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ORIGINAL PAPER

Occurrence of pathogenic *Acanthamoeba* genotypes in nasal swabs of cancer patients in Iran

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Abstract Incidences of *Acanthamoeba* granulomatous encephalitis (AGE) have been increased due to a rise in the number of high-risk people, such as immunodeficient patients. Indeed, immunosuppress situation can render the patient in acquiring opportunistic *Acanthamoeba* infections. In this study, analysis was carried out to verify the presence of free-living amoebae of *Acanthamoeba* genus in nasal swabs of cancer patients in hospitals of Tehran, Iran. Detection of isolates was based on morphotyping and PCR sequencing of the Diagnostic Fragment 3 (DF3) to identify strains at the genotype level. In addition, the pathogenic potential of the isolates was assayed using temperature and osmotolerance assays. The obtained results revealed that nine isolated strains belonging to T4 genotype-exhibited pathogenic potential. After sequencing, genotype T4 was found to be the most common one in the samples included in this study. Genotype T3 and T5 were also identified. To the best of our knowledge, this is the first study on the typing of *Acanthamoeba* strains at the genotype level in cancer patients in Iran and worldwide.

Keywords Cancer patients · *Acanthamoeba* · Genotypes · Pathogenic assay · Iran

Introduction

Free-living amoebas (FLA) are widely distributed in the environment and have been isolated from the air, soil, water, contact lenses, air conditioning units and clinical samples. Most of the information regarding FLA causing pathologies in humans is associated to *Naegleria fowleri*, *Acanthamoeba* spp. and *Balamuthia mandrillaris* (Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007; Qvarnstrom et al. 2009; Cabello-Vílchez et al. 2013). To date, *Acanthamoeba* genus are classified to 18 different genotypes based on molecular analysis approaches. Sequencing of the fragment of 18Sr DNA, ranging between 450 and 500 bp, has provided sufficient data for reliable determination of the relationships among particular genera and towards understanding their phylogeny (Gast et al. 1996; Stothard et al. 1998). Within *Acanthamoeba* genotypes, T4 is the most common type that causes amoebic keratitis (AK) and amoebic granulomatous encephalitis (AGE) disease in humans (Booton et al. 2002; Khan 2006; Ledee et al. 2009; Dart et al. 2009; Prashanth et al. 2011; Magnet et al. 2012). However, other genotypes, such as T1, T2, T3, T5, T6, T10, T11, T12 and, recently, T13 and T15, have been associated with amoebic diseases (Khan 2009; Niyyati et al. 2009; Sharifi et al. 2010; Walochnik et al. 2014; Azzam et al. 2014; Grün et al. 2014). Earlier researches exposed that some pathogenic *Acanthamoeba* strains may tolerate high temperature and osmolarity (Khan et al. 2001). This shows the characteristic adaptation of these organisms. Indeed, pathogenic *Acanthamoeba* could handle stressful situations of the human body, such as high osmolarity and temperature (Khan 2009). Thus, the possible pathogenicity of amoebae could be attributed to their thermo- and osmotolerance traits. However, additional tests (including cell culture assays and in vivo studies) are needed to confirm the pathogenicity. This is because there are many T4 strains with high thermo- and osmotolerance, which are non-pathogenic types (Dejonkheere 1980; Khan et al. 2001; Khan 2009; Mirjalali et al. 2013).

These ubiquitous protozoan parasites are opportunistic contributing agents of nasopharyngeal and skin infections and amoebic granulomatous encephalitis. An estimated 471 cases of *Acanthamoeba* AGE have been reported, although

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this number is significantly less than the actual AGE cases (Khan 2009). In recent years, incidences of these infections have increased due to a rise in the number of immunodeficient diseases, such as HIV, cancer, diabetes, hepatitis, splenectomy and steroid therapy after organ grafts (Khan 2009). Furthermore, more than 30 cases of *Acanthamoeba* and HIV coinfections have been reported (Marciano-Cabral and Cabral 2003; MacLean et al. 2007; Pietrucha-Dilanchian et al. 2012). The major route of central nervous system infection is inhalation of airborne cysts and haematogenous distribution, mostly in high-risk people (Visvesvara et al. 2007). The previous research studied isolation of *Acanthamoeba* from healthy population. This research clearly revealed that *Acanthamoeba* could be colonized in healthy people (Cabello-Vílchez et al. 2013). However, for developing AGE, people with impaired immunity are at high risk. Indeed, a main contributing factor in developing AGE is immunosuppression (Khan 2009). The majority of reported cases regarding AGE occur in patients with impaired immunity such as chemotherapy patients, HIV sufferers, graft patients and hepatitis and cirrhosis victims (Khan 2009; Walochnik et al. 2014). There are reports regarding *Acanthamoeba* encephalitis recovery after early diagnosis of infection during cytological examination of cerebrospinal fluid (Petry et al. 2006).

