

FECAL CALPROTECTIN LEVELS AND PRESENCE OF *HELICOBACTER PYLORI* CO-INFECTION IN INDIVIDUALS WITH ENTEROBIASIS

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ABSTRACT

Enterobiasis, caused by the nematode *Enterobius vermicularis*, is the most common parasitic infection in Bulgaria and worldwide. It causes damage to the colonic mucosa and impaired local immunity, which can lead to a predisposition to other intestinal infections.

Faecal calprotectin is a non-invasive biomarker that can be used to distinguish between inflammatory and non-inflammatory bowel diseases. Scientific data show varying degrees of increase in some bacterial and viral infections, but data on parasitic infections are extremely scarce. *Helicobacter pylori* is a Gram-negative bacterium that colonizes the gastric mucosa. It is one of the most common bacteria, with infection mostly occurring during childhood and remaining undiagnosed for a long time. This can lead to severe damage to the gastric mucosa.

This study aimed to determine the levels of faecal

calprotectin in patients with enterobiasis, and to assess its significance as a marker for intestinal inflammation caused by *E. vermicularis*, as well as the presence of concomitant infection with *H. pylori*.

The study included 124 individuals with confirmed *Enterobius vermicularis*, aged between 1 and 49 years. Calprotectin levels were determined using a commercial kit (LIAISON®, DiaSorin). The *Helicobacter pylori* test was performed using an automated immunoassay to qualitatively determine the presence of *H. pylori* antigen in faecal samples (LIAISON®, DiaSorin). Faecal samples from 20 healthy individuals were also tested in parallel as a control group.

H. pylori was detected in faecal samples from 14.5% (n = 18) of *Enterobius*-infected individuals (compared to none of the healthy individuals), suggesting an increased susceptibility to co-infection with this bacteria among susceptible individuals. Significantly elevated faecal calprotectin values were found in three participants with enterobiasis, and borderline values in other 16, indicating the presence of moderate intestinal inflammation in some patients with enterobiasis.

Keywords: *Enterobius vermicularis*, *Helicobacter pylori*, faecal calprotectin

INTRODUCTION

Calprotectin is a heterodimeric protein that is principally derived from the cytoplasm of neutrophils. The substance was demonstrated to possess direct antimicrobial properties, thereby contributing to the regulation of innate immune response [1]. It belongs to the S100 calcium-binding leukocyte protein family and consists of two monomers in mammals: S100A8 and S100A9. This complex is named calprotectin based on its Ca²⁺ binding and its activity against *Candida albicans* [2]. Calprotectin changes conformation when it binds to calcium, making it resistant to the proteolytic intestinal or faecal environment. [1]. The substance is initially secreted by myeloid cells in response to danger signals [3] and tissue inflammation is mediated by S100A8 and S100A9 synthesis through transcriptional induction in epithelial cells [4]. The initial triggering of the inflammatory response may result from mucosal surface infection, trauma or environmental stress. However, in case of prolonged, untreated inflammation, calprotectin can lead to mucosal damage and various pathological processes in skin, lungs and intestines [5].

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Calprotectin is produced by various cells (neutrophils, monocytes, dendritic cells, activated macrophages, oral keratinocytes and squamous mucosal epithelium) in response to inflammation [3]. Røseth et al. (1999) first proposed faecal calprotectin levels as a biomarker of mucosal inflammation, and demonstrated a close correlation between faecal leukocyte excretion and the concentration of this protein [6]. Since then, many studies have confirmed those results, and the measurement of calprotectin is now widely accepted as a validated, non-invasive way of assessing intestinal inflammation [7]. The expression of calprotectin in healthy individuals is limited to a small number of specialized cells. In various pathological conditions, measuring calprotectin shows high sensitivity in detecting inflammation, but low specificity in determining its cause [7]. For instance, significantly increased faecal calprotectin concentrations were detected in cases of infection with *Salmonella* (765 µg/g), *Campylobacter* (689 µg/g), or *Clostridium difficile* (740 µg/g), and these levels correlated with disease severity [8]. In 2014, Lam et al. found a significant positive correlation between the presence of various gastrointestinal pathogens and faecal calprotectin levels [9]. In 2019, Rady et al. reported a significant association between calprotectin levels and infection with *Cryptosporidium parvum* and *Giardia lamblia* [10]. Some authors suggested that measuring calprotectin levels is an effective way to determine the pathogenicity of *Blastocystis hominis* in human infections [11].

