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Original papers

Molecular characterization and phylogenetic analysis of Microsporidia and *Cryptosporidium* spp. in patients with multiple bowel biopsies from Fars Province, Iran

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ABSTRACT. Microsporidia and *Cryptosporidium* species are prominent agents of enteritis, capable of causing severe chronic diarrhoea in children, immunocompetent and immunocompromised individuals around the world. It is not possible to identify the parasites at species level solely on the basis of microscopy. The aim of the present study was to identify and characterize the species of Microsporidia and *Cryptosporidium* in immunocompetent humans with GI disturbances by nested PCR-RFLP, sequencing, and phylogenetic analysis. Fresh frozen and fresh paraffin-embedded biopsy specimens of the duodenum, jejunum, ileum and the cecum of 110 patients were examined. Genomic DNA was extracted from all bowel biopsies. Nested PCR targeting the 18S rRNA gene was performed by restriction endonuclease digestion of the PCR product followed by nucleotide sequencing and phylogenetic analysis. A total of three patients with chronic diarrhoea were positive for Microsporidia and *Cryptosporidium* spp. Species analysis showed the presence of *C. parvum* and *E. bieneusi* in two and one samples, respectively. This is the first PCR confirmation of the presence of *E. bieneusi* and *C. parvum* in a bowel biopsy of immunocompetent individuals in Iran. This study revealed that PCR, sequencing, and phylogenetic analysis are very powerful tools for the precise species identification of these pathogens.

Key words: *Cryptosporidium*, *Enterocytozoon bieneusi*, bowel biopsy, 18S rRNA gene, phylogenetic analysis, Iran

Introduction

Cryptosporidium is an obligate intracellular parasite closely related to Gregarines, which is known today to be the most important cause of enteritis in humans [1,2]. *Cryptosporidium* exists in the intestinal lumen, living in the parasitophorous vacuole under the plasma membrane of the host intestinal epithelial cells [3]. The parasite has a worldwide distribution, and infection occurs through the faecal-oral route, food and drinking water contaminated with its oocysts [4–6]. There are currently 31 legally acceptable species of *Cryptosporidium*. The majority of cryptosporidial infections in humans are caused by *C. parvum* and *C. hominis*, with *C. meleagridis* emerging as the third most frequent species to cause infection [7]. The prevalence of infection in immunocompetent persons and those affected with diarrhea, is about 2%, and is estimated to be about 22% in people with HIV infection [6]. In humans, symptoms include diarrhoea, vomiting, abdominal pain, and headache. In immunocompromised people, including AIDS patients, progressive gastroenteritis and persistent diarrhoea can arise, which can lead to loss of large volumes of fluid and death [8].

Microsporidia are a group of obligate intracellular parasitic fungi characterized by spore

production and known to be pathogens of invertebrates and vertebrates, including humans [9,10]. Until now, more than 1,200 species of Microsporidia are known, which are divided into 150 genera [11]. Infection with such genera as *Pleistophora* and *Nosema* is rarely diagnosed, but *Enterocytozoon bieneusi* and the *Encephalitozoon* species (*E. intestinalis*, *E. cuniculi* and *E. hellem*) are the four principle Microsporidia species infecting humans and animals [12,13]. These four species are known to be zoonotic pathogens [14,15]. In immunocompetent and immunocompromised persons, *E. bieneusi* and *E. intestinalis* invade the enterocytes of the small intestine, and infected sporoplasm is forced out of the cells [16], leading to severe and chronic diarrhoea, as well as cholangitis and cholecystitis [17]. Furthermore, *E. intestinalis* could infect other cells such as macrophages, fibroblasts and vascular endothelial cells, with infection being disseminated throughout the kidneys, liver, lungs and other organs in the body [18, 19]. The prevalence of infection in immunocompetent individuals is about 1%, and in immunocompromised patients it is between 6.5 to 27% [20,21].

