

## A Molecular Study on Intestinal Microsporidiosis (*Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*) in Renal Transplant Patients in Tabriz.

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Microsporidia are obligate intracellular protozoan. Six genera of microsporidia have been reported to infect humans. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* have been identified as important agents for chronic diarrhea and wasting syndrome in patients with AIDS. Gastrointestinal complications are frequent in immunosuppressed patients. Routine detection of microsporidia in stool specimens is usually performed by staining methods. There is no documented study in Iran about microsporidial infections in the renal transplant recipients. This study was designed to identify the microsporidial infections in renal transplant recipients Tabriz Imam Reza hospital. Sixty four renal transplant patients with or without diarrhea were included in this study. Stool samples were examined by light microscopy for the presence of ova and parasites. Samples were fixed in formalin 10% for permanent staining (Weber's method). Formalin fixed stools were used for DNA extraction. DNA was extracted by using SDS, CTAB and proteinase k. V1 and EB450 pair primers were used to amplify *E. bieneusi* and V1 and SI500 primers to amplify *E. intestinalis* DNA. Light microscopy examination of samples showed eight cases of infection with *Giardia lamblia*. Study of chromotrope stained slides showed in five patients, microsporidia spores. A study of first step PCR products using V1/PMP2 primers showed that only four samples gave a positive signal in 250 bp. By using V1/EB450 and V1/SI500 primers positive signals were achieved only by V1/EB450 primers in approximately 350bp for all five samples. Microsporidiasis in immunocompetent individuals and patients with immunodeficiency other than AIDS showed gastro-intestinal signs. Gastro intestinal system (GIS) complications like nausea, vomiting and diarrhea are quite frequent during the post-transplant and chemotherapy period. The etiology of severe diarrhea is complex. Correct diagnosis of the cause of severe diarrhea in such patients should help to protect graft survival in transplant recipients. PCR has been used to detect and identify microsporidia from clinical specimens. Study of PCR results demonstrated that four of five patients can identify by V1/PMP2 primers and one sample was negative. This study confirmed that using a combination of diagnostic methods may lead to accurate diagnosis.

**Key words:** Intestinal microsporidiasis, PCR, Iran.

Microsporidia are obligate intracellular protozoan parasites infecting a broad range of vertebrates and invertebrates (Didier, 2005). In 1857 these parasites were first recognized as pathogens in silkworms, and long before they were described as human pathogens they were recognized as a

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cause of disease in many nonhuman hosts (Franzen and Muller, 2001). The first case of microsporidiosis in humans was reported in 1959 (Matsubayashi *et al.*, 1959).

Before the AIDS pandemic, only eight cases of human microsporidial infections had been reported (Mathis *et al.*, 2005). In 1985, two years after the identification of human immunodeficiency virus (HIV) as the causative agent of AIDS, the microsporidial species *Enterocytozoon bieneusi* was discovered in HIV-infected patients with chronic diarrhea (Desportes *et al.*, 1985). By improving the diagnostic methods, microsporidia infections have been recognized in a wide range of human populations, including organ transplant recipients being treated with immunosuppressive drugs, travelers, children, contact lens wearers and the elderly (Abreu-Acosta *et al.*, 2005). The world prevalence of intestinal microsporidiasis ranges between 7 and 50%. (Cimerman *et al.*, 1999).

Six genera of microsporidia can infect human are *Enterocytozoon*, *Encephalitozoon*, *Nosema*, *Pleistophora*, *Trachipleistophora*, and *Vittaforma* (Weber *et al.*, 1994).

*Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* have been identified as important agents for chronic diarrhea and wasting syndrome in patients with AIDS (Weber *et al.*, 1994; Coyle *et al.*, 1996). Studies have demonstrated that infection with *E. bieneusi* can also cause a self-limited diarrheal syndrome in immunocompetent individuals (Weber and Bryan, 1994). Gastrointestinal complications (oral lesions, esophagitis, peptic ulcer, diarrhea, colon disorders and malignancy) are frequent in renal transplant recipients and may lead to graft loss and even patient death. Diarrhea is a frequent disorder which may be caused by pathogenic microorganisms (bacteria, viruses and parasites) or by immunosuppressive agents (immunosuppressive drugs and antibiotics) (Ponticelli and Passerini, 2005). A study showed that microsporidia can lead to graft rejection in renal transplant patients (Latib *et al.*, 2001).

