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Identification of *Enterocytozoon bieneusi* in Stool and Urine Specimens in Diarrheal Patients using PCR technique

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Abstract

Background: *Enterocytozoon bieneusi* is a major intestinal protozoan that is zoonotic and has the ability to infect both humans and several animal species. Currently, the prevalence and genotyping of *E. bieneusi* in humans in Mosul city are still unknown.

Methods: 118 stool and 177 urine samples collected from patients (males and females) in the various hospitals located around Mosul city. In this study, we used microscopic examination and PCR techniques to find *E. bieneusi* by using various types of primers to detect the target gene.

Results: The current study findings, 62.7% (74/118) of the *E. bieneusi* that were discovered in the stool specimens 65.7% (46/70) were found in males and 58.3% (28/48) in females) fit the microsporidia's shape according to microscopic analysis. The urine specimens used in this experiment did not contain any microsporidia. Additionally, the PCR technique confirmed that all of the microsporidia discovered in the current study were *E. bieneusi*, and that 54% (40/74) of them had the *SSU rRNA* gene with a molecular weight of 260 bp. Furthermore, *E. bieneusi* was found in 42.9% (12/28) of the female stool specimens and 60.9% (28/46) of the male stool specimens with a molecular weight of 400 bp. Furthermore, the prevalence rate of *E. bieneusi* was discovered in both younger and older age groups.

Conclusion: This investigation concluded that *E. bieneusi* existed in human diarrheal infections across all age groups. Males are more likely than females to have *E. bieneusi*. When looking for *E. bieneusi*, the PCR technique is more successful than other approaches.

keywords*: Enterocytozoon bieneusi,* patients (males and females), diarrhea, PCR technique

Introduction

The *Microsporidia* are obligate intracellular hosts that invade a wide variety of vertebrate and invertebrate hosts (1). Nowadays, more than 200 genera and 1,600 species of parasites exist in this category, including some rather common species including *Encephalitozoon cuniculi, Enterocytozoon bieneusi, Encephalitozoon intestinalis,* and *Encephalitozoon hellem* (2). *Enterocytozoon* (*E.*) *bieneusi* is the most serious zoonotic microsporidian

species in the world, it is capable of infecting a wide range of hosts, including humans, domestic animals, poultry, companion animals, birds, and wildlife, it is mainly transferred by the fecal-oral pathway (3, 4). Human infections with *E. bieneusi* have been becoming more prevalent since the HIV epidemic's spread in 1985, when the parasite was initially identified in HIV patients (5). More than 200 genotypes have been reported to date and divided into 11 categories (groups 1–11), *E. bieneusi* infections, whereas host adaptation seems to be more common in genotypes of Groups 3 to 11 (6). The infection with *E. bieneusi* can result in life-threatening chronic diarrhea and is associated with persistent diarrhea, malabsorption, and wasting diathesis in people with compromised immune systems, particularly those with AIDS diagnoses (7, 8). Even in individuals with healthy immune systems, self-limited diarrhea can occur and linger for up to 30 days (9). Additionally, *E. bieneusi* have also been observations regarding extraintestinal diseases and abnormalities, mainly in lung (10). Moreover, *E. bieneusi* has the potential for spreading a widespread infection that can affect the ocular, respiratory, and urogenital systems (11). Furthermore, a recent study has connected early-life *E. bieneusi* infection to limited growth in kids from low-resource environments, such as Malawi, South Africa, or Tanza, in Africa (12, 13). In addition, *E. bieneusi* is currently identified in pigs and other natural hosts (14), turkeys, pigs, chickens, cats, and goats (15), rabbits, dogs (16), and in monkeys immunised with the simian immunodeficiency virus (17). Numerous sources of infection of *E. bieneusi* can be found in the environment, particularly in surface water and wild, domestic, and agricultural animals. In addition, spores of *E. bieneusi* have been extensively identified in soil, livestock, and wildlife, and also in drinking water (18, 19). Moreover, mature spores are resistant to chlorine at doses employed to disinfect drinking water because they have powerful walls. The main way of infection is zoonotic transmission, which may occur either directly through contact with humans or animals that are diseased and do not have proper sanitation, or indirectly through eating or drinking water that has been contaminated with the pathogens (20, 21).

