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PROTOZOOLOGY - ORIGINAL PAPER

Isolation, identification, and phylogenetic analysis of potentially pathogenic free-living amoebae isolated from nasal and oral mucosa of HIV/AIDS patients in Iran

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Abstract

The burden of HIV/AIDS in Iran is not as high as in the other countries with high prevalence; however, the number of cases of HIV/AIDs is increasing in this region. According to a recent report, Iran had 5000 (1400–13,000) new cases of HIV infection with 4000 (2500–6200) AIDS-related deaths. Individuals affected by HIV/AIDS are highly susceptible for developing opportunistic infections, e.g. the cerebral complications related to pathogenic free-living amoebae and colonization of free-living amoebae (FLA) can be a serious hazard for patients living with HIV/AIDS. In the present study, a total of 70 oral and nasal mucosal samples were obtained from HIV/AIDS patients referred to the reference hospitals in Iran and tested for the presence of potentially pathogenic FLA using culture and PCR/sequencing-based methods. To discern the taxonomic status of *Acanthamoeba* genotypes a maximum likelihood phylogenetic tree was constructed and tolerance assays were performed for the positive *Acanthamoeba* strains. Among the patients with HIV/AIDS referred to the reference hospitals from 2017 to 2019, 7.1% were found positive for pathogenic free-living amoebae. Three strains (HA3, HA4, and HA5) belonged to the T4 genotype, one strain (HA1) was related to the T5 genotype assigned as *A. lenticulata*, and another strain (HA2) had high homology to *Vermamoeba vermiformis*. The tolerance assay used for *Acanthamoeb*a strains (HA1, HA3, and HA4) classified these amoebae as highly pathogenic strains. For the most part, the encephalitis cases occurring in HIV/AIDS patients in Iran remain undiagnosed due to lack of awareness of the practitioners on the available diagnostic tools for this lethal infection; therefore, the true incidence of GAE in this region is unknown. A possible colonization with FLA should be considered in the differential diagnosis of suspected cases of CNS infections among HIV/AIDS patients. This study is the first worldwide comprehensive study attempting to isolate and identify the FLA colonization in HIV/AIDS patients. This study highlights the fact that clinicians should be aware of the differential diagnosis of cerebral disease related to FLA in patients with HIV/AIDS.

Keywords HIV/AIDS patients · Free-living amoebae · Iran

Introduction

HIV-related CNS infections remain a serious challenge as such infections are considered as important causes of death among the people living with HIV (Bowen et al. 2016). Individuals affected by HIV/AIDS are highly susceptible to developing opportunistic infections such as the cerebral complications related to pathogenic free-living amoebae (FLA) (Sisson 1995; Khan and Siddiqui 2009; Ong et al. 2017).

FLA including *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri* are the most common aetiologies for FLA-causing encephalitis worldwide (Marciano-Cabral et al. 2003; Niyyati and Rezaeian 2015; Ong et al. 2017).

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Acanthamoeba consists of 20 genotypes according to the diagnostic fragment 3 region of ASA1 18S rRNA. The most prevalent genotype in the environment causing disease is the T4 genotype in Iran and around the world (Khan 2006; Lorenzo-Morales et al. 2013; Lass et al. 2014; Todd et al. 2015; Niyyati and Rezaeian 2015); Hajialilo et al. 2016; Sente et al. 2016; Lass et al. 2017). Other genotypes such as T1, T2, T3, T5, T6, T10, T11, T12, T13, T15, and recently T9 have been identified as the cause of infection as well (Hajialilo et al. 2016; Arnalich-Montiel et al. 2018).

The route of entry of *Acanthamoeba* and *Balamuthia* cysts is through inhalation or damaged skin which can lead to colonization of these amoebae in both healthy and immunocompromised individuals (Cabello-Vílchez et al. 2014; Memari et al. 2015; Memari et al. 2017). For *Naegleria*, nasal mucosa/olfactory nerves are route of infection (Visvesvara et al. 2007).

