Isolation and Genotyping of Acanthamoeba Strains from Corneal Scraps

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Abstract

***Purpose*:** To determine the prevalence and genotype of Acanthamoeba keratitis (AK) in patients referred to Farabi referral eye center

***Methods*:** In this study, corneal scraping specimens that obtained from keratitis patients examined for Acanthamoeba and its genotype identifications. Direct smears, cultivation in non-nutrient agar (NNA) followed by PCR were chosen as the basic diagnostic methods. The positive patients checked by confocal microscopy. For genotypic identifications of isolates from keratitis patients, DF3 region of rRNA gene amplified in PCR.

***Results*:** Five clinical specimens (5.6%) out of 89 collected samples showed positive by culture and PCR analysis. Then the results compared with confocal microscopy and all five patients confirmed as AK. The statistical analysis showed significant relation between infection with Acanthamoeba and wearing contact lenses (p=0.001). A logistic regression analysis showed, there is an inverse weak statistical relation between age and infection (p=0.07). Sequence analysis demonstrated that T4 is the predominant genotype in these isolates and the primary genotype in AK. All cases were unilateral and pain, redness, tearing, and light sensitivity were the most common clinical symptoms among them.

***Conclusion*:** Genotypic identification of the microorganism helps epidemiologists and parasitologists to study the transmission routes of infection in different areas. The results of this study also showed that NNA as diagnostic and economical method could be used in areas with low ophthalmic equipment such as confocal microscopy, but it is time consuming.

***Keywords*:** Acanthamoeba Keratitis, Epidemiology, DF3, Genotype

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Introduction

The free-living amoeba, Acanthamoeba spp are distributed worldwide.[1,2] These parasites feed on bacteria, fungi and algae that can be found in variety of natural and artificial habitats including water, soil, and air. This organism is the causative agent of sight-threatening infection of the cornea known as Acanthamoeba keratitis (AK).[3] The severity of the condition may fluctuate and then rapidly progress as a corneal abscess that characterized by a unique ring-shaped morphology with a relatively clear central area.[4,5] Corneal abrasions and contact lens wearers are more susceptible to AK infections as this parasite can survive across the lens and the eye. Fifteen different genotypes (T1-T15) have been related to the genus Acanthamoeba based on rRNA gene sequencing.[6] Specific genotypes of Acanthamoeba such as T4, T2B, T3, T5, T6, and T11 reported as causative agents of AK.[6] Acanthamoeba genotype T4 is the most common genotype related to AK cases worldwide. In contrast, other rare genotypes are associated only with isolates derived from brain infections.[2,3,7,8] Specific and sensitive diagnostic methods of Acanthamoeba infections can prevent severe damage to cornea such as keratitis. Corneal smear, culture and PCR are current methods for identification of this parasite. In vivo confocal microscopy is a non-invasive screening tool for diagnosis of this disease, but it needs an expert eye specialist. It is also difficult to differentiate macrophages from non double-walled Acanthamoeba cysts by use of this microscopy, so it has low sensitivity.[9] Due to the difference between genotypes of parasites, epidemiology, and routes of transmission; the geographical distribution patterns of Acanthamoeba are different. If the epidemiological ranges of infection characterized, researchers could be helped in the determination of each species in establishment of infection. The rate of this parasitic infection of cornea were reported 25%, 30% and 34.5% from Iran.[2,7,10] The incubation period varies depending on a number of host factors. If an eye infection involves previous trauma, the onset of symptoms appears to be more rapid than in infections related to the use of contact lenses.[11] The aim of this study was to ascertain the isolation and identification of Acanthamoeba genotype in scraped samples from keratitis patients and compare the results with other reports.

Methods

This is a cross-sectional study based on one-year research, that during the year 2012, eighty-nine patients with keratitis referred to Farabi research eye center (Tehran University of Medical Sciences, Tehran, Iran) from whom the specimens were collected. Samples obtained through scraping of cornea by the use of surgical blade and immersed in microtubes containing phosphate buffer saline (PBS). Meanwhile, another smear was prepared on glass slides for Giemsa staining. Then the specimens transferred to the department of parasitology, Pasteur Institute of Iran, where they were tested for the presence of Acanthamoeba.[10]

Hundred µl from each collected sample was inoculated in Petri dishes containing 1.5% non-nutrient agar (NNA) in page's saline, seeded with E. coli, and kept in incubator at 30° C. Plates scanned every day under an inverted microscope to check the presence of Acanthamoeba cysts for two weeks. The positive samples were transferred from NNA media into tubes containing PYG medium (0.75% proteose peptone, 0.75% yeast extract, 1.5% glucose with Penicillin (100 U/ml), Streptomycin (100 µg/ml), and gentamicin (40 µg/ml) (Invitrogen™) at 30° C.