Routine detection of microsporidia in stool specimens is usually performed by the trichome stain or Uvitex 2B method or even a combination of these methods (Didier *et al.*, 1995). Spores of *E. bieneusi* usually measure 1.5 by 1 mm, while the average size of spores of *E.*

*intestinalis* is 2.2 by 1.2 mm (Weber *et al.*, 1994). Indirect immunofluorescent-antibody staining methods and direct agglutination have been used to detect microsporidia (Boot *et al.*, 2000; Dowda *et al.*, 1999; Jordan *et al.*, 2006) but transmission electron microscopy is necessary for exact species differentiation of the organism (Fedorko *et al.*, 1996). The PCR has already been applied to the detection of microsporidia in clinical specimens (Ombrouck *et al.*, 1997; Katzwinkel *et al.*, 1997).

There is no documented study in Iran about microsporidial infections in immunosuppressed and immunocompromised patients. Therefore, this study was designed to identify the microsporidial (*Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*) infections in Tabriz immunosuppressed and immunocompromised patients.

## MATERIALS AND METHODS

### Patients

Sixty four patients (44 men and 20 women) who underwent renal transplantation in Renal Transplantation Center of the Imam Reza hospital of Tabriz University of Medical Sciences between 1995 and 2006 with or without diarrhea were included in this study.

### Wet mount examination

Stool samples were collected in nine months from April 2007 to January 2008. After obtaining, the samples were examined by light microscopy for the presence of ova and organisms using Lugol's iodine and 0.85% NaCl solutions. Two grams of stool were fixed in formalin 10% for chromotrope staining.

### Chromotrope staining

Ten-micro liter aliquots of a suspension of unconcentrated stool fixed in 10% formalin were very thinly spread on a slide. Smears were fixed in methanol for 5 min and stained for 90 min with the chromotrope-based stain as described by Weber *et al.* (Weber *et al.*, 1994) The stained slides were viewed at  $\times 1,000$  magnification (oil immersion) for microsporidial spores.

### DNA extraction

The stool samples fixed by Formalin were used for DNA extraction. Firstly, stools were filtered through three layer mesh and were centrifuged in 3000 g for 4 minutes. The pellet was washed three times with PBS and resuspended in 1 ml PBS. After

heating this suspension in 100°C for 15 minutes, in 1.5 ml micro tubes 300 µl of this suspension, 150 µl T/E buffer 10/1, 60 µl SDS 10 % and 5 µl proteinase K (Cinnagen, Iran) 20mg/ml were added and incubated in 56°C water bath for two days. The micro tubes were sacked several times 5-10 min by a shaker.

On the third day 100 µl NaCl 5 M and 80 µl CTAB were added, shucked and incubated for 20 min in 65°C. 700 µl Chloroform/isoamyl alcohol (24/1) was added and were centrifuged in 14000 rpm for 8 min. supernatants were collected in new micro tubes and 2-propanol were added. After 30 min holding in -20°C freeze the micro tubes were centrifuged for 15 min in 14000 rpm. Precipitated DNA was washed by 70° ethanol three times. 30 µl T/E buffer was added and kept in -20°C.

# PCR

Two sets of primers were used in this study as described in previous studies. The forward primer V1: 52 -CACCAGGTTGATTCTGCCTGAC-32 and the reverse primer PMP2: 52 -CCTCTCCGGAACCAAACCCTG-32 ) amplify the small-subunit ribosomal DNA (SSU-rDNA) of four human microsporidia, *E. bienewsi*, *E. intestinalis*, *E. cuniculi*, and *E. hellem* .

( Fedorko *et al.* ).Positive samples by V1/ PMP2 primers have been subject to two other PCR, using species-specific primers, V1/EB450 (52 - ACTCAGGTGTTATACTCAGTC-32 ) and V1/ SI500 (52 -CTCGCTCCTTTACACTCG-32 ) to differentiate *E. bienewsi* infection from *E. intestinalis* infection, respectively (Ombrouck *et al.*, 1997).