According to recent reports, the prevalence of HIV/AIDS patients in Iran is on the rise especially among the drug addicts (Joulaei et al. 2017). On the other hand, the main risk factor for developing FLA-related cerebral infections is a compromised immune system and several studies have reported of the possible co-infection with FLA in AIDS patients (Sisson 1995; Khan 2006; MacLean et al. 2007; Pietrucha-Dilanchian et al. 2012; El Sahly et al. 2017; Geith et al. 2018). Despite a recent case report describing successful treatment of an AIDS patient with encephalitis caused by *Acanthamoeba* infection, the majority of encephalitis due to potentially pathogenic FLA are fatal (El Sahly et al. 2017). A previous case report described the isolation of *Hartmannella vermiformis* from the cerebrospinal fluid of a young male patient with meningoencephalitis and bronchopneumonia (Centeno et al. 1996).

FLA may cause other diseases such as amoebic keratitis due to *Acanthamoeba*, *Vahlkampfia*, and *Vermamoeba* (Niyyati et al. 2009; Hajialilo et al. 2015; Niyyati et al. 2016). Primary amoebic meningoencephalitis (PAM) due to *Naegleria fowleri* has been diagnosed in a 6-month-old infant with a good prognosis in Iran (Movahedi et al. 2012). However, perhaps due to a lack of adequate awareness of diagnostic tools in the country, granulomatous amoebic encephalitis due to FLA has not yet been reported in Iran (Memari et al. 2015; Niyyati and Rezaeian 2015).

This study attempted to isolate and identify the FLA colonization in HIV/AIDS patients from the nasal and oral mucosa of AIDS/HIV patients referred to reference hospitals in Iran from 2017 to 2019 using available morphological and molecular tools described below. To discern the taxonomic status of *Acanthamoeba* genotypes, a maximum likelihood phylogenetic tree was constructed. The pathogenic potential of the isolated strains was also evaluated using tolerance assays.

Material and methods

AIDS/HIV patients

In this study, 70 AIDS/HIV patients admitted to reference hospitals in Iran were included. Infection of the AIDS/HIV people was confirmed using ELISA, RT-PCR tests, and western blot. These tests were performed in the laboratories of the hospitals with infectious wards in Shahid Beheshti University of Medical Sciences, Tehran, Iran. Informed consents were obtained from all patients or their relatives. Questionnaires to determine the age, sex, addiction status, clinical manifestations, and cause of hospitalization were obtained. The majority of the admitted patients had a history of pulmonary involvement secondary to the HIV/AIDS.

Culture of samples in enriched agar medium and cloning

Nasal and oral samples were obtained using sterile swaps and immediately cultured on 1.5% non-nutrient agar enriched with a layer of autoclaved *Escherichia coli*. Later, the cultures were transferred to the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The cultures were incubated at room temperature and checked for the outgrowth of amoeba every 24 h for at least 2 months. Positive plates were axenized by transferring a single amoeba to new plates according to the previous studies (Niyyati et al. 2017). Morphological identification was performed based on the page key (page 1988).

DNA extraction, PCR analysis, sequencing, and phylogenetic analysis

Trophozoites and cysts of amoebae were scraped using sterile phosphate buffered saline (pH 7.2). DNA extraction was done using InstaGene matrix kit according to the manufacturer's procedure.

PCR was performed using specific primers including JDP1 and JDP2 for *Acanthamoeba* spp. which could amplify the ASA.S1 region of 18SrRNA (JDP1: 5-GGCCCAGA TCGTTTACCGTGAA-3′ and JDP2: 5′ TCTCACAA GCTGCTAGGGAGTCA-3′) and NA1, NA2 primers set for *Vermamoeba* genus (Kong and Chung 1996; Lasjerdi et al. 2011) including (NA1: 5′-GCT CCA ATAGCG TAT ATTA A-3 and NA2: 5′-AGA AAG AGC TATCAATCT GT-3′). These primers amplify a fragment of 500 bp for *Acanthamoeba* genotypes and 700 bp for *Vermamoeba*. PCR reaction was performed in a total volume of 30 μl Taq DNA polymerase master mix red (Ampliqon, Denmark) mixed with 10 ng DNA and 0.1 M of each primer and distilled water. The PCR thermal condition included an initial denaturation step of 95 °C for 1 min followed by 35 repetitions at

94 °C for 35 s, an annealing step of 1 min duration at 56 °C for *Acanthamoeba* and 58 °C for *Vermamoeba*, and a final extension step at 72 °C for 10 min. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Sequencing of PCR products was performed using an automated sequencing machine (ABI 3130×, Iran). Obtained sequences were manually edited by chromas (version 1.0.0.1) and analysed using Basic Local Alignment Search Tool (BLASTn). The newly identified nucleotide sequences were submitted to the genetic sequence database at the National Center for Biotechnical Information (NCBI) using Bankit program (https://www.ncbi.nlm.nih.gov/WebSub/) under the following accession numbers: MK881167 and MK880399- MK880402.

