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Original Article

Occurrence of Potentially Pathogenic Bacterial-Endosymbionts in *Acanthamoeba* Spp.

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Abstract

Background: *Acanthamoeba*- bacteria interactions enable pathogenic bacteria to tolerate harsh conditions and lead to transmission to the susceptible host. The present study was aimed to address the presence of bacterial endosymbionts of *Acanthamoeba* isolated from recreational water sources of Tehran, Iran. To the best of our knowledge this is the first study regarding occurrence of bacteria in environmental *Acanthamoeba* spp. in Iran.

Methods: A total of 75 samples of recreational water sources were collected. Samples were cultured on non- nutrient agar 1.5% plates. Positive *Acanthamoeba* spp. were axenically grown. DNA extraction and PCR reaction was performed using JDP1-2 primers. All positive samples of *Acanthamoeba* were examined for the presence of endosymbionts using staining and molecular methods. The PCR products were then sequenced in order to determine the genotypes of *Acanthamoeba* and bacteria genera.

Results: Out of 75 samples, 16 (21.3%) plates were positive for *Acanthamoeba* according to the morphological criteria. Molecular analysis revealed that *Acanthamoeba* belonged to T4 and T5 genotypes. Five isolates (35.7%) were positive for bacterial endosymbionts using staining method and PCR test. Sequencing of PCR products confirmed the presence of *Pseudomonas aeruginosa* and *Agrobacterium tumefasiens*.

Conclusion: The presence of *Acanthamoeba* bearing pathogenic endosymbionts in water sources leads us to public health issues including improved sanitation and decontamination measures in recreational water sources in order to prevent amoebae-related infection. To the best of our knowledge this is the first report regarding the isolation of *A. tumefasiens* from *Acanthamoeba* in Iran and world-wide.

Introduction

Free- Living Amoebae (FLA) include potentially pathogenic protozoan parasites such as *Acanthamoeba*, *Balamuthia* and *Naegleria* (1, 2). These amoebae could lead to severe diseases such as painful keratitis, fatal encephalitis and cutaneous ulcers (3). Recently there are several reports regarding other free-living amoebae as potentially pathogenic parasites including *Paravahlkampfia francinae* and *Vahlkampfia* spp. (4, 5). These mentioned amoebae could be the cause of primary amoebic meningoencephalitis (PAM) mimicking *Naegleria fowleri*-related infection and keratitis, respectively (5). It is worthy to mention that there are also report regarding mixed infection of *Acanthamoeba* and bacteria in keratitis patients (6). These protozoan parasites are distributed in different niches such as lakes, soil, wastewater and clay (1, 2).

It should be mention that beside the direct pathogenic effect of free-living amoebae, they could be a carrier of pathogenic microorganisms such as bacteria and viruses. There are also various opportunistic pathogens such as Non-tuberculous Mycobacteria (NTM), *Pseudomonas* and *Legionella* that could exist in the same ecological niches as *Acanthamoeba* (7-9). Indeed, a wide range of bacteria could resist the intracellular killing of amoebae and they could survive and even exploit *Acanthamoeba* for their multification such as *Pseudomonas putida, Pasteurella pneumotropica, Aeromonas salmonicida, Legionella pneumophila serogroup* 1, *L. pneumophila serogroup* 3, *L. pneumophila serogroup* 6 (7, 10). Bacteria could remain in amoebae cyst form, this could be a problematic health aspect, since *Acanthamoeba* cysts are very resistance to harsh environment and could tolerate many adverse conditions such as high osmolarity, various ranges of temperature and pH (7, 11). Thus, uptake of bacteria by amoebae could lead to endosymbiosis relationship. Interestingly, pathogenic bacteria are shown to have the ability to survive in *Acanthamoeba* cytoplasm despite non-pathogenic bacteria, which they were used only as food sources. It is interesting to note that some endosymbiont environmental *Chlamydiae* alter the growth speed and/or motility of *Acanthamoeba*, this could be due to mutual relationship between amoebae and environmental *Chlamydiae* (12, 13).

