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ASSESSMENT OF FAECAL CALPROTECTIN LEVEL IN PAEDIATRIC AND GERIATRIC PATIENTS WITH CRYPTOSPORIDIOSIS

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Abstract

Cryptosporidium parasite is one of the major causes of diarrhoea. Several parasites and host factors, including the *Cryptosporidium* genotype and the host immune status and age can influence the disease severity. In the present work the faecal calprotectin (FCAL) level was assessed in *Cryptosporidium* infected patients, as a marker of intestinal inflammation in the paediatric and geriatric age groups, with correlation to *Cryptosporidium* genotype. Our results revealed that the prevalence of cryptosporidiosis in the geriatric patients was significantly higher than in the pediatric group. Genotyping revealed *Cryptosporidium hominis* as the frequently circulating species. Significantly high FCAL levels weredetected in *Cryptosporidium* positive patients denoting the ongoing inflammatory process in cryptosporidiosis. Additionally, FCAL levels were significantly greater in *Cryptosporidium hominis* compared to *Cryptosporidium parvum* infection, while unaffected by age group. Further studies are needed to investigate the impact of cryptosporidiosis in the elderly patients and to evaluate FCAL as a predictive marker of the disease outcome.

Keywords: *Cryptosporidium*, Genotypes, Faecal calprotectin, Children, Elderly

Introduction

Cryptosporidium species are apicomplexan protozoa that dwell and divide in the intestinal epithelial cells with incidence ranged from 1-3% to 10-15% of diarrhoea, in developed and developing countries, respectively, placing the parasite among the major causes of diarrhoea (Sulżyc-Bielicka *et al,* 2018). With over 37 species and 70 genotypes, *Cryptosporidium* can infect a wide variety of vertebrate hosts; the 2 main species responsible for human infection are the zoonotic *C. parvum* and the anthroponotic *C. hominis* (Deng *et al,* 2020).

In Egypt, cryptosporidiosis was encountered in man and animals (Elshahawy and Abou-Elenien, F, 2019). *C. parvum* was reported among 204/390 diarrheic children, with highest infection rate of 26/46 among children less than 2 months, 40/150 among children less than 2 years and 2/8 among children less than 7 years (Shoukry *et al,* 2009).

Cryptosporidium muris (C. muris) was first described and *C. parvum* was described two years later (Tyzzer, 1912). Like the other members of this phylum, *Cryptosporidium* has a complex life cycle with both asexual and sexual stages and invasive stages that have the characteristic apical complex from which the phylum name is derived, with infection outcome depends on several parasites and host factors, including the host immune status and age (Bouzid *et al*, 2013). Immunocompromised patients, young children and elderly are the most at-risk groups of developing severe form of the disease, being more vulnerable to infection and to its consequences. In young children, morbidity can even extend to long term impact on nutrition, growth, and development (Bourke *et al,* 2016). Cryptosporidiosis causes profuse, watery diarrhea, abdominal cramping, nausea, vomiting and low grade fever, particularly in immunocompetent patients infection may last 2-12 days but usually self-limiting. Infections may continue for two weeks or more and require fluid replacement therapy (Fayer *et al,* 1997). In congenital or acquired immune deficiencies or malnourished patients, infection can be prolonged, causing malabsorption, severe dehydration and even fatal (Xiao, 2010).

The infection is by ingestion of oocysts in unwashed vegetables or water, with subsequent sporozoite excystation, adherence, in-

ternalization, and envelopment within host enterocyte apical membrane, the disruption of the intestinal barrier with increased permeability, impaired absorption, causing excessive fluid loss, electrolytes, and nutrients (Di Genova and Tonelli, 2016). Experimental studies showed an elevated inflammatory response during *Cryptosporidium* infection (Takeuchi *et al*, 2008), and the cellular infiltration of lamina propria with lymphocytes, macrophages and neutrophils was proposed as one of the host defense mechanisms in the cryptosporidiosis (Shrivastava *et al*, 2017).

Calprotectin is a calcium and zinc-binding protein of the S100 family with proposed anti-microbial effect. It forms 60% of cytosolic protein in neutrophils and is present in monocytes and reactive macrophages at lower concentrations but rose during infections and inflammation (Konikoff and Denson, 2006). It can be as well detected in stool in active inflammatory processes in the intestine, where disruption of mucosal architecture allows the neutrophils and so calprotectin to leak to lumen and to pass in stool (Walsham and Sherwood, 2016). Since its introduction in the field of Gastroenterology, faecal calprotectin (FCAL) served as a non-invasive biomarker of inflammatory bowel conditions, with a valuable clinical utility to distinguish organic bowel conditions as inflammatory bowel (IBD) from non-organic conditions as irritable bowel syndrome (IBS) (Laserna-Mendieta and Lucendo, 2019).