Enterocytozoon bieneusi can be identified from samples using a variety of approaches, including both conventional and modern procedures. Currently, the majority of research on the epidemiology of *E. bieneusi* infections depends on utilizing conventional microscopy to directly visualize the parasites in a stool and urine sample. The main advantages of this technique are its low cost and technical simplicity. Nevertheless, the inability of microscopy to distinguish between different species limits its use. Furthermore, microscopy requires reasonably competent workers and is a laborious and costly process. The sensitivity and specificity of these techniques are not identified, and for exact species diversity ultrastructural observations by transmission electron microscopy are essential (22). Polymerase chain reaction (PCR) and, more recently, real-time PCR are molecular techniques based on the rRNA gene that have demonstrated 100% validity in parasite detection and species identification (23). To identify the species of microsporidian gastrointestinal pathogens, PCR results can be simply digested using restriction endonucleases of *E. bieneusi.*

The main goals of this study was to identify *E. bieneusi* used the molecular and microscopic techniques stool and urine samples, to look at the genetic variety of *E. bieneusi*, in order to determine the relationship between *E. bieneusi* utilizing sequencing analysis of the geographic region.

Materials and Methods

Sampling

In this study, 118 stool specimens were obtained from patients (70 males and 48 females), and 177 urine specimens were obtained from patients (71 males and 106 females). All specimens were collected from the various hospitals (Al-Salam teaching hospital, Al-Kansaa teaching hospital, Ibn-Sena teaching hospital, Al-Gumhori teaching hospital) in different parts of Mosul city between March and December 2023 (Table 2). Each specimen of stool and urine had been collected using the sterilized containers. The specimens were sent to the central laboratory of the University of Mosul, College of Science and Veterinary Medicine after being quickly cooled with CO_2 ice in a cold box.

Microscopic Examination

Enterocytozoon bieneusi in stool and urine were examined under a microscope using the modified trichrome

staining approach (24). The staining solution used in this study was made by dissolving 6 g of chromotrope 2R (CDH, New Delhi, INDIA), 0.5 g of aniline blue (Solarbio Life Sciences, Beijing, China), and 0.25 g of phosphotungstic acid in 3 mL of glacial acetic acid. This solution was allowed to rest at room temperature for thirty minutes. Then, 100 mL of distilled water and 1 M/L of HCl were added to produce a pH 2.5 solution. Methanol-fixed smears were stained for 30 minutes at 37°C employing Chromatope 2R solution. After that, acid-ahcohol (4.5 mL of acetic acid and 995.5 mL of 90% ethyl alcohol) were utilized to rinse the smears for ten seconds. After being rinsed for seconds with 95% ethyl alcohol then dipping twice in 95% alcohol, each dipping for only 5 min, the smears were left to dry and dipping for 10 min in absolute alcohol and lastly 10 min in xylene. Following, these smears were examined under a 100X microscope.

DNA extraction

Using the following protocols, the suspect *E. bieneusi* was extracted and examined. All *E. bieneusi* were stained using the adapted trichrome staining procedure in order to examine the probable *E. bieneusi* and identify the parasite's shape. *Enterocytozoon bieneusi* were employed to extract DNA in accordance the instructions supplied by the FavorPrepTM Stool DNA Isolation Mini Kit (Taiwan). The English Genova Nano instrument (Jenway/UK) was then utilized to estimate the DNA concentration of the collected DNA. This instrument provides accurate measurement. After being separated from *E. bieneusi*, the DNA was stored at -20°C to preserve its quality and purity for further testing.

