Molecular diagnosis of microsporidia strains in slaughtered cows of southwest of Iran

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Received: 15 August 2017 / Accepted: 14 November 2017 / Published online: 1 December 2017
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Abstract Microsporidia is often considered as an opportunistic infection in patients with impaired immune systems such as patients with acquired immune deficiency syndrome, transplant recipients, children and old people. Due to the ability of the parasite to transmit from animals to human as well as the increasing prevalence of parasitic infections and immune deficiency diseases; therefore, the aim of this study was to evaluate molecular diagnosis of microsporidia strains in slaughtered cows of southwest of Iran. Initially, 256 stool samples of cows were collected from 5 regions of Khuzestan province, southwest of Iran and stained by modified trichrome (weber). Then, the parasite spores were examined by optical microscope. Total DNA was extracted from samples using DNA extraction kit for stool (Bioneer) and evaluated by multiplex/nested PCR method. The products of nested-PCR were explored by RFLP method using restriction enzyme *MnII*. For genotyping, positive samples of RFLP were sequenced. Of 256, 21 and 48 samples were found positive by the staining and nested PCR tests, respectively. Of 48, 36 samples were *Enterocytozoon bienersi* with genotype frequency D (22), J (9) and M (5). Also 9 were detected as *Encephalitozoon* species among which 2 were *E. cuniculi*, 6 were *E. intestinalis* and 1 was *E. hellem* Eventually, 3

samples were found positive for both *Enterocytozoon* and *Encephalitozoon*. The results showed that cows can be a source of microsporidia infections. Due to the zoonotic importance of this parasite and its ability to transmit from animals to humans; the detection and species determination of the parasite seems essential. The highest risk of infection is for individuals with impaired immune systems.

Keywords *Enterocytozoon bienersi* · *Encephalitozoon* · Microsporidia · Genotype · Cow · Iran

Introduction

Microsporidia is a spore forming obligate intracellular parasite that can infect wide range of vertebrate and invertebrate hosts (Franzen and Müller 1999; Weber et al. 1994). The life cycle of this parasite is consists of 3 separate stages: infectious stage, replicating or growing stage or schizogony and spore formation stage or sporogony (Didier et al. 1998). Microsporidia is often considered as an opportunistic infection in patients with impaired immune systems such as patients with acquired immune deficiency syndrome (AIDS), transplant recipients, passengers, children, elderly and people with ophthalmic lenses (Omalu et al. 2006). Several new species of microsporidia was identified in AIDS patients that including *Enterocytozoon bienersi* (*E. bienersi*) in 1985, *Encephalitozoon hellem* (*E. hellem*) in 1991 and *Septata intestinalis* in 1993 (Weber et al. 1994). The symptoms spectrum caused by the virulent organism is so vast that it affects all body organs and cause symptoms such as keratoconjunctivitis, hepatitis, myositis, sinusitis and disseminated infections (Omalu et al. 2006). Clinical signs of the disease include chronic diarrhea, nausea, severe weight loss and confusion (Lindsay et al.

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2004). Probably, many human microsporidiosis have zoonotic origin and transferred by water contaminated with animal stool. However, human to human transmission has also been described (Samie et al. 2007). Although, these factors are self-limited in immunocompetent hosts, but these can be life-threatening in people with immune system dysfunction, especially AIDS patients (Franzen and Müller 1999; Samie et al. 2007). For this reason, Due to the zoonotic ability of microsporidia as well as the increasing reports of prevalence, particularly in immune deficiency cases, the present study was designed to use molecular techniques to evaluate microsporidia strains in slaughtered cows of southwest of Iran.

Methods

Sample collection

Totally, 256 stool samples of cows were collected from 5 regions of Khuzestan province (North, South, East, West and Central), southwest of Iran. The mount of 100 g of samples were collected by rectal touche method from the large intestine of the slaughtered cows. The samples were transferred to Department of Parasitology, Ahvaz Jundishapur University of Medical Sciences. Then, part of the fecal sample was used for smear preparation and staining. The rest of feces were mixed with twice the volume of potassium dichromate 2.5% and were kept at 4° C for further use (Cama et al. 2007).

