Species Identification of Acanthamoeba Strains Isolated from Patients Referring to Farabi Eye Reference Center Using PCR-RFLP Method

Mir Mostafa Ghamilouie1, *Zarrintaj Valadkhani1, Fariba Khoshzaban2

1Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran; 2Department of Parasitology, Shahed University of Medical Science, Tehran, Iran.

Received Sep 23, 2015; accepted Sep 29, 2015

INTRODUCTION

Pathogenic free-living amoebas (FLAs) can complete their life cycles in environment without entering a human or animal host. Of many FLAs, three genera and spp. are known to be associated with human disease. These microorganisms have been detected in different sources, such as soil, mud, and water. Some members of FLAs cause Acanthamoeba keratitis (AK), an infection of the eye that can lead to permanent visual impairment or blindness. However, if the infection is diagnosed at early stages, it can be treated easily without sequelae. There is a significant emphasis on Acanthamoeba as a Trojan horse of other microorganisms, including viral, bacterial, protist, and yeast pathogens [1]. Contact lenses wearers are more susceptible to eye infections due to tiny scrapes of the cornea. Due to improper disinfection of the lenses and the presence of parasite, it survives in the space between the lens and the eye and invades the corneal tissue, leading to infection.

Symptoms of AK include foreign body sensation, photophobia, decreased visual acuity, tearing, pain, and redness of the eye. Genotyping and species identification of Acanthamoeba has been reported from different geographical areas. Cysts are resistant and infectious form of Acanthamoeba. Species identification based on cyst morphology, following cultivation of isolates on non-nutrient agar (NNA), has been reported previously [2]; however, it should be considered that the morphological characteristics of the cyst can change with culture conditions [3].

With the advent of molecular techniques, various genetic markers, such as Aca 16S, 18S, Ac6/210, GP, JDP, and Nelson have been used for species identification of Acanthamoeba [4]. Acanthamoeba jacobsi (a thermophilic species of this genus) was investigated by PCR and subjected to sequence type classification by sequencing part of the 18S rRNA gene and allocated to the sequence type T15 [5].

Among different species of Acanthamoeba isolated from infected eyes, such as Acanthamoeba castellani, Acanthamoeba palestinensis, Acanthamoeba griffini, Acanthamoeba culbertsonii, Acanthamoeba polyphaga, Acanthamoeba lugdunensis, Acanthamoeba hatchetti, Acanthamoeba rhyphodes, and Acanthamoeba quina, three has been reported as the predominant ones, i.e., A. castellani, A. polyphaga, and A. griffini. Due to the increased use of contact lenses (more than 120 million individuals worldwide), AK has been rising among young individuals within the age range of 14-36 years [6]. Our previous study showed that contact lenses wearers within age group of 18-30 years were most infected with AK, which in turn indicates the correlation between age group and risk of infection [7].

*Correspondence: Zarrintaj Valadkhani
Department of Parasitology, Pasteur Institute of Iran, No. 69, Pasteur Ave, Tehran, Iran, 1316943551.
Email: valad.zarrin@gmail.com
Tel/Fax: +98 (21) 66968855

http://jommid.pasteur.ac.ir
Determination of the pathogenic potential of isolates are important for clinical purposes. In vitro cytotoxicity assays were used to determine the pathogenic potential of clinical samples isolated from keratities patients [8]. Considering the lack of use of species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in Iran, this study aimed to identify the prevalence of Acanthamoeba species among AK patients referring to Farabi Eye Reference Center, Tehran, Iran.

**MATERIAL AND METHODS**

Samples were obtained from patients referring to Farabi Eye Reference Center affiliated to Tehran University of Medical Sciences, Tehran, Iran. The patients presented with eye pain, redness, tearing, and sensitivity to light, and sensation of foreign body in the eye. In most patients, only one eye was affected, and five patients had been using contact lenses. Using a surgical blade, a corneal scraping was obtained from the infected eye of each patient by an ophthalmologist, and transferred to the laboratory. The specimens were cultured on PCA media, and any growth was transferred to PYG medium containing gentamicin (40 μg/ml) and thimerosal (100 μg/ml), streptomycin (100 µg/ml), and penicillin (100 U/ml), and incubated at 30°C. The plates were checked daily under an inverted microscope for the presence of Acanthamoeba cyst for two weeks. The positive samples were transferred to PYG media containing 1.5% NNA in page's saline, seeded with Escherichia coli, and incubated at 30°C. The plates were checked daily under an inverted microscope for the presence of Acanthamoeba cyst for two weeks. The positive samples were transferred from NNA media into tubes containing PYG medium and 1.5% NNA in phosphate buffer saline (PBS), as described previously [7]. One hundred microliters of each sample was inoculated into Petri dishes containing PYG medium and kept at 30°C.