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that colonises gastric mucosa. Since its discovery in 1982, comprehensive studies have provided growing evidence of its role in various gastrointestinal disorders [12, 13]. This pathogen is transmitted via oral-oral, faecal-oral and gastro-oral routes [14], and therefore socioeconomic and environmental factors play important role in its transmission [15]. After ingestion, *H. pylori* moves through the mucosal layer to reach the gastric epithelium, where it colonises the mucosa [16]. Through an arsenal of virulence factors, it is able to manipulate host cell signalling [17], triggering a cascade of events that disrupt cellular functions and lead to gastric pathologies [18]. The dysregulation of various cellular pathways caused by *H. pylori* leads to morphological changes in cells, disruption of cell polarity and possible initiation of oncogenic process [19]. *H. pylori* is the most common

bacterial infection in humans. It is found in around half of world's population [20], and its prevalence increases during the first ten years of life [21]. The infection occurs in early childhood and remains latent for years, with only about 30% of the infected developing noticeable symptoms. Therefore, accurate identification of *H. pylori* infection is crucial for effective treatment and limiting of its spread.

Enterobius vermicularis is a widespread intestinal parasitic pathogen whose transmission mainly occurs through ingesting infectious eggs [22]. These eggs hatch into larvae and adults once inside the body, primarily affecting the lower ileum, cecum, and ascending colon [23]. Enterobiasis is most often associated with perianal itching, but in certain cases, it can present with diarrhoea, abdominal pain and/or altered bowel habits [24]. It can also present with a clinical picture of chronic inflammatory disease [23]. We did not find any literature data on biomarkers for assessing the degree of intestinal inflammation in enterobiasis. Data on the presence of a concomitant *H. pylori* infection in patients with enterobiasis is also lacking. The present study aimed to determine the effects of *E. vermicularis* on the normal gastrointestinal function by measuring faecal calprotectin levels, and to establish the presence of concomitant *H. pylori* infection.

MATERIALS AND METHODS

Patients and Samples

Faecal samples from 124 individuals with proven enterobiasis, aged between 1 and 49 years (mean age 7.07 years), were examined. Of these, 64 (52%) were female and 60 (48%) were male. Faecal samples from 20 healthy individuals with negative laboratory results for intestinal protozoa and helminths were also examined. Of them, seven were male and 13 were female, with an average age of 3.16 years.

Methods:

Parasitological diagnosis. *E. vermicularis* was diagnosed through microscopic examination of perianal impressions taken with transparent adhesive tape (Graham's method), revealing the presence of characteristic pinworm eggs. Microscopic examination of perianal impressions and faecal samples from patients with enterobiasis excluded the presence of other intestinal protozoa and helminths.

Determination of faecal calprotectin levels. was carried out with a chemiluminescent in vitro assay

(CLIA) designed for the quantitative measurement of calprotectin in human faecal samples (DiaSorin LIAISON Calprotectin Assay), and a LIAISON analyser. The LIAISON Calprotectin test is a sandwich-type assay that uses two monoclonal antibodies to capture and detect calprotectin. Calprotectin is first extracted from faecal samples using Device Plus LIAISON® Q.S. automated extraction tubes. The sample is incubated with a calibrator or control solution containing test buffer and paramagnetic particles coated with a monoclonal antibody that recognises the calprotectin heterocomplex. A wash cycle removes unbound material. An isoluminol-conjugated monoclonal antibody that recognises calprotectin is added to the reaction mixture and incubated. Unbound conjugate is removed in a second wash step. Initial reagents are added and a rapid chemiluminescent reaction begins. The resulting light signal is measured by a photomultiplier in relative light units (RLU) and is proportional to calprotectin concentration in stool samples or calibrators and controls. The LIAISON analyser automatically calculates calprotectin concentration in the sample (in µg/g). A result of less than 50 µg/g indicates normal calprotectin level, and more than 200 µg/g - elevated level. Concentrations between 50 and 200 µg/g are considered borderline. It is recommended that faecal calprotectin levels are re-evaluated in 4–6 weeks to determine inflammatory status. The LIAISON Calprotectin Assay measures faecal calprotectin concentrations in the range of 5 to 800 µg/g. Values below 5 µg/g should be reported as <5 µg/g. The highest reportable value without sample dilution is 800 µg/g.

