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PREVALENCE AND ASSOCIATED RISK FACTORS OF *CYCLOSPORA CAYETANENSIS* AND *CYSTOISOSPORA BELLI* INFECTIONS AMONG ADULT IMMUNE-COMPETENT PATIENTS WITH DIARRHEA ATTENDING MINIA UNIVERSITY HOSPITALS, EGYPT

By

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Abstract

The prevalence of emerging human intestinal protozoan pathogens in immune-competent adults was not clearly determined in Egypt. This study assessed the prevalence of *Cyclospora cayetanensis* and *Cystoisospora belli* and their associated risk factors in Minia Governorate, Egypt. Fecal samples were collected from 190 immunocompetent patients suffering from diarrhea with or without other gastrointestinal manifestations, from June 2018 to February 2019. Questionnaires for demographic and clinical data were filled out for each patient. Fecal samples were examined macroscopically and microscopically followed by modified Ziehl–Neelsen stain to identify oocysts *Cyclospora cayetanensis* and *Cystoisospora belli*. 113 patients (59.5% with 95% CI, 52.5-66.5) out of 190 patients were infected with *Cryptosporidium* spp., *Blastocystis* spp., *Entamoeba histolytica/dispar*, *Giardia intestinalis, Cyclospora cayetanensis*, and *Cystoisospora belli*. Co-infection was detected in 22 cases (11.6%) and single parasitic infection was detected in 91cases (47.9%). *Cyclospora cayetanensis* was reported in 19 cases (10%) While, *Cystoisospora belli* was detected in 3 cases (1.7%). *Cyclospora cayetanensis* and *Cystoisospora belli* infections were significantly associated watery diarrhea and higher consumption of unwashed fruit or vegetable with no role for animal contact in their transmission. The study result implicated that *C. cayetanensis* is an important cause of adult diarrhea.

Keywords: Egypt, patients, *Cyclospora*, *Cystoisospora*, *Cryptosporidium, Blastocystis,* Diarrhea.

Introduction

Coccidia of genus *Cyclospora* and genus *Cystoisospora* (previously *Isospora*) are obligate intracellular apicomplexan parasites that parasitize the gastrointestinal tract of humans and animals causing diarrhea diseases. Although, they were initially recognized as opportunistic parasites in immune-compromised patients, they were also associated with sporadic and endemic disease in immune-competent patients (Legua and Seas, 2013). Human *Cyclospriasis and Cystoisosporasis* are caused by tcoccidian species *Cyclospora cayetanensis* and *Cystoisospora belli* respectively (Legua and Seas, 2013).

Human infection occurs by ingestion of food or water contaminated with sporulated oocysts. Man to man transmission was not reported as the excreted oocysts required 1 to 2 weeks in environment to become infective (Escobedo *et al*, 2009).

Clinical presentations mainly depended on host age and immune status. Asymptomatic or mild infections occur in older children and non-elderly adults. Severe clinical symptoms occur in infants and elderly persons. Mild-to-moderate self-limiting diarrhea is common in immune-competent patients. Patients with immune dysfunction can experience severe disease with prolonged diarrhea, anorexia, and weight loss (Almeria *et al*, 2019; Dubey and Almeria, 2019).

Microscopy using acid-fast staining is the most widely used screening technique for the diagnosis of *C. cayetanensis* and *C. belli* with or without stool concentration (Ribes *et al*, 2004). In wet mount smears, *C. cayetanensis* oocyst is spherical in shape and bet ween 7.5 & 10µm in diameter, while *C. belli* oocyst is ovoid to ellipsoid in shape, 10-40 μm x 10-30μm with only one or two immature sporoblasts. Modified Ziehl-Neelsen sta-

in technique was the gold standard, C. *cayetanensis* oocysts were stained pink to red color against green (blue) background color, while *C. belli* oocysts are stained red (Khanna *et al*, 2014).

PCR gave a limited role for detecting *C. cayetanensis* and *C. belli* in human feces due to high cost (Taniuchi *et al*, 2011) despite its high diagnostic sensitivity. However, no serological assays are commercially available for these parasites (Almeria *et al*, 2019; Dubey and Almeria, 2019).

Although, previous studies documented the presence of *C. cayetanensis* and *C. belli* in Egypt (Abdel-Hafeez *et al*, 2012; Massoud *et al*, 2012; Shehata and Hassanein, 2015; Ismail and Fadl, 2019), there was limited scientific data regarding the prevalence of these parasites among adult diarrheic patients.

