2024, 21(6): 1953-1959
https://doi.org/10.21123/bsj.2023.8059
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

Isolation and molecular characterization of *Acanthamoeba* spp. from Iraqi patients with Keratitis

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Received 06/11/2022, Revised 25/07/2023, Accepted 27/07/2023, Published Online First 20/11/2023, Published 1/6/2024

Abstract

Isolation and molecular characterization of *Acanthamoeba* spp. related corneal infections in Iraqi Patients. A total of 96 samples from corneal scraps and contact lenses were gathered from corneal patients for the current trial, which runs from November 2020 to May 2021. All plates were checked, classifying them as positive or negative morphologically identification of *Acanthamoeba* spp. where PCR analysis, which entails the targeted amplification of nucleic acids, has been carried out, and where The 18s rRNA gene's DF3 region was the focus of the isolated strains' sequences. Three patients had Acanthamoeba spp. infections, according to the findings. then the sequence analysis was performed using the NCBI tool, which showed the presence of 2different genotypes, two strains were grouped into the T3 genotype, one from T4, then they were registered in GeneBank under number accession (MZ427912, MZ349042) respectively. To our knowledge, these are the first reported cases of *Acanthamoeba* keratitis in Iraq.

Keywords: *Acanthamoeba* spp., Contact lenses, Corneal infections, Eye, keratitis.

Introduction

The protozoan of free living, *Acanthamoeba* spp., occurs in two an active trophozoite and a dormant cyst form [1]. where They have been isolated from air, soil, contact lenses, swimming pools, sea, drinking water, cleaning solutions lenses [1, 2].

Acanthamoeba pathogens mainly cause two different pathologies, one is a corneal infection, *Acanthamoebic* keratitis, and the other is a central nervous system (CNS) infection called granulomatous amoebic encephalitis (GAE) in humans and animals. *Acanthamoeba* also causes skin infections many times related to GAE [3].

Acanthamoeba Keratitis (AK), a condition that causes serious corneal infection, is difficult to diagnose and has no proven effective treatments. AK is a scarce, but potentially blinding corneal infection triggered through the trophozoite stage of the *Acanthamoeba* protozoan. *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Acanthamoeba hatchetti* are organism's species that can cause serious keratitis in contact lens wearers [1, 2].

Recently ten species (genotypes including T2, T3, T4, T5, T6, T10, T11, T13, T15, and T16) can infect human keratitis, Specificlly, *A. castellanii* and *A. culbertsoni* and *A. polyphage* show the potential

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P-ISSN: 2078-8665 - E-ISSN: 2411-7986
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strongest pathogenic, due to their ability to adhesion to the corneal tissues through specific enzymatic activity [1, 4, 5].

Acanthamoeba keratitis lasts several days to weeks, and is accompanied by characteristic symptoms, particularly by contact lens wearers or those who experience severe ocular pain, redness, photophobia, and stromal penetration, all of which can lead to a sight-threatening condition and are often misdiagnosed as Herpes simplex, fungal, or bacterial keratitis [6]. AK normally affects one eye, but in contact lens wearers, the condition can affect both eyes [7].

Materials and Methods

Sample collection:

In ophthalmology clinics and hospitals (Ibn Al-Haytham Specialist) from November 2020 to May 2021, 96 corneal scrapes and contact lens samples were obtained from patients with keratitis. All samples were cultivated on non-nutritive agar (NNA) plates with a suspension of heat-inactivated E. coli, and they were kept at 37°C for 14 days. In order to monitor the development of *Acanthamoeba* trophozoites and/or cysts for 15 days, clinical samples were investigated using PCR and direct microscopy methods.

DNA extraction and amplification:

DNA extraction, after subculturing of each sample, amoebic plaque culture was collected with 1 mL of saline buffer, transferred into a 1.5mL tube, and centrifuged for 10 min at 6000 rpm. The pellet was resuspended in 100 µL of saline buffer and used for DNA isolation. DNA extraction was performed by use of Ribo-Sorb RNA/DNA extraction kit.

For identification of the genus *Acanthamoeba*, a PCR was carried out to amplify a 18S rDNA region defined as ASA.S1 (*Acanthamoeba* Specific Amplimer) that includes the diagnostic fragment 3 (DF3), using the genus specific primers JDP1 and JDP2. The *Acanthamoeba*-specific primer pair used here included the forward primer JDP1 (5'-GGCCCAGATCGTTTACCGTGAA-3') and the reverse primer JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA-3'). Depending on the genotype, the primers amplified ca. 420 to 551 bp of 18S rDNA between reference bp 936 and 1402. These primers were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/ul as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/ul.

