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Original Article

Emerging Intestinal Microsporidia Infection in HIV+/AIDS Patients in Iran: Microscopic and Molecular Detection

Hamed MIRJALALI [1], Mehdi MOHEBALI [1,2], Hossein MIRHENDI [1], Rashid GHOLAMI [3], Hossein KESHAVARZ [1,2], Ahmad Reza MEAMAR [4], *Mostafa REZAEIAN [1,2]

1. *Dept. of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
2. *Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences, Tehran, Iran*
3. *Dept. of Infectious Diseases, Tehran University of Medical Sciences, Tehran, Iran*
4. *Dept. of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran*

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***Correspondence**
Email:
Rezaiian@tums.ac.ir

Abstract

Background: Species of Microsporidia have been known as opportunistic obligate intracellular parasites particularly in immunocompromised patients. *Enterocytozoon bieneusi* is one of most prevalent intestinal microsporida parasites in HIV+/AIDS patients. In this study, intestinal microsporidia infection was determined in HIV+/AIDS patients using microscopic and molecular methods.

Methods: Stool samples were collected from HIV+/AIDS patients during 12 months. All of the stool specimens washed with PBS (pH: 7.5). Slim slides were prepared from each sample and were examined using light microscope with 1000X magnification. DNA extraction carried out in microscopic positive samples. DNA amplification and genus/species identification also performed by Nested-PCR and sequencing techniques.

Results: From 81 stool samples, 25 were infected with microsporidia species and *E. bieneusi* were identified in all of positive samples. No *Encephalitozoon* spp. was identified in 81 collected samples using specific primers.

Conclusion: *E. bieneusi* is the most prevalent intestinal microsporidia in immunocompromised patients of Iran. On the other hand, Nested-PCR using specific primers for ssu rRNA gene is an appropriate molecular method for identification of *E. bieneusi*.

Introduction

Species of microsporidia are obligate intracellular protozoans that have been recognized among broad spectrum of invertebrate and vertebrate hosts (1, 2). Almost 1200 species of microsporidia belong to more than 140 genera were described, so far (1, 3, 4). Since the first human microsporidiosis was identified in 1959, reports of cases of immunocompromised patients who suffer from different form of microsporidia infection have been increased (5-7).

Chronic diarrhea is the most common clinical manifestation in HIV$^+$/AIDS patients with intestinal microsporidiosis, especially in individuals with less than 100 CD $_4$ + T cell per µl of peripheral blood (8-10). Prevalence of intestinal microsporidia infection has been reported from 2 to 50% and even higher depending on methods of diagnosis, geographical area or hygiene and immunity condition of study population (1, 11, 12). Although the most prevalence of intestinal microsporidiosis could be seen in immunocompromised patients with chronic diarrhea but the infection have also been reported in immunodeficient or even immunecompetent individuals with or without diarrhea, frequently (13, 14). Increasing the number of immunocompromised patients is the main reason that subtle diagnosis of opportunistic parasite such as *Encephalitozoon* spp. and *E. bieneusi* in stool has been considered for researchers. Spores of human microsporidia are very small and the size of them is varying from 1µm to 4µm (8, 13).

Many staining and serological methods have been used for diagnosis of microsporidia infection, but molecular techniques based on rRNA gene using Polymerase chain reaction (PCR) and more recently Real - time PCR have been showed 100% validity in detection and species identification of the parasite (15-19).

To date, we did not have any precise information of intestinal microsporidia infection in immunocompromised patients in Iran except rare studies that carried out by Agholi et al. (20, 21). This study aimed to determine the intestinal microsporidia infection in HIV$^+$/AIDS patients in Iran.

Materials & Methods

Sampling

This study were carried out on 81 stool samples which were collected from HIV$^+$/AIDS patients who referred to Imam Khomeini Hospital during 2012-2013. HIV infection was confirmed by Western blotting and AIDS phase also confirmed with CD $_4$ + T cell count less than 200 per µl of peripheral blood and receiving Anti-Retroviral Treatment (ART). Samples were collected actively from patients who referred to laboratory due to periodic checkup or intestinal disorders. Based on appearance of stool, each of samples was considered in one of grades consisted: formed, diarrhea and watery diarrhea. From 81 stool specimens, 58 and 23 stool samples were collected from men and women, respectively.

Parasitological study

All of stool samples suspended in PBS pH 7.5, and then the suspension filtered with sterile gases for debris exclusion. Final suspension washed three times by sterile PBS and finally supernatant removed and remained pellet divided to 2 parts and each portion re-suspended in alcohol 80% and formalin – PBS 5% for molecular and parasitological assessments, respectively.

Thin slide for all of isolates was provided and staining was carried out using Ryan blue method that described elsewhere (22), previously. Screening carried out under oil immersion lens and small (1.2- 2µm), ovoid, pinkish spores containing median belt considered as microsporidia. DNA extraction performed on positive specimens that proved with light microscopic examination.