The diagnosis of these parasites is generally based on the observation of spores and oocysts in samples under light microscope. But this method is inappropriate for determining the species of parasites. Molecular genetic techniques are powerful tools for identifying and differentiating the parasites at the species levels. Two molecular tools frequently used in the molecular study of these parasites in humans and animals, are polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP) of the 18S rRNA gene. This gene is highly polymorphic within these genera and is a useful target for the identification and differentiation of parasite species [22, 23].

The aim of the current study is to emphasize the importance of identification of these parasites in patients with chronic diarrhoea, and to characterize the phylogenetic relationship of Microsporidia and *Cryptosporidium* species in multiple bowel biopsy specimens from immunocompetent patients referred to the Department of Pathology, Shiraz Medical School.

Materials and Methods

Ethics statements. The research protocol was approved (approval no. 94-7548) by the Ethics Clearance Committee of the School of Medicine, Shiraz University of Medical Sciences (Shiraz, Iran). All study participants had given written informed consent before enrollment into the study.

Patients. In this cross-sectional study which was conducted from February to the end of December 2015, biopsy specimens of the duodenum, jejunum, ileum and the cecum of 110 patients were tested for the presence of *Cryptosporidium* and Microsporidia as coordinated with the Department of Pathology, Shiraz Medical School. A structured questionnaire was used to collect demographic data and clinical symptoms.

DNA extraction. Three to four 15 μm sections were cut from fresh frozen tissue specimens. Total genomic DNA was extracted from each clinical sample using the AccuPrep® Genomic DNA Extraction Kit (Bioneer-Daejeon-South Korea), with minor modifications of the manufacturer's protocol by subjecting samples to five cycles of freeze-thaw (freezing in liquid nitrogen for five minutes and thawing at 95°C in water bath for five minutes) to rupture the *Cryptosporidium* oocysts and Microsporidia spores. The quantification and quality control of the DNA extraction procedures were performed using a nano spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific). The DNA was stored at –20°C until use. All samples for PCR assays were prepared with aerosol-guard pipette tips to avoid contamination.

***Cryptosporidium* Nested-PCR-RFLP analysis.** Amplification of a fragment of the 18S rRNA gene locus (840 bp) was carried out using a two-step nested PCR approach. This fragment has been shown to be very specific to *Cryptosporidium*.

The primers C18PF1: 5"–TTC TAG AGC TAA TAC ATG CG-3", C18PR1: 5"–CCC ATT TCC TTC GAA ACA GGA-3", C18NF2: 5"-GGA AGG GTT GTA TTT ATT AGA TAA AG-3", and C18NR2: 5"-CTC ATA AGG TGC TGA AGG AGT A-3" were used for the first and second round nested-PCR [24]. Briefly, the PCR conditions were composed of pre-denaturation at 94°C for five minutes, then 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for one minute, followed by final extension at 72°C for 10 minutes. The secondary annealing temperature was 58°C.

RFLP of the secondary PCR product was carried out using *Ssp*I and *Vsp*I (Fermentas, Vilnius, Lithuania) for species and genotype identification of *Cryptosporidium* respectively. The reaction mixture

contained 5 µL of the secondary product, 0.5 µL of *Ssp*I or *Vsp*I (20 U), 2.2 µL of restriction buffer; this was incubated at 37°C for 120 minutes, under conditions recommended by the manufacturer. The digestion products were separated on a 2% agarose gel and visualized after GelRed (Biotium Inc., Hayward, CA) staining.

