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Original paper

Molecular diagnosis of intestinal microsporidia infection in HIV/AIDS-patients in Zahedan city, Southeast of Iran

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ABSTRACT. Microsporidia belong to the intracellular spore-like pathogen, that can cause infection in invertebrates and vertebrates, including humans. *Encephalitozoon* spp. and *Enterocytozoon bieneusi*, are important causes of chronic diarrhea, especially in patients with HIV/AIDS. Therefore, in this study, modified trichrome staining (MTS) and nested polymerase chain reaction (nested PCR) methods were used for the diagnosis of common intestinal microsporidia in faecal samples of patients with HIV/AIDS in Zahedan, southeastern Iran, for the first time. Stool samples were collected from 50 HIV/AIDS-infected patients with gastrointestinal symptoms whose infections were confirmed by serology test. Prepared smears from each stool sample were stained using the MTS method. Nested PCR was used to amplify 440 bp and 629 bp fragments of 16S rRNA genes in *E. bieneusi* and *Encephalitozoon* spp., respectively. Based on the MTS method and the nested PCR, 8 (16%) and 12 (24%) stool samples were positive, respectively. According to the results of nested PCR, eight, three, and one case were infected with *E. bieneusi*, *Encephalitozoon* spp., and both of them, respectively. Findings indicated microsporidiosis in HIV/AIDS-infected patients in Zahedan is an important health problem. Therefore, this opportunistic microorganism in HIV/AIDS-infected patients should be diagnosed using sensitive and accurate methods.

Keywords: microsporidia, HIV/AIDS, molecular, Zahedan, Iran

Introduction

Microsporidia is one of the opportunistic and intracellular pathogens that can produce spores and infect a wide range of vertebrates and invertebrates [1]. Human infections with microsporidia have been reported from all over the world and most of them involve HIV/AIDS-positive patients [2]. The first case of human microsporidiosis was reported in 1959. In 1985, *Enterocytozoon bieneusi* was reported in a patient with AIDS in France. Since then many infections have been reported from all over the world due to the development of sensitive and appropriate diagnostic methods [3]. Many human microsporidiosis infections have zoonotic origins; however, human-to-human transmission has also been reported. Determination of the routes of transmission and sources of human microsporidiosis infection seems to be difficult [4]. It should be mentioned that microsporidia can be detected in the urine, faeces, and respiratory secretions of patients. This infectious disease has clinical manifestations that include gastrointestinal, pulmonary, respiratory, ocular, muscular, cerebral, and systemic signs [2,4]. Gastrointestinal infections have been reported more frequently in patients with severe immunosuppression and those with a CD4 cell counts of < 100 cells per microliter of blood [5,6]. The present study aimed to diagnose common intestinal microsporidia in HIV/AIDS-infected patients with a medical record in the Behavioral Patients Health Center of Zahedan Deputy Health Department using modified trichrome staining and nested polymerase chain reaction (nested PCR) for the first time.

Figure 1. Area of study, Zahedan city, located in Sistan and Baluchestan province, Iran

Materials and Methods

Sample collection and microscopic examination

This study with the ethical code of IR.ZAUMS.REC.1397.171 was performed in Zahedan on HIV/AIDS infected patients who had medical records in the Behavioral Health Center of the Deputy Minister of Health (Fig. 1). Fifty faecal samples were collected from HIV/AIDS-infected individuals with clinical signs of diarrhea or gastroenteritis. To be mentioned, before stool sample collection, personal consent was obtained. Afterward, faecal samples were transferred to the parasitology laboratory. Stool samples were suspended in phosphate-buffered saline (PBS) pH 7.5, then large particles were removed using a metal strainer with 80 µl holes. The suspension was centrifuged at 3500 rpm for 15 min to remove the excess liquid. Pellet was divided into two parts. In order to perform molecular and parasitological tests alcohol 80% and formalin PBS 5% were added to each pellet of samples, respectively. Afterward, the samples were stored at room temperature until use. A thin smear was prepared from the samples fixed in formalin PBS 5% and stained by the modified trichrome-blue staining method according to the method developed by Rayan [7]. Trichrome blue dye was prepared from a combination of 6 g of chromotrope 2R, 0.5 g of aniline blue and, 0.25 g of dodecatungstophosphoric acid in 3 ml of acetic acid. The pH of the dye was adjusted to 2.5 with hydrochloric acid. The dried slides were fixed in methanol for 10 minutes and stained with the prepared dye. After destaining with alcohol acid, the slides were transferred twice in 100% ethanol for 30 seconds. Finally, the slides were transferred to xylene for 2 minutes. Each slide was examined for at least 15 min at high magnification.

DNA extraction and nested polymerase chain reaction

The DNA of faecal samples was extracted using a QIAamp DNA stool mini kit (Cat. No. 51504) according to the manufacturer's instructions. In this study, three pairs of primers designed based on the 16S rRNA gene were used to identify intestinal microsporidia (*E. bieneusi* and *Encephalitozoon* spp.) [8].

