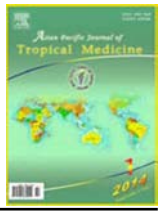
Asian Pac J Trop Med 2014; 7(Suppl 1): S74-S78

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm



Document heading doi: 10.1016/S1995-7645(14)60206-6

Genotype analysis of *Acanthamoeba* isolated from human nasal swabs in the Philippines

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ARTICLE INFO

Article history:
Received 20 Jan 2014
Received in revised form 8 Mar 2014
Accepted 26 Apr 2014
Available online 18 Sep 2014

Keywords:
Acanthamoeba
Genotype
Nasal epithelium
The Philippines

ABSTRACT

Objective: To analyze the genotypes of *Acanthamoeba* species isolated from human nasal swabs in the Philippines.

Methods: Human nasal swabs were collected from two groups: a low exposure group composed of students of the University of the Philippines-Diliman and a high exposure group composed of laborers frequently exposed to garbage, soil and dust. After isolation using non-nutrient agar plate lawned with *Escherichia coli* and DNA extraction using Chelex-100 resin, the ASA.S1 region of the gene (*Rns*) coding for nuclear, small subunit ribosomal RNA of *Acanthamoeba* was amplified through polymerase chain reaction. Purified polymerase chain reaction products were then sequenced. Neighbor-joining, maximum parsimony, and maximum likelihood phylogenetic trees were then constructed.

Results: In the low exposure group, 1 out of 70 (1.43%) students and 7 out of 110 (6.36%) in the high exposure group were culture-positive. Four soil samples were also obtained for comparison, all of which were tested culture-positive. Of the 12 *Acanthamoeba* isolates, only 9 were successfully sequenced. The basic local alignment search tool of the US National Center for Biotechnology Information was used to identify most similar sequences. Five isolates were identified as genotype T5, and 3 isolateds were genotype T4. Genotype T11 was also isolated from soil, the first to be reported in the Philippines.

Conclusions: Genotype T11 is a possible pathogenic strain and both T4 and T5 can be pathogenic to human, hence, healthy provisions, especially for high exposure groups, should be given more attention and reevaluated.

1. Introduction

Acanthamoeba sp. is an opportunistic free-living pathogen that is widely distributed in the environment including soil, water systems and air[1,2]. Currently, there are 18 known genotypes of *Acanthamoeba* based on the *Rns* gene, T1 to T18[3]. Some genotypes are pathogenic, causing human diseases like the debilitating *Acanthamoeba* keratitis that can cause blindness, and the fatal *Acanthamoeba* granulomatous encephalitis (AGE)[2,4,5].

Acanthamoeba spp. have been isolated from air samples and from nasal epithelial surfaces of healthy human, usually during the windy periods[4]. *Acanthamoeba* cysts are easily dispersed by air and can reach the nasal epithelial surfaces[6,7]. From the nasal cavity, it can travel to the central nervous system through the respiratory tract and the blood stream[1,2,5].

This present study aims to discriminate the *Rns* genotypes of *Acanthamoeba* spp. obtained from the nasal epithelium of human.

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Foundation Project: Supported by the Natural Sciences Research Institute, University of the Philippines Diliman (Grant No. BIO-12-2-02).

2. Materials and methods

2.1. Sample collection and culture

Nasal swabs from 110 volunteers were obtained from a high exposure group that frequently exposed to dust, soil, and garbage from Marikina City and Quezon City in the Philippines. A total of 70 samples were also obtained from a low exposure group composed of students of the University of the Philippines, Diliman campus. A total of 4 soil samples were also obtained (Table 1). The nasal swabs were streaked on a non-nutrient agar plate lawned with *Escherichia coli*[8].

Table 1
Source of *Acanthamoeba* isolates used in this study.

Group	Occupation	Number of samples	Number of cultures positive for *Acanthamoeba*
High-exposure	Street sweeper	44	4
	Garbage collector	37	2
	Garbage sorter	16	0
	Landscaper	6	1
	Bioreactor laborer	4	0
	Foremen and supervisors	3	0
Low-exposure	Students	70	1
Soil		4	4
Total		184	12

Table 2
Rns sequence similarities of *Acanthamoeba* isolates used in this study and reference isolates using JDP1-JDP2 primers.

Sample	GenBank accession	Genotype	Isolation source	Similar GenBank accessed reference sequences	Percent similarity (%)	Percent coverage (%)
AH48	KJ652982	T5	Human nasal swab, garbage collector	*A. lenticulata* clone CF1-249 KC164253	93	100
AH49	KJ652983	T5	Human nasal swab, street sweeper	*A. lenticulata* clone CF1-249 KC164253	93	100
AH52	KJ652986	T5	Human nasal swab, street sweeper	*A. lenticulata* clone CF1-249 KC164253	93	100
AH64	KJ652984	T5	Human nasal swab, street sweeper	*A. lenticulata* clone CF1-249 KC164253	94	100
AH73	KJ652985	T4	Human nasal swab, street sweeper	*Acanthamoeba* sp. Ac_E4c GU808286	95	100
AH77	KJ652987	T4	Human nasal swab, garbage collector	*Acanthamoeba* sp. Shi strain AF239305	96	100
AK	KJ652988	T11	Organic soil fertilizer	*A. hatchetti* BH-2 AF019068	95	100
AS1	KJ652989	T4	Soil	*Acanthamoeba* sp. Ac_E4c GU808286	95	99
AS2	KJ652990	T5	Soil	*A. lenticulata* clone CF1-249 KC164253	99	100

Table 3
Reference *Acanthamoeba* isolates included in the analyses.

