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HIGH-RESOLUTION MELTING CURVE (HRM) ANALYSIS IN GENOTYPIC DISCRIMINATION OF *CRYPTOSPORIDIUM* ISOLATES FROM STOOL OF EGYPTIAN CHILDREN

By

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Abstract

Cryptosporidiosis is a recognized child infectious killer and the second cause of diarrheal disease and death in infants. Assessing *Cryptosporidium* spp. genetic diversity is a real goal to elucidate its transmission dynamics and to design preventive measures in absence of effective treatment. *Cryptosporidium* isolates in stool of Egyptian children were detected using Acid Fast (AF) staining, copro-nPCR/RFLP assay and real time PCR high-resolution melting (HRM) curve analysis assay. Stool samples were collected from 335 children complaining of diarrhea and other GIT symptoms, attending the outpatient clinic of Abu El Reesh hospital, Kasr Al-Ainy School of Medicine, Cairo University. Two genotypes *C. hominis* and *C. parvum* were identified in 43 isolates from Egyptian children by copro-nPCR targeting COWP gene and HRM assay. Real time PCR HRM curve analysis, a closed-tube genotyping method, targeting ITS-2 gene confirmed the results of copro-nPCR/RFLP. It is simple, rapid, has more sample throughput, analysis capacities and data storage with less carry-over contamination and cost.

Introduction

Cryptosporidium spp. is a protozoan parasite, descried by WHO as one of kid killers and the second cause for diarrheal illnesses and death in newborns after Rotavirus (Kotloff *et al,* 2013). *Cryptosporidium* possesses 24 valid species and more than 44 genotypes infecting many vertebrates, including humans and animals, which differ significantly in their molecular signatures (Cama *et al,* 2008). Thirteen intestinal and gastric *Cryptosporidium* species infect immuno-competent and immuno-compromised humans have been reported until now (*C. parvum, C. hominis, C. meleagridis, C. felis, C. canis, C. suis, C. muris, C. andersoni, C. fayeri, C. cuniculus, C. ubiquitum* and *C. viatorum*) (Fayer *et al,* 2010; Elwin *et al,* 2012).

Genetic variation among *Cryptosporidium* isolates was reported and seems to be central to the control of this disease and to the understanding of the intricacies of its epidemiology (Pangasa *et al,* 2009). Though morphologically similar oocysts exist, it was difficult to accurately differentiate the *Cryptosporidium* species and genotypes by light microscopy and immunoassays that require using molecular methodologies for a precise diagnosis, and detection of parasitic species which reflects on primary & secondary prevention strategies (Jothikumar *et al,* 2008).

Analyzing selected genetic loci by PCR-based techniques or direct DNA sequencing has high sensitivity, which allows for the specific amplification of target genetic loci from small amounts of parasite DNA. PCR was integrated into different genotyping techniques, such as restriction fragment length polymorphism analysis (RFLP), random amplification method and methods detecting conformational polymorphisms or sequencing (Pangasa *et al,* 2009). Because of the increased interest in genetic analysis of pathogen, there was an extensive spotlight on

mutation scanning methods, which depend up on melting characteristics of amplicons. PCR-based melting-curve and high-resolution melting-curve (HRM) analyses significantly diminish the time, required for testing, and accomplish high mutation detection rates for small amplicons (Montgomery *et al,* 2007).

The current study aimed to determine the prevalence of *Cryptosporidium* spp. among a cohort of Egyptian children using copro AF stain, copro n-PCR/RFLP targeting the COWP gene and evaluating real time PCR-coupled HRM-curve analysis, targeting ITS-2 as the genetic marker, for detection of the predominant species of *Cryptosporidium* sp. infecting humans, and discuss the implications of this molecular-diagnostic approach.

Material and Methods

Study plan: A cross sectional prospective study was designed including 335 stool samples collected from diarrheic children. Samples were collected from cases attending the outpatient GIT clinic at Cairo University Pediatric Hospital from July 1^{st,} 2014 to January 1st 2016.

Sample collection and processing: A single fecal sample was taken from each child. Part of each stool specimen was preserved in formalin saline fixative for parasitological coproscopic examination and AF staining and other part was stored at -20^{o}C for molecular assays. Coproscopy was carried out in the Diagnostic and Research Unit of Parasitic Diseases (DRUP), copro-nPCR/RFLP assay was performed in Lab of Molecular Medical Parasitology (LMMP), Department of Medical Parasitology, Faculty of Medicine, Cairo University, Egypt and real-time PCR assay was done at Biotechnology unit of the faculty of Veterinary, Kafr El-Sheikh University, Egypt.