DNA extraction, PCR and Sequencing

For DNA extraction 250 µl from stool suspended in sterile PBS was transferred to 1.5 ml tube. After centrifuging in 5000 rpm for 10 minutes, supernatant was removed and then 400 µl of lysis buffer (100 mM Tric, 10 mM EDTA, 2% SDS, (final pH=8)), 20 µg/µl Proteinase K and acide washed Glass beads size 450 – 600 µm were added to remained pellet. Samples were vortexed for 2 minutes vigorously and incubated at 60ºC for 3 hours. Samples vortexing was repeated for 30 sec every 30 minutes. Finally, after centrifuging in 3000 rpm for 5 min, supernatant transferred to Bioneer stool DNA extraction kit (Bioneer Corporation, Daejeon, Korea). Purified DNA was stored at -20 ºC until use.

Nested – PCR was performed using genus specific primers, which were designed, based on ssu rRNA gene using online software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The first pair primers, PMicF (5´- GGTTGATTCTGCCTGACG - 3´) and PMicR (5´ - CTTGCGAGC(G/A)TACTATCC - 3´), amplified 779 bp of ssu rRNA gene of *Encephalitozoon spp* and *E. bieneusi*. The second PCR employed primers EnbF (5´- GGTAATTT-GGTCTCTGTGTG - 3´) and EnbR (5´-CTACACTCCCTATCCGTTC -3´) to amplify 440 bp and also EncepF (5´- AGTAC-GATGATTTGGTTG- 3´) and EncepR (5´-ACAACACTATATAGTCCCGTC- 3´) to amplify 629 bp fragments for *E. bieneusi* and *Encephalitozoon* spp., respectively.

First PCR reaction was performed in final volume 25 µl containing 2.5 µl of 10X PCR buffer, 2mM $MgCl_2$, 200µM dNTP, 1.5 unit of Taq polymerase (Fermentase, Thermo Fisher Scientific, Lithuania) and 10 ρM of each primers. Amplifications were carried out in PeqLab thermocycler (PEQLAB Biotechnologie GmbH, Germany) under condition, 95 ºC for 5 min followed by 35 cycles of 94 ºC for 40 sec, 55 ºC for 45 sec and 72 ºC for 45 sec and final extension of 72 ºC for 4 min. The second PCR condition consisted 95 ºC for 5 min followed by 25 cycles of 94 ºC for 35 sec, 57ºC for 35 sec, 72 ºC for 40 sec and 72 ºC for 3 min as a final extension. 5µl of PCR products were electrophoresed on 1.5% of agarose gel and were visualized after ethidium bromide staining. PCR products of positive samples were sequenced using ABI 3130 (California, USA) and the results were compared using BLAST software in GenBank database.

Statistical test

Data analyzing were performed by chi-square (X^2) test, using SPSS software (version 18, SPSS Inc., Chicago, IL). A *P*- value <0.05 was considered statistically significant.

Results

From eighty one stool samples which were collected from HIV+/AIDS patients, twenty five (30.86%) samples were positive for intestinal microsporidia infection, microscopically (Fig. 1) and all of them confirmed with Nested-PCR. Positive cases were observed in 8 (34.8%) and 17(29.31%) of women and men, respectively. No statistically significant difference was found in the both groups (*P*= 0.631). Chronic watery or moderate diarrhea were existed in 13 (52%) of positive cases. Positive cases were also seen in 12 (48%) patients with history of diarrhea. No statistically significant difference was found in the both groups (*P*= 0.986).

All the positive samples showed 440 bp fragment of *E. bieneusi* (Fig. 2). DNA amplification of *Encephalitozoon* spp. did not found by specific primers belonging to that. Sequencing results of five cases, which were selected randomly from positive samples, were compared in GenBank and consequently all of PCR results were proved. The accession numbers of sequenced samples including of KF875441, KF875442, KF875443, KF875444 and KF875445. Results of study are summarized in Table 1.

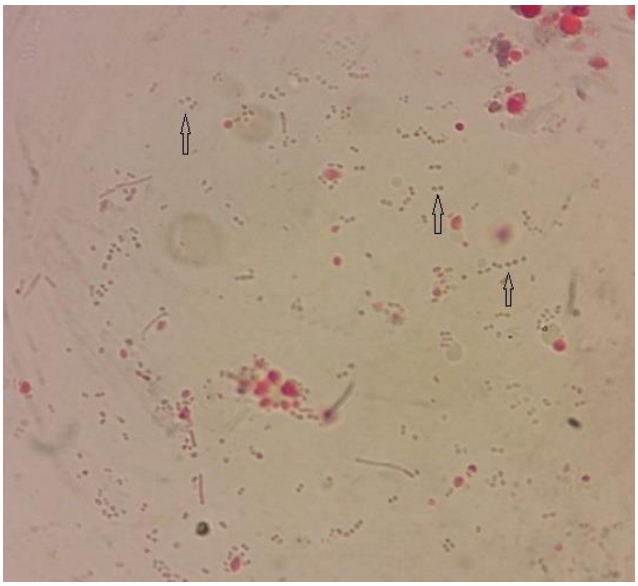

Fig. 1: *Enterocytozoon bieneusi* spores (arrows) isolated from stool of HIV⁺/AIDS patient. 1000X Magnification (Original)