**PCR and RFLP analysis.** The amoeba DNA was extracted using a commercial DNA extraction kit (HiPurA Multi-Sample DNA Purification Kit, HiMedia, India) according to the manufacturer’s instruction. Partial sequence of 18S rRNA gene was targeted using primers JDP1 (5'-GGCCCATCAGTATTACCGTGAA-3') and JDP2 (5'-TCTCACAGCTGCTAGGGAGTCA-3'), as reported previously [9]. The amplifications were performed in 20 μl reaction mixtures containing 1 U of Taq polymerase, 10 mM each of primer, 2.5 mM MgCl₂, and 200 mM dNTPs. All the reagents were provided by CinnaGen Co., Iran. The thermocycler was programmed for an initial denaturation step at 95°C for 5 min, followed by 35 amplification cycles of denaturation for 45 s at 95°C, annealing for 45 s at 57°C, extension for 50 s at 72°C, and a final elongation step at 72°C for 8 min [10]. The amplicons (<500 bp) were subjected to digestion with restriction enzymes HpyCH4IV (New England BioLab, Lithuania) and BspL I (NlaIV) (thermocience, Lithuania). An amount of 10 μl of amplicons was digested with 10 units of the restriction enzymes BspL I (NlaIV) and HpyCH4IV at 37°C for 3 h and 15 min, respectively. The digested samples were electrophoresed on a 2.5% agarose gel along with the 100 bp DNA Ladder (GeneRuler™, Fermentas, Lithuania), and then stained with DNA safe stain and visualized under a UV transilluminator [9]. The restriction fragments of the isolates were compared with the fragment sizes predicted by restriction map and CLC Genomics Workbench 3 software (Table 1).

Although, the BseDI enzyme was also used during the prediction of fragment sizes by restriction map and CLC Genomics Workbench 3 software (Table 1), the species were identified using the other two enzymes, i.e., BspL I (NlaIV) and HpyCH4IV, and there was no need to use BseDI anymore. This enzyme can also be used for the verification of the test.

**Ethical consideration.** This study was approved by the Ethics-in-Research Commission of Pasteur Institute of Iran, and a written informed consent was obtained from each participant.

<table>
<thead>
<tr>
<th>Acanthamoeba Species</th>
<th>Restriction enzyme</th>
<th>Predicted fragment sizes (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castellani</td>
<td>HpyCH4IV</td>
<td>269,122.73</td>
</tr>
<tr>
<td></td>
<td>NlaIV</td>
<td>303,97.32,31</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>240,138,61,24</td>
</tr>
<tr>
<td></td>
<td>HpyCH4IV</td>
<td>346,77</td>
</tr>
<tr>
<td>Lenticulata</td>
<td>NlaIV</td>
<td>326,96</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>244,178</td>
</tr>
<tr>
<td></td>
<td>HpyCH4IV</td>
<td>342,125</td>
</tr>
<tr>
<td>Palestinensis strain 1</td>
<td>NlaIV</td>
<td>305,97,64</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>240,138,62,26</td>
</tr>
<tr>
<td></td>
<td>HpyCH4IV</td>
<td>340,108</td>
</tr>
<tr>
<td></td>
<td>NlaIV</td>
<td>318,97,32</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>238,209</td>
</tr>
<tr>
<td></td>
<td>HpyCH4IV</td>
<td>343,105</td>
</tr>
<tr>
<td>Palestinensis strain 2</td>
<td>NlaIV</td>
<td>350,97</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>241,206</td>
</tr>
<tr>
<td></td>
<td>HpyCH4IV</td>
<td>342,120</td>
</tr>
<tr>
<td></td>
<td>NlaIV</td>
<td>332,97,32</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>240,139,62,20</td>
</tr>
<tr>
<td>Polyphaga</td>
<td>NlaIV</td>
<td>361,98</td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td>224,131,104</td>
</tr>
<tr>
<td>Culbertsoni Diamond</td>
<td>NlaIV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BseDI</td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Ten corneal scraping samples were successfully grown on NNA medium. PCR amplification of DNA extracted from the cultured samples yielded the expected bands based on the identified species. Comparison of the restricted fragments with those predicted by the restriction map and CLC Genomics Workbench 3 software revealed that 6 isolates were belonged to A. Palestinensis (Figure 1) and 4 isolates belonged to A. culbertsoni species (Figure 2).

The two bands sized 97 bp and 125 bp are weakly observed. However, according to the bands sized 305 bp and 342 bp, which have only been detected in A. Palestinensis, the sample should be A. Palestinensis. The smallest band size with NlaIV enzyme, i.e., 64 bp, were not supposed to be visible on the agarose gel due to its very small size.

The Figure 2 shows the restriction enzymes’ pattern of A. culbertsoni with estimated bands using BspL I (NlaIV) and HpyCH4V restriction enzymes. Although some bands were seen weakly, as there were no extra-suspected bands, the species were recognized correctly.