The present study aimed to assess prevalence and associated risk factors of *C. cayetanensis* and *C. belli* in immune-competent adult patients with diarrhea attended Tropical Medicine and Gastroenterology Outpatient Clinic of Minia University Hospitals.

Material and Methods

This cross-sectional descriptive study was done on 190 patients, referred to Tropical Medicine and Gastroenterology Outpatient Clinic of Minia University Hospitals from June 2018 to February 2019. All patients were suffered from diarrhea with or without other gastro-intestinal disturbances. Immune-compromised patients or patients with diarrhea due to other causes or those on antiparasitic medications 2 weeks before study were excluded. A structured questionnaire concerning required demographic and clinical data was filled out for each studied case.

Stool examinations: Single fecal sample from each participant was collected in sterile, labeled, plastic containers with tight-fitting covers, and transported to the Parasitology Department, Faculty of Medicine, Minia University within 1-3h after collection. The samples were examined following standard procedures (WHO, 2012). The stool samples were initially examined macroscopically and their characteristics such as color and consistency were recorded. Samples were processed by direct wet smear and formalin-ethyl-acetate sedimentation methods for ova and other stages (Cheesbrough, 2009). To identify oocysts of *C. cayetanensis* and *C. belli,* a permanent slide was prepared for each sample, stained with Modified Ziehl-Neelsen acid fast technique. Following fixation with methanol for 5 min, the primary stain (carbon fuchsine) was applied for 10 min. The smear was washed and decolorized with 1% acid alcohol. The counter stain (0.5% malachite green) was then applied for 30 min. The smear was washed off, dried, and examined accordingly (Garcia, 2016). Stained smears were microscopically examined with the high power and oil immersion objective.

Ethical approval: The study protocols and the consent forms were reviewed and approved by the Scientific Ethical Committee of Faculty of Medicine, Minia University on April 2018. All participants provided written informed consent after the study procedures were explained.

Statistical analysis: Data were entered in MS Excel version 2007 & analyzed using SPSS 24.0 software. Numbers, percentages & mean SD were calculated. Fisher's exact test compared proportions. Multivariate Binary Logistic Regression determined associated risk factors of *C. cayetanensis* and *C. belli* infection with significance at $p < 0.05$.

Results

A total of 190 patients with diarrhea were investigated to assess the prevalence of *C. cayetanensis* and *C. belli.* The mean age of the participants was 33.6±4.8 years, ranging 20-67 years. Most participants were males (117, 61.6%), and the majority come from rural areas (126, 66.3%). Frequent reported clinical manifestations associated with diarrhea were abdominal pain followed by vomiting where they were reported in 86.3% and 61.5% of total patients, respectively. The total prevalence rate of intestinal parasitic

infections in the present study was 59.5% (113/190) (95% CI, 52.5-66.5). The infection rates of male and female patients were 61.5% (72/117) and 56.2% (41/73).

Two or more parasites infection were 22/190(11.6%), but 91/190 (47.9%) with a single parasite. *C. cayetanensis* was 19/190 (10%) those with *C. cayetanensis, Cryptosporidium* spp. coinfection was 3/19 (15.8%), & *C. cayetanensis, E. histolytica/dispar* 1/19 (5.2%). *C. belli* was 1.6% (3/190) with *Blastocystis* spp. co-infected in two of them. *Cryptosporidium* sp., *Blastocystis* sp., *E. histolytica/dispar, G. intestinalis* and *E. coli* were 27.4%, 17.9%, 7.4%, 6.3%, 1.6% respectively. Clinical features were not significantly associated with *C. cayetanensis* or *C. belli* except for diarrhea. *C. cayetanensis* or *C. belli* patients had watery diarrhea (87.5%) than negative patients (50.6%).

C. cayetanensis or *C. belli* was significantly associated with low probability of animal contact and higher consumption of unwashed fruit or vegetables.

Details were given in tables (1, 2, & 3) and figures 1 & 2).

Table 1: Distribution of single and mixed intestinal parasitic infections among patients with diarrhea.