Microsporidian species characterization by PCR. Microsporidian 18S rRNA coding regions were amplified using the following primers: EBIEF1 (5"-GAA ACT TGT CCA CTC CTT ACG-3") and EBIER1 (5"-CCA TGC ACC ACT CCT GCC ATT-3") to amplify a 607 bp fragment from *E. bieneusi*, SINTF (5"-TTT CGA GTG TAA GGA GTC GA-3") and SINTR (5"-CCG TCC TCG TTC TCC TGC CCG-3") to amplify a 520 bp fragment from *E. intestinalis*, ECUNF (5"-ATG AGA AGT GAT GTG TGT GCG-3") and ECUNR (5"-TGC CAT GCA CTC ACA GGC ATC-3") to amplify a 549 bp fragment from *E. cuniculi*, and EHELF (5"-TGA GAA GTA AGA TGT TTA GCA-3") and EHELR (5"-GTA AAA AGA CTC TCA CAC TCA-3") to amplify a 547 bp fragment from *E. hellem* [25].

Concisely, the PCR conditions included an initial denaturation at 94°C for five minutes, followed by 35 cycles, each consisting of 94°C for 30 s, 55°C for 45 s, 72°C for 90 s, and a final extension step at 72°C for 10 minutes. Amplified products were resolved on 2% agarose gel and stained with GelRed (Biotium Inc., Hayward, CA).

All primers were synthesized by Macrogen Genomics Laboratories (Macrogen, Seoul, South Korea).

DNA sequence analysis. For confirmation, the secondary PCR products were purified by using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced on both ends through the sequencing service of Macrogen Genomics Laboratories (Seoul, South Korea). The resulting sequences were edited and aligned with the BioEdit Sequence Alignment Editor, version 7.2.5. [26]. Multiple alignments were performed with GenBank copies of Microsporidia and *Cryptosporidium* species data from Iran and other countries. A phylogenetic tree was constructed with the Maximum Composite Likelihood option of the Neighbor-Joining method using MEGA-7 [27]. The reliability of the NJ tree was assessed by the bootstrap method with 1,000 replications, and 95% site coverage cutoff as the statistically significant value.

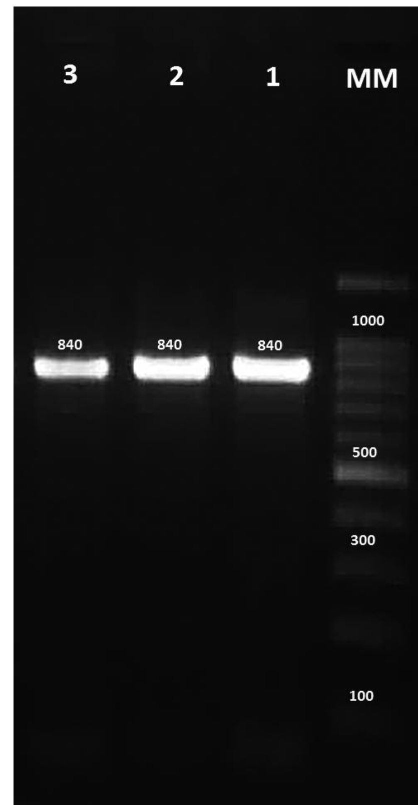

Fig. 1. Agarose gel electrophoresis of 18S rRNA secondary PCR products (840 bp) of *Cryptosporidium* spp.
Lanes are: MM Molecular Marker; 1 and 2 Positive samples; 3 Positive control.

Nucleotide sequence accession numbers. The partial sequences of the 18S rRNA genes of *C. parvum* and *E. bieneusi* obtained in this study were deposited in the GenBank database under accession numbers KX216598- KX216599.

Results

Molecular analysis of *Cryptosporidium* 18S rRNA gene

Nested-PCR was accomplished for amplification of estimated sizes of 1325 bp and 840 bp fragments (Fig. 1) in the first and second PCR reaction, respectively. Two biopsy samples were positive in nested PCR. Species recognition was performed by digesting the secondary PCR product with an *Ssp*I enzyme that cut in locations 11, 12, 108, 268, and 448, of which 108, 268, and 448 were visible after electrophoresis on agarose gel (Fig. 2). Genotype identification was carried out by *Vsp*I restriction enzyme, which demonstrated three cuts in locations 104, 116, and 628, of which 104 and 628 were visible after electrophoresis on agarose gel