Previous studies in Iran were mainly focused on isolation of *Acanthamoeba* spp. from water sources, however there were no previous researches regarding survey of *Acanthamoeba* in chlorinated water an also Amoebae-endosymbionts in this region and thus the main aim of the present research was to address the occurrence of bacterial endosymbionts of *Acanthamoeba* isolated from man-made recreational water sources using staining and molecular based methods.

Material and Methods

Study area and sample collection

A total of 75 samples of recreational water sources including 40 samples of ponds and 35 samples from indoor swimming pools were collected from Tehran, Iran. All samples were transferred to Dep. Parasitology and mycology, School of medicine, Shahid Beheshti University of Medical Sciences within few days.

Sample processing (filtration, cultivation and cloning)

Four hundred milliliters of water sample filtered using nitrate membranes. Each sample was cultured into non nutrient agar plates. All plates were then sealed and incubated at room temperature for up to 1 month. Positive plated were then submitted to cloning according to our previous study (14). Briefly, a single cyst was transferred to a fresh medium and adopted to axenic situation within weeks. This allows no contaminants in the medium. Cleaned plates were then examined for intra-

cellular bacteria using inverted microscopy and gram staining as following.

Microscopic bacterial - endosymbionts detection

Cloned plates washed with sterile normal saline and slides were then prepared from *Acanthamoeba*-positive plates. Gram staining was applied according to previous researches (15).

DNA extraction, PCR amplification and nucleotide sequencing of Acanthamoeba spp.

DNA extraction was performed using modified phenol-chloroform method according to our previous study (14). For genotyping of *Acanthamoeba* spp. PCR analysis was done using genus-specific primer pairs called JDP1-2 primers. These primers could detect a diagnostic fragment of all 18 genotypes of *Acanthamoeba* spp. (16). The PCR reaction was prepared in 30 µl Ampliqone (Taq DNA Polymerase Master Mix Red, Denmark). Briefly, 25 µl of master mix with 10 ng DNA templates and 20 pmol primers were mixed to achieve a volume of 30 µl. PCR product were then submitted to sequencing.

Molecular identification of bacterial-endosymbionts

All *Acanthamoeba*-positive samples were submitted to PCR using bacteria-universal primers. These primes could amplify a fragment of 16S rRNA gene of various bacteria including *Pseudomonas*, *Agrobacterioum* and several other genera. The nucleotide sequences of the primers were as following: Forward: 5-TCG ACA ACA GAG TTT GAT CCT GGC TCA G -3 and Reverse: 5-ATC CAA GCT TAA GGA GGT GAT CCA GCC-3. These primers correspond to 1100 bp DNA sequence of 16S rRNA gene of several bacteria (17).

The PCR amplification was carried out in a total volume of 30 µl. Thermal profile involved 40 s at 94°C, 90 s at 59.6°C and 120 s at 72°C for 35 cycles.

Sequences analysis for Acanthamoeba and bacterial endosymbionts

Sequences derived from the amoebae and their endosymbionts were tested against all available nucleotide sequences in the GenBank database. The DNA sequences have been deposited in the Genetic sequence database at the National Center for Biotechnical Information (NCBI) using the Sequin program (version 10.3). (GenBank ID for *Acanthamoeba*: KJ504214-KJ504227 and GenBank ID for bactertia: KJ563278- KJ563281). Multialign were performed for *Acanthamoeba* genotypes.

Results

Out of 75 samples, 16 (21.3%) plates were positive for *Acanthamoeba* according to the morphological criteria. *Acanthamoeba* trophozoites were flat in shape and cysts were double walled with star shape endocysts. Ten samples (25%) of pond and 4 samples (11.4%) of swimming pool waters were found to be positive for *Acanthamoeba* genus in non-nutrient agar medium.