Amplification of the Genes

The PCR technique was employed for amplifying each gene sequence (Table 1). The entire 30 µl PCR reaction was carried out. The reaction mixture contained 15 µl of 2× GoTaq (Green Mix Master) from Promega Corporation (USA), 1 µl of primer F and R, 9 µl of double distillate water from Promega Corporation (USA), and 4 µl of the *E. bieneusi* DNA template. The target sequence amplicons were then seen utilizing gel electrophoresis.

A 2% agarose gel was prepared by Peqlab (Germany) and placed into wells together with DNA samples and a 100 bp ladder DNA marker in order to conduct gel electrophoresis. Through electrophoresis, the DNA that had been amplified fragments were separated and seen, and their sizes were determined by comparing them to the DNA ladder. The volume was adjusted to 30 µl in an Eppendorf tube after the entire mixture was added. The optimum heat cycling temperatures were used during the PCR amplification. Depending on the method of PCR used, various variables were used, such as denaturation, annealing, and extension temperatures and times. These are typically designed to operate optimally with the specific primer set and amplified DNA template.

Results:

The results of the current investigation revealed that 62.7% (74/118) of the *E. bieneusi* found in the stool specimens fit the morphology of the microsporidia based on microscopic analysis. Additionally, the present investigation reported that 65.7% (46/70) of microsporidia was detected in males, whereas 58.3% (28/48) was detected in females. However, no microsporidia was discovered in the urine specimens used for this investigation (Figure 2). Furthermore, the PCR technique utilized in this investigation revealed findings indicating that 54% (40/74) of microsporidia possessed the *SSU rRNA* gene with molecular weight 260 bp and all microsporidia found in the current study was *E. bieneusi* (Figure 3, Table 2). Additionally, *E. bieneusi* detected in 60.9% (28/46) of the male stool specimens and 42.9% (12/28) of the female stool specimens with molecular weight 400 bp (Figure 4, Table 2).

According to the table 3, a high prevalence rate of microsporidia was found by microscopic inspection in the age range of 12 to 18 years (83.3%) and in the age range of 51 to 65 years (75%). Subsequently, the age groups of 0 to 10 years and 31 to 50 years had a prevalence rate of microsporidia of 60% and 57.1%, respectively. In the 19–30 age group, the prevalence percentage of microsporidia was lower (44.4%), additionally, similar letters indicate that there is no significant difference between the groups, while different letters indicate that there is a significant difference between the groups ($P < 0.05$). (Table 3).

According to the *SSU rRNA* gene in microsporidia was detected in the present investigation, and the results

further demonstrated that all microsporidia discovered in feces with a molecular weight of 260 bp as shown as figure 3.

Furthermore, based on the *ITS* outer gene using the primer, the current study's results indicated that all of the microsporidia were *E. bieneusi*, which has a molecular weight of 400 bp, (Figure 4).

According to the *Small subunit ribosomal RNA* gene in microsporidia was not detected in the current investigation in patients with a molecular weight of 1265 bp as shown as figure 5.