DISCUSSION

Acanthamoeba was suggested to be pathogenic in human for the first time in 1958 [11]. Some members of this genus can cause vision-threatening keratitis [1]. Immuno-compromised patients, including AIDS patients and immunosuppressive drug users are particularly susceptible to Acanthamoeba infections. [12].

In the present study, based on PCR-RFLP-based-species identification of 10 Acanthamoeba isolates from corneal scrapings, 6 isolates were identified as A. Palestinensis and 4 isolates as A. culbertsoni. All the isolates belonged to the genotype T4, which has been shown to be the predominant genotype in Iran [13]. Therefore, it can be implied that all the isolates of our study belonged to pathogenic species of Acanthamoeba. The results of this study are similar to those of Niyyati et al., who based on the genotypic identification of Acanthamoeba species, demonstrated that most of the Acanthamoeba strains belonged to the genotype T4 both in clinical and environmental samples; however, genotype T11 was also reported for the first time in their clinical samples [7].

In a study in Taiwan, Acanthamoeba spp. concentrations were determined according to TaqMan real-time qPCR method, through which 10 different genotypes of Acanthamoeba could be detected simultaneously. Acanthamoeba genotypes of all isolates were determined as T4 and the species were categorized to A. culbertsoni, A. polyphaga, A. castellanii, and A. hatchetti [14]. Most of the human Acanthamoeba infections have been associated with the isolates of the genotype T4. For example, more than 90% of AK cases belong to this genotype, suggesting that the abundance of T4 isolates in human infections can be due to their greater virulence and/or other properties that increase their transmissibility as well as their low susceptibility to chemotherapeutic agents [1].

Michalek et al. reported acanthaporin as the first pore-forming toxin to be described from Acanthamoebae that
causes severe and often fatal diseases [15]. Contact lenses wearers are more susceptible to AK, so that about 80% of this infection occurs in young and healthy people who use medical or colored contact lenses; however, eye trauma and poor water supply are other major risk factors associated with AK [16]. Our previous study also showed that contact lenses wearers within age group of 18-30 years were most infected with AK [7]. Rezaeian et al. [17] and Maghsoud et al. [18], reported that most of the AK isolates were from contact lenses wearers. De Jonckheere reported that 15 strains of Acanthamoeba were isolated from contact lenses, boxes, and saline solutions of 9 keratitis patients. All their isolates belonged to T4 that has different subtypes. They also found a strain of Hartmannella from a cornea and two vahlkampfiiids isolates from contact lenses, which had no connection with keratitis. They concluded that only Acanthamoeba genotype T4 is responsible for keratitis in Belgium [19].

The assignment of Acanthamoeba species to a series of 18S rRNA gene sequence types is being widely accepted as a genetic classification system [5]. Booton et al. examined partial 18S ribosomal DNA (Rns) sequences of Acanthamoeba isolates in Hong Kong. They examined the links between strains isolated from corneal scrape specimens, contact lenses, lens cases, lens case solutions, and home water-supply faucets of patients with Acanthamoeba and also looked for evidence of mixed infections. They found 12 Rns genotype T4 and one Rns genotype T3 [10]. In a study in Malaysia, 21 cloned Acanthamoeba isolates, designated as IMU1 to IMU21, were established from positive primary cultures. Five species were identified based on the morphological criteria of Pussard and Pons, i.e., A. castellanii, A. culbertsoni, A. griffini, A. hatchetti, and A. polyphaga, however, 5 species could not be determined morphologically [20]. It should be considered that the morphological characteristics of the cyst can change with the culture conditions, so it is not an appropriate criteria for species identification [3]. In another study from southwestern Korea, ribosomal DNA PCR-RFLP and mitochondrial DNA-RFLP analysis were performed for 43 Acanthamoeba environmental isolates from contact lens storage cases. These isolates were compared to American Type Culture Collection (ATCC) strains and clinical isolates from patients with keratitis. Their results showed that the most predominant (48.8%) species was A. castellanii. They also found that 38 (88.4%) out of 43 isolates from contact lens storage cases were identical to those of clinical isolates, and were considered as potential keratopathogens [21]. Also, the results of our study indicated that contact lenses could increase the susceptibility of individuals to infection with Acanthamoeba species, which is consistent with the results of the above mentioned studies. Although, the results of the present study have similarity with those of other studies, geographical conditions is also a factor that can affect the results.

To the authors' knowledge, no report is available on the species identification of Acanthamoeba by PCR-RFLP method. From the results of this study, it can be concluded that predominant species of the Acanthamoeba vary with geographical locations and the methods used in different studies. Given that this pathogen is emerging as a risk for human health in Iran and worldwide, identification of pathogenic species could be important for clinical purpose, such as diagnosis and treatment.

The limitation of this study was the cost effectiveness of the method used.

ACKNOWLEDGEMENT

This work was supported by Grant No. 598 from Pasteur Institute of Iran, Tehran. The authors greatly appreciate the support and guidance provided by Dr. F. Rahimi from Farabi Eye Research Center.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

REFERENCES