Of 16 *Acanthamoeba* isolate, 14 were cloned successfully. Sequences analysis revealed that *Acanthamoeba* belonged to T4 and T5 genotypes (Fig. 1) (Table 1). T5 genotype has been isolated from swimming pools. Five isolates (35.7%) of *Acanthamoeba* were positive for bacterial endosymbionts in their cytoplasm using microscopic observation and gram staining method. Gram staining revealed the presence of bacteria readily in the host amoebae cytoplasm (Fig. 2). Many rod shaped bacteria were localized in the cytoplasm. It should be mention that 4 isolated bacteria showed an approximately 1100 bp band (Fig. 3). However, one strain were failed in sequencing even with repeated PCR reaction. Sequencing of PCR products verified the presence of three *Pseudomonas aeruginosa* and one *Agrobacterium tumefasiens* in *Acanthamoeba* T4 genotypes (Table 1). The result of multialign is shown in Fig. 4 which it shows >5% dissimilarity in 18S rRNA gene. Isolation of *Agrobacterium tumefasiens* from *Acanthamoeba* is for the first time worldwide.

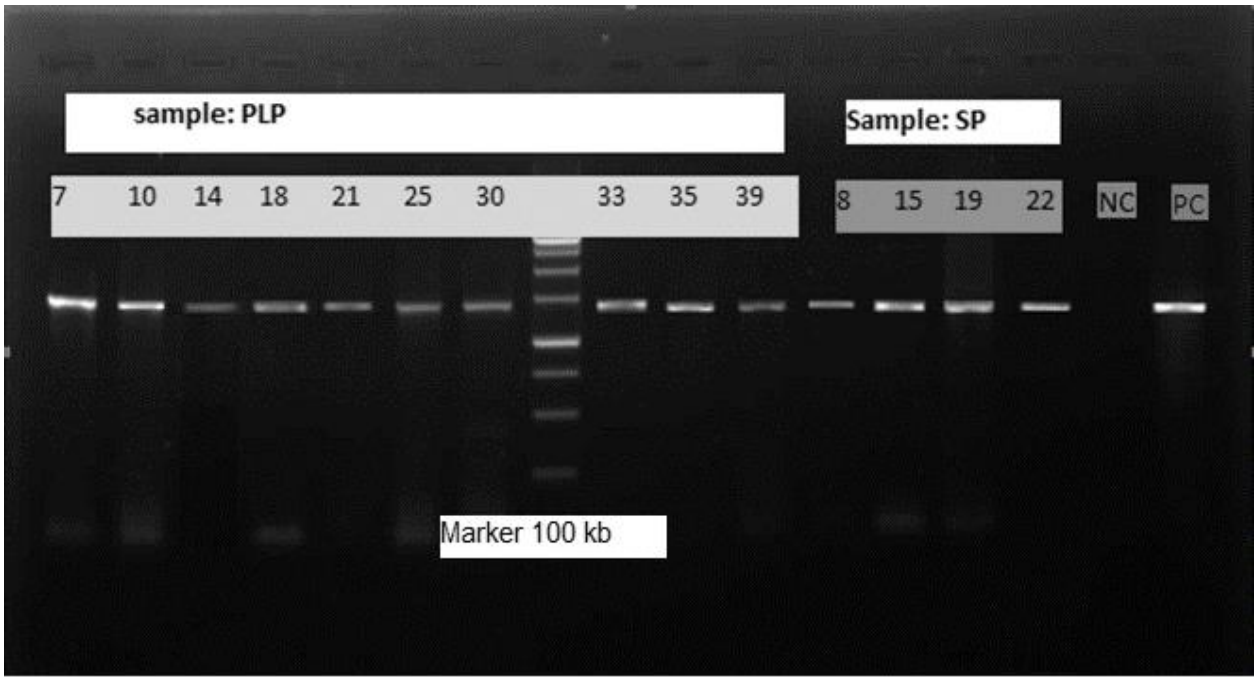

Fig. 1: 500 bp PCR product electrophoresis of positive *Acanthamoeba*
(Marker: 100 bp, NC: Negative control, PC: Positive control, SP: Swimming pool, PLP: pond water)

