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Original Article

Comparison of a PCR-Based Method with Culture and Direct Examination for Diagnosis of *Acanthamoeba keratitis*

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Abstract

Background: The aim was to compare three different methods (direct examination, culture and PCR methods) for the diagnosis of *Acanthamoeba keratitis* (AK) in corneal scrapes.

Methods: Twenty eight corneal scrapes and contact lenses were collected from keratitis patients and referred to the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences. Corneal scrapes were divided in three parts for direct examination, culture on non-nutrient agar and PCR analysis. PCR analysis was also performed using a 18S rRNA gene primer pair (DF3 region). DF3 (Diagnostic fragment 3) is a region of the nuclear small subunit ribosomal RNA gene which is specific for detecting *Acanthamoeba* strains.

Results: *Acanthamoeba* was the causative agent of keratitis in 50% of the patients. Direct smear of all prepared corneal scrapes in AK patients was negative and culture was positive in only 14.3% of the isolates. PCR analysis was positive in 71.4% of AK patients. These three methods were negative in corneal scrapes of non-AK patients. The sensitivity and specificity of PCR technique for the detection of *Acanthamoeba* sp. were calculated as 71.4% and 100%, respectively.

Conclusion: According to high sensitivity and specificity of PCR-based method, this study confirmed that PCR using 18S rRNA gene primers (DF3 region) is more useful for detecting AK cases compare to culture and direct microscopy methods.

Key words: Acanthamoeba, *Keratitis, Diagnosis, Corneal scrapes*

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Introduction

Free- living amoebae of *Acanthamoeba* genus are very common in environmental sources such as water (mineral water, tap water and pool water), soil, sewage, dust and air (1). These amebas are the causative agents of a painful and sight threatening amoebic keratitis (AK) (1-3). According to previous studies, AK continues to rise in Iran and worldwide (3-6). It should be mentioned that genotype T4 is the most isolated genotype in clinical and environmental samples in this region (6, 7). Remarkable increase in the number of AK cases is mainly due to improper use of contact lens and lack of knowledge within contact lens wearers, especially in Iran (5, 6, 8). Prognosis of this corneal infection is very dependent on the time of accurate diagnosis and initiation of proper treatment (9).

Previous researches revealed that early diagnosis and treatment would lead to better visual outcome (9). Laboratory diagnosis of AK is mainly based on culture of corneal scrapes, lenses and lens cases solution on non-nutrient agar and direct smear methods (9). Despite of high specificity of culture based methods; these assays need a long incubation time (14 days in average). Direct smears also can lead to misdiagnosis of *Acanthamoeba* in 60-70% of AK cases (9, 10). DNA-based methods have shown to be more applicable in diagnosing AK cases, since they can lead to more rapid results compared to culture based-methods (10-12). It should be mentioned that PCR-based methods, which amplify different part of 18S rRNA gene, have shown sensitivity up to 88% (10). To date, the promising primers for genotyping purposes is 18S rRNA gene primers (Diagnostic Fragment 3 region, DF3) (11). These primers could amplify as few as 1 *Acanthamoeba* cells in the samples (11).

The main goal of the present study was to compare three different methods for the diagnosis of AK in corneal scrapes of AK patients. We also analyzed the sensitivity and specificity of PCR-based method compared with our defined gold standard.

Material and Methods

Sampling

This research was conducted as a comparative study. Overall, corneal scrapes and contact lenses of 28 keratitis patients referred to the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Iran were collected during 2006-2008. Diagnosis of AK was based on culture of contact lenses, ophthalmological examination and clinical symptoms (gold standard). Ophthalmologic examinations were performed in an Ophthalmology Center of Tehran University of Medical Sciences and different clinics.

Direct smear and culture examinations

Contact lenses were examined by both direct examination and culture on 2% (w/v) non-nutrient agar overlaid with heat-killed *Escherichia coli* according to previous studies (6, 7). Corneal scrapes were divided in three parts for different examination: direct examination, culture and PCR analysis. The third part of corneal samples was kept at 4^0C for further molecular analysis.

