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Original Article

Possible Correlation between *Giardia duodenalis* Genotypes and Fecal Calprotectin in Children with Diarrhea

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Abstract

Background: Giardiasis is one of the leading causes of diarrhea, particularly among children under the age of five in developing countries. Fecal calprotectin (FC) is an important biomarker for diagnosis and monitoring of inflammtory bowel disease, but other diagnoses should be considered in light of its elevation. We aimed to evaluate FC level in patients diagnosed with giardiasis and elucidate a possible correlation between *Giardia* genotypes and FC levels.

Methods: Overall, 120 fecal samples were collected from children aged 4-12 years and tested for giardiasis by light microscopy. Out of which, 50 samples were enrolled within two groups: group I "cases" and group II "controls" and then subjected to PCR amplification, sequencing of the beta-giardin (bg) gene of the parasite, and FC evaluation.

Results: Assemblage B was identified in 75%, and assemblage A in 25% of samples. FC levels were statistically elevated in "group I" in comparison to "group II". Likewise, there was a statistically significant difference between FC levels in patients infected with assemblage A and assemblage B with a mean of 114 µg/gm and 202 µg/gm, respectively.

Conclusion: The study highlighted the possible association between *Giardia* genotype B and elevated FC levels, further detailed studies are necessary to clarify these finding.

Introduction

Giardia duodenalis (syn. *G. lamblia* and *G. intestinalis*) is a widespread intestinal parasite of humans and mammals, and is considered as one of the most common parasitic infection with 200 million people infected worldwide posing a serious public health threat (1). Eight assemblages have been identified in *G. duodenalis*, including assemblages A–H, each of which has a distinct host range (1,2). Most targeted genes for molecular studies of *G. duodenalis* depended upon the analysis of glutamate dehydrogenase (gdh), beta-giardine (bg), small subunit ribosomal RNA (SSU rRNA), and triosphate isomerase (tpi) genes have shown that the majority of human infections are caused by the two main genetic assemblages A and B (2). A small number of infections with assemblage E have also been reported (3). Genomic analysis of assemblage A and B isolates have indicated a substantial discrepancy between the two groups suggesting that assemblage "A" and "B" *Giardia* isolates are distinct *Giardia* species (4).

The quest for associations between certain assemblages and well-defined patterns of symptoms has evolved secondarily to the development of tools dissecting the molecular biology of various *Giardia* isolates and the understanding of symptoms spectrum associated with giardiasis (5). The possibility of a link between *G. duodenalis* assemblages and virulence, as evidenced by the likelihood of causing diarrhea and other clinical symptoms, has yielded inconclusive results (6). Nevertheless, differences in assemblage A and B isolates pathogenicity were demonstrated in mice in vivo studies (7).

Since the parasite was first identified, *Giardia* pathogenicity has been debated. At the peak of infection, *Giardia* trophozoites induce pathophysiological processes that result in malabsorptive diarrheal disease. It induces various symptoms ranging from asymptomatic carriage to chronic diarrheal disease (8,9). The mechanisms underlying clinical giardiasis consequences are still unknown, but recent research indicates that *Giardia* strain variability, microbiota composition, co-infecting enteropathogens, host genetically determined mucosal immune responses, immune modulation, and host nutritional status are all important factors (9).

Furthermore, some human studies and experimental evidence indicated that giardiasis might cause pro-inflammatory intestinal responses (10). In adults and even in children, FC, a protein known to be present in stool in neutrophil-mediated intestinal mucosal inflammation, is commonly used as a surrogate marker for intestinal inflammation (10,11). It is suggested as a marker for primary screening and follow-up of inflammatory bowel disease (IBD) patients. The cut-off value of 50 ng/ ml was used to differentiate between IBD and other diarrheal causes (12). However, elevated FC levels have been also identified in infectious diarrhea, including some intestinal parasitic diseases, which correlates with the clinical severity of infectious diarrhea (13). FC level in infectious diarrhea is significantly higher than those shown in irritable bowel syndrome, which has values consistent with the healthy controls (14,15).

