Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

Diagnosis of *Acanthamoeba* keratitis in Mashhad, Northeastern Iran: A Gene-Based PCR Assay

Nazgol KHOSRAVINIA [1], *Abdolmajid FATA [1,2], *Elham MOGHADDAS [2], Bibi Razieh HOSSEINI FARASH [1,2], Mohammad Reza SEDAGHAT [3], Ali Raza ESLAMPOUR [3], Lida JARAHI [4]

1. *Department of Medical Parasitology and Mycology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
2. *Cutaneous Leishmaniasis Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*
3. *Khatam-al-Anbia Haspital, Mashhad University of Medical Sciences, Mashhad, Iran*
4. *Community Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

Received 19 Mar 2020
Accepted 15 Jun 2020

Keywords:
PCR;
Genotype;
Acanthamoeba keratitis;
Diagnosis;
Iran

***Correspondence Emails:**
FataA@mums.ac.ir
moghaddas.elham@yahoo.com

Abstract

Background: The genus *Acanthamoeba* is a free-living opportunistic protozoan parasite, which widely distributed in soil and fresh water. *Acanthamoeba* keratitis, which causes a sight-threating infection of the cornea, is going to rise in Iran and worldwide. The aim of this study was to compare direct microscopy, culture and PCR for detection of *Acanthamoeba* spp. in clinical samples and to determine the genotypes of *Acanthamoeba* spp. by sequencing 18SrRNA gene.

Methods: Among patients clinically suspected to AK referred to a tertiary ophthalmology center at Mashhad, northeastern Iran. During 2017-18, twenty corneal scrapes specimens obtained. The samples were divided into three parts, subjected to direct microscopic examination, culture onto non-nutrient agar and PCR technique. Sensitivity, specificity, accuracy and likelihood ratio were evaluated.

Results: Among 20 persons clinically suspected to amoebic keratitis, 13(69.2%) patients definitely diagnosed as *Acanthamoeba* keratitis. Wearing contact lens, eye trauma due to foreign particle and swimming in fresh water were the main predisposing factors. Most of patients suffered from pain and photophobia. Corneal ring infiltration and epithelial defect were common clinical sings. Direct examination had the lowest sensitivity and sensitivity of both Nelson-PCR and JDP-PCR methods were equal and highest. In addition, the results of sequencing identified that all strains belonged to T4 genotype.

Conclusion: Amoebic keratitis is a sporadic parasitic keratitis, which is mainly seen in contact lens user in Mashhad. PCR based on 18S ribosomal DNA with JDP primers is a reliable and highly sensitive method for diagnosis of *Acanthamoeba* keratitis in clinically suspected cases.

Introduction

The genus *Acanthamoeba* is a free-living amoeba distributed ubiquitously in various environments such as soil, air and fresh water sources (1). Even, it can be found frequently in artificial habitats among the public swimming pools, surgical instruments, and contact lens solution. Some species of *Acanthamoeba* can cause potentially sight-threatening infection due to *Acanthamoeba* keratitis [AK] (2-4). Up to now, more than 20 genotypes of *Acanthamoeba* have been identified based on 18S ribosomal DNA (rDNA) sequencing method (designated T1 to T20) (3). Of these, 8 species of *Acanthamoeba* have been implicated in eye ocular infections: *A. castellanii, A. polyphaga, A. hatchetti, A. culbertsoni, A. rhysodes, A. lugdunensis, A. quina, and A. griffini* that most of keratitis causing isolates belongs to type T4 (1, 5).

The gradual increases in wearing contact lenses, misdiagnosis of AK and its slow response to medical treatment due to its encystation in harsh conditions are the most important factors that make *Acanthamoeba* critical for human health (3, 6). Furthermore, *Acanthamoeba* can play the role of carrier for pathogenic bacteria (e.g. *Legionella*, *Pseudomonas* and *Helicobacter*) that these bacterial pathogens lead to severe human infections (5, 7). Usually, an early and reliable AK diagnosis does not occur because it is easily confused with other infectious keratitis. Actually, primary clinical signs of AK are nonspecific and indistinguishable from *Herpes simplex* virus infection or fungal keratitis (8, 9). Because of AK delayed diagnosis, it is associated with poor prognosis and subsequently prolonged course of treatment, as far as *Acanthamoeba* spp. has been able to infiltrate deeply into the corneal stroma (10-12).