In Iran, there are no reports of CNS infection relating to *Acanthamoeba* and *Balamuthia*. However, there is a single investigation that reports the isolation of *N. fowleri* in a 5-month-old child with a good prognosis (Movahedi et al. 2012). The absence of reports regarding AGE is mainly due to the lack of research in this subject area. Furthermore, in this region, there are no laboratory tests for diagnosis of this fatal central nervous infection. Previous research conducted in Iran showed a high occurrence of *Acanthamoeba* in tap water sources and dust and biofilms in wards serving for those with immunosuppressed situation (Lasjerdi et al. 2011).

The main aim of the present study was to determine the occurrence of *Acanthamoeba* spp. in cancer patients of Tehran, Iran, by using morphological and molecular typing of the isolated strains. This is the first study to determine *Acanthamoeba* genotypes in immunosuppressed patients of hospitals in Iran and worldwide.

Materials and methods

Samples

In this study, 80 nasal swabs were collected from cancer patients. These patients were undergoing chemotherapy for approximately 6 months in reference hospitals of Iran. The samples were transferred to the Dept. of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran. Informed consent was obtained from all the individuals whose nasal swabs were included in this study. The privacy rights of the individuals are respected in this study. Swabs were then cultivated in 1.5 % non-nutrient agar (NNA) covered with a layer of a heat-killed *Escherichia coli* in Page's solution. The plates were incubated at 30 °C for up to 2 months, and the cultures were monitored every 24 h. The clones of concern were then transferred to the new culture plates for axenification and further molecular analyses.

Axenification of isolates

All of the positive isolates were cloned. To achieve this, we transferred one single amoeba to a fresh medium. Several replicates were performed to achieve a plate without bacterial and fungal contamination. However, axenification in axenic medium of protease peptone, yeast extract and glucose (PYG) was only successful in some of the *Acanthamoeba* isolates.

DNA extraction

DNA was extracted by using the phenol-chloroform method (Niyyati et al. 2009). Primer sets were applied for molecular identification of the strains as follows: JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 ′ TCTCACAAGCTGCTAGGGAGTCA-3′, which could amplify the ASA.S1 region of 18S rRNA gene. These primers yield a fragment of approximately 500 bp (Schroeder et al. 2001). PCR was performed in 30 μl Taq DNA Polymerase Master Mix Red (Ampliqone, Denmark) as a ready-made mixture. Briefly, 25 μl of Taq Master mix were used with 10 ng template DNA, 0.1 μM of each primer and distilled water. The thermal cycling conditions were an initial denaturing step of 94 °C for 1 min and 35 repetitions at 94 °C for 35 s. Annealing and extension steps were 56 °C for 45 s and 72 °C for 1 min, respectively.

The extension time was prolonged for 5 min at 72 °C. PCR products were electrophoresed on a 2 % agarose gel stained with a solution of ethidium bromide and visualized under UV light. Sequencing and molecular identification of isolates PCR products were resolved by using the ABI 3130X automatic sequencer in the Takapozist. The sequences were manually edited using Chromas (version 1.0.0.1) and analysed against all eukaryotic nucleotide sequences available in the GenBank database. The DNA sequences for the new isolates were deposited in the genetic sequence database at the National Centre for Biotechnical Information (NCBI) using Sequin program (version 10.3).

Diagnostic Fragment 3 sequences for the new isolates were deposited in the GenBank database under the accession numbers KJ786503–KJ786526.