The present work aimed to assess the level of calprotectin in *Cryptosporidium* infected, both in paediatric and geriatric patients' age groups, with correlation to *Cryptosporidium* genotype.

Material and Methods

Study setting: The study was conducted during the period from September 2020 to June 2021, at the Medical Parasitology department, the Clinical and Chemical Pathology department, Faculty of Medicine, Cairo University, and the gastroenterology outpatient clinics in Abul-Reesh Paediatric, Cairo University Hospitals.

Population and sample collection: A total of 355 patients complaining from diarrhoea were included in the study. Patients were divided into 2 groups: G1: Paediatric group consisted of 165 children aged from 4 to 12 years, of which 95 were females & 70 were males. G2: Geriatric group consisted of 190 elderlies aged above 60 years, of which 110 were females and 80 were males. All patients were subjected to medical history taking and clinical examination.

Ethical consideration: An informed consent was obtained from all participants in the study, including the elderly patients and the parents of the young patients. All study procedures were performed according to the Ethical Guidelines of Faculty of Medicine, Cairo University, and in agreement with the 2000 Helsinki Declaration. *Cryptosporidium* positive patients were notified.

Laboratory procedures: All morning stool sample were divided into 2 parts, one part was examined fresh as part of stool examination and used in permanent staining, while the other part was frozen for later use in immune and molecular techniques.

Morning stool samples were subjected to concentration by formalin ethyl acetate sedimentation, then staining using Kinyoun's Acid-Fast (KAF) permanent stain (Garcia, 2007).

FCAL level measurements: Frozen stool samples were used for the quantitative measurements. A solid phase sandwich ELISA, the DRG: HYBRiDXL Calprotectin kit (Cat. No. HYE-5767) was done according to the manufacturer instruction. For tested results interpretation, FCAL values higher than 200µg/g were considered positive (Rady *et al,* 2019).

Nested PCR and RFLP for *Cryptosporidium* genotyping: Frozen stool of the *Cryptosporidium* positive samples by KAF were subjected to DNA extraction using the Favour Prep stool DNA isolation Mini Kit. (Fav-orgen Biotech Corp. Cat. No. FAFST00). The eluted DNA was used as template for nested

PCR amplification targeting *Cryptosporidium* oocyst wall protein (COWP) gene using the following primers in 1st run BCOWPF and BCOWPR, and in the 2nd run Cry-15 and Cry-9 (Spano *et al*, 1997). Using RFLF technique, enzymatic digestion with the RsaI enzyme was then performed on the nested PCR products (Ibrahim *et al*, 2021).

Statistical analysis: Data were computerized and analyzed using the statistical package for the Social Sciences (SPSS) program version 26 (IBM Corp., Armonk, NY, USA). Categorical data was summarized using frequency (count) and relative frequency (percentage); and analyzed using Chi square (χ^2) test. Exact test was used instead when the expected frequency is less than 5. Quantitative variables were presented as mean and standard deviation; and compared using the non-parametric Mann-Whitney test. P-values < 0.05 were considered significant.

Results

In a total of 355 patients, *Cryptosporidium* oocysts were detected in 43 (12.1%) samples, of which 32 (74.4%) samples were identified as genotype 1 and 11 (25.6%) samples were identified as genotype 2, while FCAL was detected in 43 (12.1%) cases of the participants. Comparison between the study groups revealed a significantly higher frequency of cryptosporidiosis in the geriatric group (15.8%) versus the pediatric group (7.9%), but without significant difference in *Cryptosporidium* genotypes and FCAL between pediatric (G1) and geriatric (G2).

Conferring to *Cryptosporidium* infection, FCAL was detected in 29/43 (67.44%) positive cases versus 14/312 (4.48%) negative cases, revealing a significant association between FCAL and cryptosporidiosis (P-value <0.05). Regarding *Cryptosporidium* genotyping, The FCAL was detected in 25/32 (78.12%) genotype 1 samples, and in 4/11 (36.36%) genotype 2 samples. A statistically significant association was observed between FCAL and *Cryptosporidium* genotype 1. The mean FCAL level was 645.1±332.5, and 359.9 ±324.1 in *Cryptosporidium* positive cases and *Cryptosporidium* genotype 1, respectively.

Details were given in tables (1 & 2) and figure (1).