DNA extraction

Using a sterile scraper, Acanthamoeba harvested from the surface of NNA positive plates and kept in microtube containing PBS, pH 7.2. Then samples centrifuged at 5000 rpm for 8 min followed by a total genomic DNA extraction from pellets using HiPureA™ multi-sample DNA purification kit.

PCR analysis

PCR was performed using JDPs (JDP1-JDP2) primer pair to amplify rRNA gene Diagnostic Fragment 3 region (DF3 region).[8] PCR amplification, carried out in a volume of 20 µl containing 1.25 U Taq polymerase, 10-15 ng DNA template, 200 µM dNTPs, 2.5 mM $MgCl_2$, and 10 ρmol of each primer. PCR thermal cycling conditions were set as follow: initial

denaturing phase at 95 °C for 5 min continued by 35 repetitions at 95° C for 45 sec, 57.6 °C for 45 sec and 72 °C for 50 sec with a final extension step of 8 min at 72 °C in the last cycle. The amplification product was detected using gel electrophoresis on a 1.5% agarose gel, which was prepared with 1 µl/40ml DNA safe stain (Cinaclon) and visualized under UV light.

Confocal microscopy

In order to confirm the results of culture and PCR methods, the infected patients with A. keratitis, requested for checking with confocal microscopy.

DF3 region of rRNA Gene sequencing

DF3 region of rRNA Gene that amplified as the DNA products, directly sequenced by the sequencing department of the Pasteur Institute of Iran. Raw sequences manually edited by Chromas 2.4 sequence viewer and identified at the genotype level based on the sequence analysis of the DF3 by comparison with the available Acanthamoeba DNA sequences reference in GenBank.

Statistical analysis

The results of this study analyzed by using the SPSS software version 16. Descriptive data reported by descriptive analysis and χ^2 test used for evaluation of two-way communications.

Results

Five clinical specimens (5.6%) out of 89 collected samples, showed positive by culture in NNA and PCR analysis. The cyst of A. keratitis shown on NNA cutre media (Figure 1) and stained with Giemsa method (Figure 2).

The PCR product of all positive samples by amplification of rRNA gene Diagnostic Fragment 3 region after running on agarose gel (470 bp) is shown in figure 3.

By using confocal microscopy, all five patients confirmed for AK. The positive samples were the cases with contact lens, thus statistical analysis showed significance relation between infection with Acanthamoeba and wearing contact lenses (p=0.001). Results showed that contact lens could increase 3.43 times the chance for getting infection (odds ratio: 3.44; 95% CI: 2.47-4.78).

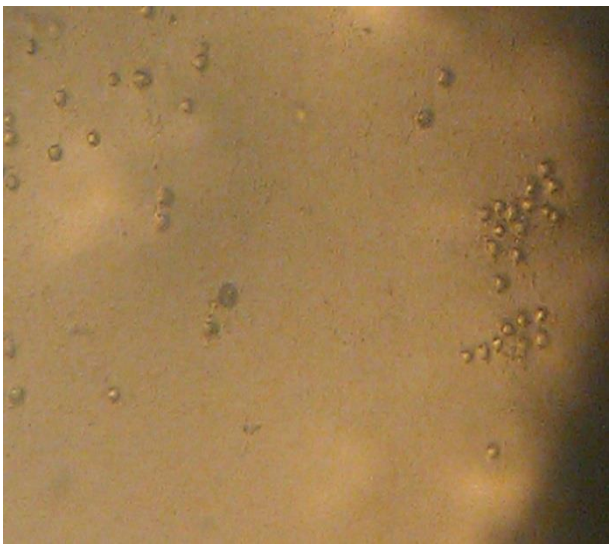

Figure 1. Growth of A. keratitis from scraping samples on non-nutrient agar culture media in cyst stage

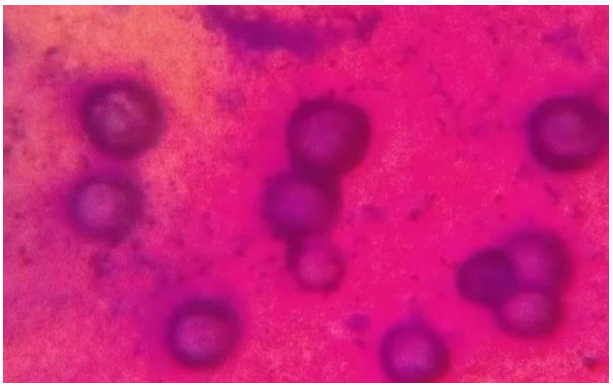

Figure 2. The cyst of A. keratitis stained with Giemsa method

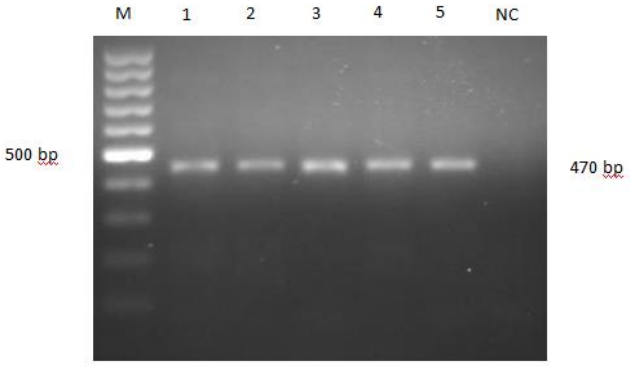

Figure 3. PCR product of Acanthamoeba from culture media, M: Marker, NC: Negative control, 1, 2. 3, 4, and 5, indicate the 5 positive samples