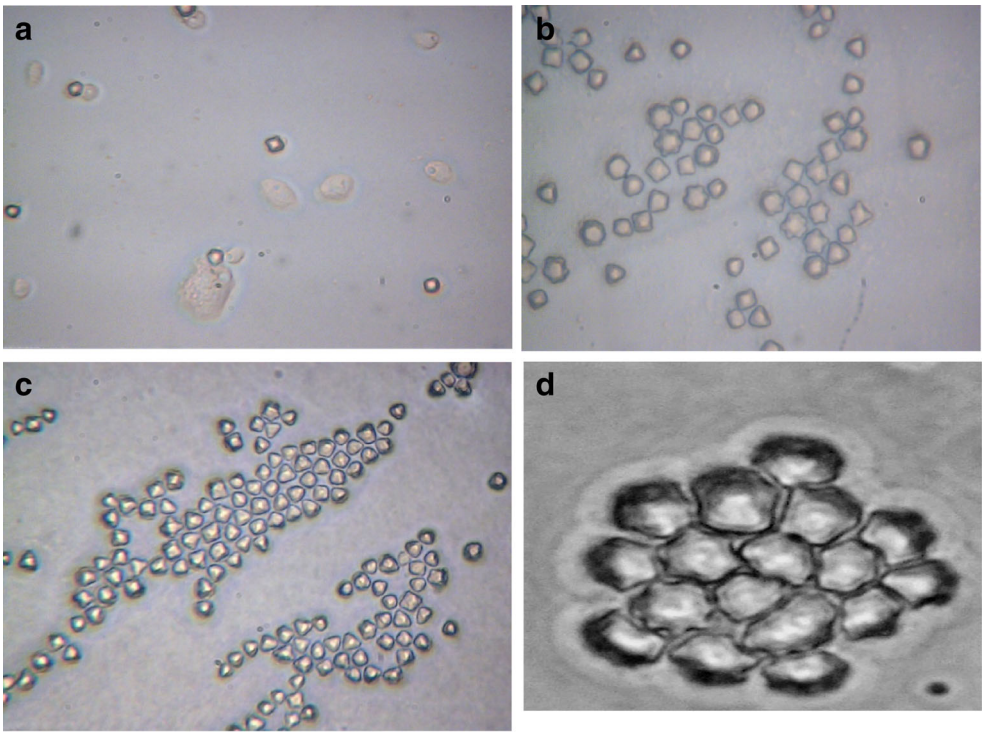

Fig. 1 **a** Trophozoites of *Acanthamoeba* (×100). **b**–**d** Star-shaped cyst of *Acanthamoeba* belonging to T4 type (×400) isolated from mucosal tissue of cancer patients in hospital of Tehran, Iran

Table 1 Isolated genotypes from cancer patients and their pathogenic potential

Code	Genotype	Accession number	Growth 30 °C	Growth 37 °C	Growth 40 °C	Growth mannitol 0.5 M/1.5 M
MN-IS-P-21	T4	KJ786505	+	−	−	−/−
MN-IS-P-79	T4	KJ786515	+	+	+	+/+
MN-IS-P-72	T4	KJ786514	+	+	+	+/+
MN-IS-P-148	T4	KJ786526	+	+	+	+/+
MN-IS-P-125	T5	KJ786518	+	−	−	−/−
MN-IS-P-128	T4	KJ786519	+	+	+	+/+
MN-IS-P-71	T4	KJ786513	+	+	+	+/+
MN-IS-P-68	T4	KJ786512	+	−	−	−/−
MN-IS-P-156	T4	KJ786525	+	−	−	−/−
MN-IS-P-84	T4	KJ786516	+	+	+	+/+
MN-IS-P-153	T4	KJ786524	+	−	−	−/−
MN-IS-P-58	T4	KJ786508	+	+	+	+/+
MN-IS-P-20	T4	KJ786504	+	−	−	−/−
MN-IS-P-66	T4	KJ786510	+	−	−	−/−
MN-IS-P-67	T4	KJ786511	+	+	+	+/+
MN-IS-P-56	T4	KJ786507	+	−	−	−/−
MN-IS-P-136	T4	KJ786522	+	+	+	+/+
MN-IS-P-19	T3	KJ786503	+	−	−	−/−
MN-IS-P-64	T5	KJ786509	+	−	−	−/−
MN-IS-P-145	T4	KJ786523	+	−	−	−/−
MN-IS-P-124	T4	KJ786517	+	−	−	−/−