Genotype	Name of isolate/strain	GenBank accession	Source/location
T1	*Acanthamoeba* sp. CDC V548	DQ339096	Granulomatous amoebic encephalitis, human brain, USA
T1	*A. castellani* CDC:0981:V006	U07400	Granulomatous amoebic encephalitis, human brain, Georgia, USA
T2	*A. palestinensis* Reich ATCC 30870	U07411	Soil, Israel
T3	*A. polyphaga* Panola Mountain strain ATCC 30487	AF019052	Soil, Georgia, USA
T3	*Acanthamoeba pearcei* strain ATCC 50435	AF019053	Sewage dump, Atlantic Ocean, USA
T3	*Acanthamoeba griffini* S-7 ATCC 30731	U07412	Beach bottom, Connecticut, USA
T4	*A. polyphaga* Page-23 CCAP1501 strain ATCC 30871	AF019061	Freshwater pond, WI, USA
T4	*Acanthamoeba* sp. OSU 04-023 clone 2	DQ451162	Infected toucan liver tissue
T4	*Acanthamoeba* sp. OSU 04-023 clone 3	DQ451163	Infected toucan liver tissue
T4	*A. castellani* CDC:0981:V006	U07401	Keratitis, India
T4	*Acanthamoeba* sp. ATCC 50497 strain BCM:0288:37	U07410	Keratitis, Texas, USA
T5	*A. lenticulata* strain 45 ATCC 50703	U94730	Nasal mucosa, Germany
T5	*A. lenticulata* JC-1 ATCC 50428	U94739	Freshwater stream, NY, USA
T5	*A. lenticulata* PD2S ATCC 30871	U94741	Swimming pool, France
T6	*A. palestinensis* strain 2802	AF019063	Swimming pool, France
T7	*Acanthamoeba astronyxis* Ray & Hayes strain ATCC 30137	AF019064	Lab water, Washington, USA
T8	*Acanthamoeba tubiashi* OC-15C strain ATCC 30867	AF019065	Freshwater, Maryland, USA
T9	*Acanthamoeba comadoni* strain Comadon & de Fonbrune	AF019066	Soil, France
T10	*Acanthamoeba culberstoni* Lilly A-1 strain ATCC 30171	AF019067	Human cell culture, Indiana, USA
T11	*A. hatchetti* BH-2	AF019068	Brackish water, Maryland, USA
T11	*Acanthamoeba* sp. PN15	AF333607	Clinical sample, Pakistan
T12	*Acnathamoeba healyi* strain CDC 1283:V013	AF019070	Granulomatous amoebic encephalitis, Barbados
T13	*Acanthameoba* sp. UWC9	AF132134	Contact lens case
T13	*Acanthamoeba* sp. UWET39	AF132136	Soil, USA
T14	*Acanthamoeba* sp. PN15	AF333607	Clinical sample. Pakistan
T15	*A. jacobsi* AC080	AY262361	Water supply, Pakistan
T15	*A. jacobsi* AC194	AY262362	Freshwater sediment, UK
T15	*A. jacobsi* AC304	AY262364	Untreated water, Australia
T16	*Acanthamoeba* sp. cvX	GQ380408	Environmental sample
T17	*Acanthamoeba* sp. TSP07	JF325889	Soil sample, Brazil
T18	*Acanthamoeba* sp. CDC:V621 clone 10	KC822470	Granulomatous amoebic encephalitis, human, USA
-	*B. mandrillaris* strain CDC:V039	AF019071	No source given

Acanthamoeba palestinensis: *A. palestinensis*; *Acanthamoeba polyphaga*: *A. polyphaga*; *Acanthamoeba jacobsi*: *A. jacobsi*;

2.2. DNA extraction, PCR and DNA sequencing

Genomic DNA was extracted using Chelex®-100 resin (Sigma, Missouri, USA) extraction procedure[9] and then used as a template in PCR as described previously[10]. Primers JDP1 and JDP2 were used to amplify the ASA.S1 region of the *Rns* gene coding for *Acanthamoeba*'s nuclear, small subunit ribosomal RNA[11]. PCR product was purified using QIAquick® gel extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. The purified PCR products were then sent to Macrogen (Seoul, South Korea) for direct sequencing.