To authenticate genetic associations among identified genotypes of *Acanthamoeba* inferred by the 18S rRNA gene, a phylogenetic tree was generated by MEGA 5.05 software based on maximum likelihood algorithm and Kimura 2-parameter model. The distance scale was estimated 0.02. *Vermamoeba vermiformis* was addressed as an out-group branch. The accuracy of the phylogenetic tree was assessed by 1000 bootstrap replications. Bootstrap values of higher than 70% were supported the topology on each branch.

Tolerance assays

Thermotolerance and osmotolerance assays were carried out for *Acanthamoeba* strains. Briefly, 10^5 trophozoites were inoculated in two fresh medium and the incubation was performed at 37 °C and 40 °C for 7 days. For the osmotolerance assay, the isolated strains were cultured on two plates containing 0.5 and 1 M D-mannitol and the outgrowth of the amoebae was checked during a week (Khan 2001).

Results

Out of 70 oral and nasal samples obtained from HIV/AIDS subjects, 5 (% 7.1) specimens were positive for potentially pathogenic FLA based on the morphological criteria and molecular tools explained above. Four isolates (HA1, HA3, HA4, and HA5) belonged to *Acanthamoeba* spp. and one sample (HA2) was identified as *Vermamoeba* genus. The age range of the patients with positive samples was 43–65 years and the patients with negative FLA samples were 13–85 years. Two patients were drug users (Table 1). Four patients with positive samples for FLA had neurological signs. Sequencing of the DF3 region of *Acanthamoeba* genus revealed that three *Acanthamoeba* strains (isolate codes: HA3, HA4, and HA5) belonged to the T4 genotype. The other strain (isolate code: HA1) showed high identity (97%) with genotype T5 assigned as *A. lenticulata* (Table 2). Interestingly, the obtained genotypes demonstrated high homology with the strains previously isolated from water and dust in Iran (Accession numbers: KM823784, MK192791). One strain showed high homology with the amoebic keratitis strain isolated in Poland (accession number: MF671963). Another strain (isolate code: HA2) which was morphologically identified as *Vermamoeba*, showed high homology with *V. vermiformis* (accession number: LC422060). The patient with positive sample for *V. vermiformis* died due to Pneumocystis pneumonia (PCP) (Table 2). Tolerance assay showed that three *Acanthamoeba* strains (HA1, HA3, and HA4) were highly pathogenic as they grew at high temperature and osmolarity (Table 2).

To discern the taxonomic status of *Acanthamoeba* genotypes, a maximum likelihood phylogenetic tree was constructed. The topology of identified isolates are shown in clade I (T5 genotype; *Acanthamoeba lenticulata*; accession number; MK880399), clade IV (T4 genotype; *Acanthamoeba* sp.: accession numbers; MK880400-MK880402), and clade VI (*V. vermiformis*; MK881167) (Fig. 1).

Discussion

This is the first worldwide comprehensive study isolating and identifying the colonization of potentially pathogenic FLA belonging to the *Acanthamoeba* T4 and T5 genotypes and *Vermamoeba vermiformis* in HIV/AIDS patients. Moreover, the present study is the second report

Table 1 Biographic data and infection history of HIV/AIDS patients with positive free-living amoeba colonization in oral and nasal mucosa

Isolate code	Age	Sex	Occupation	Literacy	Infection history	Neurological signs	Drug user
HA1	45	F	Housewife	High school	Respiratory infection	+	–
HA2	65	M	Self-employed	Illiterate	Pneumocystis pneumonia (PCP)	+	+
HA3	43	M	Employee	Diploma	Respiratory infection	+	–
HA4	54	F	Retired	University degree	Respiratory infection	+	–
HA5	NA	F	NA	NA	–	–	+

NA, not available

Table 2 Data showing the characteristics of free-living amoeba isolated from HIV/AIDS patients in Iran