Discussion

Microsporidia are a type of unicellular eukaryotic creature that specialize in intracellular parasitism (30). As far as we know, *E. bieneusi* is the most significant species of microsporidiosis, causing over 90% of cases in humans and having an infection distribution over the whole world (31, 32). Based on other calculations, more than 520 million people worldwide roughly 7.9 billion people may infect *E. bieneusi* in 2021 (33). The current investigation shows that the prevalence rate of *E. bieneusi* in patients was 54%. This study found a lower prevalence rate of *E. bieneusi* than earlier research that found a 71.4% prevalence rate of the infection in Iranian individuals (34), in Uganda was 76.9% (35), and in USA was 78% (36). In addition, the current study's findings revealed that the prevalence rate of *E. bieneusi* in patients was greater than that of earlier research, which indicated that the prevalence rate of *E. bieneusi* was 30% in USA (37), in Spain was 17% (38), in UK was 14.3% (39), and in Niger was 10.5% (40). The reasons for the variations in the findings of the different research include factors such as close contact between humans and animals, inadequate living circumstances, feces from animals contaminating drinking water, and a lack of knowledge about preventative measures, all of which raise the risk of contracting *E. bieneusi* infection (41). The most often used section of the rRNA gene for genotyping *E. bieneusi* isolates is the internal transcribed spacer (ITS). In addition to people, it has been isolated from pigs, macaques, wild boars, cats, dogs, livestock llamas, horses, gorillas, monkeys, raccoons, muskrats, beavers, foxes, otters, poultry, pigeons, parrots, sparrows, aquatic birds, and falcons thus far (42, 43, 44). In addition, pathogens-infected people and animals shed spores into the surrounding environment via their feces, urine, and respiratory systems, all of which may serve as possible infection sources (45). Despite the fact that pathogens have been linked to one waterborne outbreak, it is unknown how animals related to the contamination. Fecal-oral pathogens are linked to surface contamination in food, and some studies indicate that animal wastes are a significant source of contamination (46). Moreover, eating vegetables contaminated with *E. bieneusi* contributed to a foodborne spreading gastrointestinal sickness that affected over 100 individuals in Sweden (47). Furthermore, males have a greater prevalence rate of infected *E. bieneusi* (60.9%) than females have (42.9%). The results of our study were in agreement with the previous studies, which showed that males are more infected with *E. bieneusi* than females (32, 48, 49). The high male infection rate among humans might be related to bad lifestyle choices and a greater interest in animal husbandry. Simultaneously, a high level of engagement in animal husbandry may also be associated with the high positive rate of the people in rural areas (50). Beliefs related to gender impact perceptions and behaviors, influencing several facets of society's behavior, self-inflicted harm, and ecosystem management (51). The presence of gender-related differences in perceptions of the places people prefer, the activities they prefer, and their frequency of participation all affect potential exposure to infection (52). Comparing females to males, it is often observed that the former have stronger innate and adaptive (cellular and humoral) immune responses. The reasons why females have a higher immune response than males could be attributed to both psychosocial and physiological elements, such as differences in gender and differences in sex, such as genetic and epigenetic factors, sex hormones.

Furthermore, a significant prevalence rate of microsporidia was identified in the age groups ranging from 12–18 years (83.3%) and 51–65 years (75%). *Enterocytozoon bieneusi* is a prevalent infection that produces persistent diarrhea in humans, particularly in those with decreased immunity or immunodeficiency (HIV patients, recipients of transplants of organs, recipients of bone marrow transplantation, young people, the elderly, and cancer sufferers) (53, 54). The data analysis of humans at various stages of life found that the

infection rates of the elderly and young were greater. According to previous studies, both young and old are risk factors for infection with *E. bieneusi* (55). Similar findings have been reported in a variety of searches, suggesting that individuals with diarrhea had notably higher rates of *E. bieneusi* infection than patients without diarrhea, infection risk is higher in those who have diarrhea (56, 57). Furthermore, variances in testing techniques, objectives, and logistics progress could account for the disparity in the infection rate caused by *E. bieneusi* (58). PCR-based molecular methods were recently employed to identify and characterize *E. bieneusi*, providing more accurate results than prevalence rates ascertained by optical microscopy of stained biological smears (59). The most accurate and sensitive diagnostic instruments now in use are PCR-based techniques.

Conclusion

Enterocytozoon bieneusi has been identified as an important cause of microsporidiosis in both young and old individuals and all the age categories in Mosul city, Iraq, with immunesuppressivity and infancy serving as the main risk factors. The young and older humans are more exposed to infection to *E. bieneusi*. Microsporidia species can be recognized utilizing a variety of molecular biology techniques, including the traditional PCR method. When compared to staining, the PCR approach is more accurate and faster at identifying *E. bieneusi* in patient stool.

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Conflict of Interest

I promise no conflicts of interest.