DNA extraction

DNA extractions of corneal scrapes in all 28 patients were performed by phenol-chloroform method according to our previous study (7). Five hundred µl DNA lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and 30 µl proteinase K (0.25 mg/ml) were directly added to corneal scrapes and

incubation was done at 60°C, overnight. DNA concentration (ng/µl) was assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain).

DF3 PCR amplification

PCR was performed using 18S rRNA gene Primers (DF3 region) (Forward: 5'-GGCCCA-GATCGTTTACCGTGAA-3' and Reverse: 5'-TCTCACAAGCTGCTAGGGGAGTCA-3') (13). 18s rRNA gene primers (DF3 region) were examined by different researchers and was proved to be genus specific for *Acanthamoeba* sp. (13). PCR reaction was performed in a 30 µl volume containing 1.25 U Taq DNA polymerase, 30 ng DNA, 1.5 mM $MgCl_2$, 200 µM dNTP and 0.2 µM each primer. Cycles of PCR were set up as following: pre denaturation step at 95 °C for 2 min and 30 cycles of denaturation at 95 °C for 30 S, annealing at 50 °C for 30 S and extension at 72 °C for 30 S with an elongation step of 5 min at 72 °C at the last cycle.

Gel electrophoresis

PCR-products (500bp) were electrophoresed on 2% (w/v) agarose gel, stained with ethiduim bromide solution (0.5 µg/ml) and visualized under UV light using a Chemi-doc Image Analyzer (Biorad, Madrid, Spain).

Sensitivity and specificity assessments

Sensitivity, specificity and confidence interval of PCR-based method and culture were calculated in comparison to the gold standard.

Results

According to the defined gold standard in the present study, 14 of 28 patients (50%) were suffered from AK. All patients were female soft contact lens wearers and their ages were between 18-25 years old. The reported clinical symptoms in these AK patients were severe pain and photophobia. Cultures of contact lenses were positive in all of AK patients (14/28), while culture of corneal scrapes were positive in only 2 of 14 AK cases (14.2%). Positive cultures of lenses and corneal scrapes were obtained after two weeks. Additionally, direct examination of all corneal scrapes obtained from AK patients, were negative. It should be noted that all of corneal scrapes which were obtained from non AK patients were negative in direct examination as well as their culture. PCR with 18S rRNA gene primers (DF3 region) were positive in 10 out of 14 AK corneal scrapes (71.4%) (Fig. 1). All of corneal scrapes that obtained from non-AK cases were negative by PCR-based methods. The sensitivity and specificity of PCR-based method was calculated as 71.4% and 100% (95% C.I, 65.5%-74.3%), respectively. In addition, sensitivity and specificity of corneal scrapes culture was calculated as 14.3% and 100% (95% C.I, 12.5%-16%), respectively. Besides, concordance of two methods was calculated as 48.8%, which is rather low.