Results and Discussion

Four samples of 96 of *Acanthamoeba* spp. were used in current study. The samples were morphologically recognized based on active trophozoite and cyst form features Fig. 1.

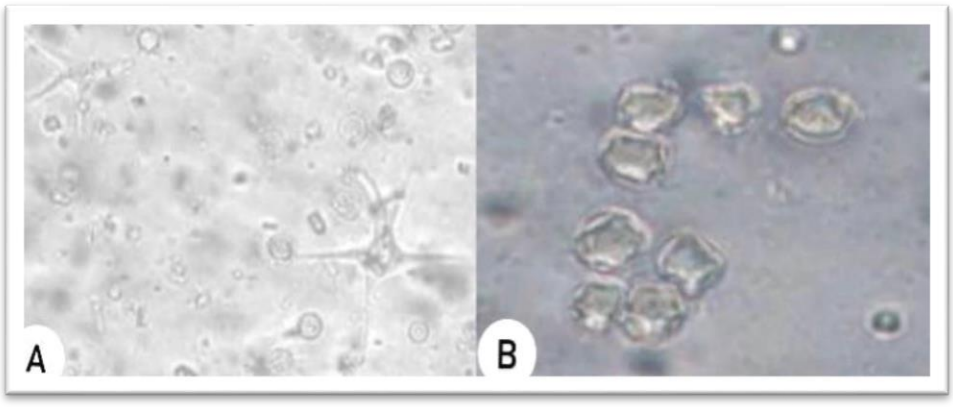

Figure 1. (A) Fresh unstained *Acanthamoeba* trophozoites, showing spine-like structures (x100)., (B)Fresh unstained *Acanthamoeba*, star shape cysts isolated from cornea (x100).

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Acanthamoeba Specific amplimer (ASA.S1) gene was amplified by using PCR technique via JDP primer. The PCR products were subjected to electrophoresis, 100 bp DNA ladder where 423 to 551 bp. The results shown in Fig. 2, proved that all the introduced isolates were belonging to the genus *Acanthamoeba*.

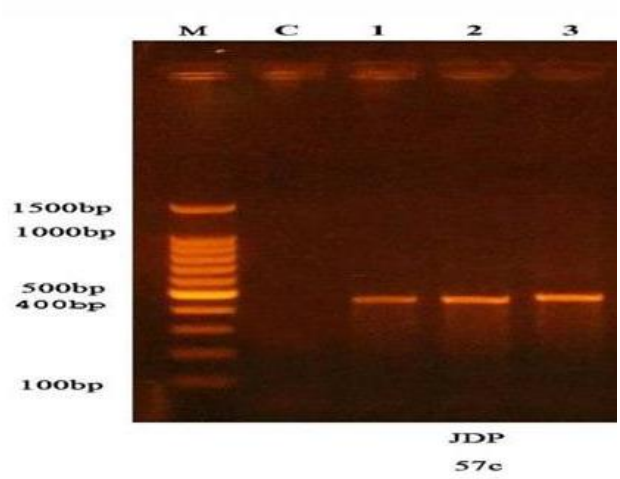

Figure 2. Agarose gel electrophoresis of *Acanthamoeba* spp. PCR amplified DNA product.

Lane M: 100 bp DNA molecular weight marker.
Lane C: Negative control.
Lane 1-4: Positive samples.

The amplified PCR product of three samples of Iraqi isolates was sequenced for the partial 18 S rRNA gene. The isolates were classified as T3, T4, after modifying the sequences with Sequencer software and comparing them to sequences in the GenBank database identified and they were registered in GenBank under accession numbers MZ427912, MZ349042 respectively. The study showed that T3 was detected in two corneal infected patients, T4 from washing water of a contact lens female wearer.

In recent years, the traditional morphological classification of *Acanthamoeba* species based mostly on cyst shape and size has become inconsistent. Following that, the disparity between the morphological method and the molecular biological discoveries prompted a reclassification of species, which began with the examination of portions of the 18S rRNA gene [8] . Where [9] clarifies that trophozoites were pleomorphic and mobile, having a large number of vacuoles of various sizes and contents.

Acanthamoeba spp. is a type of free-living amoeba that can be found in the air, water and soil. Ocular trauma is the most common risk factor in the globe, followed by contact lens wear and swimming [1, 10] . In this study, there were cases associated to contact lens use. Although the trophozoites of *Acanthamoeba* spp. varies in size and pseudopod structure, the trophozoites of *Acanthamoeba* spp. When observed under a light microscope, they appear to be quite similar, but a thorough observation (particularly at the cyst stage) led to the identification of a number of species and the development of a categorization system [11].

