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ORIGINAL PAPER

In vitro sensitivity of *Blastocystis hominis* to garlic, ginger, white cumin, and black pepper used in diet

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Abstract To determine the growth pattern and in vitro susceptibility of *Blastocystis hominis* to metronidazole (MTZ), garlic, ginger, white cumin, and black pepper. Stool specimens were collected from 16 irritable bowel syndrome (IBS) and 10 controls between July–November 2010. Stool microscopy and culture for *B. hominis* was performed. Drug susceptibility assays was done using 0.01 and 0.1 mg/ml of MTZ, garlic, ginger, white cumin, and black pepper. Effect was assessed on *B. hominis* culture after 48 h. Stool DNA was extracted using stool DNA extraction kit (Qiagen) and polymerase chain reaction (PCR) done using subtype-specific sequence-tagged-site primers. *B. hominis* genotype 3 and coinfection of 1 and 3 tended to grow well in culture compared to isolated type 1 infection. Exposed to MTZ at a concentration of 0.01 mg/ml, 38% (6/16) *B. hominis* from IBS did not grow in culture compared to 100% (10/10) of *B. hominis* from control (p=0.001). When they were exposed to MTZ at 0.1 mg/ml, 56% (9/16) *B. hominis* from IBS did not grow in cultures compared to 100% (10/10) from control (p=0.01). Forty-four percent (7/16) *B. hominis* from IBS did not grow in culture compared to 100% (10/10) *B. hominis* from control when exposed to garlic at a concentration of 0.01 mg/ml (p=0.003) and following exposure to garlic at 0.1 mg/ml, 38% (6/16) *B. hominis* from IBS did not grow in cultures compared to 100% (10/10) from control (p=0.001). *B. hominis* isolates from IBS had a cell count of 6,625 at a MTZ concentration of 0.01 mg/ml that reduced to 1,250 as MTZ concentration was increased to 0.1 mg/ml (p=0.08). *B. hominis* from IBS with a mean cell count of 3×10^5 at baseline decreased to 1×10^4 when exposed to garlic at 0.01 mg/ml (p<0.001) and to 1×10^3 (p<0.001) when garlic was 0.1 mg/ml. *B. hominis* from IBS cell count decreased to 1×10^5 when exposed to white cumin at 0.01 mg/ml (p=0.01) and to 1×10^5 (p<0.001) when white cumin was 0.1 mg/ml. Exposed to black pepper at 0.1 mg/ml, cell count of *B. hominis* from IBS decreased to 1×10^5 (p=0.01). *B. hominis* from IBS decreased to 1.3×10^5 exposed to ginger at 0.01 mg/ml (p=0.001). *B. hominis* isolates were mostly genotypes 3, type 1 and 3 coinfection, and non-typeable *B. hominis* isolates. *B. hominis* isolates from IBS mostly genotype 1 demonstrated an increased sensitivity to garlic at 0.01 mg/ml with a *B. hominis* cell count of 3,714 compared to 6,142 when exposed to 0.01 mg/ml of MTZ. However, this sensitivity did not increase as garlic concentration was increased to 0.1 mg/ml, for *B. hominis* cell count was 6,000 compared to 1,428 as MTZ was increased to 0.1 mg/ml.

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Introduction

Blastocystis hominis is one of the most common intestinal protozoa which is found in large intestine in humans. It appears to infect both immunocompetent and immunocompromised individuals. Although several reports have suggested that *B. hominis* could cause gastrointestinal disorders, the specific pathogenicity of this organism has not yet been defined. The clinical consequences of *B. hominis* infection are mainly diarrhea or abdominal pain with nonspecific gastrointestinal symptoms such as nausea, anorexia, vomiting, weight loss, lassitude, dizziness, and flatulence. It has been speculated that thick-walled cysts might be responsible for external transmission. The various mechanisms suggested for *B. hominis*-mediated gastroin-

testinal symptoms include adherence of *B. hominis* to the gut epithelium, triggering a lysis mechanism as shown for *Entamoeba histolytica*, *Giardia lamblia*, and existence of a diarrheagenic toxin.