Samples examination by staining

Part of the fecal sample was stained by modified trichrome (weber). At first, the slides were fixed with methanol. Then, the samples were placed in trichrom color for 240 min. After decolorization with acid-alcohol, and washing with 95% ethanol, the slides were placed in absolute ethanol and for clarification in xylene. At the next stage, to identify the spores of microsporidia, the slides were examined by an optical microscope at magnification of $\times 1000$ together with immersion oil. The positive samples were identified by dorsal vacuoles in microsporidia spore (Pirestani et al. 2011).

Extraction of DNA

The DNA was extracted by DNA extraction kits for stool (Bioneer) and the extracted DNA was stored at -20°C .

Molecular detection

The extracted DNA was used for identification of different microsporidia genera by multiplex/nested PCR (Katzwinkel

Table 1 The primary and secondary primers used for multiplex/nested PCR

The primary primers	The secondary primers
MSP-1: tgaatgkgtccctgt	MSP-3: ggaattcacaccgcccgctcrtat
MSP-2A: tcactcgccgctact	MSP-4A: ccaagcttatgcttaagtymaargggg
MSP-2B: gttcattcgactact	MSP-4B: ccaagcttatgcttaagtcaggagg

et al. 1996). Table 1 indicated the primary and secondary primers used for multiplex/nested PCR. Flanking regions of the small subunit (SSU) and large subunit (LSU) of the ribosomal DNA (rDNA) and internal transcribed spacer (ITS) were the targets for the multiplex/nested-PCR method. Upstream primers MSP-1 and MSP-3 target the 3 region of the SSU and recognized a broad range of microsporidian species, including *Encephalitozoon* spp. and *E. bienersi*. Downstream primers MSP-2B and MSP-4B target the 5 region of the LSU of *E. bienersi*, while MSP-2A and MSP-4A specifically recognize *Encephalitozoon* spp. and some other microsporidia but not *E. bienersi* (Sokolova et al. 2011). The amplified fragment length by the primers was 500 bp and 300 bp for gender of *Enterocytozoon* and *Encephalitozoon*, respectively. At first, the samples were examined by the primary primers. Then, for differentiating the species of *Enterocytozoon* and *Encephalitozoon*, the positive samples were explored by the secondary primers and by multiples/nested PCR method. Ultimately, for further confirmation, the nested PCR positive samples were subjected with RFLP using *MnII* enzyme..

Sequencing

For genotyping, the PCR–RFLP positive samples were sequenced by Bioneer Company (Daejeon, South Korea). The sequenced nucleotides were compared with published sequences and homology was analyzed using BLAST and DNA sisMax 3.0. Finally, the phylogenetic tree was drawn using the MEGA (version 3) and Neighbor-Joining software (Fig. 4).

Results

The staining analysis

Figure 1 shows microsporidia spore in the stool samples stained by modified trichrome (weber). Of 256, 21 samples were positive by the staining.

The molecular and RFLP analysis

Figure 2 indicates electrophoresis of PCR products on a 1.5% agarose gel. In addition, Table 2 demonstrates the

Fig. 1 Microsporidia spore in stool samples that stained by modified trichrome (weber), the slides were examined by optical microscope at magnification of $\times 1000$ together with immersion oil (spore size was 4–6 μm)

Fig. 2 The electrophoresis of PCR products on 1.5% agarose gel, M: DNA marker 100 bp, sample 1: *Encephalitozoon* spp (300 bp), sample 2, 3, 4, 5 and 7: negative sample, sample 6: *E. bieneusi* (500 bp)

results of molecular analysis of the collected fecal samples in the cities of Khuzestan province, southwest of Iran. According to the finding, the nested PCR revealed that 48 (18.7%) were positive out of 256 samples screened, in which 36 (14.1%) were *E. bieneusi* and nine (3.5%) cases were detected as *Encephalitozoon* species. Lastly, three samples were found positive for both *Enterocytozoon* and *Encephalitozoon*. Figure 3 shows electrophoresis of the RFLP products on a 2% agarose gel. Based on the results of RFLP, *Encephalitozoon* species were contains *E. cuniculi* (Weber et al. 1994), *E. intestinalis* (Samie et al. 2007) and *E. hellem* (Franzen and Müller 1999).