Table 1: Distribution of *Cryptosporidium* infection, *Cryptosporidium* genotype and calprotectin in population

Materials	Variables	G1 (N=165) No. (%)	G2 (N=190) No. (%)	Total (N=355) No. (%)	P-value
Cryptosporidium	Positive	13 (7.9%)	30 (15.8%)	43 (12.1%)	0.023*
	Negative	152 (92.1%)	160 (84.2%)	312 (87.9%)	
	Genotyping**				
	Genotype 1	8 (61.5%)	24 (80.0%)	32 (74.4%)	0.262
	Genotype 2	5 (38.5%)	6 (20.0%)	11 (25.6%)	
FCAL	Positive	17 (10.3%)	26 (13.7%)	43 (12.1%)	0.330
	Negative	148 (89.70%)	164 (86.3%)	312 (87.9%)	

*significant, **Genotyping of *Cryptosporidium* positive cases (N=43)

Table 2: Distribution of *Cryptosporidium* infection, *Cryptosporidium* genotype in relation to FCAL

Item	Variables	FCAL Positive (N=43) No. (%)	FCAL Negative (N=312) No. (%)	Total(N=355) No. (%)	P value
Cryptosporidium	Positive	29 (67.4%)	14 (4.5%)	43 (12.1%)	<0.001*
	Negative	14 (32.6%)	298 (95.5%)	312 (87.9%)	
	Genotyping**				
	Genotype 1	25 (86.2%)	7 (50.0%)	32 (74.4%)	0.022*
	Genotype 2	4 (13.8%)	7 (50.0%)	11 (25.6%)	

*significant, **Genotyping of *Cryptosporidium* positive cases (N=43)

Discussion

The present study revealed the overall *Cryptosporidium* prevalence rate was 12.1% among diarrheic patients, with significantly higher prevalence in the geriatric patients than in the paediatric patients. In Egypt Youssef *et al.* (2008) among nineteen studies on immunocompetent individuals with diarrhea presenting to inpatient or outpatient clinics showed cryptosporidiosis prevalence

ranged from 0% - 47% (median 9%, IQR 3-15%). Dong *et al*, (2020) reported that the global pooled prevalence of *Cryptosporidium* infection was 7.6 % (95% CI: 6.9-8.5). The highest estimated infection prevalence was in Mexico (69.6%, 95% CI 66.3-72.8), Nigeria (34.0%, 95% CI 12.4-60.0), Bangladesh (42.5%, 95% CI 36.1-49.0) and Republic of Korea (8.3%, 95% CI 4.4-13.2) among general residents, patients, school children and healthy population, respectively. The prevalence was high in people from low-income country, people with gastrointestinal symptoms, and children < 5 years old

Over the past two decades, successive epidemiological studies have identified children as a vulnerable group to *Cryptosporidium* infection, with variable prevalence rates according to the used diagnostic tool and the geographic location (Abdel-Messih *et al*, 2005; Gatei *et al*, 2006; Tahira *et al*, 2012; Shalaby and Shalaby, 2015; Gargala *et al*, 2017; El-Bahnasawy *et al,* 2018; CDC, 2018; Shaposhnik *et al*, 2019). Although elderly persons were described as "at increased risk" to cryptosporidiosis, few epidemiological data are available regarding *Cryptosporidium* infection prevalence in the geriatric age group (Naumova *et al*, 2003; Mor *et al*, 2009; Amin *et al*, 2021).

In the present study, *Cryptosporidium* genotyping showed a higher prevalence of genotype 1 (74.4%) over genotype 2 (25.6%). Generally, genotypes 1 and 2 were described as human genotype and cattle genotype, respectively, allocated to one species the *C. parvum* (Molloy *et al,* 2010). But, genotype 1 was acknowledged as a distinctive species "*Cryptosporidium hominis*" and genotype 2 as "*C. parvum*" (Hashim *et al,* 2006). Several molecular epidemiological studies proved a higher prevalence of *Cryptosporidium hominis* than *C. parvum* (Gatei *et al*, 2006; Naguib *et al*, 2018). A higher impact of the anthroponotic cryptosporidiosis over zoonotic transmission was determined in endemic areas, mainly in developing countries (Krumkamp *et al*, 2021).

Pathogenesis of cryptosporidiosis, as the rest of infectious diseases, is directed by the complex interplay between the parasite characteristics involving the phenotype and genotype which define its virulence, and the host factors such as age and the immune status. These factors contribute to both the severity as well as the risk of infection (Bouzid *et al*, 2013).

In the present study, FCAL was assessed in symptomatic *Cryptosporidium* infection taking into account the age of the host and the genotype of the parasite. There was a significantly higher FCAL level in *Cryptosporidium* positive diarrheic patients versus *Cryptosporidium* negative diarrheic patients, as well as a significantly higher FCAL level in genotype 1 compared to genotype 2, but there was no significant difference in FCAL level between the age groups. Salman *et al.* (2017) reported positive FCAL in 41.75% of *Entamoeba histolytica* infections, 21.27% of *Giardia lamblia* infections, and 3.22% of *Blastocystis hominis* infections. Also, high FCAL levels were identified in 2 different case reports of *Giardia lamblia*, one a 32-year-old patient from Iran (Gol *et al*, 2018) and the other a 6-year-old patient from USA (Shapiro *et al*, 2021) co-infected with *Cryptosporidium.* For the non-pathogenic protozoa the reported data were contradictory, Salman *et al.* (2017) didn't find any FCAL levels in *Dientamoeba fragilis* and *Chilomastix mesnili* infections, but Aykur *et al*. (2020) reported elevated FCAL in *Dientamoeba fragilis* infected patients.