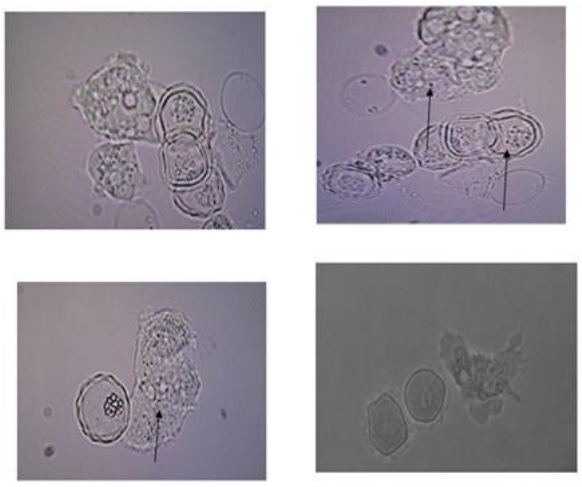

Fig. 2: Cloned *Acanthamoeba* spp. cysts and trophozoites containing rod shaped-bacteria (magnification x400)

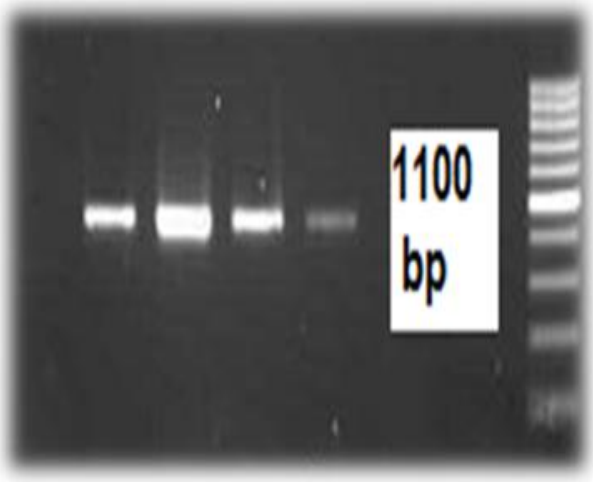

Fig. 3: 1100 bp PCR product electrophoresis of positive endosymbionts (four isolates) (Marker: 1kb)

Table 1: *Acanthamoeba* genotypes isolated from recreational waters and their related endosymbionts

Isolates code	Genotypes	Accession Number	Endosymbiont code	Isolated bacteria	Accession Number
MN(AC)7-PW-IR	T4	KJ504214	MN-Endo5	*P. aeroginosa*	KJ563278
MN(AC)10-PW-IR	T4	KJ504215	--------		--------
MN(AC)14-PW-IR	T4	KJ504216	--------		--------
MN(AC)18-PW-IR	T4	KJ504217	MN-Endo32	*P. aeroginosa*	KJ563279
MN(AC)21-PW-IR	T4	KJ504218	--------		--------
MN(AC)25-PW-IR	T4	KJ504219	--------		--------
MN(AC)30-PW-IR	T4	KJ504220	--------		--------
MN(AC)33-PW-IR	T4	KJ504221	MN-Endo1	*P. aeroginosa*	KJ563280
MN(AC)35-PW-IR	T4	KJ504222	--------		--------
MN(AC)39-PW-IR	T4	KJ504223	--------		--------
MN(AC)8-SPW-IR	T5	KJ504224	MN-Endo11	*A.tumefacience*	KJ563281
MN(AC)15-SPW-IR	T4	KJ504225	-	No sequenc-ing	--------
MN(AC)22-SPW-IR	T5	KJ504226	--------		--------
MN(AC)19-SPW-IR	T4	KJ504227	--------		--------