Standard laboratory method for the diagnosis of AK is mainly based on direct microscopy of corneal scrape smears and contact lens solution. Culture of these specimens on non-nutrient agar overlaid with *Escherichia coli* can be successful (13). In spite of high specificity of conventional methods; it requires technical experience with morphology of cysts and trophozoites of *Acanthamoeba* spp. (14, 15). In addition, these assays are limited by poor sensitivity. Therefore, the sensitivity of direct smears for diagnosis of AK can be less than 50% (16). Recently, DNA-based methods including polymerase chain reaction (PCR) has been applied as alternative diagnostic modalities to detect *Acanthamoeba* spp. in corneal specimens with high sensitivity ranging from 77 to 88% (15, 16). PCR analysis in order to amplify of 18S rDNA in diagnostic regions is appropriate for distinguishing most *Acanthamoeba* genotypes (17). In addition to being more rapid compared to culture based-methods, this procedure also donates the valuable phylogenetic information (18).

We aimed to detect *Acanthamoeba* spp. in corneal scrapings samples of patients clinically suspected to AK by Nelson-PCR, JDP-PCR, direct microscopic examination, culture on non- nutrient medium and to evaluate these three different methods.

Martials and Methods

Ethical approval

The study was approved by the Ethics Committee, Mashhad University of Medical Sciences with the ID code: IR.MUMS.FM.REC.1396.92.

Sampling

During 2017-2018, twenty specimens of corneal scrapes were obtained from patients suspected to AK, who referred to the Khatam-al-Anbia Eye Hospital, Mashhad University of Medical Sciences, Mashhad, Iran. All clinical samples were obtained using special eye scalpels, by an ophthalmologist. The specimens were transferred to phosphate buffer

saline (PBS) in order to do fresh smear, culture and PCR examination according to previous studies.

Direct microscopic examination

Direct microscopic examination was performed by putting one-third part of the specimen into a drop of PBS solution on a glass slide. Smears were investigated under a standard light microscope with × 40 magnifications to identify of *Acanthamoeba* trophozoites or cysts based on morphological features (19).

Culture

Briefly, 100 micro liters of each sample were inoculated onto 1.5 % non-nutrient agar (NNA) overlaid with *E. coli*, and incubated at room temperature. In order to detect the outgrowth of the *Acanthamoeba* trophozoites and/or cysts, Plates were checked every 2 days for approximately 1 month by using an inverted microscope (20, 21). To achieve a media culture of *Acanthamoeba* from fungal or bacterial contamination, a piece of agar was cultivated on a fresh plate (5, 22).

DNA extraction, PCR and DNA sequencing

The third part of the specimens stored at −20 °C, was used for the molecular technique. Primarily, the samples were melted at room temperature then DNA extraction was performed by QIAamp DNA Mini kit (Cat. no. 51304) according to the manufacturer's protocol. Two specific primer pairs were used for detection of *Acanthamoeba* spp. A pair of primers JDP1 (fwd) (5'-GGCCCAGA TCGTTTACCGTGAA-3') and JDP2 (rev) (5'-TCTCACAAGCTGCTAGGGAGTCA-3') was applied to amplify a fragment of 423 to 551 bp of ASA.S1 region in18S rRNA gene (23, 24). The second primer set was Nelson primers (fwd) (5'-GTTTGAGGCAATAACAGGT-3') and (rev) (5_ GAATTCCTCGTTGAAGAT-3') for amplification of a 229 pbs fragment of genomic rRNA (12). The conventional PCR reaction by both primer pairs was performed on the thermo cycler (Bio-RAD, T100 thermo cycler) using a ready-made mixture of Amplicone (Taq DNA Polymerase Master Mix RED, Denmark) in a 30-µl final volume containing 5µl of DNA and 0.3µM concentration of each primer in accordance with standard procedure. Thermal cycling conditions were as follows initial denaturation at 94 ˚C for 5 min, 35 cycles of denaturation at 94 °C for 35 S, annealing at 56 °C for 45 S and extension at 72 °C for 60 S and a final extension step at 72 °C for 10 min (6, 25). To visualize PCR-products, they were electrophoresed on 1% (w/v) agarose gel, stained with Green viewer substance (1 µl/10ml) and then the presence of bands (nearly 500bp) were confirmed under UV light using a Gel-doc Image Analyzer.

Sequencing analysis

To determine the genotype, a 500 bps fragment of ASA.S1 region was Amplified from 18s rRNA gene of *Acanthamoeba* strains by JDP primers, and then gel purified (PCR products purification kit, parstous Corporation, Iran, Cat. No. A101221) and finally sequenced by Macrogen Company (Korea). The DNA sequences were edited manually with MEGA6.06 software and compared with BLAST Gen Bank database.