Various antibiotics that include metronidazole, furazolidone, nitazoxanide, sulfamethoxazole/trimethoprim, etc. have been previously used for the treatment of diarrhea and enteritis associated with *B. hominis* as the sole identified pathogen in children and adults (Dunn and Boreham 1991). In a randomized, single blind study, Dinleyici et al. (2010) compared no treatment to the efficacy of *Saccharomyces boulardii* or metronidazole (MTZ) for the duration of diarrhea and the duration of colonization in children with gastrointestinal symptoms and positive stool examination for *B. hominis*. On day 15, clinical cure was observed in 77.7% in group A (n=18); in 66.6% in group B (n=15); and 40% in group C (n=15) (p<0.031, between groups A and C). At the end of the first month after inclusion, clinical cure rate was 94.4% in group A and 73.3% in group B (p=0.11). Parasitological cure rate for *B. hominis* was very comparable between both groups (94.4% vs. 93.3%, p=0.43) (Dinleyici et al. 2010). Metronidazole is an antimicrobial drug with high activity against *Trichomonas vaginalis*, *E. histolytica*, *G. lamblia*, etc. However, resistance to ciproxin and metronidazole was demonstrated previously compared to furazolidone (Yakoob et al. 2004).

Garlic (*Allium sativum*) contains a wide range of the thiosulfinates which are responsible for the antibacterial activity (Jonkers et al. 1999; Iimuro et al. 2002; Sivam et al. 1997). The antimicrobial activity of garlic has been attributed to the presence of thiosulfinates (e.g, allicin). The amino acid, allicin, is metabolized by allinase (a cysteine sulfoxide lyase) to allicin and other thiosulfinates (Block 1985). Allicin acts by totally inhibiting RNA synthesis and partially inhibiting DNA and protein synthesis (Feldberg et al. 1988). Bacterial susceptibility to garlic may also depend on structural differences of the bacterial strains. The cell wall of content of Gram-positive and -negative microorganisms contain a variable quantity of polysaccharides 15–60% and 0–20% lipid, (Carpenter 1968). The polysaccharide and lipid contents of the cell wall have an effect on the permeability of allicin and other garlic constituents; this may be responsible for the difference in susceptibility to garlic between Gram-negative e.g., *Helicobacter pylori* and Gram-positive e.g., *Staphylococcus aureus* (Cellini et al. 1996; Sivam et al. 1997).

Cumin (*Cuminum cyminum*) is a popular spice in Middle Eastern and South Asian cuisine. It is used for the treatment of dyspepsia, diarrhea, flatulence, and as an appetite stimulant in traditional medicine. Cumin seeds are also rich in calcium, magnesium, iron, zinc, and some of the vitamins B (http://www.vegetarian-nutrition.info/herbs/cumin.php). Both aqueous and ethanolic extracts of black pepper (*Piper nigrum*) contain an alkaloid piperine that has demonstrated antibacterial activity (Pundir and Jain 2010; Singh et al. 2005; Khajuria et al. 2002). Black pepper finds an extensive use in traditional antibacterial preparations. A number of piperidine and pyrrolidine alkamides are known to occur in *P. nigrum* (Parmar et al. 1997), the most important being piperine, known to possess a variety of biological properties like analgesic, antipyretic, antibacterial, etc. (Miyakado et al. 1979). Black pepper finds an extensive use in traditional antibacterial preparations.

An extensive genetic variability has been described recently in *B. hominis* isolates. In a previous study, we demonstrated the predominant genotype of *B. hominis* in IBS-D was type 1, while in healthy control, it was genotype 3 (Yakoob et al. 2010a). Infection with single genotype of *B. hominis* was present in 73% with IBS-D and in 27% in control group while with multiple genotypes in 25 (64%) in IBS-D and 14 (36%) in control group (p=0.30), respectively (Yakoob et al. 2010b). In this study, we evaluated the in vitro effect of dietary herbs such as garlic, ginger, white cumin, and black pepper in two different concentrations on the parasite counts at 48 h using a reproducible method previously described (Yakoob et al. 2004). Metronidazole was used as a control for its known anti-*B. hominis* effect. These strains of *B. hominis* were isolated from the stool specimens obtained from irritable bowel syndrome (IBS) patients and healthy controls in our previous study (Yakoob et al. 2010a).