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Table 1: The sequence Primers and PCR program used for detecting of the genes

Microsporidia SPP.	**Gene**	**Primers**	**Sequence of the primers (5- to 3-)**	**Size (bp)**	**Reference**
Enterocytozoon bieneusi Nested PCR	*ITS* outer primers	EBITS3	5-GGTCATAGGGATGAAGAG-3	400	(25)
		EBITS4	5-TTCGAGTTCTTTCGCGCTC-3		
	ITS inner primers	EBITS1	5-GCTCTGAATATCTATGGCT-3		
		EBITS2	5-ATCGCCGACGGATCCAAGTG-3		
E. bieneusi and *Encephalitozoon*	*SSU rRNA*	FP	5-CAGGTTGATTCTGCCTGACG-3	260	(26)
		RP	5-ATCTCTCAGGCTCCCTCTCC-3		

E.bieneusi and *E.intestinalis*	*Small subunit ribosomal RNA*	PMP1 PMP2	5'-CACCAGGTTGATTCTGCCTGAC-3' 5'-CCTCTCCGGAACCAAACCTG-3'	250-270	(27)
Microsporidian species Nested pcr	*Small subunit ribosomal RNA*	F1 R1 F1 R1	5-CACCAGGTTGATTCTGCCTGACG-3 5-TTATGATCCTGCTAATGGTTCTCC-3 5-GCCTGACGTAGATGCTAGTC-3 5-ATGGTTCTCCAACTGAAACC-3	1300 1265	(28) (29)
Enc. cuniculi, Enc. hellem, Enc. İntestinalis and E. Bieneusi	Small subunit ribosomal RNA	PMP1 PMP2	5-CACCAGGTTGATTCTGCCTGAC-3 5-CCTCTCCGGAACCAAACCCTG-3	*E. bieneusi* 250 bp, *E. cuniculi* 268 bp, *E. intestinalis* 270 bp *E. Hellem* 279 bp	(27)

PCR program**:** EBITS1 = 35 cycles (94 °C – 60 s, 57 °C – 60 s, 72 °C – 60 s), EBITS2 = 35 cycles (94 °C – 60 s, 55 °C – 60 s, 72 °C – 60 s), P = 35 cycles (94 °C – 60 s, 58 °C – 60 s, 72 °C – 60 s), PMP = 35 cycles (94 °C – 60 s, 60 °C – 60 s, 72 °C – 60 s), MI 1 = 35 cycles (94 °C – 60 s, 57 °C – 60 s, 72 °C – 60 s), MI 2 = 35 cycles (94 °C – 60 s, 55 °C – 60 s, 72 °C – 60 s)

Table 2: The number and percentage rate of microsporidia and *E. bieneusi* identified in stool and urine specimen of males and females using microscope and PCR examination

Gender	No. samples	Stool specimen		.No. specimen	Urine specimen	
		Results of positive microscopy exam No. (%)	Result of positive PCR exam No. (%)		Results of positive microscopy exam No. (%)	Result of positive PCR exam No. (%)
Male	70	46 (65.7%)	28 (60.9%)	71	-	-
Female	48	28 (58.3%)	12 (42.9%)	106	-	-

Total	118	74 (62.7%)	40 (54%)	177

Table 3: number and percentage of patients in stool by microsporidia

Age range	No. of samples	No. of Patients	Percentage of Patients
0 -10	50	30	(30/50) 60% [ab]
12 -18	12	10	(10/12) 83.3% [a]
19 -30	18	8	(8/18) 44.4% [b]
31- 50	14	8	(8/14) 57.1% [ab]
51 -65	24	18	(18/24) 75% [a]
Total	118	74	62.7%