Pathogenicity tests

Osmotolerance assay

Two concentrations of mannitol (0.5 M and 1 M) in a non-nutrient agar medium used for each strain and incubation were done at the room temperature. Growth of *Acanthamoeba* were followed up (were assessed) daily for a period of 7 days (26, 27).

Thermotolerance assay

Acanthamoeba isolates were obtained from non-nutrient agar containing *E. coli*, inoculated into new culture medium at two different temperatures 37 °C and 40 °C. Growth of amoebae was evaluated by invert microscopy

every 24 h for a period of 1 week. The positive control was used concomitant with each culture for elimination of any probable error (28, 29).

Statistical analysis

One specimen was considered positive for *Acanthamoeba* sp. when the results for any two direct examinations, culture, or PCR with either primer set were positive. We defined this reference standard upon which each of the methods was compared for the statistical analysis. Statistical tests were performed using Med Calc (https://www.medcalc.org/calc) and SPSS (Chicago, IL, USA) software (version 11.5). *P* value <0.05 was considered significant.

Results

Among 20 patients suspected to AK, 13 (65%) samples of 4 (30.8%) males and 9 (69.2%) females were positive based on defined gold standard (positive results for two of four tests). Most of the patients were in the age group 25-35-yr-old. The results showed that all of the patients had history of at least one of the following risk factors: wearing contact lens, eye trauma due to entrance of foreign particles or swimming inside fresh water. Only soft lenses have been used by those patients. Among the patients, 2 (15.4%) individuals had only history of swimming inside private garden pool sand, in 4 (30.8%) persons, infection started after an eye trauma (Table 1). Most of patients suffered from pain and photophobia. Corneal ring infiltration and epithelial defect were common clinical sings.

Based on the defined gold standard diagnostic method, cultures were positive in 6 of 13, while only 5 samples obtained positive result by direct examination (Fig. 1). The results of Nelson-PCR were positive for 14 cases (one false positive) (Fig. 2). Finally, JDP-PCR detected *Acanthamoeba* parasite in 13 of 13 (Fig. 3). Among diagnostic methods, direct examination had the lowest sensitivity (38.46% with 95% confidence interval 13.86% to 68.42%). The sensitivity of culture was 46.15% (CI 74.87% to 19.22%) and the sensitivity of the Nelson-PCR and the JDP-PCR methods was equal to 100% (CI 75.29% to 100%). In fact, all positive samples of *Acanthamoeba* were detected by PCR method with 2 primer pairs used in this study. The specificity of JDP-PCR, Nelson-PCR, direct examination and culture was 100% (CI 59% to 100%), 85.71% (CI 42.13% to 99.64%), 100% (CI 29.04% to 96.33%) and 100% (CI 59.04% to 100%) respectively (Table 2).

The existence of one false positive case in the Nelson-PCR indicates a lower performance than other compared diagnosis methods. Agreement between PCR method with each of the Nelson and JDP primers compared to gold standard was calculated as 100% (*P* value <0.001).

Table 1: The frequency of risk factors among the patients with *Acanthamoeba* keratitis

No. of patients	***Risk factors***		
	Contact lens	Trauma	Swimming
2			+
4		+	
3	+		+
4	+	+	

Table 2: Sensitivity, specificity, NPV and PPV of four diagnostic methods compared to defined gold standard

Assay	*No. positive*	*No. negative*	*Sensitivity (%)*	*Specificity (%)*	*PPV (%)*	*NPV (%)*
Direct –smear	5	15	38.46	100	100	46.7
Culture	6	14	46.15	100	100	50
JDP_PCR	13	7	100	100	100	100
Nelson_PCR	14	6	100	85.71	92.86	100

PPV, positive predictive value; NPV, negative predictive value
Sensitivity: number of true positives divided by number of true positives plus false negatives ×100. Specificity: number of true negatives divided by the number of true negatives plus false positives ×100