The exact cause of elevated FC levels in only some giardiasis cases, whereas other patients may have low levels, is not yet evident. Additionally, there is no apparent correlation between the level of FC and *Giardia* genotypes. To fill some of the above-mentioned knowledge gaps, this study aimed to assess the level of FC in patients diagnosed with giardiasis in comparison to the control group and to elucidate a potential correlation between FC levels and *G. duodenalis* genotypes reported.

Methods

Study design and samples collection

Overall, 120 fecal samples were collected from children aged 4-12 years during the period from October 2019 to March 2020 and tested for giardiasis by light microscopy. Fifty fecal samples only were enrolled within the two studied groups. Group I (cases) included 30 children suffering from diarrhea with or without other gastrointestinal manifestations, their stool examination was positive for *G. duodenalis* by light microscopy. Patients with gastrointestinal bleeding and those receiving anti-inflammatory, antibiotics, or antidiarrheal medications were excluded from the study. Group II (controls) included 20 children of similar social and environmental backgrounds with no known abdominal symptoms and negative fecal samples by light microscopy for *G. duodenalis*.

Both groups were selected from Ain shams University's Hospitals outpatients' clinics and samples were transferred to Ain Shams University's Faculty of Medicine's Parasitology Diagnostic Unit. Any associated–enteric parasites were excluded by direct microscopic examination and Modified Zeihl Neelson (MZN) stain. Stool samples were sent to a microbiology laboratory to perform stool culture to exclude bacterial causes. Any sample with associated bacterial or parasitic infections other than giardiasis was excluded from the study. Collected samples were stored at – 20 °C without adding any preservatives for further analysis.

Ethical Approval

In accordance with informed consent, privacy and confidentiality of patients sampled and analyzed anonymously during the study, all participants were enrolled.

Molecular genotyping

Following the manufacturer's instructions, genomic DNA was extracted using the QIAamp® Stool mini kit (Qiagen, Germany). DNA extracts were quantified using a spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE) to contain at least one ng of DNA/µL and kept at – 20 °C until further usage. For molecular identification of *G. duodenalis*, a 511-bp fragment of the bg gene was amplified using nPCR Lalle et al. (16) and Caccio et al. (17). Positive (known *G. duodenalis* PCR-positive sample) and negative (no DNA template) controls were routinely integrated in all PCR runs. Secondary PCR products were analyzed using 2% agarose gel (Vivantis) electrophoresis stained with SYBR stain (Invitrogen, Auckland, New Zealand).

Among the positive samples detected by PCR, twelve samples were sequenced using Applied Biosystems 3500XL (Life Technologies, USA) on an ABI 3730 Genetic Analyzer (Applied Biosystems). Acquired sequences were visually inspected using Geneious 9.0.5 software program for quality control and presence of double peaks. Using the BLAST tool, consensus sequences were compared to those previously deposited at the National Centre for Biotechnology Information (NCBI) for assemblages and sub-assemblages assignment (http://www.ncbi.nlm.nih.gov/blast).

Quantitative FC ELISA test

The assessment of FC was conducted using the PhiCal Calprotectin ELISA Kit (Immundiagnostik AG, Bensheim, Germany). The stool sample was processed, and different dilutions from the stool extract were obtained according to the manufacturers' instructions. The optical density of all the kit standards was calculated, and a standard curve was obtained. The values for each sample were plotted on that curve, and the ELISA test was performed in duplicate. A result below 50 ug/gm was considered negative as reported by the manufacturer (18).

Statistical analysis

Data were coded and entered using the statistical package of social science (IMB SPSS) version 11.5 (Chicago, IL, USA) for statistical analysis. The data both qualitative and quantitative were presented. When applicable, the Chi-squared test and Fisher's exact test were used to compare groups. *P*-value was statistically significant if the $P<0.05$. Diagnostic yield (specificity and sensitivity) of the utilized diagnostic tests were conducted.