Materials and methods

This prospective study was conducted at the Aga Khan University Hospital, Karachi, Pakistan. Stool specimens were collected from 16 irritable bowel syndrome (IBS) patients attending the gastroenterology clinic and 10 (38%) healthy controls volunteered stool specimens between July and November 2010. These *B. hominis* were cultured from fecal samples of 16 (62%) with IBS, of these, 14 (54%) were males and 12 (46%) females. There was no history of antibiotic use in these patients. Stool microscopy and culture for *B. hominis* were done as described before (Zaman and Khan 1994).

Microscopy of fecal smear

Briefly, fecal sample microscopy was done as described before (Zaman and Khan 1994). Briefly, direct wet mount in which approximately 2 mg of feces was thoroughly emulsified on a glass slide in one drop of physiologic saline and covered with a cover slip. A similar preparation was made on another slide using Lugol's iodine. These preparations were examined under both the low power (×10) and high dry (×40) objectives.

Culture of feces

Cultures were done, by inoculating approximately 50 mg of feces into 5 ml of Jones' medium. For culturing *B. hominis*, Jones medium without starch was used, as it supports good growth of the parasite as described before (Zaman and Khan 1994). The cultures were incubated at 37°C and examined after 24, 48, 72, and 96 h. If no *B. hominis* were seen up to the end of this period, they were regarded as negative. The sediment was examined under both the low power (×10) and high dry (×40) objectives.

Plant extracts

Extracts were prepared of garlic, white cumin, black pepper, and ginger. Spices were measured, grounded, and soaked in double distilled water for about 48 h at room temperature to have a stock solution of 100 mg/mL. Solution was filtered through Grade 1 filter paper (Whatman, UK). Infusions were neutralized to pH 7.0. Extracts were stored in the dark at −20°C until use. Infusion of dried spices was used except for garlic which was used fresh. Herb extracts and MTZ stock solution of 1 mg/ml was prepared and added to media containing falcon tubes to give final concentration of 0.01 and 0.1 mg/ml.

Drug susceptibility assays

In vitro susceptibility assays were performed using a method previously described (Zaman and Zaki 1996). Briefly, in each media tube, 50 μl of cultures containing 200,000 *B. hominis* was added, which had been counted in a Neubauer chamber. These were incubated for 48 h at 37°C with different concentrations of dietary herbs and metronidazole each added as a solution in water. The calculated amount of metronidazole was first added to media containing tubes to achieve 0.01 and 0.1 mg/ml of the drug. Same method was used for the various plant extracts. The media tubes with *B. hominis* culture and without drug were used as controls. The effect of the drug was assessed after allowing the *B. hominis* to grow for 48 h. For this, 4 ml of the supernatant medium from each tube was carefully removed without disturbing the culture pellet at the bottom that contained the *B. hominis.* The sediment containing *B. hominis* was then gently agitated to obtain a uniform distribution and counts again made in a Neubauer chamber and the percentage increase or decrease in growth between the control and the test tubes was calculated for each strain.

Extraction of genomic DNA

Genomic DNA of *B. hominis* was extracted by using Stool DNA Extraction kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was stored at −20°C until PCR was carried out for *B. hominis* genotyping.