Parasitological coproscopy and AF stain: Fecal samples were examined directly by wet mount and after concentration using a modified Ritchie's biphasic method (Garcia, 2007), and then were permanently stained using cold Kinyoun's AF stain (Biostain Ready Reagents Ltd Manchester, England) to detect *Cryptosporidium* sp. copro-oocyst.

Extraction of the genomic DNA: Genomic DNA was extracted utilizing Favor Prep stool DNA isolation Mini Kit (Favorgen Biotech corporation ping-Tung 908, Taiwan, Cat. No. FASTI001) with modification in the prolongation of incubation after 56^{O}C at 10 minutes to 95^{O}C for one hour after thermal shock (cycling of deep freezing in liquid nitrogen for 5 min & immediately transferred into water bath 95°C for 5 min. repeated for 5 cycles) the obtained DNA was measured for concentration and purity.

Copro n-PCR / RFLP analyses: Copro-nPCR was done targeting COWP gene. PCR amplification was performed in a 25µl volume containing 12.5 µl master mix, 200 n*M* from each primer, and 3µl of the template DNA for the primary reaction and 1µl for the secondary one. The cycling conditions were 35 cycles at 94 °C for 1 min, (63°C for 1 min for 1ry & 54°C for 30 s for nested 54) and 72°C for 1 min, followed by 10 min at 72°C. The amplified products were visualized with 1.5% agarose gel electrophoresis after ethidium bromide staining. Amplified products of nPCR of positive sample of COWP gene were digested using *Rsa I* endonuclease after manufacture instruction and resolved using 3% Metaphor electrophoresis after ethidium bromide staining.

Enzymatic amplification and HRM curve analysis: The ITS-2 region (440-450 bp) was PCR-amplified from genomic DNA samples using primers AP58SF (forward: 5'-ATC TCT CAA GCG AAA TAG CAG TAA-3') and APITS-2R (reverse: 5'-CCC ATT GAT AAA CGG ATT TCC-3') (Pangasa *et al,* 2009). PCR was performed in 25ml volumes containing 200 nM of each primer, 12.5µl of Luminaris color HRM master mix (Thermo scientific), 4.5µl nuclease-free water and 3 µl of the template DNA using the thermo cycling conditions (Pangasa *et al,* 2009). In every run, positive and negative controls were included, and the cycle threshold and melting-curve values were recorded.

Statistical analysis: Data were coded and entered using the statistical package SPSS version 17 (Chicago, IL, USA) for statistical analysis.

Results

Demographic, clinical and initial laboratory data of study samples: Cases presented variable GIT symptoms were assessed medically. The mean age of subjects under study was 4.59 years ±3.38 standard deviation (SD), 56.1% of them were males and 43.9% were females with male/female ratio 1:3. Total of 326 (97.3%) children used tap water and 81 (24.2%) had a history of animal contact. Main symptom of all was diarrhea in 141(42.1%) cases.

Occurrence and Genotyping of *Cryptosporidium*: Out of 335 examined stool samples with AF stain (Figure 1), *Cryptosporidium* oocysts were found in 12 samples (3.6%). Further assessment by nPCR targeting COWP gene revealed positivity in 43 (12.8%) stool samples (Figs. 2 & 3).

The specific categorization of samples using PCR-coupled HRM identified 38 cases for which *C. hominis* presented two peaks (profile 1), and 5 cases were *C. parvum* with one peak (profile 2) (Tab.1; Fig. 4).

Cryptosporidium genotypes and risk factors: The study showed no significant correlation between *Cryptosporidium* species and demographic and clinical variants. However, there was a positive correlation between *Cryptosporidium* species and with symptomatic patient (P<0.05).

Table 1: Used primers and their sequences.

		Primers	*Sequence*	*Expected product size (bp)*	*Reference*
COWP gene	1ry PCR	BCOWPF	5'ACCGCTTCTCAACAACCATCTTGTCCTC-3'	769-bp	(Pedraza-Díaz *et al*, 2001)
		BCOWR	5'-CGCACCTGTTCCCACTCAATGTAAACCC-3'		
	nPCR	Cry-15	5́-GTAGATAATGGAAGAGATTGTG-3̀	553-bp	
		Cry-9	5́-GGACTGAAATACAGGCATTATCTTG-3̀		

Table 2: Number of samples and the profile of PCR-coupled melting-curve analysis targeting ITS-2 amplicons.