Results

Overall, 30 *G. duodenalis* positive fecal samples were enrolled as cases (Group I), and 20 samples were as controls (Group II). Microscopic examination and stool culture showed no other parasitic or bacterial infection in any stool sample. The mean age of group I was (6.53 ± 2.240), and the mean age of group II was (7.85 ± 2.540) yr. Group I included 17/30 (56.7%) boys and 13/30 (43.3%) girls, group II included 12 /20 boys (60%) as well as 8/20 girls (40%). A non-significant correlation was found between sex and age distribution between the two groups ($P>0.05$). The main presentation of group I was diarrhea, 10 of them suffered from diarrhea less than two weeks (acute diarrhea), while 20 presented with diarrhea more than two weeks (chronic diarrhea). PCR was done for all samples in both groups (Group I & Group II). Group II was confirmed to be negative for giardiasis by PCR. While in-group I, among the 30 microscopy *Giardia* positive samples, 24 generated the expected products (511 bp).

Genotyping of Giardia duodenalis

Among the 24 positive samples by PCR, 12 were randomly selected for genotyping. Out of the twelve bg sequences analyzed, three were assigned to assemblage A and nine to assemblage B. Correlation between *Giardia* assemblage and patient data demonstrated that assemblage B showed higher incidence in female (55%) and was associated with chronic diarrhea. In contrast, assemblage A patients were presented with acute diarrhea (Table 1).

Table 1: Data of the twelve *G. duodenalis* patients isolates

Sample N	Sex	Age (yr)	Fecal calprotectin	Type of Diarrhea	Assemblage
1	Female	6	193	Chronic	B
2	Female	5	190	Chronic	B
8	Female	4	195	Chronic	B
9	Male	10	230	Chronic	B
14	Male	6	210	Chronic	B
15	Female	6	187	Chronic	B
17	Male	8	110	Acute	A
21	Male	5	205	Chronic	B
22	Female	4	115	Acute	A
23	Male	6	220	Chronic	B
27	Female	5	190	Chronic	B
30	Male	4	118	Acute	A

Among the sequenced samples, neither A+B mixed infections nor host-specific assemblages of feline, canine, nor livestock (C-F) origin were identified. Genetic heterogeneity was more evident at the subtype level in assemblage B. One of the three A sequences was identical to a previously described reference sequence (GenBank accession number AY072723), while the other two varied by three to four single-nucleotide polymorphisms (SNPs) with X85958. There were 4–7 SNPs in the nine B sequences, some of which coincid-

ed to polymorphic (double peaks) positions with AY072725 (Table 2).

Table 2: Genotypic characterization of *G. duodenalis* patients isolates

Isolate	*Genbank accession no*	*Assemblage*	*Reference sequence*	*SNP*
L58	MT641254	A	X85958	4
L78	MT641255	A	X85958	3
L89	MT641256	AII	AY072723	-
L13	MT641257	B	AY072725	5
L16	MT641258	B	AY072725	4
L43	MT641259	B	AY072725	5
L49	MT641260	B	AY072725	5
L53	MT641261	B	AY072725	6
L57	MT641262	B	AY072725	7
L59	MT641263	B	AY072725	5
L69	MT641264	B	AY072725	4
L83	MT641265	B	AY072725	6

SNP: single-nucleotide polymorphisms

FC level Evaluation

FC was tested among enrolled samples within the two groups; a level lower than 50 µg /gm was considered negative. FC means level was 164.03 (37.67) and 48.45 (17.06) µg/gm in group I and group II, respectively, with a statistically significant correlation ($P<0.05$). The cut-off level of FC for predicting giardiasis was perceived to be 92 µg/gm using the receiver operator characteristic (ROC) curve. This cut-off level could predict cases diagnosed by giardiasis using microscopy with 100% sensitivity and 100% specificity. In contrast, patients diagnosed with nPCR showed 80% sensitivity, 100% specificity, 100% PPV, 76.9% NPV, and area under the curve (AUC) 0.893 (Table 3 and Fig. 1). The uppermost level of FC in group I in this study was 230 µg /gm. FC level was elevated in chronic cases with a mean of (186.9 µg /gm) than in acute cases of diarrhea with a mean of (118.3 µg /gm) ($P>0.05$). Moreover, there was a difference between FC in patients infected with assemblage A and assemblage B with a mean of 114 µg/gm and 202 µg/gm, respectively.