The genotyping analysis

Based on the results of genotyping, the genotype frequency of *E. bieneusi* was D (22), J (9) and M (5). Figure 4 indicates the phylogenetic tree of *E. bieneusi* based on ITS, SSU and LSU regions of bovine isolates. The sequence of M genotype (M1–M4) identified in the present study has 100% similarity with published sequence of bovine origin (Accession No. AB897497.1). Also, the isolates of J-1–J-7 were 100% similar to the registered isolates of bovine in GenBank with accession number AF135837.1. Two isolates of *E. cuniculi* and one isolate of *E. hellem* were 100% similar to the registered isolates of bovine in GenBank with accession numbers AB897506 and AB897505.1, respectively. Comparison of the nucleotide sequence of *E. intestinalis* with the registered sequence in GenBank with accession number AB 897501.1 showed that of 6 positive samples, 2 cases had a nucleotide difference with the registered sequence in GenBank. This difference was in position 80 that guanine (G) base was replaced by thymine (T) base. Also, the nucleotide sequence comparison of 12 positive samples of *E. bieneusi* with the registered sequence in GenBank with accession number AB897492.1 indicated that there was a significant nucleotide difference and cytosine (c) base was replaced by adenine (A) base in position 251. On the other hand, in the sample No. 7 and 10, thymine (T) and guanine (G) were replaced by adenine (A) and thymine (T) in position 306 and 319, respectively. Also, in the sample No. 6 was a difference in 320 position of ITS region and thymine (T) base was replaced by adenine (A). In 330 position, two nucleotide differences were observed in the sample No. 4 and 12 that adenine (A) and guanine (G) were replaced by cytosine (c) and adenine (A), respectively. Eventually, *E. bieneusi* sequence had 80% homology with the registered sequence in GenBank with accession number AB897492.1.

Discussion

Opportunistic infections are one of the major factors in mortality of patients with acquired immune deficiency syndrome. Reports related to microsporidia infections in patients with chronic diarrhea are increasing and its prevalence has reached from 22 to 32% in developing countries (Samie et al. 2007; Ignatius et al. 1997). Accordingly in this study, we investigated intestinal microsporidiosis in the slaughtered cows of 5 regions of Khuzestan province (North, South, East, West and Central), southwest of Iran. Because of direct and indirect relationship with humans, cow was selected. In this research, we used from the modified trichrome (weber) staining based on studies of Ryan et al. (1993). In this

Table 2 The results of molecular analysis of the collected fecal samples in the cities of Khuzestan province, southwest of Iran

City (number)	Genus of parasite	Total number	Species and genotypes (number)	Percentage
Ahvaz (2)	<i>Encephalitozoon</i>	9	<i>E. cuniculi</i> (Weber et al. 1994)	3.5%
Dezful (1)			<i>E. intestinalis</i> (Samie et al. 2007)	
Bavi (3)			<i>E. hellem</i> (Franzen and Müller 1999)	
Mahshahr (1)				
Andimeshk (1)				
Abadan (1)	<i>E. bieneusi</i>	36	D (22)	14.1%
Ahvaz (5)			J (9)	
Andimeshk (4)			M (5)	
Mahshahr(1)				
DashtAzadegan(2)				
Dezful (8)				
Baghmalek (5)				
Bavi (4)				
Ramhormoz (2)	Mix	3	<i>Enterocytozoon</i> and <i>Encephalitozoon</i>	1.1%
Behbahan(1)				
Hoveyze(4)				
Ahvaz (1)				
Hoveyze(1)				
Dezful (1)

Fig. 3 Electrophoresis of the RFLP products on a 2% agarose gel, M: DNA marker 100 bp, sample 1: Negative control, sample 2: Positive control without enzyme, sample 3: *E. cuniculi* (210 and 90 bp) sample 4: *E. intestinalis* (160 and 60 bp), sample 5: *E. Hellem* (180 and 80 bp)

staining, the background color of blue aniline is stable and it has better contrast with other bacterial and fungal agents. Also, amount of its phosphotungstic acid is down that improves the background color (Ryan et al. 1993). On the other hand, the PCR technique is a successful method that can be detect microsporidia infections with small numbers

of spore in clinical and environmental samples. According to various studies, the detection threshold of microsporidia in faecal samples is 100 and 10,000–1,000,000 spores per gram of feces by the PCR and light microscopy, respectively. It represents the high sensitivity of the technique in comparison with the staining methods (Müller et al. 2001; Garcia 2002). For this reason, we observed that of 256 samples, 21 cases were positive by the staining but in nested-PCR method, 48 samples were positive.