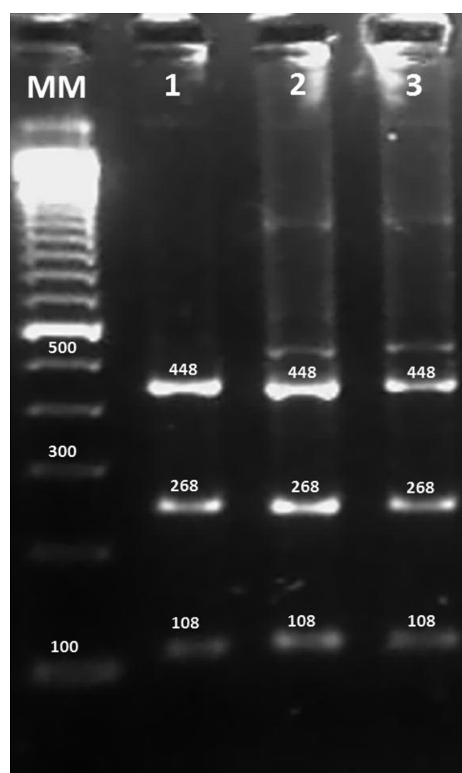

Fig. 2. Shows the profile of the RFLP analysis for characterization of *Cryptosporidium* species in positive bowel biopsy samples based on 18S rRNA gene, using *Ssp*I restriction enzyme that recognized *C. parvum* in all of the positive samples
Lanes are: MM Molecular Marker; 1 Positive control; 2 and 3 *C. parvum* (108, 268 and 448 bp bands).

(Fig. 3). The two positive biopsies were characterized as *C. parvum*.

Molecular analysis of Microsporidial 18S rRNA gene

Amplification of the DNA yielded from all biopsy specimens with the specific primers given above enabled one positive case to be characterized by PCR. Single-species infection was identified for *E. bieneusi* in a biopsy sample; with a diagnostic band of 607 bp visible in the agarose gel (Fig. 4). When the templates obtained were assayed with the specific primers for the three species of *Encephalitozoon* known to infect humans, no amplification was obtained in any of the samples studied.

18S rRNA gene sequencing and phylogenetic analysis

The achieved sequences confirmed the presence of the *C. parvum* (two isolate) and *E. bieneusi* (one isolate) that were identified by the PCR analysis. The sequences of two isolates of *C. parvum* were 100% identical (GenBank accession no. KX216598).

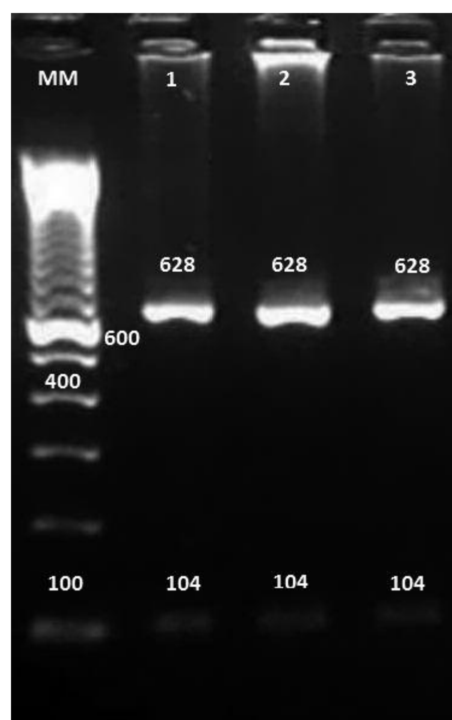

Fig. 3. Shows the profile of the RFLP analysis for characterization of *Cryptosporidium* genotype in positive bowel biopsy samples based on 18S rRNA gene, using *Vsp*I restriction enzyme that recognized *C. parvum* in all of the positive samples
Lanes are: MM Molecular Marker; 1 and 2 *C. parvum* (104 and 628 bp bands); 3 Positive control