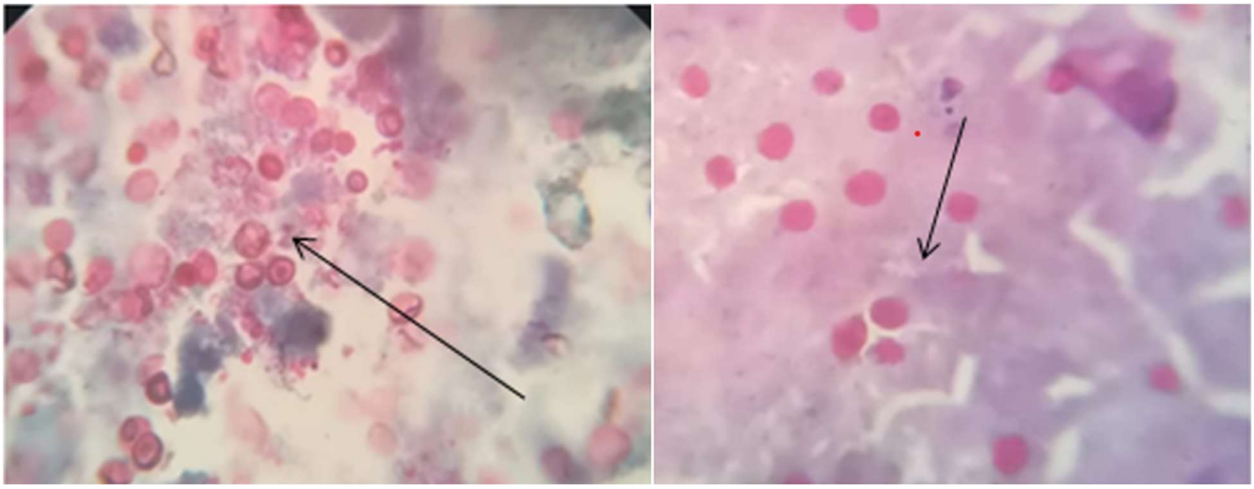

Figure 1: Microscopic examination revealed the presence of microsporidia in the stool samples. The microsporidia spores are indicated by the arrows. The spores were pink, shaped like an oval, and stayed in clusters

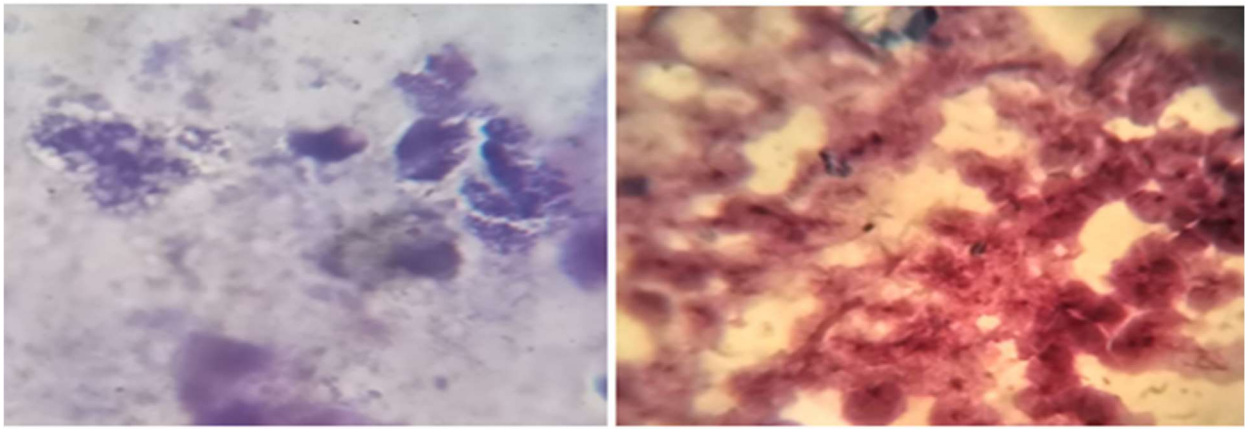

Figure 2: Microscopic examination revealed that there are no microsporidia presence in the urine specimens