Strain code	Isolated amoebae	Species and/ or genotype	Identity/ query coverage (%)	Thermo-tolerance (37/ 42 °C)	Osmotolerance (0.5/1 M)	Accession number
HA1	*Acanthamoeba*	*lenticulata* (T5 genotype)	97/98.7	+/+	+/+	MK880399
HA2	*Vermamoeba*	*vermiformis*	98/90	N/A	N/A	MK881167
HA3	*Acanthamoeba*	T4	98/99	+/+	+/+	MK880400
HA4	*Acanthamoeba*	T4	95/99.5	+/+	+/+	MK880401
HA5	*Acanthamoeba*	T4	94/99.7	+/−	+/−	MK880402

in Iran of isolation and identification of *A. lenticulata* (T5 genotype) from mucosal tissues.

The burden of HIV/AIDS in Iran is not as high as in the countries with high prevalence, yet the number of cases is on the rise. According to recent data reported by UNAIDS, Iran had 5000 (1400–13,000) new HIV infection cases and 4000 (2500–6200) AIDS-related deaths. In 2016, the estimated number of people living with HIV was around 66,000 (37000–120,000) (UNAIDS 2016). Intravenous drug users, female sex-workers, and incarcerated persons were the most prevalent at-risk groups for many years (Joulaei et al. 2017). The co-infection of HIV and *Acanthamoeba* has been previously described (Marciano-Cabral and Cabral 2003); however, *Balamuthia mandrillaris* GAE in HIV-infected persons is rarely described in the literature (Yohannan and Feldman 2019). Additionally, only one case report from Iran described the occurrence of *Acanthamoeba* belonging to the T4 genotype in the mucosal tissue of a 15-year-old male with HIV

