Ghallab *et al*, 2016), and in adult patients in Ismalia Governorate (El-Hamshary *et al*, 2008) and Zagazig (El-Settawy and Fathy 2012).

PCR-based methods proved higher sensitivity and must replace the conventional methods for detection of many protozoa, including *Cryptosporidium* (Tumwine *et al*, 2003; El-Hamshary *et al*, 2008; Fathy *et al*, 2014; El-Badry *et al*, 2015; Ghallab *et al*, 2016), because of high sensitivity, specificity, easy performance of copro-PCR amplification technique and as an obvious choice for improved detection of *Cryptosporidium* in stool samples.

Conclusion

There was a relatively low prevalence of *Cryptosporidium*, with a predominance of child and female cases. The conventional microscopic and staining techniques used for the diagnosis of *Cryptosporidium* spp. are limited by its low sensitivity, while PCR assays are more sensitive and specific. There is under-diagnosis of *Cryptosporidium* as most labs rely on stained fecal smears for detection and physicians do not request stool analysis for *Cryptosporidium*.

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Explanation of figures

Fig.1: *Cryptosporidium* spp. oocysts stained with AF stain and viewed by oil immersion lens (x1000).
Fig.2: Agarose gel electrophoresis for products of nPCR targeting COWP gene of *Cryptosporidium* spp. at 553 bp.
Lane 1, 2, 3, 5: negative cases, Lane 4, 6, 7, 8: positive cases, Lane 9: positive control, Lane 10: negative control.