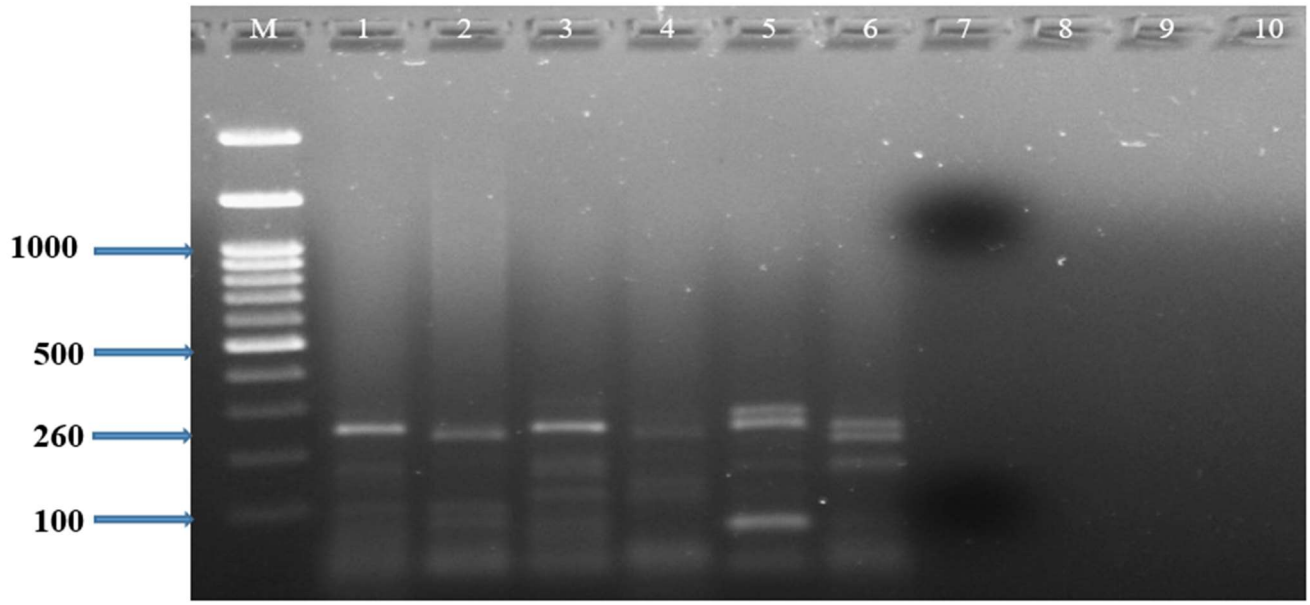

Figure 3: Agarose gel electrophoresis (2%) showing the typical amplicon of the *SSU rRNA* gene product of microsporidia in feces. The amplification of DNA appears as a ladder-like pattern. Lanes 1–6 represent positive microsporidia isolated from feces, Lane 7-10 represent negative of microsporidia, Lanes M are DNA Marker 100 bp ladder (GeneDirex, Korea).