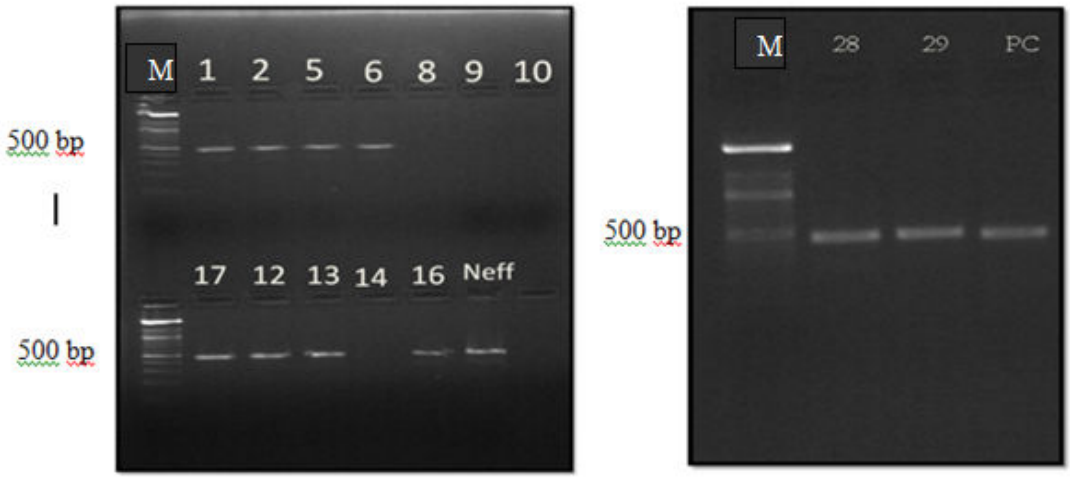

Fig.1: PCR-product of corneal scrapes obtained from Amoebic keratitis patients (M= Molecular weight marker (100bp), Neff= positive control, PC=Positive Control, Numbers= patients code)

Discussion

Laboratory diagnosis of AK is based on different methods such as direct examination (staining methods), culture and DNA-based methods (10, 13). Among these methods, direct examination and DNA-based assays can provide a rapid detection of *Acanthamoeba* (9, 10, 12). However, culture is the mainly used technique for detecting *Acanthamoeba* in contact lenses, corneal scrapes and lens solution (9). This is due to its low cost and simplicity of culture. In the present study, all of lens cultures from AK patients were positive after two weeks. This is in agreement with previous studies, which revealed that culture usually needed a long incubation time (1, 9). Additionally culture of lenses can lead to false positive results as a result of lens case contamination with *Acanthamoeba*. Since rapid diagnosis and initiation of proper treatment is crucial in prognosis of this painful infection, culture can lead to delay in diagnosis of the etiological agent of keratitis (9, 10). On the other hand, there are several reports regarding false positive results of lens cultures due to contamination of contact lenses with non-pathogenic *Acanthamoeba* (14). In 2007, 8% of contact lenses were positive in culture within asymptomatic contact lens wearers (14). This can attributed to eye immunity and also different strains of *Acanthamoeba* (14). Therefore, corneal scrapes are the best sources for identification of the causative agent.

Interestingly, this study revealed that all of corneal scrapes from AK patients were negative by direct examination. Previous studies also demonstrated that *Acanthamoeba* could misdiagnose in 60-70% of direct examinations (14). Therefore, direct examinations cannot be applicable test for confirming AK infection. Detecting of *Acanthamoeba* with culture of corneal scrapes has also shown a low sensitivity (9, 10, 15, 16). Our finding proved that culture of corneal scrapes was not reliable (sensitivity was calculated as 14.2%). This can be due to superficial sampling of cornea as well as invasion of amoeba to deeper corneal layer (9). It should be

emphasized that previous studies showed that PCR-based methods using primers targeting different part of 18S rRNA genes are useful for rapid detection of amoeba in corneal samples (9, 12). Studies by researchers have shown that 18S rRNA gene primers (DF3 region) are promising and these primers introduced as *Acanthamoeba*-genus specific and they are not able to amplify DNA of other free-living amoeba such as *Balamuthia* and *Naegleria* (11).

Our result confirmed that PCR by using 18S rRNA gene primers (DF3 region) could provide a high sensitivity and specificity for detection of *Acanthamoeba* in corneal scrapes in a short period. These primers are also able to detect a very small amount of *Acanthamoeba* DNA (1-2 ρg) (11, 14) and were widely used for genotyping purposes on cultured amoeba which are very superabundant in number (6, 7, 10).

In conclusion, according to our result it has been confirmed that PCR-based method by using 18S rRNA gene primers (DF3 region) is useful for laboratory diagnosis of AK and it should set up in ophthalmological centers as a diagnostic tool.

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