The etiological diagnosis of *Acanthamoeba* keratitis relies on the detection of trophozoites, pre-encystment trophozoites, mature cysts, and empty cysts by corneal smear. *Acanthamoeba* cysts were recognized by the presence of well-defined double cyst walls (ectocyst and endocyst). The exterior wall of an *Acanthamoeba* cyst was wrinkled or smooth (ectocyst) while the inner wall was a stellate, polygonal, star–like, or even reasonably smooth (endocyst) and measured 12 to 25m in diameter. Previous researchers utilized the same criteria to distinguish between *Acanthamoeba* and other organisms [11, 12] .

In this study, *Acanthamoeba* infection diagnosis and *Acanthamoeba* keratitis prognosis in non-contact lens wearers were both greatly improved by the use of PCR tests. It allows more rapid diagnosis with small samples, and although it increases the sensitivity diagnostic, requires specialized laboratories [13, 14] .

According to the results of the present study [15] , which included 26 cases of *Acanthamoeba* keratitis, *Acanthamoeba* positivity rates for direct microscopy were 15.3%, 92.3% for PCR, 46.1% for culture, and 100 percent for real-time PCR. Other kinds of keratitis, such as bacterial, herpes simplex virus, and fungal infection, have clinical characteristics that are comparable to *Acanthamoeba* keratitis. Another study by [16] reported that PCR technique was used to detect AK from 61, 64 patients with corneal infection. Also [17] found that PCR technique is more sensitive than the culture technique which is positive only between 30% and 60% of AK samples.

In Egypt, [18] detected a lower incidence of *Acanthamoeba* spp. 43.3% in freshwater samples using a specific primer. Rayamajhee-et al [19] observed that 100% of freshwater samples presented

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P-ISSN: 2078-8665 - E-ISSN: 2411-7986
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Acanthamoeba spp. by using specific primers. In Iran, [20] molecularly identified *Acanthamoeba* environmental samples from mountain pool water samples and they also identified the same *Acanthamoeba*. from clinical samples of *Acanthamoeba* keratitis patients. The classification of species belonging to the genus *Acanthamoeba* previously was done according to the morphology of the cyst as a practice then, it has been replaced by the classification based on the study of the genotype according to the gene sequence for the 18S subunit of ribosomal RNA. Recently, twenty genotypes (T1-T20) are described, nine of them (T2, T3, T5, T6, T10, T11, T12, T15 and T18) are causing the pathology in man [1].

T4 genotype is the most frequent in both samples environmental as clinics [1]. The findings of the present study of alignment revealed that all isolates belonged to the *Acanthamoeba* genus, with two genotypes identified: T3, T4. *Acanthamoeba* genotype T3 was isolated from two patients who were suffering from redness of the eye with eyelids edema and pain. Contact with contaminated soil, water, dust, or other elements can result in infection, as can inhaling cysts [1, 2] . It can also be contracted by activities such as bathing or contact with warm waters. There is a study was agreeing with current results that isolated the *Acanthamoeba* T3 genotype from different water sources [21].

The current study is agreeing with Aykur & Dagci [22] when they studied Acanthamoeba isolates and they showed five genotypes (T2, T3, T4, T9 and T11) in 138 patients as well as they reported the T4 genotype was the most prevalent T4 genotype was the most common in corneal scrapes and contact lenses samples.

Recently [23] *Acanthamoeba* spp. was shown to be present in 30.3% of 56 corneal infected patients. According to the sequencing analysis study, 94.1% of amoebic keratitis isolates belonged to the T4 genotype, with only one patient sample belonging to the T11 genotype 5.8%.

Nowadays [24] were detected with *Acanthamoeba* Keratitis genotype T2 (*A. castellani*) according to PCR and sequence analysis in two cases, one of them is contact lenses wearer and the other with no history of trauma or wearing contact lenses. The most important risk factors for AK promotion are trauma, contact lens wear and contact with contaminated water.

In other countries of the world, other workers recorded the T11 genotype is the predominant source of AK over the world [25] . In Egypt few investigations on *Acanthamoeba* genotyping have been conducted. The presence of *Acanthamoeba* T1, T2, T3, T4, and T7 genotypes in water sources was originally reported by [25] . These results are nearly similar to the result that was recorded in the present study. Based solely on clinical symptoms, smear, culture, and in-vivo confocal microscopy, 42 corneal scraping samples were recently reported to be infected with (AK). AK was connected to contact lens use in 29 eyes. A significant source of infection is improper contact lens use [26] .