Bustinduy *et al.* (2013) in Ugandan children with *Schistosoma mansoni* reported the raised FCAL in heavy infections strongly correlated with egg patient infection. Besi des, Johansson *et al.* (2013) as well reported high FCAL level in a 22-year-old Swedish patient infected with *Enterobius vermincularis*; however this case was associated with mucosal ulcerations mimicking Crohn's disease.

Nevertheless, De Gier *et al.* (2018) didn't report significant association between FCAL

levels and some helminthiasis (*Ascaris lumbricoides, Trichuris trichiura* and *Ne-cator americanus*) in Cuban and Cambodian schoolchildren. Likewise, Patel *et al.* (2021) in Tanzania didn't find significant association between FCAL and *Trichuris trichiura* infection in their case-control study.

The elevated FCAL sensitively reflected intestinal mucosal inflammation without denoting the specific cause. It can be attributed mainly to inflammatory diseases, as well as neoplastic lesions, in addition to a number of cases of infectious diarrhoea (McMahon and Chhabra, 2018). FCAL can be released in intestinal infections associated with marked cellular infiltration of the mucosa, involving largely the neutrophils and macrophages. Therefore, bacterial infections, which usually elicit an immune response with neutrophils as the prevailing cell type, show higher FCAL levels than viral infections (Chen *et al*, 2012).

The mucosal immune response to parasitic infections differs according to the type of parasite whether helminthic and protozoal agent (Kasper and Buzoni-Gatel, 2001). Thus, it encompasses the activation of variety of cells including eosinophils, mast cells mostly in the former, macrophages, dendritic cells, and neutrophils mainly in the latter type of parasite, and the FCAL was expected to be in detectable amounts in intestinal protozoa infections rather than in helminthic infections, as well as, the nature of the infectious agent, the disease activity and severity can impact the amount of the FCAL released, as neutrophils have been proposed to play a role not just in host protection but also in the development of intestinal lesions (El-Naccache *et al*, 2020).

For instance, in the IBD patients the use of FCAL has been beneficial for the screening and diagnosis, in addition to follow up the disease activity (Yamamoto *et al*, 2014). Consequently, FCAL levels can be predictive of future episodes of relapse, it was found in study on patients with quiescent ulcerative colitis that a FCAL cut-off level of 170µg/g was a 76% sensitive and 76% specific predictor of relapse (Peretz *et al*, 2016). Similarly, in infectious diarrhoea, such as bacterial infections FCAL levels were found to correlate to disease severity and consequently influencing the treatment regimen (McMahon and Chhabra, 2018).

The differences between the diverse *Cryptosporidium* population, species, subtypes, and subtype families were indicated as regard the disease severity and clinical manifestations outcome (Cama *et al*, 2007). On the species level, various studies implied the higher severity of *Cryptosporidium hominis* infection compared to *C. parvum.* Bushen *et al*. (2007) compared *Cryptosporidium hominis* and *Cryptosporidium parvum* course of illness in Brazilian children, they concluded that *Cryptosporidium hominis* results in heavier infections with higher risk of stunted growth. Chappell *et al.* (2006) investigated the experimental challenge of healthy volunteers with *Cryptosporidium hominis* oocysts, they reported that the occurrence of diarrhoea and oocysts shedding increased with the increase of the infecting dose, and no cases asymptomatic shedding were observed unlike *Cryptosporidium parvum* exposure ones. Moreover, *Cryptosporidium hominis* infection was suggested to be associated with a higher risk of extraintestinal sequalae in immunocompetent patients rather than *C. parvum* (Hunter *et al,* 2004). However, in the immunocompromised patients, Dey *et al.* (2016) reported that the *C. hominis* infection with was accompanied with higher parasite burden and more frequent nausea and vomiting compared to *Cryptosporidium parvum.*

Conclusion

The prevalence of cryptosporidiosis in the geriatric patients was significantly higher than in pediatric ones. Genotyping showed that *Cryptosporidium hominis* is frequently circulating species. A high global burden of cryptosporidiosis was mainly among children mainly immunocompromissed ones or malnourished people.

Significantly high FCAL levels in *Cryptosporidium hominis* positive diarrhea compared to negative ones denoted the ongoing inflammatory process in cryptosporidiosis, but unaffected by ages.

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Explanation of figure

Fig. 1: Boxplot representing FCAL levels in *Cryptosporidium* positive and negative cases (A), and in *Cryptosporidium* genotype 1 & 2 (B). (Significant P-value)