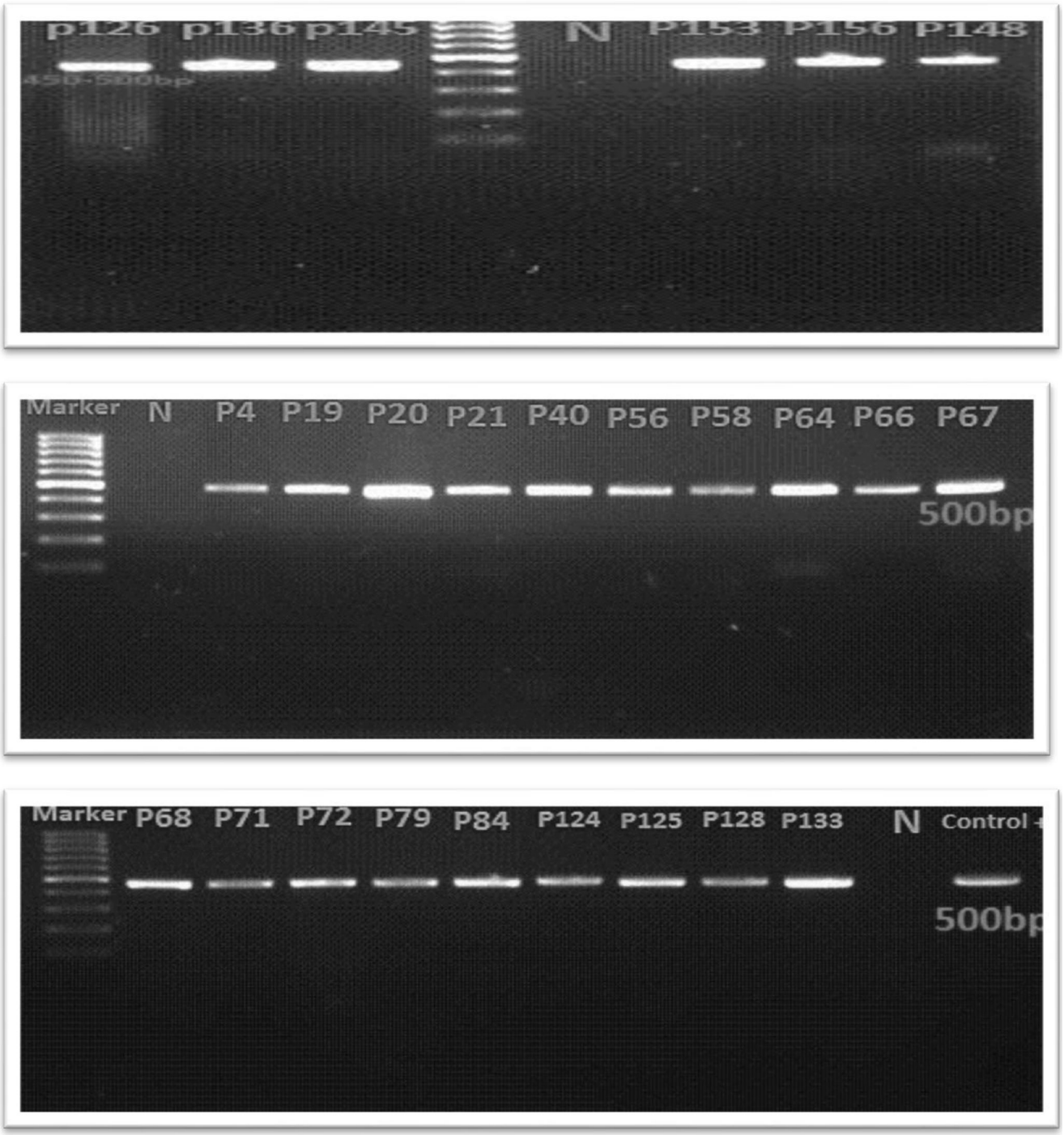

Fig. 2 Gel electrophoresis of the 500-bp PCR product of *Acanthamoeba* spp. isolated from mucosal tissue of cancer patients in hospital of Tehran, Iran (*N* negative control)

Temperature and osmolarity assays

For temperature tolerance assays, all of the successful axenic cultures (21 strains) were incubated for up to 72 h at 30, 37 and 40 °C. To examine the effect of osmolarity on the growth of *Acanthamoeba* trophozoites, 21-well plates containing 0.5 and 1.5 M mannitol were seeded, as previously described (Martín-Navarro et al. 2008). Approximately 1000 trophozoites were inoculated onto plates and incubated for up to 72 h at 28 °C. During this period, we checked the growth and survival of amoebae (Khan et al. 2001; Martín-Navarro et al. 2008).