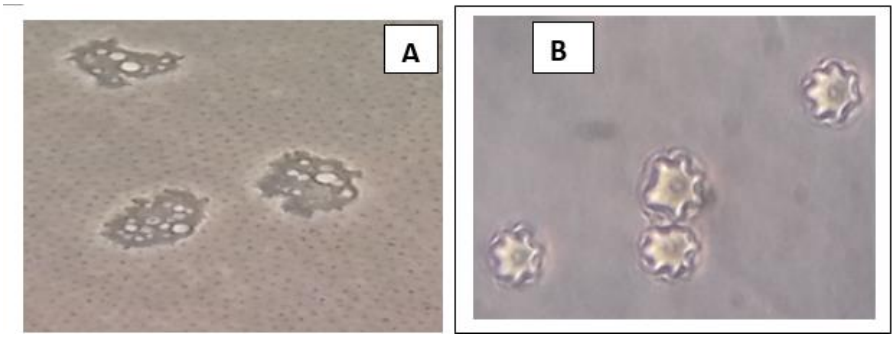


Fig. 1: Invert microscopy photographs of *Acanthamoeba* trophozoite (A) and cysts (B), cultured on non-nutrient agar

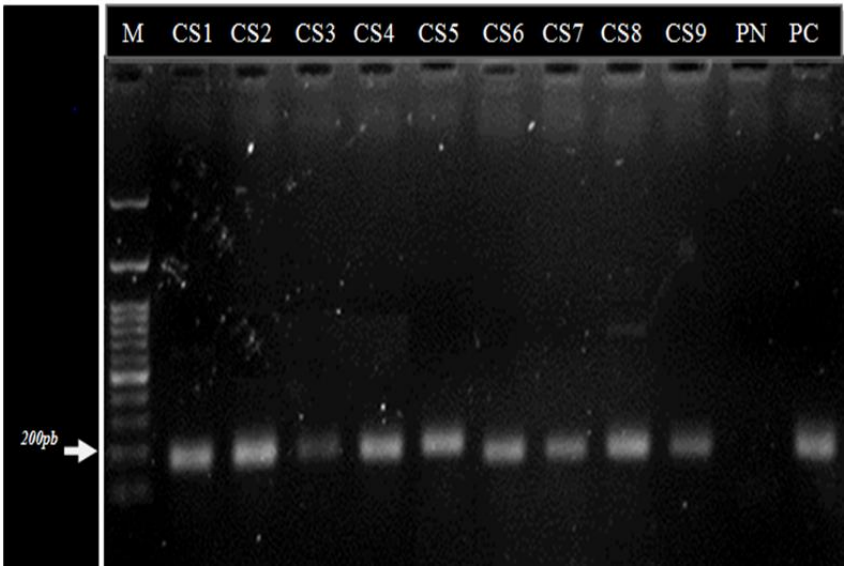


Fig. 2: Agarose gel electrophoresis of PCR products using Nelson primers: lanes CS1-CS4: corneal scrape, Lane PC: Positive control, lane PN: lane Negative control, lane M: standard DNA marker (100 bp DNA size)

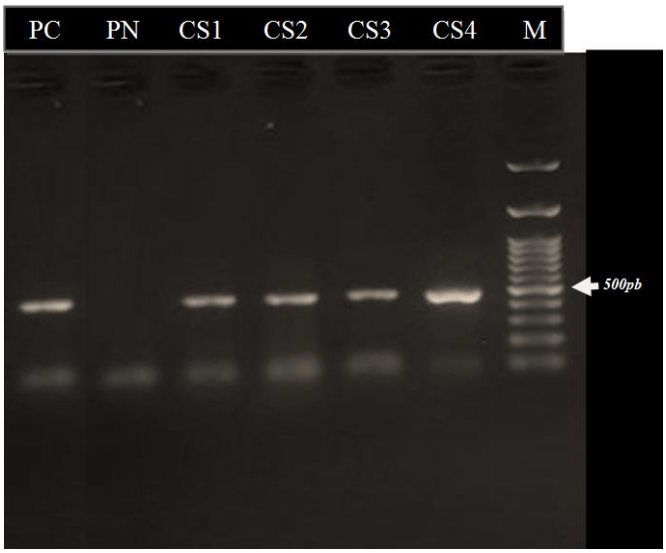
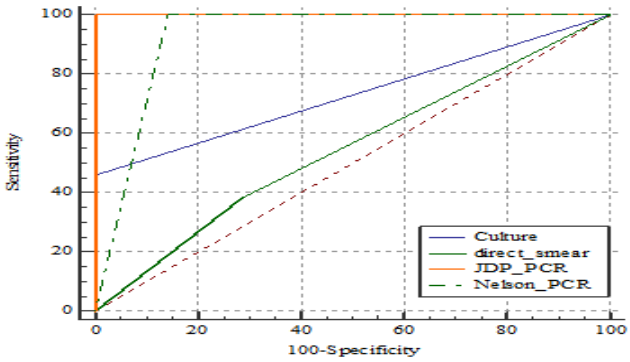