Genotyping by PCR with sequence-tagged-site primers

Seven kinds of subtype-specific sequence-tagged-site (STS) primers developed for typing the *Blastocystis* isolates were used as described previously (Abe et al. 2003a, b, c; Li et al. 2007a, b; Yan et al. 2006; Yoshikawa et al. 1998, 2000, 2003). Seven standardized subtype-specific STS primers were used, namely SB83 (351 bp) for subtype 1, SB340 (704 bp) for subtype 2, SB227 (526 bp) for subtype 3, SB337 (487 bp) for subtype 4, SB336 (317 bp) for subtype 5, SB332 (338 bp) for subtype 6, and SB155 (650 bp) for subtype 7 (Yoshikawa et al. 2004), according to a recent classification terminology (Stensvold et al. 2007). Typing of the *Blastocystis* isolates was conducted through PCR amplification on the basis of the presence or absence of the products within parallel control PCR amplification. The PCR conditions consisted of one cycle denaturing at 94°C for 3 min, 30 cycles including annealing at 59°C for 30 s, extending at 72°C for 60 s, denaturing at 94°C for 30 s, and additional cycle with a 5-min chain elongation at 72°C (PCR System 9700, Perkin Elmer, USA). The PCR products and molecular markers were electrophoresed in 2% agarose gel with Tris-acetate-EDTA electropheresis buffer. The size markers were 100 base-pair ladder (Promega, USA). The PCR amplification for each primer pair was repeated at least thrice. Bands were visualized by the imaging system (Gel Doc 2000, Gel Documentation System, Bio Rad, UK) after being stained with ethidium bromide.

Statistical analysis

Results are expressed as mean+standard deviation for continuous variables (e.g., age) and number (percentage) for categorical data (e.g., gender, stool culture, diarrhea etc.).

Univariate analysis was performed by using the independent sample *t* test, Pearson Chi-square test, and Fisher Exact test were also used whenever appropriate. A p value of <0.05 was considered as statistically significant. All p values were two sided. Statistical interpretation of data was performed by using the computerized software program SPSS version 16.0.

Result

Culture characteristics of *B. hominis* genotypes

B. hominis genotypes 3 and 1 and 3 tended to grow well in culture when present as a coinfection compared to isolated type 1 infection.

Table 1 Effect of metronidazole and garlic on *Blastocystis hominis*

	Irritable bowel syndrome $n=16$	Control $n=10$	p value
Metronidazole (0.01 μg/ml)			
No growth	6 (38)	10 (100)	0.001
B. hominis	10 (62)	0 (0)	
Metronidazole (0.1 μg/ml)			
No growth	9 (56)	10 (100)	0.01
B. hominis	7 (44)	0 (0)	
Garlic 0.01 μg/ml			
No growth	7 (44)	10 (100)	0.003
B. hominis	9 (56)	0 (0)	
Garlic 0.1 μg/ml			
No growth	6 (38)	10 (100)	0.001
B. hominis	10 (62)	0 (0)	

Univariate analysis was performed by using the independent sample t test, Pearson Chi-square test, and Fisher Exact test were also used whenever appropriate. A p value of <0.05 was considered as statistically significant. All p values were two sided. Number and percentage=n (%)

Metronidazole

B. hominis (38% (6/16)) isolated from IBS patients did not grow in culture compared to 100% (10/10) *B. hominis* from control when exposed to MTZ at a concentration of 0.01 mg/ml ($p=0.001$). When they were exposed to MTZ at 0.1 mg/ml, 56% (9/16) *B. hominis* isolated from IBS patients did not grow in cultures compared to 100% (10/10) from control ($p=0.01$) (Table 1).

Garlic

B. hominis isolates (44% (7/16)) from IBS patients did not grow in culture compared to 100% (10/10) *B. hominis* from control when exposed to garlic at a concentration of 0.01 mg/ml ($p=0.003$). When they were exposed to garlic at 0.1 mg/ml, 38% (6/16) *B. hominis* isolated from IBS did not grow in cultures compared to 100% (10/10) from control ($p=0.001$) (Table 1; Fig. 1).