Type of infection	Intestinal parasites	Number & (%)
Single infection	*Cryptosporidium* spp.	37 (19.5)
	Blastocystis spp.	20 (10.5)
	Entamoeba histolytica/dispar	10 (5.2)
	Giardia intestinalis	8 (4.2)
	Cyclospora cayetanensis	15 (7.9)
	Cystoisospora belli	1 (0.5)
Total		91 (47.9)
Mixed infection	*Cyclospora cayetanensis*+ *Cryptosporidium* spp.	3 (1.6)
	Cyclospora cayetanensis+ *Entamoeba histolytica /dispar*	1(0.5)
	Cryptosporidium spp. + *Blastocystis* spp.	6 (3.2)
	Cryptosporidium spp.+ *Giardia intestinalis*	2 (1.1)
	Cryptosporidium spp.+ *Entamoeba coli*	2 (1.1)
	Blastocystis spp. + *Entamoeba histolytica/dispar*	2 (1.1)
	Blastocystis spp. + *Cystoisospora belli*	2 (1.1)
	Blastocystis spp. + *Giardia intestinalis*	1 (0.5)
	Blastocystis spp. + *Entamoeba coli*	1 (0.5)
	Cryptosporidium spp.+ *Blastocystis* spp.+ *Giardia intestinalis*	1 (0.5)
	Cryptosporidium spp.+ *Blastocystis* spp.+ *Entamoeba histolytica/dispar*	1 (0.5)
Total		22 (11.6)

Table 2: Relation between *C. cayetanensis* or *C. belli* infection and clinical presentations among patients with diarrhea.

Clinical presentations	Total patients (n=1840	*C. cayetanensis* or *C. belli*[a] Positive (n=16)	*C. cayetanensis* & *C. belli*[b] Negative (n=168)	P value [c]
Abdominal pain: Yes	160 (87%)	12 (75%)	148 (88.1%)	
No	24 (13%)	4 (25%)	20 (11.9%)	0.233
Nausea: Yes	83 (45.1%)	9 (56.3%)	74 (44%)	
No	101 (54.9%)	7 (43.7%)	94 (56%)	0.433
Vomiting: Yes	113 (61.4%)	10 (62.5%)	103 (61.3%)	
No	71 (38.6%)	6 (37.5%)	65 (38.7%)	0.928
Fever: Yes	95 (51.6%)	5 (31.3%)	90 (53.6%)	
No	89 (48.4%)	11 (68.8%)	78 (46.4%)	0.074
Diarrhea: Watery	99 (53.8%)	14 (87.5%)	85 (50.6%)	
Semi-formed	85 (46.2%)	2 (12.5%)	83 (49.4%)	0.007

[a] Patients with single *C. cayetanensis* or *C. belli* only included, [b] Cases with *C. cayetanensis* or *C. belli* and other intestinal parasites excluded, [c] P-value by Fisher's Exact test

Discussion

Despite substantial global effort and programs organized by the World Health Organization (WHO), intestinal parasitic infections are among the most important causes of morbidity and mortality, particularly in developing countries and one of the most important etiologies of diarrheal diseases (Berhe *et al*, 2018). Since *C. cayetanensis* and *C. belli* caused diarrheal disease in immune-competent and immune-suppressed patients worldwide (Çiçek *et al*, 2011; Ud Din *et al*, 2011), this cross-sectional descriptive study was performed to provide more

informative epidemiological data about the two parasites in Minia Governorate.

Table 3: Risk factors of *C. cayetanensis or C. belli* among patients with diarrhea.

Risk factors	Total patients (n=184) No. (%)	Positive *C. cayetanensis* or *C. belli* [a] (n=16)	Negative *C. cayetanensis* & *C. belli* [b] (n=168)	OR (95%CI) [c]	P value [d]
Male	115 (62.5)	11 (68.8%)	104 (61.9%)	1.09 (0.33-3.64)	
Female	69 (37.5)	5 (31.2%)	64 (38.1%)		0.886
20-35(years)	84 (45.7)	6 (37.5%)	78 (46.5%)	0.58 (0.15-2.19)	
36-50(years)	57 (31)	4 (25%)	53 (31.5%)	0.50 (0.12-2.14)	0.424
> 50(years)	43 (23.3)	6 (37.5%)	37 (22%)		0.351
Residence: Rural	123 (66.8)	11 (68.8%)	112 (66.7%)	2.69 (0.53-13.71)	
Urban	61 (33.2)	5 (31.2%)	56 (33.3%)		0.234
Animal contact: Yes	119 (64.7)	8 (50%)	111 (66.1%)	0.12 (0.02-0.61)	
No	65 (35.3)	8 (50%)	57 (33.9%)		0.011
Raw fruits & vegetables: Yes	89 (48.4)	12 (75%)	77 (45.8%)	7.16 (1.77-28.94)	
No	95 (51.6)	4 (25%)	91 (54.2%)		0.001