In the first step of the nested PCR, PMic F: 5'-GGTTGATTCTGCCTGACG-3' and PMic R: 5'-CTTGCGAGCRTACTATCC-3' primers were used to amplify 779 bp fragment of *E. bieneusi* and *Encephalitozoon* spp. At the second stage, Enb F: 5'-GGTAATTTG GTTCTCTGT GTG-3 'and Enb R: 5'-CTACACTCCCTATCCGTTC-3' primers for amplification of 440 bp fragment of *E. bieneusi* and the pairs primers of Encep F: 5'-AGTACGATGAT TTGGTTG-3' and Encep R: 5'-ACAACACTATAT AGTCCCGTC-3' were used to amplify the 629 bp fragment of *Encephalitozoon* spp.

The DNA amplification was performed in a thermocycler at 95°C for 5 min followed by 35 cycles at 94°C for 40 sec, at 55°C for 45 sec, at 72°C for 45 sec, and final extension at 72°C for 4 min. The second stage of amplification was performed at 95°C for 5 min, followed by 25 cycles at 94°C for 35 sec, at 57°C for 35 sec, at 72°C for 40 sec, and the final extension at 72°C for 3 min.

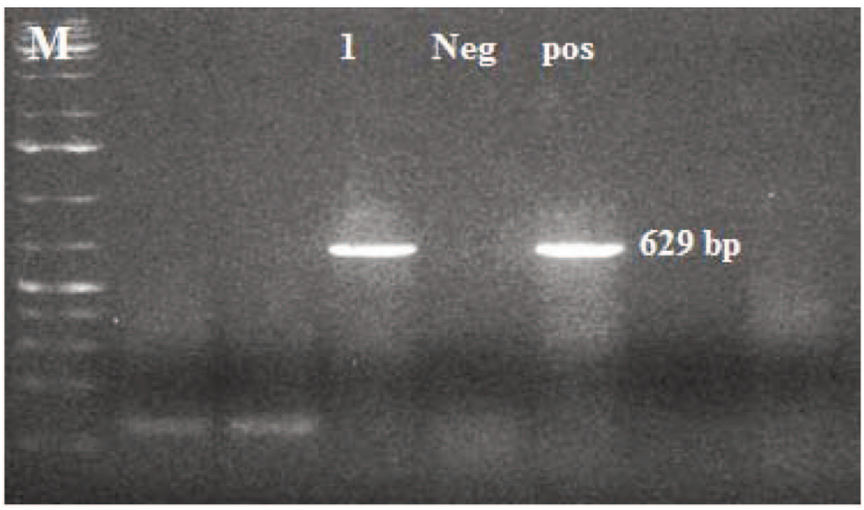

Figure 2. Gel electrophoresis of 629 bp fragment of *Encephalitozoon* spp. amplified using nested PCR, M: 100 bp marker, 1: *E. bieneusi*, Neg: negative control, pos: positive control

Finally, 5 µl of PCR products were electrophoresed on 1.5% agarose gel and after staining with Gel Red, bands were observed under UV illuminator.

CD4 T lymphocyte cell count

The number of CD4 T lymphocyte cell was counted by the flow cytometry technique.

Statistical analysis

Collected data were analyzed by Fisher's exact test and Cohen's kappa coefficient test, using SPSS software (version 22).

Results

Based on modified blue trichrome staining and nested PCR method, 8 (16%) and 12 (24%) stool samples were positive, respectively. The *Encephalitozoon* spp. infection was observed in three (6%) samples. Figure 2 shows amplified 629 bp fragment of *Encephalitozoon* spp. In addition, one (2%) sample was infected with both *Encephalitozoon* spp. and *E. bieneusi*. Furthermore, eight (16%) samples were infected with *E. bieneusi*. Figure 3 shows amplified 440 bp fragment of *E. bieneusi.* It should be noted that all cases that were positive for microsporidia based on staining method results, were found to be positive by nested PCR test as well. Besides, a moderate agreement was found between nested-PCR and modified blue trichrome staining, regarding the detection of microsporidia (Kapp value: 0.75). In this study, positive cases were found ranged in age from 18 to 42 years. Microsporidia infection was detected in 5 (42%) males and 7 (58%) females.

Also 8 (67%) and 4 (33%) microsporidia infected samples had semi-formed and diarrhea consistency, respectively. Also, no significant relationship was found between sex, stool consistency, and microsporidiosis (P-value > 0.05).

Based on CD4 T lymphocyte cell count, samples were divided into two groups. 7 (14%) and 43