Results

Out of the total of 80 samples using morphological criteria, 36 (45 %) plates revealed *Acanthamoeba* trophozoites and double wall cysts (Fig. 1). Axenification was only successful in 21 (26.25 %) of the 36 *Acanthamoeba* isolates. After sequencing the DF3 region, three genotypes belonging to T4, T3 and T5 were identified (Table 1, Fig. 2). Genotype T4 was the most common genotype (18; 50 %), followed by T3 (1; 2.8 %) and T5 (2; 5.6 %) (Table 1). Additionally, 12 isolates belonging to T4, T3 and T5 were not able to tolerate the high temperature and osmolarity. Thus, they are classified as non-pathogens. Nine isolates, all belonging to the T4 genotype (MN-IS-P-79, MN-IS-P-72, MN-IS-P-148, MN-IS-P-128, MN-IS-P-71, MN-IS-P-84, MN-IS-P-58, MN-IS-P-67, MN-IS-P-136), showed growth in high temperature (37 and 40 °C) and osmolarity (0.5 and 1.5 M). Thus, they are classified as isolates with high pathogenicity

Discussion

The present research is the first investigation regarding the occurrence of *Acanthamoeba* in high-risk people, including cancer patients from hospital wards in Iran. In a previous study conducted by Lasjerdi et al., free-living amoebae were detected in 37 (52.9 %) of 70 dust and biofilm samples from immunodeficiency hospital wards in Iran. Morphological identification of 33 (89.1 %) plates revealed *Acanthamoeba*. Furthermore, T4 was the predominant genotype in the samples (Lasjerdi et al. 2011). The aforementioned research highlights the presence of free-living amoebae in the hospital environment. Thus, our study aimed to evaluate the presence of *Acanthamoeba* in immunosuppressed patients. A similar study has been documented in Peru which showed that out of 74 nasal swap samples from healthy individuals, 21 (28.4 %) were positive for *Acanthamoeba* spp. and two genotypes belonging to T4 and T15 were reported. In their samples, genotype T4 was the most common genotype (76.2 %) and one *Acanthamoeba* strain belonging to T15 (4.8 %) was also isolated (Cabello-Vílchez et al. 2013). However, in the Peru research, the studied population was healthy people. In contrast, our study revealed that there is a high occurrence of *Acanthamoeba* in cancer patients, and T4 genotype is the most common genotype within this population. It is important to note that all of the patients included in our study were immunocompromised for at least 2 months, due to chemotherapy. Colonization of *Acanthamoeba* with pathogenic potential exposes high-risk people to the development of amoebic granulomatous encephalitis. On the other hand, there are many reports regarding the death of cancer patients due to unidentified encephalitis in Iran. Compared with our study, previous studies focusing on the isolation of *Acanthamoeba* strains from nasal swabs recorded a lower percentage of isolated FLA (Cerva et al. 1973; Simitzis et al. 1979; Michel et al. 1982; Badenoch et al. 1988). These studies reported FLA isolation ranging between 0.86 and 9.3 %. In our study, 45 % of the samples were positive for *Acanthamoeba* and 21 isolates were genotyped and, thus, allowed the identification of T4, T3 and T5 isolates within the samples. This study highlights the existence of potentially pathogenic *Acanthamoeba* strains in the nasal passages of cancer patients from hospitals in Iran.

The ability of pathogenic *Acanthamoeba* to covert high level of heat shock proteins (HSP60 and HSP70) have led researchers to set up a simple plating assay for detection of pathogenic *Acanthamoeba* from non-pathogenic strains (Khan et al. 2001). This plating test is mostly based on different tolerance ability of *Acanthamoeba* strains to variable osmolarity and temperature (Khan 2009). Interestingly, all of the pathogenic strains were within T4 genotype. However, more tests, including cell culture assay and in vivo studies, are required to carry out pathogenic evaluation of the isolated amoebae.

Overall, the present research highlights that clinicians should be more aware of this lethal disease and diagnostic options, particularly within suspected encephalitis. This is because there is a high occurrence of potentially pathogenic amoebic strains in cancer patients.

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