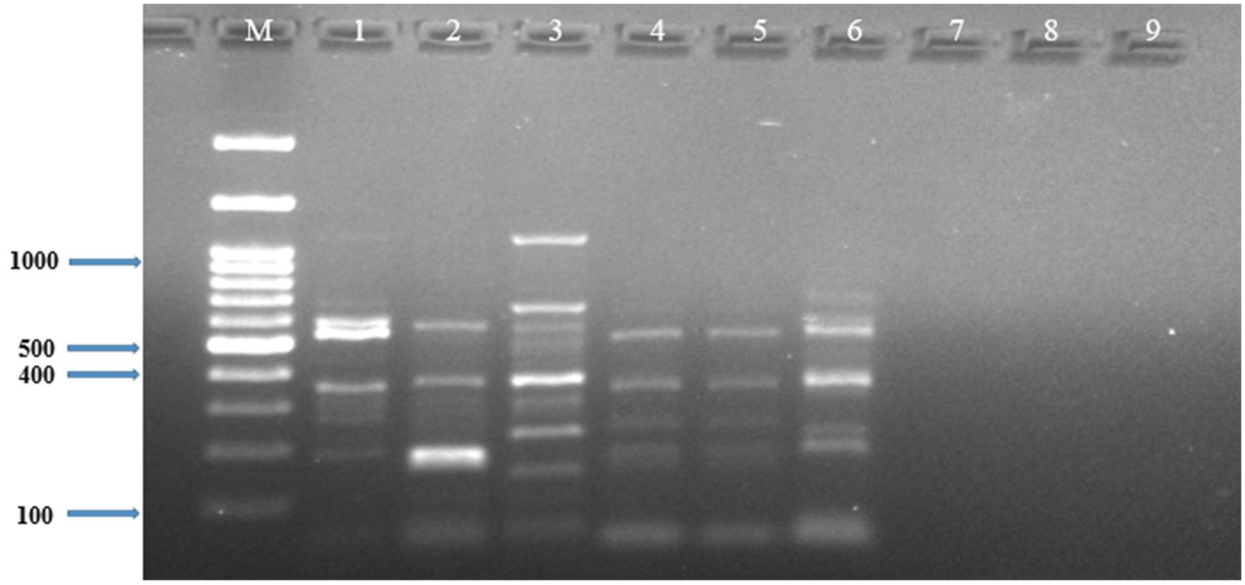

Figure 4: Agarose gel electrophoresis (2%) showing the typical amplicon of the *ITS* outer gene to detect *E. bieneusi* in patients. The amplification of DNA appears as a ladder-like pattern. Lanes 1–6 represent positive *E. bieneusi* isolated from patients, Lanes 7–9 represent negative *E. bieneusi*. Lanes M are DNA Marker 100 bp ladder (GeneDirex, Korea).

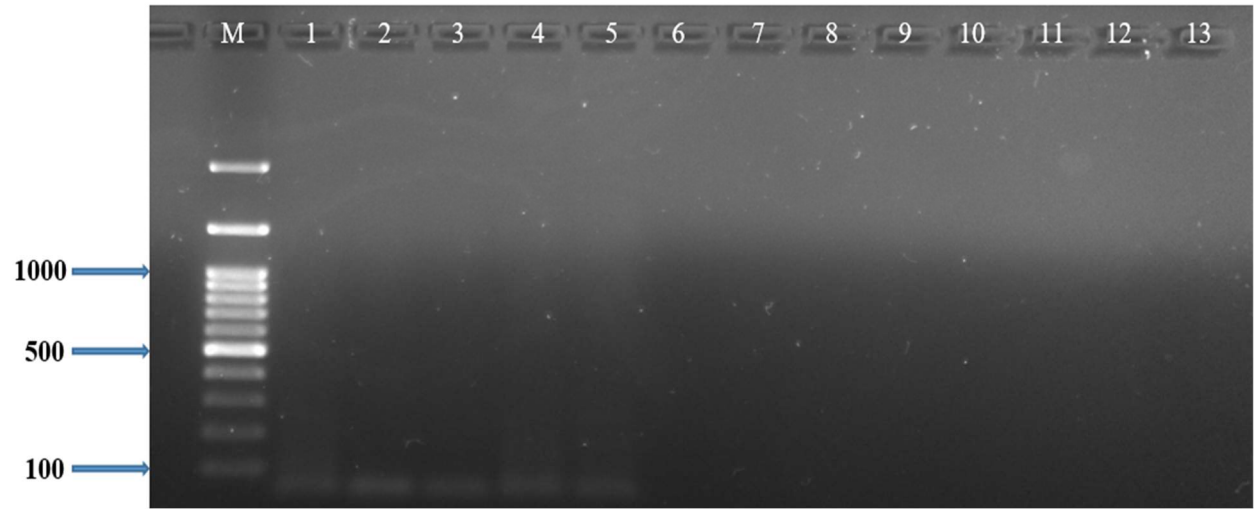

Figure 5: Agarose gel electrophoresis (2%) showing the typical amplicon of the *Small subunit ribosomal RNA* gene to detect microsporidia in patients. Lanes M are DNA Marker 100 bp ladder (GeneDirex, Korea).