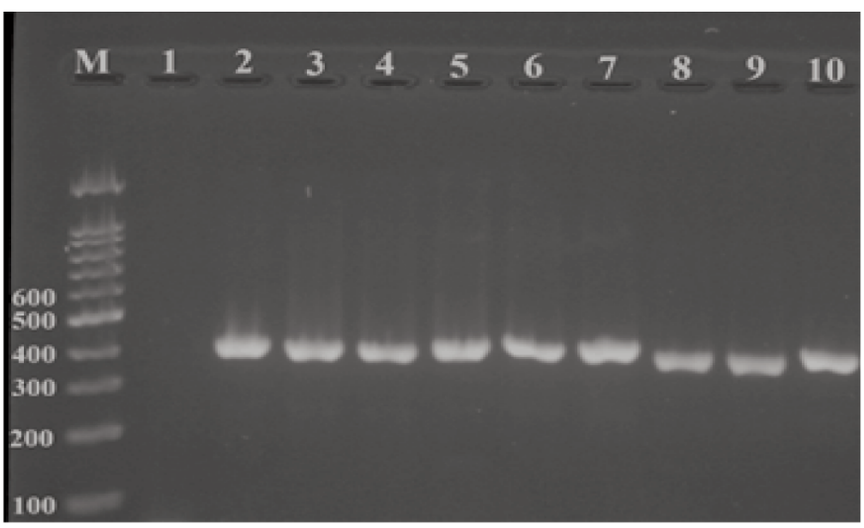

Figure 3. Gel electrophoresis of 440 bp fragment of *E. bieneusi* amplified using nested PCR, M: 100 bp marker, 3 to 10: samples of *E. bieneusi*, 1: negative control, 2: positive control

(86%) HIV/AIDS-infected patients had CD4 T lymphocyte cell count of < and > 200 cells/µl, respectively. Microsporidia were detected in all of the patients who had CD4 T lymphocyte cell count of < 200 cells/µl. A significant difference was found in both groups and microsporidia infection (*P*-value < 0.05).

Discussion

Opportunistic infections are one of the major causes of death in patients with immunodeficiency syndrome. The number of microsporidia-infected patients has increased due to the enhancement of the sensitivity and accuracy of diagnostic tests [9].

In this study, 24% and 16% of the HIV/AIDS patients (n=50) were positive by nested PCR and microscopy methods, respectively. The prevalence of human intestinal microsporidiosis has been reported 30.9% in Tehran, 6.8% in Fars, 18.5% in Chaharmahal and Bakhtiar, 0.5% in Hamedan, 2% in Isfahan, and 9.8% in Kerman [10–15]. In different parts of the world, the prevalence of microsporidiosis in patients with immunodeficiency was 7–50% [16]. Based on GenBank data, microsporidia is more prevalent in 92 countries such as India, China, Russia, and Thailand than other countries including Syria, Switzerland, and Romania [17]. In Iran's neighboring countries, including Turkey and Iraq the prevalence rate of human infection has been reported 13.4% and 10.3%, respectively [18,19]. The human prevalence rate of this disease is affected by important factors such as climate, sources of drinking water and, infected animals. Contaminated water with animal faeces can play a significant role in the transmission of microsporidia; hence, this issue should be taken more seriously in deprived areas of the country, including Sistan and Baluchestan province. Careful attention should be paid to the hygiene and safe water supply, especially regarding HIV/AIDS-infected individuals who often do not have good living conditions or might be addicts and homeless.

Until now, several trichrome staining methods have been designed to detect microsporidia, one of which is modified trichrome staining designed by Weber for the first time. This method was then modified by Ryan, who used aniline blue instead of fast green for the background staining [7,9,20]. In this study, modified trichrome-blue staining was used based on the study of Ryan et al. [7], and aniline blue background dye was used which has high stability and better differentiation with other bacterial and fungal agents. To date, several genes have been used in molecular studies, including the 16S rRNA gene, alpha-tubulin, beta-tubulin, Hsp 70, reverse transcriptase, and chitin synthase. Among these genes, the most molecular and phylogenetic studies have been performed on the 16S rRNA gene since its locus provides the most complete and reliable information and the sequence of this locus is available for most genus and species of microsporidia [21–23].

The nested PCR technique is a suitable method that can be used to detect microsporidia infection with a low number of spores in clinical specimens. According to various studies, the sensitivity of the molecular method is much higher than the microscopy method for detecting microsporidia in fecal samples. In line with other studies, in the present study, the nested PCR detected more positive cases, compared to the microscopy method [24–27].

In the present study, the most commonly identified microsporidia in patients with HIV/AIDS were *E. bieneusi* and *Encephalitozoon* spp., respectively. *E. bieneusi* is a more prevalent species of microsporidia in HIV/AIDS-infected patients. In this respect, present study was consistent with other studies conducted on HIV/AIDS infected patients in Iran and the world [16,27,28]. In various studies, conflicting results have been reported about the association of microsporidiosis and the CD4 T lymphocyte cell count in HIV/AIDS-infected patients. The results of this study showed a significant difference between both groups of CD4 T lymphocytes cell count and microsporidia infection (*P*-value < 0.05). In this respect, the results were consistent with other studies [8,16]. In the present study, no significant relationship was found between microsporidiosis and diarrhea. In this regard, this research is in line with other studies [8,22].

Due to the high prevalence of microsporidiosis in HIV/AIDS-infected patients in Zahedan city, the prevalence of this disease in other susceptible and immunocompromised groups such as cancer patients and transplant recipients should be more considered.

The findings of the current study indicated the high number of HIV/AIDS-positive patients in Zahedan city, infected with intestinal microsporidia. It is important to consider the correct and timely diagnosis of these patients with a powerful method

such as nested-PCR. Moreover, the study is required to determine the genotypes of Microsporidia spp. in HIV/AIDS- patients in Zahedan city.

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