Comparison of effect of herbs on *B. hominis*

B. hominis isolates from both IBS and control were sensitive to garlic, ginger, black pepper, and white cumin (Figs. 2, 3, 4, and 5). *B. hominis* isolates from IBS had a cell count of 6625±66 at a MTZ concentration of 0.01 mg/ml that reduced to 1,250±20 as MTZ concentration was increased to 0.1 mg/ml ($p=0.08$). *B. hominis* from IBS with a mean cell count of 3×10^5 at baseline decreased to 1×10^4 when exposed to garlic at 0.01 mg/ml ($p<0.001$) and to 1×10^3 ($p<0.001$) when garlic was 0.1 mg/ml. *B. hominis* from IBS cell count decreased to 1×10^5 when exposed to white cumin at 0.01 mg/ml ($p=0.01$) and to 1×10^5 ($p<0.001$) when white cumin was 0.1 mg/ml. Exposed to black pepper at 0.1 mg/ml, cell count of *B. hominis* from IBS decreased to 1×10^5 ($p=0.01$). *B. hominis* from IBS decreased to 1.3×10^5 exposed to ginger at 0.01 mg/ml ($p=0.001$).

Correlation of *B. hominis* genotype and effect of herbs

B. hominis isolates from control exhibited equal sensitivity to both MTZ and garlic (Table 1). They were mostly type 3, type 1 and 3 coinfection, and non-typeable *B. hominis* isolates. *B. hominis* isolates from IBS mostly genotype 1 demonstrated an increased sensitivity to garlic at 0.01 mg/ml with a *B. hominis* cell count of 3,714 compared to 6,142 when exposed to 0.01 mg/ml of MTZ. However, this sensitivity did not increase as garlic concentration was increased to 0.1 mg/ml, for *B. hominis* cell count was 6,000 compared to 1,428 as MTZ was increased to 0.1 mg/ml.

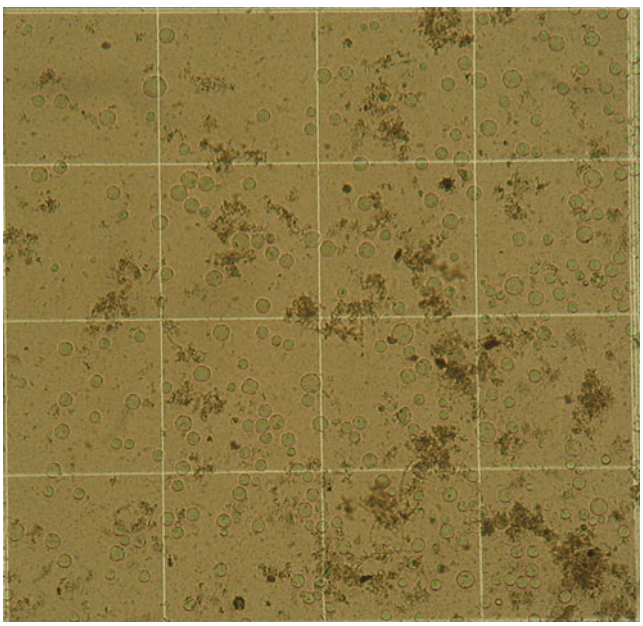

Fig. 1 *Blastocystis hominis* without exposure to extracts or metronidazole (control)

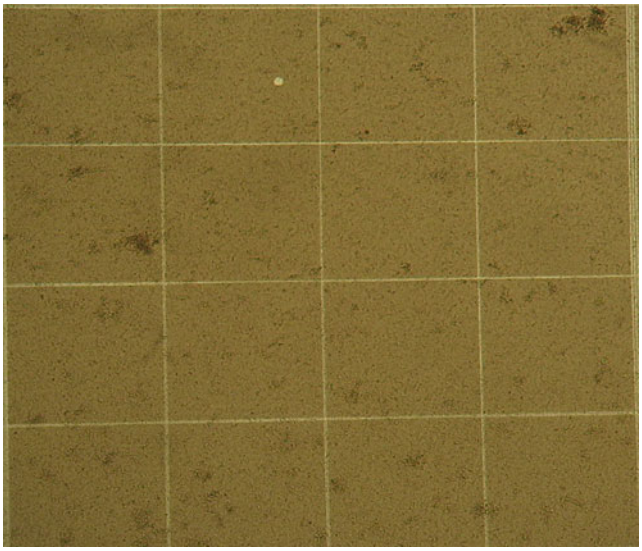

Fig. 2 *Blastocystis hominis* with exposure to garlic extract (0.01 μg) showing no growth