Fig. 3: Agarose gel electrophoresis of PCR products using JDP primers: lanes CS1-CS4: corneal scrape, Lane PC: Positive control, lane PN: lane Negative control, lane M: standard DNA marker (100 bp DNA size)

Although it was for direct examination 83% but there was no statistically significant relationship (*P* value = 0.65). A very low agreement between the methods of culture and Gold standard was observed (37% with *P* value = 0.03). The accuracy of the methods was calculated using measuring the area under the curve of ROC (Receiver Operating Characteristic) diagram. The analysis of these results shows the accuracy of the Nelson-PCR and JDP-PCR tests was excellent (ACU). Accuracy of culture test was acceptable and direct examination had a low accuracy (Fig. 4).



Test Method	AUC*	SE	95% CI**
Culture	0.731	0.0720	0.489 to 0.901
Direct smear	0.692	0.117	0.449 to 0.876
JDP_PCR	1.000	0.000	0.832 to 1.000
Nelson-PCR	0.929	0.0714	0.721 to 0.995

*AUC: Area under the ROC Curve **CI: confidence interval

Fig. 4: ROC analysis of molecular and traditional methods used in this study compared to gold standard for the laboratory diagnosis of *Acanthamoeba* keratitis

The results of sequencing identified that all strains belonged to T4 genotype with a more than 95% similarity. Sequencing of isolates was deposited in Gen Bank database under accession numbers MK297910.1 to MK297916.1 and MK297918.1 (Fig. 5).