	N. of samples	Peak 1	Peak 2
Profile I (*C. hominis*)	38	72.7^0C	74.9^0C
Profile II (*C. parvum*)	5	77.8^0 C	-

Discussion

In the last decades in Egypt, there has been a reported varying *Cryptosporidium* preva-lence, a high of molecular prevalence rate of infection was shown in many studies up to 25% of the examined patients (El-Settawy and Fathy, 2012; Fathy *et al,* 2014; Ghallab *et al,* 2014). In contrast, much lower results (4.6%) reported (Abd El-Kader *et al*, 2011). The diminished sensitivity of AF stain in the study might be due to the high threshold ne-cessary for oocyst detection in watery stool where the detection rate could be accom-plished at a concentration of 10,000 oocysts/ g stool (Weber *et al,* 1991).

Genotypic discrimination, PCR-RFLP analysis yielded *C. hominis* predominance in 38 samples (88.4%) and *C. parvum* in 5 cases (11.6%). Mixed infection wasn't detected, indicating that the main source of *Cryptosporidium* infection was anthroponotic rather than zoonotic. These findings agreed with two Egyptian studies (Abdel-Kader *et al*, 2011; Ibrahim et al, 2016) and differred from two studies (Eida *et al,* 2009; Sadek, 2014). In industrialized nations, *C. hominis* predominates among human cryptosporidiosis, as in Spain (65.7%), USA (67%), Japan (68%), Australia and Canada (76%). As well as, in developing countries, as Peru (79%), South Africa (82%) and Thailand (83%), indicating that anthroponotic species were the main cause of human infection. The re-lation of *C. parvum*/*C. hominis* was however more balanced in other countries, as England and Wales (49.2%), France (51%) and Bel-gium (54.2%), while *C. parvum* dominated in the Netherlands (72%) and Italy (90%) (Ong *et al,* 2002; Samie *et al,* 2006; Liorente *et al*, 2007; Geurden *et al,* 2009).

PCR coupled HRM curve analysis targeting ITS-2 gene confirmed the results of

copro-nPCR/RFLP. PCR coupled HRM curve achieve parasite specific identification and differentiation (Monis *et al*, 2005). The presence of multiple peaks in HRM is diagnostically informative, giving a unique profile with an increased number of characters for the resolution of species and/or genotypes (Robinson *et al*, 2006). PCR-coupled HRM-curve analysis is suited for the quick screening of many *Cryptosporidium* oocyst DNA samples as there was no requirement for the handling, pouring, and/ or scanning of electrophoretic gels, thus con-siderably diminishing overall time and cost. It is a closed-tube genotyping method that eliminates risk of contamination. The qualit- a tive approach, exhibited certain advantages over other electrophoretic ones (Gasser *et al*, 2001), mainly in relation to sample through-put, analysis time, analysis capacities and data storage. Risk of carry-over contamina-a tion and materials' cost were much lower than that for others (Zhang *et al*, 2012).

HRM master mix used in this study contained a third-generation florescent dye (Eva green). This dye proved ideal with good stability and instrument compatibility coupled with optimal DNA-binding characteristics, including low or no affinity for ssDNA, and short DNA fragments and a just-right affinity for dsDNA, thus increasing the specificity of HRM reaction (Mao *et al*, 2007).

In the present study, only children with diarrhea and other GIT symptoms showed significant cryptosporidiosis association. In contrast, El-Helaly *et al*. (2012) and Jiang *et al*. (2014) reported a higher infection rate in males than females, Park *et al*. (2006) found a significant correlation between cryptosporidiosis and sexes, as boys were more susceptible than girls to infection. Untreated water, animal contact and farm visit have been reported as risk factors for infection in many studies. Others did not find relationship between infection and drinking water or animal contact (Khalili and Mardani, 2009).

GIT symptoms including diarrhea, abdominal pain, flatulence, itching, vomiting, or appetite loss (Chauret *et al*, 1999) are known risk factors for cryptosporidiosis.

Clinical manifestations in cryptosporidiosis might be correlated to different species and subtypes. Cama *et al*. (2008) reported that cases with *C. parvum*, *C. felis*, *C. canis*, and *C. meleagridis* infection were associated with diarrhea only, but diarrhea, nausea, vomiting, and general malaise were associated with cryptosporidiosis. Mumtaz *et al*. (2010) reported that environmental, clinical and host behavioral factors acted as important risk factors for infection but neither affecting the pathogenicity nor the disease course. In the present study, the high population densities with greater chance of person-to-person transmission and variation in socioeconomic population status within same geographical areas might play an important role in the differences reported in the prevalence of the disease and may explain the obtained results in the present study.