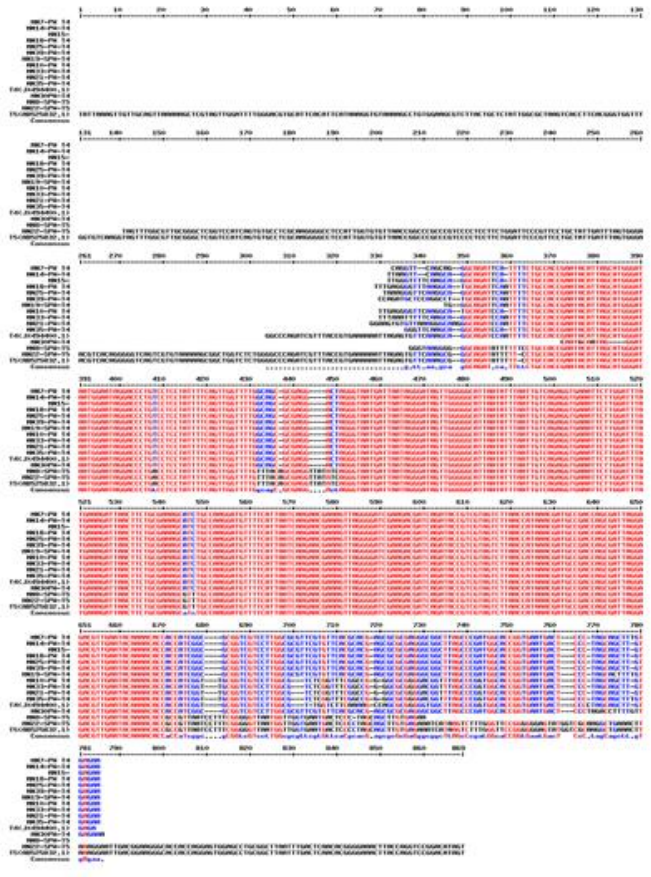

Fig. 4: Multialign of isolated *Acanthamoeba* belonginig to T4 and T5 genotypes (multalign software, France)

Discussion

The present study revealed that *Acanthamoeba* could harbor potentially pathogenic bacteria in recreational water sources such as pond and chlorinated water including swimming pools.

This is the first study regarding detection of *Acanthamoeba* in swimming pools in Iran and the result showed that contamination of pools are less than ponds. The present study showed that 35.7% of amoebae contain intracellular bacteria. Other researches showed that 24% of environmental amoebae could harbor bacteria (18). Additionally, out 24% of environmental *Acanthamoeba* and 26% of clinical *Acanthamoeba* contain intracellular bacteria (19). In accordance, several bacteria have been reported that could live in the free-living amoebae as endosymbionts including *Acanthamoeba* and *Naegleria* (20-22), however this is the first report of *Agrobacterium tumefasience* in *Acanthamoeba* spp. worldwide. *Agrobacterium* is a potentially pathogenic Gram-negative bacterium responsible for systematic human infection especially in imunocompromised individuals (23, 24). *Agrobacterium* spp. are ubiquitous in natural and man-made water sources and basically they are harmful for plants (24).

The present study confirmed that *P. aeroginosa* presents in cytoplasm of the host amoebae. This is important since both of organisms are corneal pathogen and they may increase the chance of AK in appropriate situation (25). To this end there are several reports regarding severe keratitis due to mixed *Acanthamoeba* and *Pseudomonas* infection in cosmetic contact lens wearers with poor prognosis (26, 27). Complicated cases of AK also may be due to coexistence of corneal bacterial pathogens such as *Pseudomonas* with *Acanthamoeba* and lack of response to proper treatment may reflect the presence of amoebae-endosymbionts. In addition, according to previous studies this interaction may lead to increased virulence of bacteria and also may affect the pathogenicity of amoebae (25, 26, 28). This issue needs to clarify by more researches. To this end, the focus of recent researches has shifted from direct pathogenic effects of *Acanthamoeba* toward their role as carriers of pathogenic bacteria. Overall, amoebae could be an ideal replicative niche for bacterial communications and could act as reservoir. The long interaction between amoebae and bacteria could lead to adaptation behavior towards an intracellular lifestyle (29). A previous research also revealed that although addition of disinfectants may influence amoebal density, but it seems that FLA can re-colonize in treated waters within a short period of time (30).

Conclusion

The present study reports for the first time the occurrence of novel endosymbiotic bacteria (such as *A. tumefasciences*) in environmental *Acanthamoeba* strains. Further researches regarding the relationship between bacteria and their host amoeba and their effect on pathogenicity of either amoebae or bacteria is of utmost importance.

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