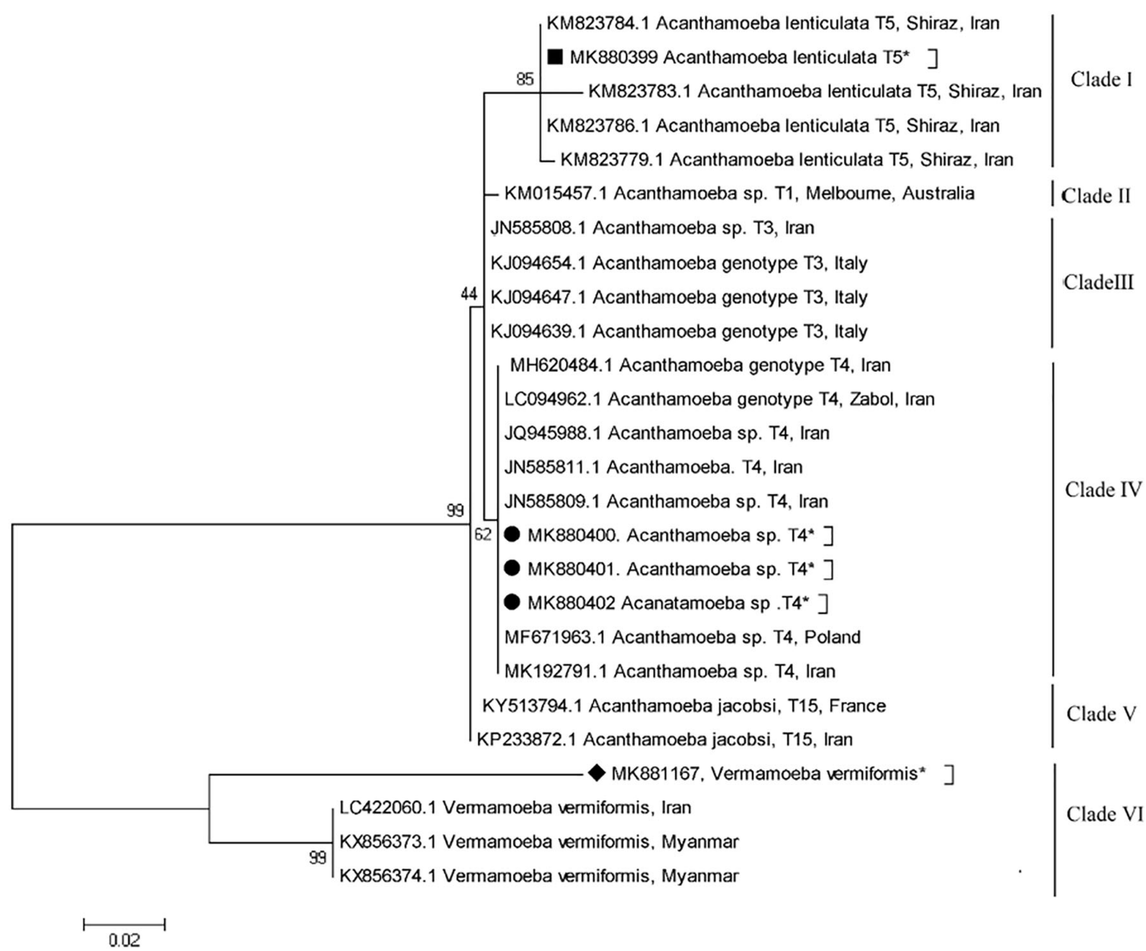

Fig. 1 The taxonomic status of identified *Acanthamoeba* isolates was shown in a phylogenetic tree based on the multiple sequence alignment of 18S rRNA gene. The characterized isolates (T4, T5, and *V. vermiformis*) marked by asterisk (*)

infection (Memari et al. 2017). Limited studies have shown the colonization of FLA in the mucosal tissue of patients with an incidence rate estimated to be 4.8–45% (Memari et al. 2015; Niyyati et al. 2017). In our study, 7% of patients living with HIV/AIDS referred to reference hospitals in Iran from 2017 to 2019 were positive for colonization with FLA. The tolerance assay classified the strains as highly pathogenic strains, although more tests such as in vivo tests are needed to clarify the pathogenicity of the strains. There are no previous reports regarding the occurrence of GAE in this region; however, many HIV/AIDS patients die due to unidentified encephalitis in which FLA may be the etiological agent. Thus, awareness of clinicians of this potentially lethal infection and the available diagnostic tools is of high priority. Four patients (HA1, HA2, HA3, and HA4) in our study showed neurological signs; nevertheless, the investigation of CSF was not possible due to the patients' health condition.

Other reports show the colonization of potentially pathogenic amoebae within clinical settings including ophthalmology hospital wards, the units involved in caring of immunocompromised patients, and haemodialysis units which can put the immunocompromised patients at risk of acquiring opportunistic infection (Lasjerdi et al. 2011; Niyyati et al. 2017). *Acanthamoeba* spp. is also isolated from healthy individuals although immunosuppression is a risk factor for fatal CNS disease from opportunistic infections (Cabello-Vílchez et al. 2014).

Acanthamoeba lenticulata isolated from one patient (HA1) was also shown to grow at high temperature and osmolarity, indicating the pathogenic nature of this strain. This genotype was previously isolated from cornea and environmental sources. However, this is the first report of the occurrence of this genotype in an HIV patient. Previous study showed that *Mycobacterium avium* survives but does not efficiently replicate in these strains (Ovrutsky et al. 2013).

One patient (code: HA2) contaminated with *Vermamoeba vermiformis* developed PCP, the most frequent opportunistic infection among people with AIDS. Interestingly, *Vermamoeba* may act as a vehicle to transfer *pneumocystis* to susceptible persons. Cabello-Vílchez et al. previously described the presence of endosymbiotic *Mycobacterium chelonae* in a *Vermamoeba vermiformis* strain isolated from the nasal mucosa of an HIV patient in Lima, Peru (Cabello-Vílchez and Núñez-Ato 2018). Moreover, a previously published study demonstrated that a *V. vermiformis* strain obtained from a case of human keratitis had the potential to produce a cytopathic effect on keratocytes in vitro (Kinnear 2003). Most cases of CNS *Acanthamoeba* infection are diagnosed post-mortem (El Sahly et al. 2017). To our knowledge, there are only three reported cases of an AIDS patient with CNS *Acanthamoeba* infection who were successfully treated and survived (Martinez et al. 2000; Carter et al. 2004; El Sahly et al. 2017;)

Colonization of highly pathogenic FLA in patients living with HIV/AIDS is a serious risk factor for fatal CNS infections. This study highlights the fact that clinicians should be more aware of the differential diagnosis and available diagnostic tools for cerebral disease related to FLA in patients with HIV/AIDS.

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Compliance with ethical standards

The Ethics Committee of Shahid Beheshti University of Medical Sciences in Iran approved this study (ethics committee code: 1396.1122).

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