The primers V1 (5'- CACCAGGTTGATTCTGCCTGAC-3') and EB450 (5'-ACTCAGGTGTTATACTCAGTC-3') were used to amplify *E. bienewsi* DNA. The primers V1 and SI500 (5'-CTCGCTCCTTTACACTCGAA-3') were used to amplify *E. intestinalis* DNA.

Amplification of DNA was performed in a total volume of 20 µl. The PCR mixture consisted of 10× reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 mM (each) deoxynucleotide triphosphate, 300 pM (each) primer, and 1 U of *Taq* polymerase (Cinnagene, Iran).The PCR conditions for the V1/PMP2 sets of primers was 10 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; a final phase of extension

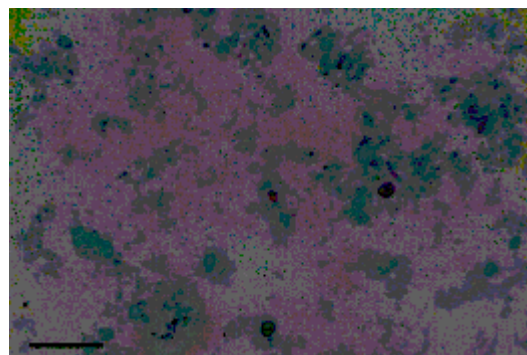
at 72°C for 10 minutes. The PCR conditions for the other two sets of primers was described by Ombrouck *et al.* (Ombrouck *et al.*, 1997). PCR products were assessed by electrophoresis in 1.5% (w/v) Tris-acetate/EDTA (TAE) agarose gel and staining with 0.5 µg/ml ethidium bromide. The size of the fragments was determined by using known size markers.

# RESULTS

Sixty four renal transplant recipients were included in this study. Light microscopy examination of samples using normal saline and Lugol's iodine only showed eight cases of infection with *Giardia lamblia* and saprophytes such as *Entamoeba coli*.

In five patients, chromotrope stained slides showed bright pink bodies with size 1-2.5 µm in diameter. These were accepted as positive samples for intestinal microsporidiasis (Fig-1).

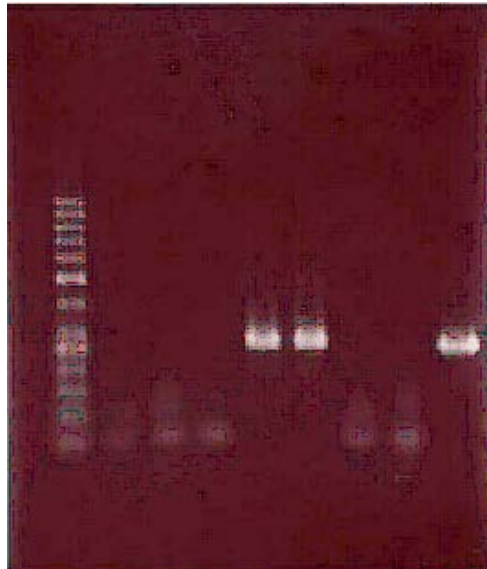
The first step in molecular diagnosis is DNA extraction. Microsporidian DNA was easily extracted from cultured organisms, but spores in stool specimen required harsh conditions employing both mechanical and chemical disruption and a laborious 4-day procedure. A dilution series using stool specimens spiked with *E. cuniculi* DNA demonstrated that fresh stool strongly inhibited the PCR assay but treatment of stool with 0.5% sodium hypochlorite or 10% formalin removed the PCR inhibition (Fedorko *et al.*, 1995). In this study formalin fixed stools were used for removing the PCR inhibitors also to yield a sufficient amount of pure DNA. Before DNA extraction procedure, the samples were heated in



**Fig. 1.** Microscopic photography of stool samples after Weber's staining (×100)

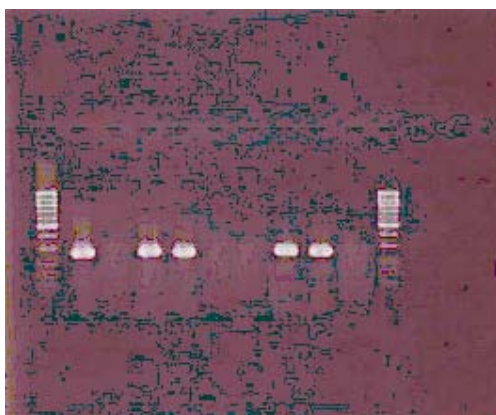
100°C as described by Ombrouck *et al.* (Ombrouck *et al.*, 1997).