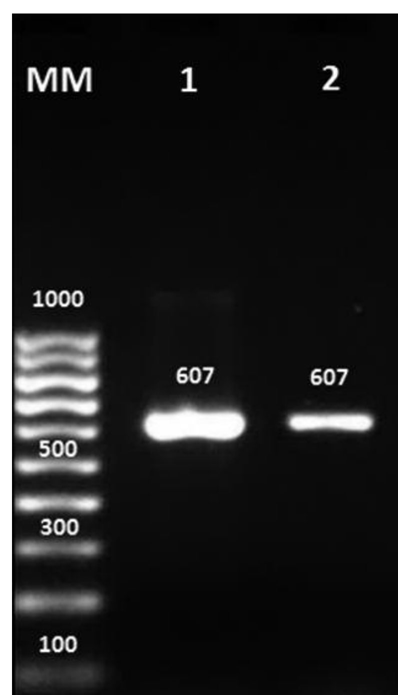

Fig. 4. PCR amplified product of Microsporidia spp. based on 18S rRNA gene from one positive biopsy sample, which shows a diagnostic band of 607 bp fragment specific for *E. bieneusi*
Lanes are: MM Molecular Marker; 1 *E. bieneusi*; 2 Positive control.

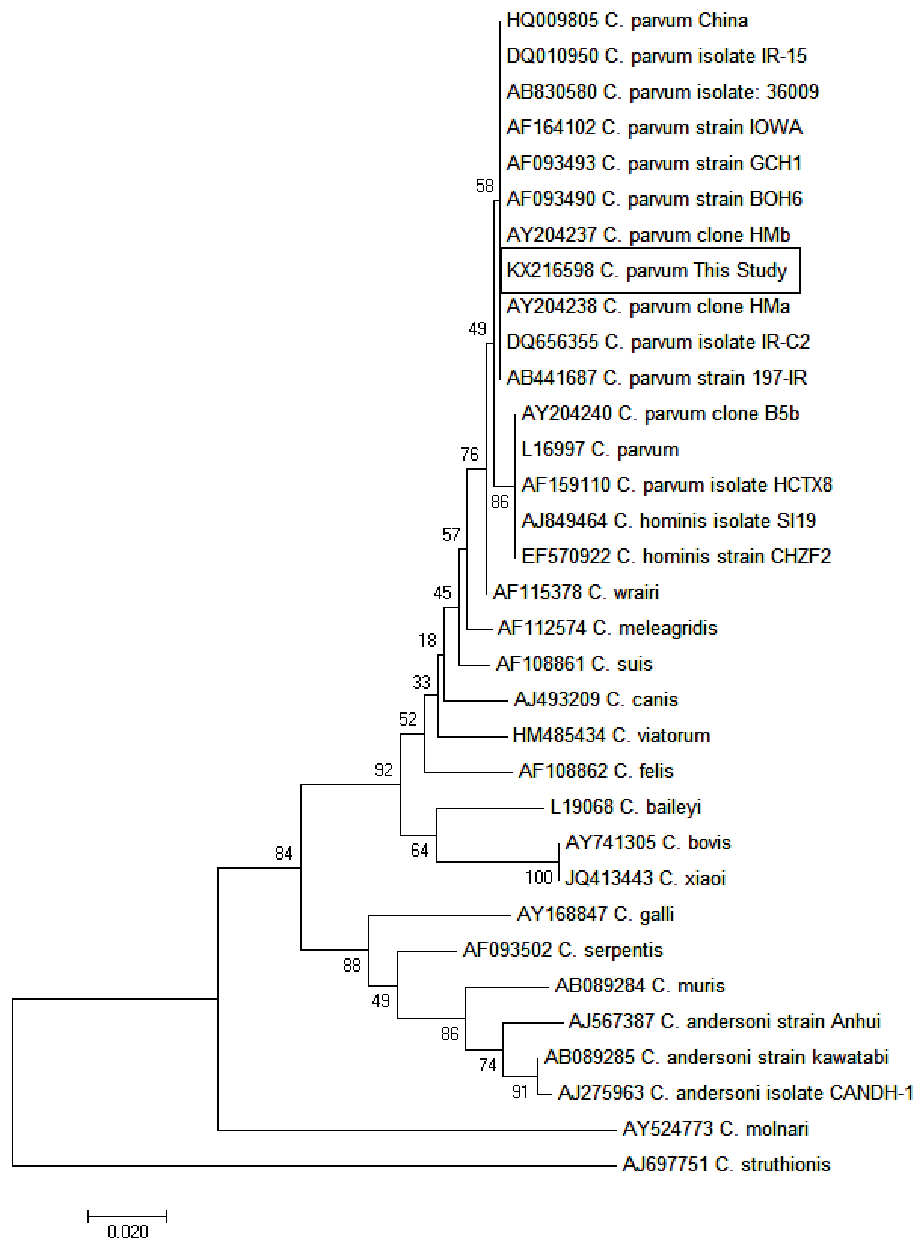

Fig. 5. Phylogenetic relationship among various *Cryptosporidium* isolates to each other as inferred by Neighbor-Joining tree based on 18S rRNA gene
Numbers on branches are percentage bootstrap values of 1,000 replicates. All positions with less than 95% site coverage were eliminated. The evolutionary distances between sequences were computed using the Maximum Composite Likelihood method. The scale bar indicates an evolutionary distance of 0.020 nucleotides per position in the sequence. The reference sequences accession numbers are inserted. Evolutionary analyses were conducted in MEGA-7.

Each of the two *C. parvum* isolates showed 100% identity to the published genotype II HMa and HMb isolates (Accession numbers AY204238-AY204237 respectively). One *E. bieneusi* isolate showed 100% identity to the published isolates (Accession numbers AF023245, AF024657 and L16868).

The phylogenetic relationships of the isolates were evaluated using the NJ phylogram method (Figs. 5 and 6). In the NJ method, the two *C. parvum* and one *E. bieneusi* examined in this study formed one clade with the published isolates mentioned above, with full statistical reliability (100%).