The qualitative detection of *H. pylori* antigen in faecal samples was performed using the DiaSorin LIAISON Meridian *H. pylori* SA test. This one-step, delayed-release sandwich assay uses monoclonal antibodies to detect *H. pylori* antigen in stool. The test uses a 200 µL sample consisting of a mixture of sample diluent and *H. pylori* antigen extracted from stool. The sample is incubated with paramagnetic particles that are coated with a capture antibody for this antigen. Following this, an isoluminol-conjugated antibody for the *H. pylori* antigen is added to the reaction and left to incubate. Following the second incubation, unbound material is removed via a wash cycle. The starting reagents are then added and a rapid chemiluminescent reaction begins. The resulting light signal is measured by a photomultiplier in relative light units (RLU) and is

proportional to the concentration of *H. pylori* antigen in the stool samples or the calibrators and controls. The analyser automatically calculates *H. pylori* antigen levels in stool samples and expresses the results as index values. The cut-off value used to distinguish the presence of *H. pylori* antigen from its absence is set at an index value of 1.1. The detection limit for *H. pylori* antigen in stool samples is 4.0 ng/ml.

Statistical methods:

The Statistical package SPSS, Version 19 was used. Indicators such as frequencies, means, and χ^2 were determined. Values of $p \leq 0.05$ are statistically significant.

RESULTS

Fecal Calprotectin Levels in Patients with Enterobiasis and in the Studied Control Group

Determination of fecal calprotectin concentrations in healthy individuals ($n = 20$) showed elevated levels: 189 µg/g in one child (a two-year-old girl) and a borderline result of 63.5 µg/g in one five-year-old boy. The remaining 18 individuals (90%) in the control group had normal faecal calprotectin values (see Table 1).

Table 1. Faecal calprotectin levels in healthy controls and in individuals with enterobiasis.

Studied Group	Fecal calprotectin levels (number of subjects)			Total
	Unmagnified	Borderline	Increased	
Healthy individuals ($n=20$)	18 (90%)	1 (5%)	1 (5%)	20 (100%)
Persons with enterobiasis ($n=124$)	105 (85%)	16 (13%)	3 (2%)	124 (100%)

Within the cohort of *E. vermicularis*-infected subjects, a total of 19 individuals (15.3%) exhibited elevated or borderline calprotectin concentrations. No statistically significant difference in this indicator existed in comparison with the control group ($\chi^2 = 0.39$, $p = .531$).

The three subjects with elevated fecal calprotectin values (over 200 µg/g) were children without clinical complaints: a 4-year-old girl (267.6 µg/g), a 4-year-old boy (667.6 µg/g) and an 11-year-old boy (485 µg/g). The mean concentration of calprotectin in the faeces of patients with increased levels was 473.4 µg/g. Borderline concentrations (between 50 and 200

µg/g) were reported in 16 infected individuals (12.9% of those examined), all of whom were children. Of these, 9 (56.2%) were boys and 7 (43.8%) were girls, with no statistically significant sex-associated difference in calprotectin levels ($p = 0.674$). Levels ranged from 54.5 to 194.1 µg/g, with a mean of 93.7 µg/g. Of the individuals with borderline faecal calprotectin levels, eight (50%) reported clinical symptoms (seven with perianal itching and one with abdominal pain), while the rest had no such symptoms (Table 2).

Table 2. Clinical manifestations in individuals with enterobiasis and borderline levels of faecal calprotectin.

Male sex	Female sex	Age (years)	Clinical symptom	Fecal calprotectin concentration µg/g
	1	1	perianal itching	108,5
1		3	asymptomatic	194,1
	1	7	perianal itching	100,2
	1	8	perianal itching	154,2
	1	18	abdominal pain	95,8
	1	5	asymptomatic	54,5
1		6	perianal itching	104,0
1		8	asymptomatic	65,7
	1	9	asymptomatic	114,0
1		9	asymptomatic	61,6
	1	7	perianal itching	69,5
1		6	perianal itching	69,4
1		4	asymptomatic	104,0
1		5	asymptomatic	71,7
1		5	asymptomatic	68,3
1		6	perianal itching	63,8

Presence of *Helicobacter pylori* infection in healthy and *Enterobius*-infested individuals

Helicobacter pylori was not detected in any of the tested healthy individuals.

Among patients with enterobiasis, concomitant *H. pylori* infection was detected in 18 individuals (14.5%). (Table 3). The positive index values obtained for *H. pylori* ranged from a minimum of 1.16 to a maximum of 46.2 (average 8.38 ± 45.04).

Ten female individuals (56%) and eight male individuals (44%) were found to have co-infection, with no

Table 3. Characteristics of patients with enterobiasis co-infected with *Helicobacter pylori*.

	Number	%
Total persons with co-infection	18	14.5
Sex:		
- female	10	56
- male	8	44
Age group:		
- children	16	89
- adults	2	11
Presence of clinical symptoms:		
- yes	9	50
- no	9	50

statistically significant difference between the two groups ($p = 0.571$). Sixteen out of the 18 co-infected individuals (89%) were children and two (11%) were over 18 years of age (Table 3).