Patients aged between 18 to 30 years old of whom three were male (60%) and wore corrective lenses and two others were females (40%) and used colored contact lenses. A logistic regression analysis showed, there is an inverse weak statistical relation between age and infection (p=0.07). As mean (SD) age of those infected ones was 2/20 (92/1) and for healthy individuals was 91/41 (62/22). All the patients complain from one eye only, and pain, redness, tearing, and light sensitivity were the common clinical symptoms among them.

Sequence analysis demonstrated that T4 is the genotype in these isolates and that genotype T4 is the primary genotype in keratitis Acanthamoeba infections.

Discussion

The genus Acanthamoeba is the causative agent of granulomatous amebic encephalitis, which occurs in immune compromised patients, while keratitis reported in healthy individuals.[12] As mentioned before, the rate of this parasitic infection of cornea by Acanthamoeba reported between 25-34% from Iran.[1,2,6,10] However results in this study showed, 5.6% of the patients with keratitis were determined positive for Acanthamoeba infections and all the cases were among contact lens consumers. Our result is not consisted with previous report from Iran. The reasons may be due to, 1st, these samples have been collected from everybody who referred to the eye clinic with sign of keratitis, but the others may had collected the samples from those who were suspected to AK. Different microorganisms that are able to infect the eyes may cause keratitis, such as bacteria, fungi and viruses. The 2nd point may be the increasing knowledge of the self care, specially in cases of contact lenses users. The factors such as virulence of parasites strains and water sanitation are influential to the occurrence of the infection. Early diagnosis is an essential step for effective treatment of AK. Improper sterilization procedures identified as the cause of a large percentage of AK infections.[13] The infection usually diagnosed by an ophthalmologist based on symptoms, growth of the parasite from eye-scraping sample in culture media, and/or observing the ameba by a process called confocal microscopy. This study showed that NNA culture media as a simple and economical method can be used for diagnosis of AK, which is considered less frequent in developing countries compared with fungal and bacterial keratitis. According to the report of Patel and coworkers AK has increased, and the main risk factor is attributed to the wear of contact lenses, which represent approximately 85 to 90% of the AK cases.[14] Few reported studies have been on isolated smears from the patients, but most reports are from the environmental sources.[1,15] During our study in Farabi referral eye center only one suspected A. keratitis patient by confocal microscopy requested for culture and PCR methods to confirm the diagnosis. Results of both methods were reported as negative. The diagnostic sensitivity of confocal microscopy for A. keratitis is reported to be 90.6%, in patients with both clinical and objective evidence.[16] Thus, it is recommended that confocal microscopy and culture be requested simultaneously in order to increase accuracy.

In the present study, we examined genotypes of Acanthamoeba obtained from AK infections to assess which genotype is the most common one. Among 13 clinical isolates of Acanthamoeba reported by Maghsood et al, eight isolates belonged to T4, two belonged to T3, and three belonged to the T2 genotype.[17] Although the place of collecting samples of this study is the same as theirs which was done ten years earlier. Our findings demonstrated that T4 is accessible and adaptable for humans in this region. In another study by Niyyati et al, results demonstrated that most of the Acanthamoeba strains belonged to the genotype T4; however, genotype T11 was also reported for the first time in their clinical samples.[7] Consistent with previous studies our results also showed that the T4 genotype is predominant type. No non-AK infections has been found to be associated with T3 or T11 genotypes that has been occasionally reported in AK infections.[18] Acanthamoeba has been isolated worldwide from disparate environments such as fresh and salt water, drinking water, contact lens washing solutions, ventilation systems, dialysis apparatus, hydrotherapy areas in hospitals, and dental irrigation systems. In addition, outbreaks of contact lens associated AK have been reported in the USA.[3,19]

Conclusion

The main focus in this study was to determine the prevalence and genotype of AK in patients referred to Farabi referral eye center. Epidemiologists and parasitologists suggest that for accurate incidence estimation of the parasitic infection and determining the role of each species in transmission, identifying the genotypes is essential. All isolates in this study were T4 genotype, and concluding that it is the primary genotype in AK infections. The results also showed that NNA culture media as diagnostic and economical method could be used in the regions with limited ophthalmic equipment such as confocal microscopy or used along with it, but it is time consuming.

Acknowledgments

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