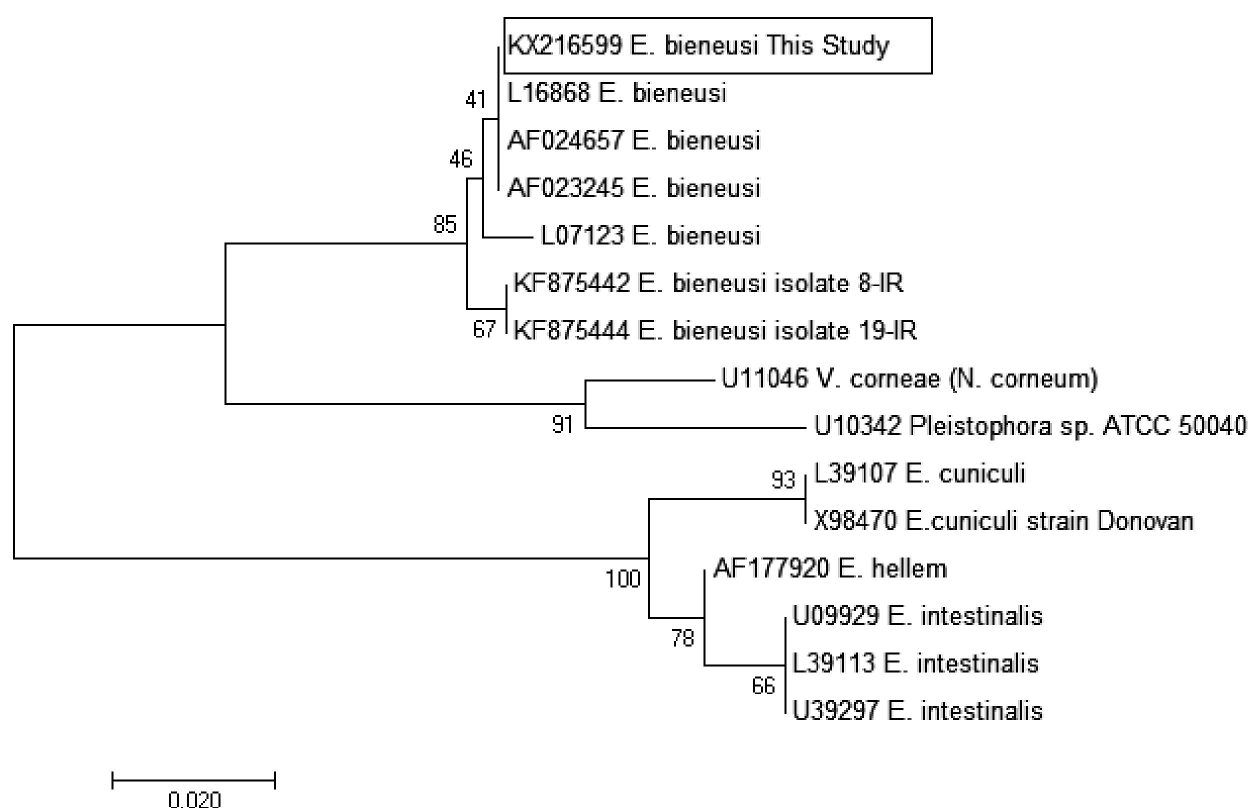

Fig. 6. Phylogenetic relationship among various Microsporidial isolates to each other as inferred by Neighbor-Joining tree based on 18S rRNA gene
Numbers on branches are percentage bootstrap values of 1,000 replicates. All positions with less than 95% site coverage were eliminated. The evolutionary distances between sequences were computed using the Maximum Composite Likelihood method. The scale bar indicates an evolutionary distance of 0.020 nucleotides per position in the sequence. The reference sequences accession numbers are inserted. Evolutionary analyses were conducted in MEGA-7.

Discussion

Opportunistic agents such as *Cryptosporidium* and Microsporidia cause gastroenteritis in children, immunocompetent or immunocompromised individuals. Molecular genetic techniques are authoritative tools used to distinguish the different species of these parasites. Until now, several molecular markers have been employed to discriminate species within the genus *Cryptosporidium*. Nested PCR-RFLP for 18S rRNA gene has been found to be suitable for the differentiation of *Cryptosporidium* species [28]. In the present study, the species responsible for cryptosporidiosis in two positive patients were both verified as *C. parvum* genotype II by sequencing and phylogenetic analysis. The prevalence of infection in immunocompetent persons affected with diarrhoea, has been found to be about 2% [6], which is in line with our findings. In addition, the fact that the predominant form in the present study was *C. parvum* is in agreement with previous studies of human cryptosporidiosis in other developing countries [29]. A study characterizing isolates of *Cryptosporidium* spp. from human and animal hosts in Iran on the basis of the 18S rRNA gene by Meamar et al. found *C. parvum* to be the predominant species in humans [30]. Pirestani et al. [31] also studied the molecular determination of *C. parvum* in human and cattle with 18S rRNA gene in the Shahriar region. Their study revealed that *C. parvum* isolated from cattle was associated with infection transmission to humans in that region. Rafiei et al. [32] found *C. parvum* to be the most prevalent species infecting humans in a study on *Cryptosporidium* spp. isolated from immuno-compromised patients and children. Similarly, Vejdani et al. [33] found that PCR-RFLP was 2.1% more sensitive than IFA for examining human faecal samples, and that *C. parvum* was the most predominant species in humans.