Of the individuals with enterobiasis and concomitant *H. pylori* infection, nine (50%) had anamnestic data of clinical symptoms, while the remaining nine had no symptoms (Table 4).

Table 4. Demographic and clinical data for individuals with enterobiasis and a concomitant infection with *Helicobacter pylori*.

Number	Positive index (PI CLIA)	Sex	Age	Clinical symptom
1.	2.57	female	49	asymptomatic
2.	1.64	female	37	diarrhea
3.	25.7	male	6	abdominal pain
4.	3.84	female	1	perianal itching
5.	17.10	male	3	asymptomatic
6.	7.24	female	5	asymptomatic
7.	12.7	female	7	diarrhea
8.	2.37	female	4	asymptomatic
9.	3.09	male	5	bruxism
10.	4.62	female	9	asymptomatic
11.	4.78	female	6	asymptomatic
12.	4.69	female	6	perianal itching
13.	5.52	male	3	bruxism
14.	46.2	male	6	perianal itching
15.	2.21	male	12	asymptomatic
16.	1.16	female	5	asymptomatic
17.	2.92	male	4	asymptomatic
18.	2.56	male	3	abdominal pain

The analysis of results showed that, the values of faecal calprotectin in were not increased in 14 (78%) and were borderline in 4 (22%) of the individuals co-infected with *Helicobacter pylori*. Increased calprotectin values in faeces were not observed in any of the co-infected cases, and no association was established between the two indicators ($p=0.360$) (Table 5).

Table 5. Fecal calprotectin levels in patients with enterobiasis, according to the presence or absence of a *Helicobacter pylori* infection.

	Fecal calprotectin levels			Total
	Unmagnified	Borderline	Increased	
<i>Helicobacter pylori</i> (-)	91 (86%)	12 (11.3%)	3 (2.8%)	106
<i>Helicobacter pylori</i> (+)	14 (78%)	4 (22%)	0 (0%)	18
Total:	105	16	3	124

DISCUSSION

Since its identification and characterisation in 1980, calprotectin has been recognised as a highly validated, non-invasive biomarker for assessing intestinal inflammation. Its levels are used to guide diagnostic and therapeutic decisions in a number of intestinal diseases [7]. Furthermore, calprotectin allows the monitoring of intestinal inflammation and can rule out the diagnosis of inflammatory bowel disease in symptomatic patients. Methods for testing faecal calprotectin are simple and painless to perform [1]. According to the majority of available techniques, a concentration of 50 µg/g of faeces is considered normal in adults and children over four years of age, with no gender-based differences [25]. However, a moderate increase in levels up to 100 µg/g has been observed in individuals aged over 65 [26]. Faecal calprotectin levels are also physiologically higher in newborns and young children, probably due to their initial exposure to food allergens [27].

Studies have found increased levels of faecal calprotectin in cases of bacterial and viral enteritis [8]. In parasitic infections, faecal calprotectin was shown to be significantly associated with the presence of *C. parvum* (53% positive), *G. lamblia* (46% positive) and *H. pylori* (21% positive at over 200 µg/ml) [10]. Aykur et al. (2020) found increased concentrations of this protein in individuals infected with the parasite *Dientamoeba fragilis*, indicating the parasite's pathogenicity [28]. Shapiro et al. reported very high levels of faecal calprotectin in a child with giardiasis and concluded that it can increase significantly in cases of persistent or severe giardiasis [29].

Of the patients with enterobiasis in our study, only three had elevated levels of faecal calprotectin and 16 had borderline values for this indicator. Together with the lack of a statistically significant difference regarding the number of individuals with elevated or borderline concentrations of faecal calprotectin in the uninfected control group, this finding suggests that enterobiasis is characterised by a relatively weak increase of this protein. Similar results have also been reported for faecal calprotectin levels in patients with certain viral infections [8]. According to several authors, slight elevations in faecal calprotectin concentrations should be interpreted with caution. Some reports suggest that measuring faecal calprotectin levels is an effective way of determining the pathogenicity of certain intestinal parasites,

such as *Blastocystis hominis* [11]. Rady et al. (2019) investigated the effects of intestinal parasites on faecal calprotectin levels and concluded that intestinal protozoa induce high levels of faecal calprotectin, unlike intestinal helminths. The authors suggested that this may be a strategy to evade the host immune response [10]. Research on the impact of helminths on gut microbiota and host immunity is currently limited, and their role in the development of autoimmune and inflammatory bowel diseases is not yet fully understood. Further targeted studies are required in order to achieve a comprehensive understanding of this interaction [30].