[a] Patients with *C. cayetanensis* or *C. belli* only were included, [b] Double infection of *C. cayetanensis* or *C. belli* and other parasites were excluded, [c] OR: Odd's ratio, CI: Confidence interval, [d] P-value of multivariate binary logistic regression.

Overall parasitic prevalence among immune-competent patients with diarrhea was 59.5% (with 95% CI, 52.5-66.5), this prevalence rate was like that reported by Abdel-Hafeez *et al,* (2012) in immune-competent children from the same governorate. The identified intestinal parasites from this study's participants were only protozoan parasites (*Cryptosporidium* spp., *Blastocystis* spp., *Entamoeba histolytica/Dispar*, *Giardia intestinalis, Cyclospora cayetanensis*, *Cystoisospora belli* and *Entamoeba coli*) and many papers from Egypt reported higher protozoan infections than helminthic infections with higher prevalence of *Cryptosporidium* spp. (Baiomy *et al*, 2010; Abdel-Hafeez *et al*, 2012). No helminthic infections were detected in this study might be due increase the awareness of helminthic infections transmission and regular mass treatment of anti-helminthic drugs in governmental schools (Dahesh, 2018).

Co-infection with two or more parasites was recognized in 22 patients, as these parasites almost have the same mode of transmission which is waterborne and foodborne routes. Waterborne protozoan diseases in Minia district were reported by Khalifa *et al,* (2014) especially *Cryptosporidium* spp. *Blastocystis* spp. or *Cryptosporidium/C. cayetanensis.*

C. cayetanensis and *C. belli* were detected in 10% and 1.6% of the participants of this study respectively, while in Minia district was (2.1%) in children in 2012 (Abdel-Hafeez *et al*, 2012). Other papers from Egypt reported a prevalence rate of *C. cayetanensis* ranged from 17 % to 19.6% in the immune-competent children (Abdel-Wahab *et al*, 2008; Massoud *et al*, 2012). The prevalence rate of *C. belli* in Minia district was 6.3% in 2012 (Abdel-Hafeez *et al*, 2012). Reportable data on the prevalence of *C. cayetanensis* and *C. belli* in adult immune-competent were insufficient.

Patients with positive *C. cayetanensis* or *C. belli* more likely had watery diarrhea than negative patients (P=0.007). Several studies and human case reports stated *C. cayetanensis* and *C. belli* as a cause of watery diarrhea in immune-competent and immune-compromised patients (Ozdamar *et al*, 2010; Kim *et al*, 2013; Jiang *et al*, 2018; Almeria *et al*, 2019).

No significant association was between the prevalence of *C. cayetanensis* and *C. belli* and risk factors, including sex, age, residence, and animal contact. However, *C. cayetanensis* and *C. belli* infections were significantly associated with consumption of unwashed fruits and vegetables. This agrees with (Mahmoudvand *et al*, 2019). In addition, the meta-analysis studies done by Almeria *et al*, (2019); Li *et al*, (2019) and Dubey and Almeria (2019) confirmed this association. Furthermore, the *C. cayetanensis* and *C. belli* sporulated oocysts can remain in the environment for a longtime maintaining in-

fectivity. This agreed with Li *et al*. (2020) who reported that *C. cayetanensis* and *C. belli* were frequently recovered from raw vegetables.

Conclusion

Cryptosporidium spp., *Blastocystis* spp. and *Cyclospora cayetanensis* were prevalent among immunocompetent adult patients admitted to Minia University hospital. *C. belli* was infrequently detected in this study. Consumption of unwashed fruit or vegetable is a main route of *C. cayetanensis* and *C. belli* transmission.

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Explanation of figures

Fig. 1: Frequency of intestinal parasites among adult patients with diarrhea.

Fig. 2: *Cyclospora cayetanensis* oocyst and *Cystisopora belli* oocyst in stools stained with Modified Ziehl-Neelsen stain, X100.