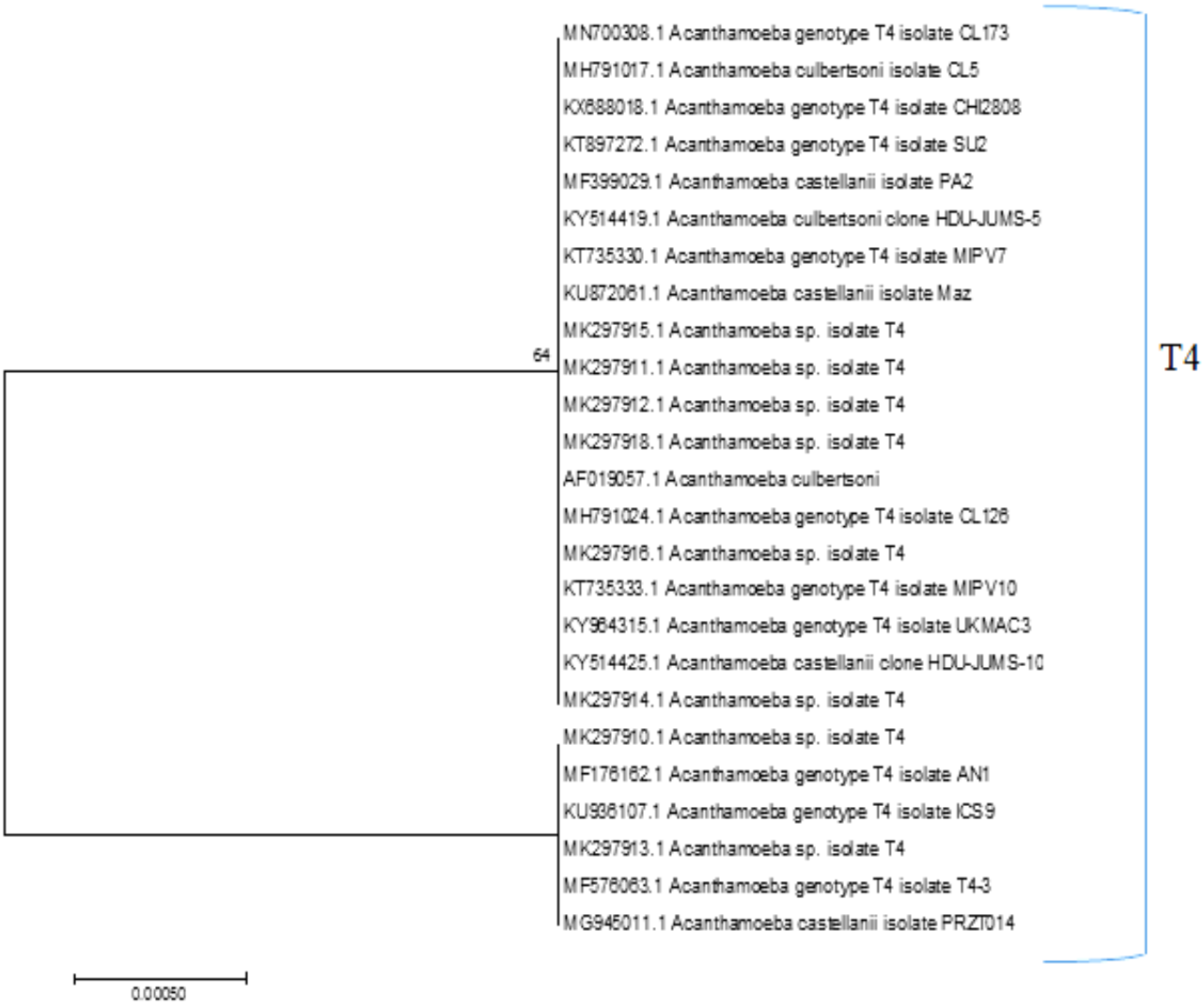


Fig. 5: Phylogenetic tree of *Acanthamoeba* species analyzed by Neighbour- joining method based on 18S rRNA gene sequences using MEGA6.06 software. Number sat the nodes represent Bootstrap values based on 1000 replicates (MK297910.1 to MK297916.1 and MK297918.1 are Genbank accession numbers of 18s rDNA sequences of the *Acanthamoeba* isolates in this study)

Positive Likelihood Ratio for JDP-PCR and culture were calculated unlimited while this ratio for the Nelson-PCR was calculated as 7. Negative Likelihood Ratio for PCR with both Nelson and JDP primers were equal to zero. NLR of direct examination and culture were 0.62 and 0.54, respectively (Table 3).

Table 3: Positive and Negative likelihood ratio of four diagnostic methods compared to defined gold standard

Assay	*PLR(95%CI)*	*NLR(95%CI)*
Direct – smear	∞	062(0.4 - 0.9)
Culture	∞	0.54(0.33-0.89)
JDP_PCR	∞	0
Nelson_PCR	7(1.14-42.97)	0

Positive likelihood ratio: ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease (True positive rate / False positive rate = Sensitivity / (1-Specificity))
Negative likelihood ratio: ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease (False negative rate / True negative rate = (1-Sensitivity) / Specificity)

Discussion

This is the first study on accurate isolation and diagnosis of *Acanthamoeba* keratitis in Mashhad, Iran. The present study compared and evaluated conventional methods with molecular technique for detection of *Acanthamoeba* in specimens obtained from patients suspected to AK, based on JDP-PCR and Nelson-PCR. The results showed that molecular methods were the most sensitive and direct microscopic examination was the least sensitive one. Niyyati et al obtained similar results, whereas Boggild et al stated that culture method is more sensitive than JDP-PCR (12, 15). In spite of the high specificity of direct examination and culture, they have a high false negative rate; therefore, the mentioned conventional methods are less valuable than molecular methods in diagnosis of AK. The low number of positive cases detected in the direct examination can be due to little sampling obtained from cornea, because of possible danger of pathology lesion and new infection. The number of high false negative cases in the culture method is a result of superficial sampling. Because *Acanthamoeba* penetrates in to deeper layers of the eye and superficial sampling decrease the chance of amoeba isolation (14). However, the proper sampling for isolation of *Acanthamoeba* spp. from the cornea is necessary for molecular methods. But here is one point to mention that molecular techniques with high sensitivity can detect small number of amoeba (less than 1 amoeba per micro liter), while the culture method in such situation is not so successful to proliferate of *Acanthamoeba* trophozoites, which is a slow-growing protozoan and requires long incubation period (8).

Regarding ROC chart, this study showed that JDP-PCR has higher accuracy than Nelson- PCR, which contradicted the study, performed by Boggild et al (12). On the other hand, molecular method is fast, reducing the response time to the patient thus rapid patient treatment, decreases complications. The results of present study indicated most of our patients were young women and more than half of the patients who suffered from AK were the contact lens wearers, which corresponds with others studies (21, 30). The difference between the results of this study and other studies could be related due to sample size and sampling time. In general, women are more susceptible to AK due to the greater use of cosmetic or so-called decorative contact lenses in order to have more beautiful and eliminate eyes imperfections. Contact lenses are one of the main risk factors in AK and even fungal keratitis due to use of home remedies or boiling water for lenses maintenance and the ineffectiveness of the disinfection solution of contact lens in removal of *Acanthamoeba* cysts (11). The contact lenses, espe-