Conclusion

There is *C. hominis* predominance among Egyptian children. HRM curve analysis, a closed-tube genotyping method, confirmed the results of copro-nPCR/RFLP. It is simple, without probe, rapid, has more sample throughput, analysis capacities and data storage with less carry-over contamination and cost.

Contribution of each author: All manuscript authors contributed to every activity: idea of paper, study design, collection of materials, methodology, writing and revising the paper.

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References

Abd El-Kader, MN, Blanco, M, Tammam, M A, Abd El Ghaffar, AB, Osman, A, *et al*, 2011: Detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* in human patients in Cairo, Egypt. Parasitol. Res. 110, 1:2465-70.

Cama, VA, Bern, C, Roberts, J, Cabrera, L, Sterling, CR, *et al*, 2008: *Cryptosporidium* species and subtypes and clinical manifestation in children, Peru. Emerg. Infect. Dis. 14, 10: 1567-74.

Chauret, C, Springthorpe, S, Sattar, S, 1999:

Fate of *Cryptosporidium* oocysts, *Giardia* cysts, and microbial indicators during waste water treatment and anaerobic sludge digestion. Can. J. Microbiol. 45, 3:257-62.

Eida, AM, Eida, MM, El-Desoky, A, 2009: Pa-thological studies of different genotypes of human *Cryptosporidium* Egyptian isolates in ex-perimentally mice. J. Egypt Soc. Parasitol. 39, 3: 975-90.

El-Helaly, NS, Aly, MA, Attia, SS 2012: Dete-ction of *Cryptosporidium* Infection among children with diarrhea. New York Sci. J. 5, 7:68-76.

El-Settawy, MA, Fathy, GM, 2012: Evaluation and comparison of PCR, coproantigen ELISA and microscopy for diagnosis of *Cryptospori dium* in human diarrheic specimens. J. Am. Sci. 8, 12:1385-78.

Elwin, K, Hadfield, SJ, Robinson, G, Crouch, ND, Chalmers, RM, 2012: *Cryptosporidium viatorum* sp. among travellers returning to Great Britain from the Indian subcontinent, 2007-2011. Int. J. Parasitol. 42:675-82.

Fathy, MM, Abdelrazik, NM, Hassan, FA, El-Badry, AA, 2014: Molecular copro-preva-lence of *Cryptosporidium* in Egyptian Children and evaluation of three diagnostic methods. Res. Brief. 51:727-9.

Fayer, R, Santín, M, Macarisin, D, 2010: *Cry-ptosporidium ubiquitum* sp. in animals and humans. Vet. Parasitol. 172:23-32.

Garcia, LS, 2007: Diagnostic Medical Parasi-tology, 5th ed. ASM Press, Washington D. C.

Gasser, RB, Zhu, XQ, Caccio,`S, Chalmers, R, Widmer, G, *et al,* 2001: Genotyping *Crypto-sporidium parvum* by single-strand conforma-tion polymorphism analysis of ribosomal and heat shock gene regions. Electroph. 22, 3:433-7.

Geurden, T, Levecke, B, Cacció, SM, Visser, A, De Groote, G, et al, 2009: Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhea in human patients in Belgium. Parasitol. 136, 10:1161-8.

Ghallab, MMI, Abdel Aziz, IZ, Shoeib, EY, El-Badry, AA, 2014: Laboratory utility of co-0proscopy,copro-immunoassays & copro-nPCR assay targeting Hsp90 gene for detection of *Cryptosporidium* in children, Cairo, Egypt. J. Parasit. Dis. 40:901-5.

Ibrahim, MA, Abdel-Ghany, AE, AbdelLatef, GK, Abdel-Aziz, S, Aboelhadid, S, 2016: Epi-demiology and public health significance of *Cryptosporidium* isolated from cattle, buffaloes, and humans in Egypt. Parasitol. Res. 115, 6:2439-48

Jiang, Y, Ren, Y, Yuan, Z, Liu, A, Zhao, H, *et al,* 2014: *Cryptosporidium andersoni* as a novel predominant *Cryptosporidium* species in outpatients with diarrhea in Jiangsu Province, China. B.M.C. Infect. Dis. 14:1-6.

Jothikumar, N, da Silva, AJ, Moura, I, Qva-rnstrom, Y, Hill, VR, 2008: Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays. J. Med. Microbiol. 57, 9:1099-105.