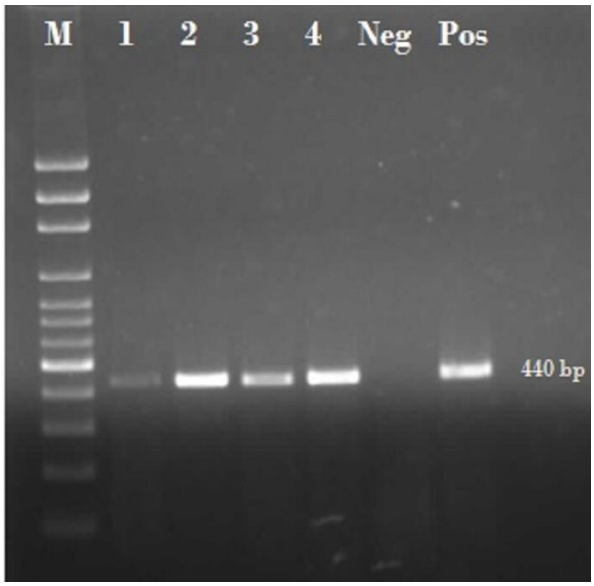

Fig. 2: Gel electrophoresis of 440 bp fragment of *E. bieneusi* using Nested- PCR, M: 100bp marker, 1 to 4: samples of *E. bieneusi*, Neg: negative control, Pos: positive control

Table 1: General characteristic of 81 HIV⁺/AIDS patients co-infected with *Enterocytozoon bieneusi*

		No. of collected samples	No. (%) of infected samples
Gender	Male	58	17(29.31)
	Female	23	8(34.8)
Age (yr)	20- 35	41	16(39.02)
	36-50	33	6(18.18)
	51-65	7	3(42.85)
Stool appearance	Formed	39	12(30.77)
	Diarrhea	24	7(29.16)
	Watery diarrhea	18	6(33.33)

Discussion

In our study, *E. bieneusi* obtained from 30.86% of HIV⁺/AIDS patients. The percent of infected patients in both genders was approximately equal and chronic sever or moderate diarrhea was seen in more than half of samples.

Although, more recently Agholi et al. (21) described microsporidiosis in some HIV⁺/AIDS patients (356/8) but there are not any precise information about microsporidia infection and also genus or species that are involve in transmission cycle of infection in those patients in Iran. Our findings clearly show that the microsporidiosis could be seen in more HIV⁺/AIDS patients than that was mentioned in previous study (21). Differentiation in findings likely related to manners of stool preparation or DNA extraction. As most researchers have been declared, DNA extraction from microsporidia spores in stool has many complexities. *E. bieneusi* and *Encephalitozoon* spp. spores have very small size, rigid double layer wall and also low counts in stool samples. As a result, stool preparation and

strong DNA extraction methods can impress the quality and quantity of molecular results (17, 23, 24).

Our results also show that *E. bieneusi* is the most important agent of intestinal microsporidia infection in HIV$^+$/AIDS patients in Iran. The finding is in agreement with other studies from different areas of the world. It is interest to mentioned that *E. bieneusi* is one of most prevalent microsporidia parasites which were reported from immunocompromised individuals such as HIV$^+$/AIDS patients in most countries (11) and probably play an important role in intestinal involvement and subsequently chronic diarrhea in HIV$^+$/AIDS patients (21, 25-27).

In our study *E. bieneusi* was detected in 12 (48%) of those patients who had not any severe or moderate diarrhea indication, at the sampling time. Although no statistical relationship between diarrhea and microsporidiosis were seen in this study but it is interest to mention that according to recent research, intestinal microsporidiosis could be seen in individuals with intermittent diarrhea or rarely without history of acute or chronic diarrhea (13). However, all of HIV$^+$/AIDS patients in our study, received Anti-Retroviral Treatment (ART) and consequently their CD $_4$ + T cell count improved. On the other hand, some patients received different drug for signing treatment of diarrhea that probably both of reasons could be efficacious in diarrhea remission at the sampling time.

So far, prevalence of different genus of Microsporidia has not been known completely in Iran and probably the cases of involvement with various forms of microsporidiosis are more than our expectancy. Furthermore, it is need to assessment the infection get noticed in susceptible patients such as transplant recipients and cancer patients who are under immunosuppressing treatment and chemotherapy, respectively or patients with other congenital or acquired immune system failures as at risk individuals.

Conclusion

E. bieneusi is probably the most prevalent intestinal microsporidia genus particularly in HIV$^+$/AIDS in Iran. Strong stool preparation and molecular methods such as Nested-PCR could be useful for detection of intestinal microsporidia infection.

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