Table 3: Receiver operator characteristic curves and predictive value of fecal calprotectin for diagnosis of giardiasis

Variable	*Microscopy*	*n PCR*
Cut off value (ug/gm)	92	92
Sensitivity	100%	80%
Specificity	100%	100%
PPV	100%	100%
NPV	100%	76.9%
AUC	1.000	0.893

PPV: Positive predictive value; NPV: negative predictive values; AUC: Area under the curve

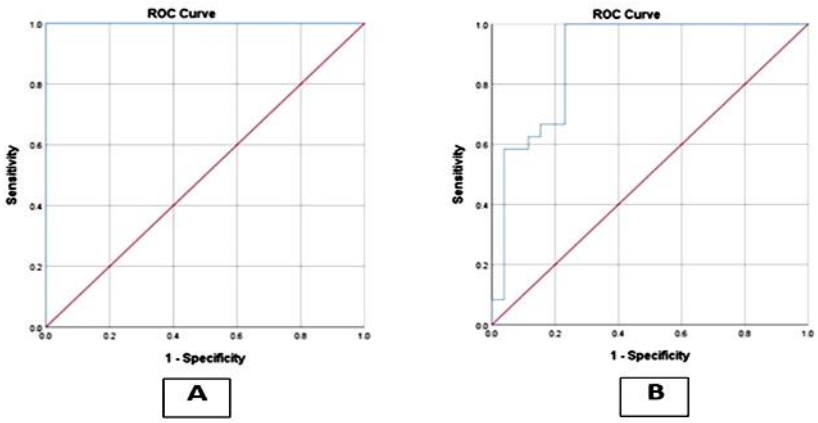

Fig. 1: Receiver operator characteristic (ROC) curves to demonstrate the ability of fecal calprotectin (FCP) to predict giardiasis as diagnosed by microscopy (A) and nPCR (B)

Discussion

High FC level is an indicator of inflammation of the intestinal mucosa resulting from neutrophil migration toward the intestinal tract. It correlates to the degree of intestinal inflammation (19). It is mainly used as a marker of IBD but can also be elevated in some cases of bacterial and viral enteritis (20, 21). The gastrointestinal tract symptoms caused by intestinal parasitic infections can correlate with inflammatory bowel symptoms, which lead to difficulties with its diagnosis and treatment (12). There are only few studies that evaluate the relationship between intestinal inflammatory markers and parasitic infections, and the evidence regarding the importance of FC in parasitic infections is conflicting (11, 15, 22, 23). However, there might be a link between FC levels and severe infections caused by intestinal parasites such as *Schistosoma mansoni* and *G. intestinalis* (11, 15).

G. duodenalis is an essential protozoan parasite that causes intestinal manifestations. Some researchers discussed the possibility of *Giardia* infection to cause an inflammatory response resulting in the elevation of FC levels (24). In contrast, others indicate a less role of pathogenicity attributed to giardiasis (25). Unfortunately, there is a lack of research that correlates FC elevation with the genotypes of *Giardia*. Moreover, there is a lack of reference ranges for FC levels obtained specifically in giardiasis patients (24). Thus, one of the aims of the present work was to highlight a possible correlation between the level of FC and *Giardia* genotypes.