Discussion

B. hominis has been associated with diseases in immunocompetent and immunocompromised subjects (Dunn and Boreham 1991; Jonkers et al. 1999). These patients are treated with MTZ when *B. hominis* is suspected of causing disease. Currently, metronidazole is the drug of choice for treating protozoal infection. Previously, an aqueous extract of *Nigella sativa* at concentrations of 100 and 500 μg/ml showed a potent lethal effect on both *B. hominis* isolates. There was no significant difference between the inhibitory effect of *N. sativa* and metronidazole on the *B. hominis* living cell count (El Wakil 2007).

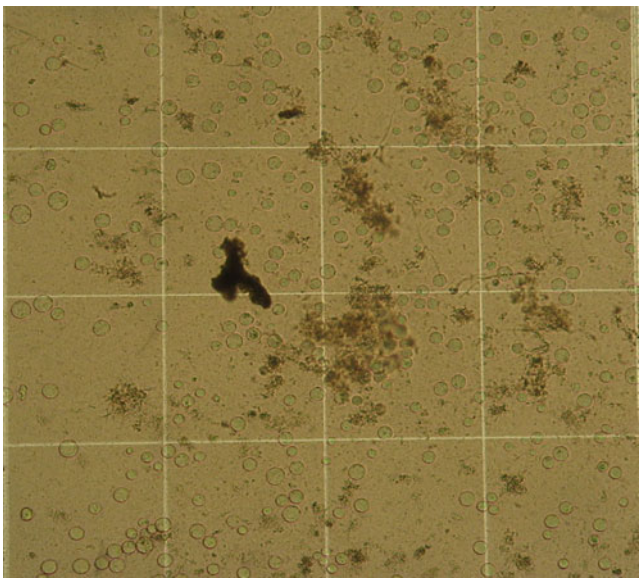

Fig. 3 *Blastocystis hominis* with exposure to ginger extract (0.01 μg) showing profuse growth

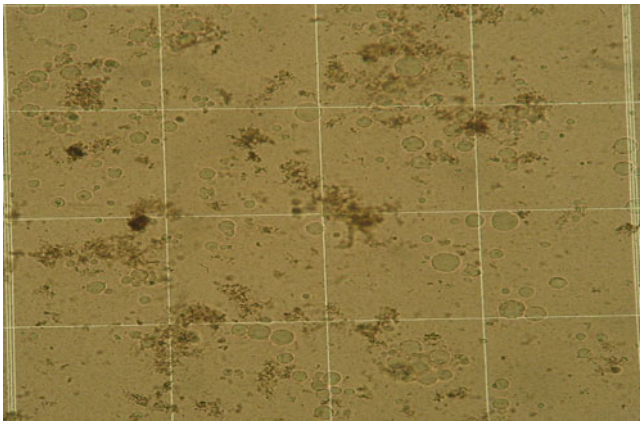

Fig. 4 *Blastocystis hominis* with exposure to black pepper (0.01 μg) showing no inhibition of growth

This study demonstrated that clinical isolates of *B. hominis* sensitivity to garlic was proportional to MTZ in suppressing the growth of *B. hominis*. Garlic was equally effective in both tested concentrations. *B. hominis* isolates were not sensitive to ginger, black pepper, and cumin compared to garlic and MET. They were unable to suppress *B. hominis* growth whereas ginger appeared to be promoting the growth of *B. hominis* isolates at higher concentration. *B. hominis* genotype 3 from control was more sensitive to metronidazole compared to genotype 1, and this was also demonstrated when there was a coinfection of genotype 1 and 3. *B. hominis* genotypes 1 and 3 as coinfection and non-typeable genotypes flourished in in vitro culture, and their growth was sustained over time as compared to that of isolated genotypes 1 or 3.