Likewise, most PCR assays performed in other

countries have led to the confirmation of *C. parvum* as the major cause of cryptosporidiosis in humans. Gatei et al. [34] performed a thorough molecular analysis of the 18S rRNA gene of *Cryptosporidium* isolates from patients with or without HIV Infections living in Kenya, Malawi, Brazil, the United Kingdom and Vietnam. Altogether, 75 percent of isolates were found to be *C. parvum*. In a study of *Cryptosporidium* species distribution amongst 2414 patients with diarrhoea in England between 1985 and 2000, Leoni et al. identified *C. parvum* and *C. hominis* in the majority of cases [35]. Trotz-Williams et al. [36] genotyped 11 human cases of cryptosporidiosis in the Ontario province by PCR-RFLP analyses of the *Cryptosporidium* oocyst wall protein (COWP) and 18S rRNA genes and found *C. parvum* to be the predominant isolate from humans. Their findings suggest that cattle and other ruminants may be a source of human infections in Ontario. An investigation of sporadic human cryptosporidiosis in 261 human faecal samples in New South Wales, Australia by Waldron et al. [37] found *C. hominis* and *C. parvum* to be the most frequent causes of human cryptosporidiosis, constituting 59% and 16% of infections, respectively. Coupe et al. [38] found the 18S rRNA gene sequence to be the most informative and practical method of examining 68 stool samples from patients with microscopically-proven cryptosporidiosis. Their genotyping study confirmed that *C. hominis* and *C. parvum* were the predominant species in both immunocompetent and immunocompromised patients.

Cryptosporidiosis in our study was significantly associated with the occurrence of diarrhoea and inflammatory reaction. This relationship was largely attributable to *C. parvum* genotype II. This is in agreement with previous studies [39]. In children and immunocompromised persons, differences in clinical manifestations have been observed between *Cryptosporidium* species, especially between *C. hominis* and *C. parvum*. Besides, infections with *C. parvum* were associated with chronic diarrhea and vomiting in children more frequently than *C. hominis* [40, 41].

In addition, our present findings identify one positive case of *E. bieneusi* by sequencing and phylogenetic analysis. To our knowledge, this is the first report of *E. bieneusi* infection in an immunocompetent patient in Iran, verified by molecular methods and sequencing. Only very limited data is available in Iran on human microsporidiosis, and until the present time only limited studies have been achieved. Until now, only one report of Microsporidia infection in patients from Iran has been submitted to PubMed. Mirjalali et al. [42] report that 25 of 81 stool samples collected from HIV positive patients were infected with Microsporidia species, and *E. bieneusi* were identified in all positive samples, which concurs with our findings.

The positive case of *E. bieneusi* identified in this study shows that this organism can cause chronic diarrhoea in immunocompetent individuals. Other studies of immunocompetent individuals refer to isolated cases or cases among groups of young individuals who live in European countries [43–45]. In most of these studies, *E. bieneusi* was the Microsporidia identified, as have other studies performed in South Africa, India and China [46–48]. The three patients described in the present study represent the first cases of Microsporidiosis and Cryptosporidiosis identified in immunocompetent individuals in Iran, and, more interestingly, the first of such cases diagnosed by use of PCR in bowel biopsy specimens.

In conclusion, the results of our study reveal that 18S rRNA-based PCR and PCR-RFLP tools can be used impressively in the analysis of clinical samples and can be used to supplement routine detection methods. Correspondingly, our results provide new data on Microsporidia and *Cryptosporidium* infection in immunocompetent patients with chronic diarrhoea in Iran. The possibility that infection with *E. bieneusi* and *C. parvum* is associated with symptomatic disease in patients with a competent immune system needs to be clarified in further studies; however, it is noteworthy that *E. bieneusi* and *C. parvum* were the only parasitic pathogens detected in three patients with chronic diarrhoea. Hence, the presence of chronic intestinal microsporidiosis and cryptosporidiosis should be suspected in patients with persistent diarrhoea, malabsorption and progressive weight loss.

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