cially the soft type, are completely attached to corneal and decrease oxygenation of the cornea cells, which lead to desquamation of superficial cell of cornea, provide easier penetration of *Acanthamoeba* into the corneal stroma. Since the prevalence of a disease in a population can affect some of the characteristics of diagnostic tests, especially sensitivity and specificity; the positive and negative Likelihood Ratio was measured. The Likelihood ratio is one of the most important characteristics of diagnostic tests that help to accept or reject the presence of a disease in a person. This study revealed that all species of *Acanthamoeba* isolated from patients with AK belonged to the genotype T4.

According to previous studies in Iran, T4 genotype has been identified as the predominant type among patients with AK (31). However, Niyyati et al and Haji-Alilu et al reported genotypes of T3, T11 and T9 as causal agents of AK (6, 19). It seems that the high frequency of T4 genotype is due to its high virulence leading to high transmission and low susceptibility to chemical agents (32). T4 genotypes in northeastern Iran had 100% homology with recorded genotypes from north, central, west and south of this country (Table 4). Pathogenicity test showed that only one strain had a high pathogenic ability. It seems that the use of more pathogenic tests such as in vivo tests is more accurate (6).

Table 4: BLAST Results of 18S rRNA of *Acanthamoeba* isolated from nine keratitis patients, Northeast of Iran

Accession numbers in this study	***Accession numbers in Gene bank***	***Country***	***References***
MK297910 (100%)	MG917706.1	Iran	6
MK297911 (100%)	MF139792	Iran	Unpublished
MK297912 (100%)	LC373015	Iran	Unpublished
MK297913 (100%)	MK192801	Iran	Unpublished
MK297913 (100%)	MH539781	Iran	Unpublished
MK297914 (99%)	KY514425	Iran	Unpublished
MK297915 (99%)	MG825466	Brazil	35
MK297916 (99%)	MH100806	Chile	36
MK297917 (95%)	MG969816	Iran	Unpublished
MK297918 (99%)	MH100806	Chile	36

Conclusion

Amoebic keratitis is a sporadic parasitic eye infection, which is mainly seen in contact lens wearers in Mashhad. PCR based on 18S ribosomal DNA with JDP primers is a reliable and highly sensitive method for diagnosis of AK in clinically suspected cases.

Therefore, the molecular PCR test with JDP primers is the most accurate and sensitive method in the diagnosis of AK in clinically suspected cases.

Acknowledgements

This study is a part of student MS thesis No. 1294-A in relation to a Research Project Code No. 940612. We thank them, for their support. We also would like to thank the entire lab. Staffs of Khatam-Al-Anbia Hospital in Mashhad specially Miss. Amininejad for their sincere cooperation.

Conflict of interest

The authors declare there is no conflict of interest.