In Tunisia, and North Africa, the first AK isolated from 5 100% of patients who suffered from corneal abscess was T4 genotype [27] . Therefore, the prevalence of AK was obtained in 14 cases of 230 corneas scraped samples. 10 {14} and 92.8% of cases were between the ages of 19 and 27. The majority of cases had a history of contact lenses [28] . Other workers in other countries like [29] in the United Kingdom recorded an outbreak of AK in contact lens wearers, [30] in Austria and [31] in the Netherlands, everyone agrees that the misuse of contact lenses, the poor cleaning of the lenses and the omission of the use of sterile ophthalmological solutions are among the most important reasons for the outbreak of Acanthamoebiasis. These results have similarities that were recorded in the present study.

T3 genotype was found in two individuals who had no prior history of trauma or contact lens wear, perhaps as a result of exposure to environmental variables like water washing or swimming in a pool . Many studies have shown that domestic and pool water is responsible for *Acanthamoeba* spp. spreader, [32] in Malaysia in which they found *Acanthamoeba* in the stairs, walls, wax of the edge and in the center of the pool. Recently [19] was obtained T3 and T4 in domestic bathroom water in Sydney[33] showed that *Acanthamoeba* was isolated from bathroom and kitchen spouts. In conclusion, T3 and T4 genotypes were shown to be the primary causative agents of *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), and the most frequently described as a causal agent of amoebic illnesses and regarded the most pathogenic. Early diagnosis is important in preventing complications in those infected with AK. Many studies clarify that the delay in diagnosis of

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P-ISSN: 2078-8665 - E-ISSN: 2411-7986
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precocious leads to the progression and increased damage produced by *Acanthamoeba* spp.

Conclusion

The current study documents the presence of *Acanthamoeba* in corneal samples from Baghdad neighborhoods with a high human population density, which may be dangerous to human health.

Acanthamoeba was first identified in genotypes using PCR and sequencing analysis (including T3 and T4).

The *Acanthamoeba* sequences obtained in the present study were submitted to GenBank (MZ427912 and MZ349042).

Acknowledgment

Authors would like to Very special gratitude and thanks go to the College of Science for Women, I would like to express my deepest appreciation to Asst. Prof. Dr. Zahraa Abdul-Raheem Ahmed for their help and support. I must also thank the staff of Ibn Al Haitham Teaching Eye Hospital, and the laboratory staff assistant in the hospital, for their support and help throughout the process of the research.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

M.B: Sample collection, and Laboratory methodology include culture media prepared, prepared slides and Photographing samples, molecular detection. N.M: She gave a title to the research, developed a plan of action, supervised the work, we discovered the *Acanthamoeba* parasite. K.A: Contribute to sample collection.

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العزل والكشف الجزيئي لطفيلي *Acanthamoeba* spp. في المرضى العراقيين المصابين بالتهاب القرنية

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الخلاصة

تعتبر *Acanthamoeba* من أكثر الطفيليات انتشارًا وهي على اتصال دائم مع الانسان والحيوان تتكون دورتها من مرحلتين هما الطور الخضري (الناشطة) والطور المتكيس. تضمنت الدراسة الحالية على عزل طفيلي *Acanthamoeba* الموجودة في قرنية العين. تم جمع ستة وتسعين عينة سريرية من مرضى التهاب القرنية من مستشفى (ابن الهيثم التخصصي) في بغداد للفترة من تشرين الثاني 2020 إلى نهاية أيار 2021 لعزل وتشخيص طفيلي *Acanthamoeba*، ولقد تم زرع جميع العينات على أوساط تفريقيه، فبعد أخذ العينات عن طريق قشط القرنية أو المسحات، تم نقلها إلى اوساط أجار غير مغذية (ANN) تحتوي على معلق *E. coli* المعطلة بالحرارة وحضنت لمدة 14 يومًا عند 37 درجة مئوية. ثم تم فحص جميع الاطباق وتصنيفها على أنها تحديد شكلي إيجابي أو سلبي للتعرف شكليا على *Acanthamoeba* spp. ولم يلاحظ وجود الفطريات فيها. ومن ثم تم إجراء تحليل تفاعل البلمرة المتسلسل(PCR) وتسلسل لتحديد أنواع عزلات *Acanthamoeba*. بعد تحليل (PCR)، وتحليل جزء DF3 جعل من الممكن تصنيف السلالات الثلاثة من جنس *Acanthamoeba*، ثم تم إجراء تحليل التسلسل باستخدام أداة NCBI، والتي أظهرت وجود طرازين وراثية مختلفة، تم تجميع سلالتين في النمط الجيني T3، واحدة من T4، ثم تم تسجيلها في GeneBank تحت أرقام المدخلات على التوالي (MZ427912، MZ349042).

الكلمات المفتاحية: الاكانثاميبا، حافظة العدسات اللاصقة، اصابات القرنية، العين، التهاب القرنية.