The present study included 30 *Giardia*-positive cases and 20 *Giardia*-negative controls by light microscopic examination. Out of the 30 *Giardia*-positive samples, 24 (80%) were confirmed using bg nPCR. The sensitivity of PCR in detecting *G. duodenalis* is known to be higher than that of conventional microscopy (26). The discrepancy between PCR amplification of DNA and some positive microscopy samples in the current study could be attributed to the presence of inhibitors in stool samples, cyst quantity and quality, sample storage conditions, DNA extraction technique, number of copies of the target genes, and primer and cycling conditions selection (27). Twelve *Giardia* positive samples were genotyped at the bg locus and two genetically distinct assemblages A and B of *Giardia* infections were detected. Assemblage B was predominant (9/12 or 75%) in comparison to assemblage A (3/12 or 25%), indicating that the transmission of human giardiasis in the current study was primarily anthroponotic. Our results were closely related to assemblage B distributions previous-

ly reported in Egypt (28, 29), and in contrast to other studies in Saudi Arabia, Iran, and Yemen where assemblage A was predominant (30-32).

Attempts to ascribe clinical variability and pathogenicity based on assemblage designation have been inconclusive, despite genetic divergence (10). Few studies have found an association between symptomatic assemblage B infection and asymptomatic assemblage A infection (30, 33). While strong correlation of persistent diarrhea with assemblage B and intermittent diarrhea with assemblage A was described in some reports (34). Similar findings were also found in our results as cases presented with chronic diarrhea were infected with assemblage B, while assemblage A cases were presented with acute diarrhea.

In the present study, the FC levels were elevated in *Giardia* positive cases (Group I) exceeding 50 ug/gm, with a significant difference between this group and the control group (Group II). In agreement, *G. duodenalis* could raise the FC level (11). Moreover, an increased FC level was reported in a case of chronic giardiasis attributed it to the inflammatory response caused by the chronic exposure to infection (24). In contrast, there was a non-significant association between the presence of *G. duodenalis* in stool and high FC (35). In this study, the cut-off value of FC was 92 µg /gm with an uppermost level reaching 230 µg /gm, which is lower than the cut-off value demonstrated by IBD in other studies. Jha et al., (36) reported a cut-off value of 188 µg/gm as a predictor for differentiating inflammatory bowel syndrome from ulcerative colitis. With an uppermost level reaching 3000 µg /gm in case of active ulcerative colitis. This difference between the cut value in *Giardia* infection and IBD may be attributed to the degree and extent of mucosal inflammation caused by different diseases. FC level is considered the only marker that can significantly differentiate between different endoscopic grades of infections (36).

In this study, the FC level (92 µg /gm) had a higher sensitivity for predicting *G. duodenalis* infection by microscopy than nPCR, while the specificity was the same using both techniques. The discrepancy might be since microscopy detected more *Giardia*-positive cases than PCR in our results. Our results also showed that FC levels were higher in patients with chronic diarrhea (186.9 µg /gm). In agreement, FC could be used to screen patients newly referred for chronic diarrhea (37). The mean FC level in assemblage B patients (202 µg/gm) was higher than assemblage A patients (114 µg/gm) indicating a possible higher pathogenic role for assemblage B. Similarly, assemblage B is considered more virulent in recent reports and more common in outbreaks (3). Likewise, the parasite genotype might determine the degree of intestinal inflammation and that *G. duodenalis* assemblage B can cause a high degree of mucosal damage associated with infiltration of inflammatory cells (38, 39). Assemblage B might cause more duodenal inflammation in adults with elevated FC levels as well (11). Elevated FC level in this study could be attributed to the degree of intestinal inflammation as most patients had more prolonged exposure to infection-causing chronic diarrhea. Moreover, it may be due to *Giardia* genotyping, as genotype B was more evident.

Conclusion

This study highlighted a possible correlation between the elevated FC levels and *Giardia* assemblage B and the usability of FC with an estimated Cut-off value for assessing gastrointestinal tract infection caused by giardiasis. However, an accurate estimation of giardiasis long-term effect on child growth is a complex task, due to the number of potential factors that should be considered. Extensive comparative genomics studies on several assemblages A and B isolates are required to ascertain strain-specific pathogenicity.

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None

Conflict of interest

The authors declare that there no competing interests.

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