References

1. Aboul-Magd LA, Abaza BE, Nada WM, et al. Evaluation of polymerase chain reaction (PCR) as a diagnostic technique for acanthamoebic keratitis. Parasitol United J. 2016;9(2):87.
2. Grün A-L, Stemplewitz B, Scheid P. First report of an *Acanthamoeba* genotype T13 isolate as etiological agent of a keratitis in humans. Parasitol Res. 2014;113(6):2395-400.
3. Lorenzo-Morales J, Khan NA, Walochnik J. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite. 2015;22:10.
4. El-Sayed NM, Younis MS, Elhamshary AM, et al. *Acanthamoeba* DNA can be directly amplified from corneal scrapings. Parasitol Res. 2014;113(9):3267-72.
5. Khan NA. *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev. 2006;30(4):564-95.
6. Hajialilo E, Behnia M, Tarighi F, et al. Isolation and genotyping of *Acanthamoeba* strains (T4, T9, and T11) from amoebic keratitis patients in Iran. Parasitol Res. 2016;115(8):3147-51.
7. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. Parasit Vectors. 2012;5:6.
8. Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev. 2003;16(2):273-307.
9. Tay-Kearney ML, McGhee CN, Crawford GJ, et al. *Acanthamoeba* keratitis: a masquerade of presentation in six cases. *Acanthamoeba* keratitis: a masquerade of presentation in six cases. Aust N Z J Ophthalmol. 1993;21(4):237-45.
10. Orosz E, Farkas Á, Kucsera I. Laboratory diagnosis of *Acanthamoeba* keratitis in Hungary. Acta Microbiol Immunol Hung. 2016;63(3):293-9.
11. Hammersmith KM. Diagnosis and management of *Acanthamoeba* keratitis. Curr Opin Ophthalmol. 2006;17(4):327-31.
12. Boggild AK, Martin DS, Lee TY, et al. Laboratory diagnosis of amoebic keratitis: comparison of four diagnostic methods for different types of clinical specimens. J Clin Microbiol. 2009;47(5):1314-8.
13. Bhosale NK, Parija SC, Mandal J, et al. Utility of Polymerase chain reaction in diagnosis of *Acanthamoeba* and Microsporidial keratitis. Int J Curr Microbiol App Sci. 2016;5(2):854-60.
14. Lehmann OJ, Green SM, Morlet N, et al. Polymerase chain reaction analysis of corneal epithelial and tear samples in the diagnosis of *Acanthamoeba* keratitis. Invest Ophthalmol Vis Sci. 1998;39(7):1261-5.
15. Niyyati M, Lorenzo-Morales J, Mohebali M, et al. Comparison of a PCR-based method with culture and direct examination for diagnosis of *Acanthamoeba* keratitis. Iran J Parasitol. 2009;4(2):38-43.
16. Yera H, Zamfir O, Bourcier T, et al. Comparison of PCR, microscopic examination and culture for the early diagnosis and characterization of *Acanthamoeba* isolates from ocular infections. Eur J Clin Microbiol Infect Dis. 2007;26(3):221-4.
17. Pasricha G, Sharma S, Garg P, et al. Use of 18S rRNA gene-based PCR assay for diagnosis of *Acanthamoeba* keratitis in non-contact lens wearers in India. J Clin Microbiol. 2003;41(7):3206-11.
18. Khoushzaban F, Deylamiasl A, Jabarvand M, et al. Diagnosis of *Acanthamoeba* Keratitis by Polymerase Chain Reaction. 2004; J Curr Ophthalmol. 2004:17(1);30- 36.
19. Niyyati M, Lorenzo-Morales J, Rezaie S, et al. Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. Exp Parasitol. 2009;121(3):242-5.
20. Hajialilo E, Niyyati M, Solaymani M, et al. Pathogenic Free-Living Amoebae Isolated From Contact Lenses of Keratitis Patients. Iran J Parasitol. 2015;10(4):541-6.
21. Rezeaian M, Farnia S, Niyyati M, et al. Amoebic keratitis in Iran (1997-2007). Iran J Parasitol. 2012;2(3):1-6.
22. Schuster FL. Cultivation of pathogenic and opportunistic free-living amoebas. Clin Microbiol Rev. 2002;15(3):342-54.
23. Schroeder JM, Booton GC, Hay J, et al. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba*e from humans

with keratitis and from sewage sludge. J Clin Microbiol. 2001;39(5):1903-11.
24. Khairnar K, Tamber GS, Ralevski F, et al. Comparison of molecular diagnostic methods for the detection of *Acanthamoeba* spp. from clinical specimens submitted for keratitis. Diagn Microbiol Infect Dis. 2011;70(4):499-506.
25. Lasjerdi Z, Niyyati M, Haghighi A, et al. Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. Parasitol Res. 2011;109(3):575-80.
26. Khan NA. Pathogenesis of Acanthamoeba infections. Microb Pathog. 2003;34(6):277-85.
27. Khan NA, Jarroll EL, Paget TA. *Acanthamoeba* can be differentiated by the polymerase chain reaction and simple plating assays. Curr Microbiol. 2001;43(3):204-8.
28. Mirjalali H, Niyyati M, Abedkhojasteh H, et al. Pathogenic assays of *Acanthamoeba* belonging to the t4 genotype. Iran J Parasitol. 2013;8(4):530-5.
29. Walochnik J, Obwaller A, Aspöck H. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of Acanthamoeba spp. Appl Environ Microbiol. 2000;66(10):4408-13.
30. Garate M, Cubillos I, Marchant J, et al. Biochemical characterization and functional studies of *Acanthamoeba* mannose-binding protein. Infect Immun. 2005;73(9):5775-81.
31. Niyyati M, Lorenzo-Morales J, Rahimi F, et al. Isolation and genotyping of potentially pathogenic *Acanthamoeba* strains from dust sources in Iran. Trans R Soc Trop Med Hyg. 2009;103(4):425-7.
32. Maghsood AH, Sissons J, Rezaian M, et al. *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. J Med Microbiol. 2005;54(Pt 8):755-9.