The highest prevalence of intestinal microsporidiosis in humans is related to *E. bieneusi*. In the current study, also, this parasite had the highest prevalence in cows. The results of this study showed that 36 (14.1%) of the fecal samples were infected with the parasite and the highest prevalence was related to genotype D. Consistent with these results, in 2006, Leelayoova et al. in Thailand indicated that the highest prevalence of genotype of *E. bieneusi* was related to genotype D (36.4%) in fecal samples of AIDS patients. The phylogenetic analysis of the data suggests the zoonotic importance of *E. bieneusi* (Leelayoova et al. 2006). Also, in 2008, Lee et al. examined 180 milk specimens of dairy cows by molecular analysis and showed that of 180 milk specimens, 50 samples were found positive for *E. bieneusi*, of which 3 were identical to the human *E. bieneusi* types. This study suggests that some the parasite isolates from milk can infect humans as well as cows' milk may play an important role at the transmission of the parasite infections to humans (Lee 2008).

Fig. 4 The phylogenetic tree of *E. bieneusi* based on ITS, SSU and LSU regions of bovine isolates

According to the results of this study, of 36 *E. bieneusi* species, 9 cases were identified as genotype J. In 2005, for the first time, Haro et al. (2005) identified genotype J of *E. bieneusi* in non-mammalian host (chicken). This study along with our study shows that there is the transmission risk of certain genotypes to new host that this genotype previously was not seen in the host and was seen in a specific host. The identification of this genotype in cow increases geographical and zoonotic range the parasite.

Based on the results of RFLP in our study, *Encephalitozoon* species were detected in nine samples that including *E. cuniculi* (Weber et al. 1994), *E. intestinalis* (Samie et al. 2007) and *E. hellem* (Franzen and Müller 1999). In the past, this view was that *E. bieneusi*, *E. intestinalis* and *E. Hellem* can be seen only in humans but afterwards, *E. bieneusi* and *E. intestinalis* were also detected in a wide range of domestic and wild animals (Reetz et al. 2004). On the other hand, *E. Hellem* was identified in the birds (Gatti et al. 1997). There are also *E. cuniculi* in various animals. This data indicated that four main species infecting humans have zoonotic sources (Haro et al. 2003). Due to the importance of microsporidia zoonotic and its ability to transmit from animals to human, as well as, due to the increasing prevalence of parasitic infections and immune deficiency diseases such as patients with acquired immune deficiency syndrome (AIDS), transplant recipients; the detection and species determination of the parasite seems essential for adoption of health strategies.

Finally, this study is important in terms of health; because the opportunistic pathogens such as human microsporidia were isolated from the slaughtered cows of Khuzestan province, southwest of Iran. Because of the close relationship with humans, the source of milk and

meat for humans, the use of the feces for fertilizing; this animal is as an important source of pollution. Therefore, in order to the design of appropriate prevention for the parasite, it is essential that the high-risk individuals such as patients with impaired immune systems should be receive the accurate information about the risk of direct relationship with these animals. Also, it is recommended to further researchers, to examine the different hosts such as the domestic and wild animals and the role of these animals in infecting high-risk individuals and other people. Hence, it is need to examine the whole range of epidemiological and molecular studies on the hosts.

Conclusion

The results showed that cows can be a source of microsporidia infection. Due to the zoonotic importance of this parasite and its ability to transmit from animals to humans; the detection and species determination of the parasite seems essential for adoption of health strategies. The highest risk of infection is for individuals with impaired immune systems.

Acknowledgements We would like to express our gratitude towards Ahvaz Jundishapur University of Medical Sciences for sponsoring this research project as a master's thesis in parasitology registered under OG-92128. Thanks also go to the Department of Parasitology, Jundishapur University of Ahvaz for its assistance in laboratory tasks.

Author's contribution All authors contributed to the study design. Mehdi Tavalla and Moluok Beiromvand were leader of the research. Elham Kord-Sarkachi carried out experimental tests and prepared the Manuscript. All authors read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

Human and animal rights All animal experimental procedures were approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Iran (with code of OG-92128).

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