Khalili, B, Mardani, M, 2009: Frequency of *Cryptosporidium* and risk factors related to cryptosporidiosis in under 5-year old hospitalized children due to diarrhea. Iranian J. Clin. Infect. Dis. 4, 3:151-5.

Kotloff, KL, Nataro, JP, Blackwelder, **WC, et al, 2013:** Burden and etiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382, 9888:209-22.

Liorente, MT, Clavel, A, Goñi, MP, Varea, M, Seral, C, *et al,* 2007: Genetic characteriza-tion of *Cryptosporidium* species from humans in Spain. Parasitol. Int. 56, 3:201-5.

Mao, F, Leung, WY, Xin, X, 2007: Characteri-zation of EvaGreen and the implication of its physicochemical properties for qPCR applica-tions. B.M.C. Biotechnol. 7:76-9.

Monis, PT, Giglio, S, Saint, PC, 2005: Comp-arison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investiga-tion of the effect of dye concentration on amplification and DNA melting curve analysis. Anal. Biochem. 340:24-34.

Montgomery, J, Wittwer, CT, Palais, R, Zh-ou, L, 2007: Simultaneous mutation scanning and genotyping by high resolution DNA melting analysis. Nat. Protoc. 2:59-66.

Mumtaz, S, Ahmed, J, Ali, L, 2010: Frequency of *Cryptosporidium* infection in children under five years of age having diarrhea in the North west of Pakistan. Afr. J. Biotechnol. 9, 8:1230-5.

Ong, CS, Eisler, DL, Alikhani, A, Fung, VW, Tomblin, J, *et al,* 2002: Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: first report of human infections with a cervine genotype. Emerg. Infect. Dis. 8, 3:263-8.

Pangasa, A, Jex, AR, Campbell, BE, Bott, NJ, Whipp, M, *et al,* 2009: High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. Mol. Cell Probes 23, 1: 10-5. **Park, JH, Kim, HJ, Guk, SM, Shin, EH, Kim, JL, *et al,* 2006:** A survey

of cryptosporidiosis among 2,541 residents of 25 coastal islands in Jeollanam-Do (Province), Republic of Korea. K.
J. Parasitol. 44, 4:367-72.
Robinson, BS, Monis, PT, Dobson, PJ, 2006: Rapid, sensitive and discriminating identification of *Naegleria* spp. by real-time PCR and melting-curve analysis. Appl. Environ. Microbiol. 72:5857-63.
Sadek, GM, 2014: Use of nested PCR-RFLP for genotyping of *Cryptosporidium* parasites iso-lated from calves and children suffering from di-arrhea. J. E.P.U. 7, 2:129-37.
Samie, A, Bessong, PO, Obi, CL, Sevilleja, J E, Stroup, S, *et al*, 2006: *Cryptosporidium* species: preliminary descriptions of the prevalence and genotype distribution among school children and hospital patients in the Venda region, Limpopo Province, South Africa. Exp. Parasitol. 114, 4:314-22.
Weber, R, Bryan, R, Juranek, D, 1991: Improved stool concentration procedures for detection of *Cryptosporidium* oocysts in in fecal specimens. J. Clin. Microbiol. 30, 11:2869-73.
Zhang, P, Liu, Y, Alsarakibi, M, Li, J, Liu, T, *et al*, 2012: Application of HRM assays with EvaGreen dye for genotyping *Giardia duodenalis* zoonotic assemblages. Parasitol. Res. 111: 2157-63

Explanation of figures

Fig. 1: *Cryptosporidium* oocysts stained with cold Kinyoun's AF stain.
Fig.2: Agarose gel electrophoresis for products of nPCR targeting COWP gene of *Cryptosporidium* at 533 bp., Lane 1: 100 bp DNA molecular weight marker, Lane 2: Positive control, Lanes 3: Negative control &Lanes 4- 6: Positive samples.
Fig. 3: Agarose gel electrophoresis for COWP RFLP products after digestion with *RsaI* endonuclease. Lane 1: 100 bp DNA marker ladder, Lanes 2&3: *C. hominis* genotype I digestion products at 34, 106, and 410 bp using *RsaI* & Lane 4&5: *C. parvum* gentype II digestion products at 34, 106, 125 and 285 bp.
Fig. 4: Representative profiles in melting-curve analysis of ITS-2 amplicons for *C. hominis* (red) (A), *C. parvum* (black) (B).