It is well-documented that humans are frequently exposed to multiple pathogens, either concurrently or in succession, leading to co-infections [31]. Co-infecting pathogens have the capacity to form synergistic or antagonistic relationships, which may affect host immune defences and the severity of diseases. In certain instances, co-infections have been observed to modify the metabolic microenvironments of both the host and the pathogen. This modification has the potential to compromise host immune status and influence microbial pathogenicity by affecting tissue colonization. The immunosuppressive effects of parasitic invasions was demonstrated to reduce the efficacy of vaccines, hinder the development of immunological memory and consequently diminish protection against co-infecting pathogens [31].

H. pylori belongs to the *Helicobacteraceae* family [32]. After ingestion, this transmissible, pathogenic, Gram-negative, spiral bacterium becomes a true colonizer of the gastric mucosa [15]. The presence of *Helicobacter pylori*, is associated with the development of a range of upper gastrointestinal disorders including chronic gastritis, peptic ulcer, MALT (gastric mucosa-associated lymphoid tissue) lymphoma and gastric cancer [33]. There are various diagnostic methods of this infection, including a blood test to determine the presence of *Helicobacter pylori* antibodies (which can persist for years after complete eradication of bacterium), urea breath tests, endoscopic biopsies, and stool antigen tests. The latter detect *Helicobacter pylori* antigens in faeces, indicating the presence of the bacterium in stomach. The faecal test can be used to determine the effectiveness of treatment. In recent years, highly sensitive molecular biological methods were also developed for the rapid and precise detection of this bacterium in various

clinical materials [34]. *Helicobacter pylori* infection is widespread, affecting more than half of the world's population [35]. It most often infects the stomach during childhood. The prevalence of *H. pylori* infection varies significantly in childhood, with reported rates of up to 90% in some underdeveloped countries, whereas in developed countries, the rate ranges from 1.8% to over 65% [36, 37]. Two studies show higher positive rates in boys than in girls, but data on gender association are contradictory [38]. The average prevalence among symptomatic children in Bulgaria is 24.5% [39]. Poor hygiene, consumption of unwashed fruit and vegetables, and failing to wash hands after school are important risk factors for *H. pylori* infection [40].

Helicobacter pylori infection is usually asymptomatic, meaning that undiagnosed infection can lead to chronic inflammation [41]. This chronic inflammation can lead to persistent infection of the gastric mucosa, resulting in gastric atrophy and intestinal metaplasia [42]. According to Rady et al. (2019), the severity of gastric inflammation is determined by multiple host and bacterial factors, such as the presence of comorbidities. They found that faecal calprotectin levels were significantly associated with *H. pylori* infection (21% of those infected had levels above 200 µg/ml), and that *H. pylori* co-infection with intestinal parasites is common [10].

We could not find any data on co-infection with *Helicobacter pylori* in patients with enterobiasis in the literature. Our study showed a high prevalence of this pathogen among individuals infected with *Enterobius vermicularis* – 14.5%, compared to no *H. pylori* infection in the control group. Enterobiasis is widespread, affecting over 1 billion people worldwide [43], and has been reported in both temperate and tropical regions. This parasitic helminthiasis primarily affects young children and usually occurs within families or groups, particularly in crowded living conditions combined with poor personal hygiene [44]. The main factors that can lead to infection with both *Enterobius vermicularis* and *H. pylori* are poor socioeconomic conditions, poor hygiene, and the consumption of contaminated food and unfiltered water [32, 43]. The common risk factors and widespread prevalence of these pathogens, particularly among children, highlight the importance of identifying and treating them. It is widely acknowledged within the scientific community that intestinal parasitic infections have the

potential to result in secondary immunodeficiency. [45]. The exact mechanisms of interaction between *Enterobius vermicularis*, *Helicobacter pylori*, and the diseases they cause are difficult to determine. Nevertheless, there is evidence suggesting that the interaction between *Helicobacter pylori* and intestinal parasites can have serious health consequences, as it can have a significant impact on tissue integrity and the intestinal microbiome balance [31].

Co-infections involving bacteria and parasites are common due to their overlapping ecological niches and inadequate disease control [46]. Co-infection with multiple pathogens often leads to a lack of proper diagnosis due to overlapping symptoms that resemble those caused by the individual agents. This can result in delayed treatment, allowing the pathogens to multiply, and leading to disease progression. Investigating the presence of concomitant infections in patients with enterobiasis is important because it contributes to their complete recovery and limits the further spread of pathogens. Due to the prolonged natural course of these infections and the necessity of treatment, parallel examination is recommended, particularly within family and children's groups.

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