Only four samples showed positive signal of 250 bp through first PCR (Fig-2), and after using V1/EB450 and V1/SI500 primers, all of them showed an approximately 350bp band that correspond to V1/EB450 primer (Fig. 3).



Lane 1: 50-basepair DNA ladder  
Lane 2: negativecontrol  
Lane 3-9: Patients samples

**Fig. 2.** Agarose gel electrophoresis of PCR products with V1/PMP2 primers



Lane 1: 100-basepair DNA ladder  
Lane 2-8: Patients samples  
Lane 9: negativecontrol  
Lane 10: 100-basepair DNA ladder

**Fig. 3.** Agarose gel electrophoresis of PCR products with V1/EB450 primers

## DISCUSSION

Microsporidia are obligatory intracellular protozoan parasites. They are released into the environment via stool, urine, and respiratory secretions. Persons or animals infected with microsporidia are possible sources of infection (Franzen and Muller, 2001). Studies suggested that water can be a source of infection for intestinal microsporidiosis (Dowda *et al.*, 1999; Fournier *et al.*, 2000).

The clinical signs of microsporidial infection include intestinal, ocular, muscular, and systemic disease (Weber *et al.*, 1994). The most prevalent microsporidian-associated disease in HIV-infected patients is chronic diarrhea with wasting syndrome (Cimerman *et al.*, 1999), whereas immunocompetent individuals and patients with immunodeficiency other than AIDS showed gastro-intestinal signs (Wichro *et al.*, 2005). Gastrointestinal system (GIS) complications like nausea, vomiting and diarrhea are quite frequent during the post-transplant period (Ponticelli and Passerini, 2005). Diarrhea is one of the most frequent and important complication in organ transplant recipients. The etiology of severe diarrhea following transplantation is complex. In addition to bacterial, viral and parasitic agents, almost all of the administered immunosuppressive agents might lead to diarrhea (Ponticelli and Passerini, 2005; Altinparmak *et al.*, 2002). Correct diagnosis of the cause of severe diarrhea in such patients can help to graft survival in transplant recipients (Maes *et al.*, 2006). There are few documented studies of microsporidiosis in renal transplant patients. *Encephalitozoon* species produce more invasive disease than others (Latib *et al.*, 2001; Mohindra *et al.*, 2002). In a study in Iran concerning intestinal parasitic infections in renal transplant recipients *Blastocystis hominis* and *Giardia lamblia* were the most frequent parasites (Nateghi Rostami *et al.*, 2007). Diagnosis of human microsporidiosis depends on the identification of spores in clinical samples. The identification of microsporidia by light microscopy has greatly improved during the last few years, but species differentiation is usually impossible by these techniques (Muller *et al.*, 1999). The sensitivity and specificity of microscopy method highly depend on the expertise of the examiner



(Katzwinkel *et al.*, 1997). PCR has been used to detect and identify of microsporidia in the clinical specimens. It has been recommended that multiple diagnostic methods may be required to diagnose a microsporidial infection, particularly when fecal specimens are examined (Garcia, 2002).

Although, in previously reported study PCR using V1/PMP2 primers showed more sensitivity than light microscopy to detect intestinal microsporidiosis (Muller *et al.*, 1999). In four of five samples that were positive through chromotrope staining method we could produce a 250 bp band through first PCR using V1/PMP2 primers and one sample was negative. The same result was reported previously (Katzwinkel *et al.*, 1997), and as described there "in the absence of either a gold standard for the detection of microsporidia in clinical samples or pathogenomonic clinical features of microsporidiosis, the true status of these discrepant cases cannot be resolved". All five microscopic positive samples were detected by using V1/EB450 *E. bienersi* specific primer and no product was seen using V1/SI500 primer. This finding is concordant with other reports that indicate predominance of *E. bienersi* in HIV-infected patients (Coyle *et al.*, 1996).

In conclusion, this study confirmed that diagnosis of microsporidial infections should be based on parasitological and molecular methods. Although, parasitological methods can be used in peripheral laboratories, species detection needs molecular methods.

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