In this study, the viable cell count method provided a reliable method to determine the activity of the herbs against clinical isolates of *B. hominis* (Singh et al. 2005). The ability of the *B. hominis* isolates to grow at a

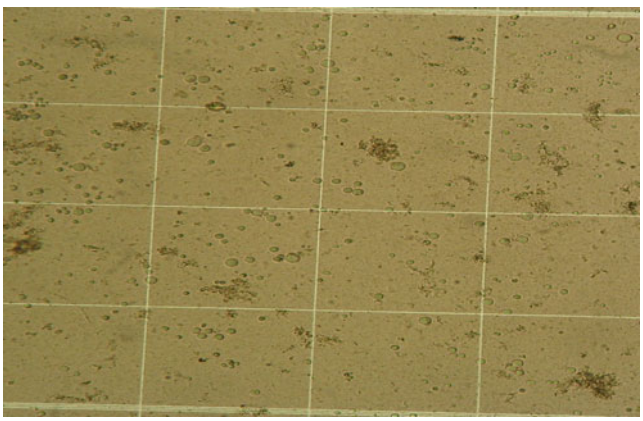

Fig. 5 *Blastocystis hominis* with exposure to white cumin extract (0.01 μg)

concentration of 0.1 mg/ml showed that these herbs had no effect on *B. hominis* isolates (Khajuria et al. 2002). However, it is possible that their active ingredients might demonstrate activity against *B. hominis* when obtained by another method. There are previous reports of *B. hominis* trophozoites and cysts demonstrating resistance to MTZ (Khajuria et al. 2002; Yakoob et al. 2010a, b; Zaman and Zaki 1996; Haresh et al. 1999; Germani et al. 1998). However, Dinleyici et al. (2010) demonstrated clinical efficacy of both MTZ and *S. boulardii* in symptomatic children with *B. hominis* infection presenting with abdominal pain, diarrhea, nausea-vomiting, and flatulence for more than 2 weeks. Both MTZ and *S. boulardii* had beneficial effects on symptoms and presence of parasites (Dinleyici et al. 2010). Similarly, Moghaddam et al. (2005) evaluated the effects of metronidazole and trimethoprim/sulfamethoxazole (TMP/SMX) on persons infected with *B. hominis*. A total of 104 non-immunocompromised subjects were monitored for 1 year after treatment. Of the 104 infected individuals with *B. hominis* infection, 28 had large numbers of *B. hominis* present in stool before treatment. Of these 28 severely infected individuals, 12 were treated with metronidazole/250–750 mg at a dose of 3×/day/10 days, and 4 of the 12 were eradicated. Nine individuals were treated with TMP/SMX 1 tab 3×/day/10 days, and 2 of the 9 were eradicated. For severe *B. hominis* infections, it appeared that metronidazole and TMP/SMX were effective in some individuals but not in all (Moghaddam et al. 2005). Possible mechanisms for apparent failure include extensive use of MTZ, TMP/SMX in the local community for various indications, inadequate dosage, patient noncompliance, and inactivation of the drug by the normal bacteria flora. Similar explanation might be offered for these herbs which are commonly used in cooking recipes. In this study, garlic was the most potent plant extracts against *B. hominis*. Other natural herbs that have been tried against *B hominis* include oregano, etc. (Force et al. 2000). Oil of Mediterranean oregano *Origanum vulgare* was orally administered to 14 adult patients whose stools tested positive for *B. hominis*. After 6 weeks of supplementation with 600 mg emulsified oil of oregano daily, there was complete disappearance of *B. hominis* in eight cases. Also, *B. hominis* scores declined in three additional cases. Gastrointestinal symptoms improved in 7 of the 11 patients who had tested positive for *B. hominis* (Force et al. 2000).

The strains of *B. hominis* used were isolated before experiment so they reflected their natural susceptibility. There are metronidazole-resistant *B. hominis* infections, and treatment of such cases by metronidazole might not provide eradication. However, whether garlic extract can be used in such cases needs to be looked at. If a protozoal cause of diarrhea is suspected, MTZ use should be reviewed if symptoms persist. In conclusion, this study has shown that *B. hominis* isolates vary in their degree of susceptibility to ginger, garlic, cumin, and black pepper common constituents of diet. However, further study is required to search for drugs that can be effective against *B. hominis*.

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