2.3. Genotype identification, alignment and phylogenetic analysis

The basic local alignment search tool of the US National Center for Biotechnology Information was used to identify most similar sequences. The sequences obtained were aligned using ClustalW of Bioedit, with gaps and ambiguous sequences identified by visual inspection. The trees, rooted on a suitable outgroup [*Balamuthia mandrillaris* (*B. mandrillaris*)], were constructed using neighbor-joining (NJ) and maximum likelihood (ML), which were based on the best model as well as the non-model-based maximum parsimony (MP). Bootstrap re-sampling was also carried out with 1 000 replicates for NJ and MP and 100 replicates for ML. PAUP version 4.0b10 was used for MP and NJ[12], while sequences were uploaded into the website PhyML (http://www.atgc-montpellier.fr/phyml/) for the ML analysis. NJ, MP and ML consensus trees were generated from all clusters that had a bootstrap support of >50% from at least two of the models.

2.4. GenBank references

Sequences determined in this study were deposited in

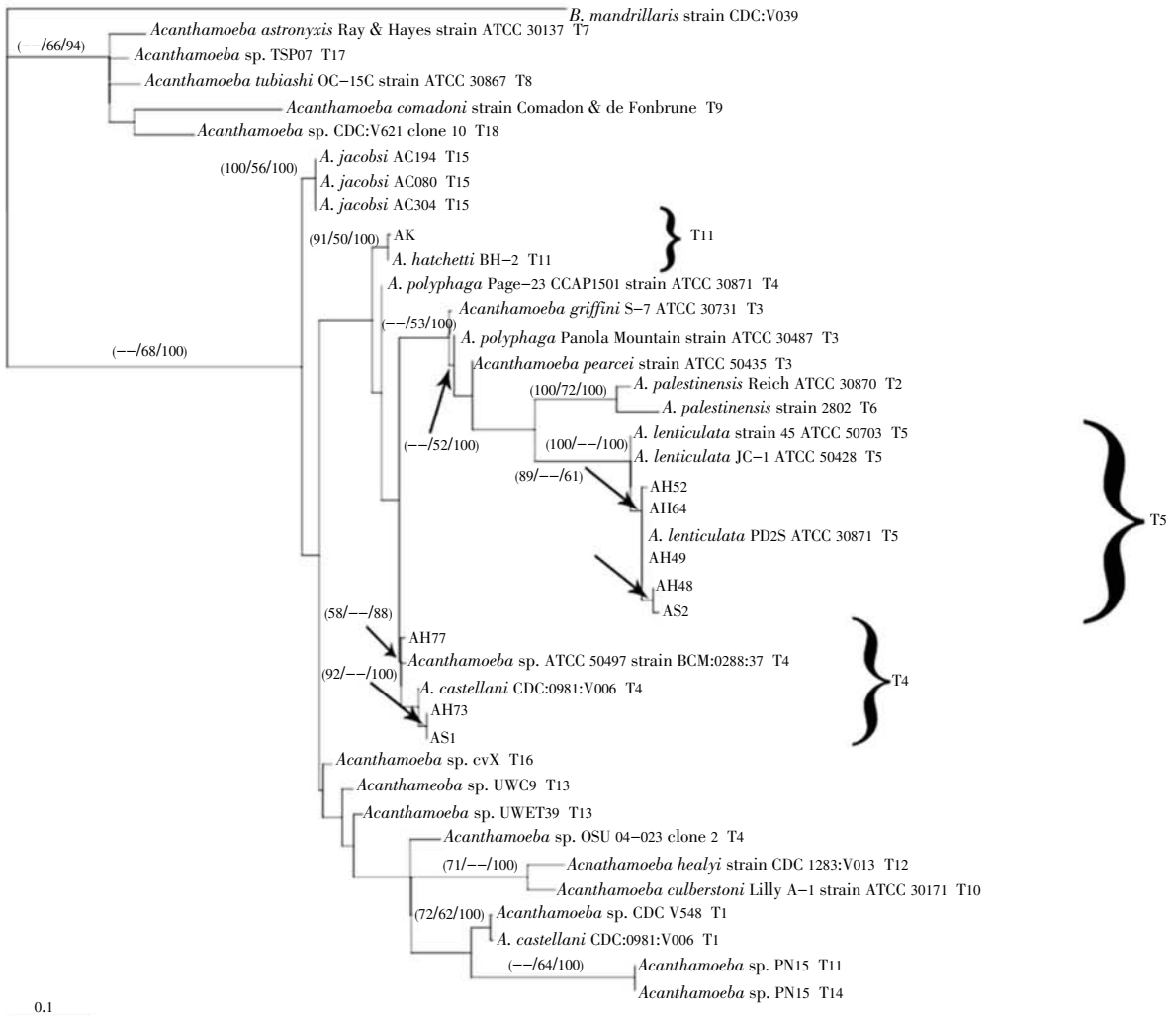

Figure 1. Phylogenetic tree of the nasal swab and soil isolates from this study and reference *Acanthamoeba* isolates, based on 296 aligned nucleotide base pairs of the ASA.S1 region of the *Rns* gene.

The tree, constructed using the maximum likelihood method using the GTR+G+I model, was rooted on *B. mandrillaris*. The three values at each node represent the bootstrap support from ML, NJ, and maximum parsimony analyses, respectively. Bootstrap support <50% are not shown. The scale bar on the lower left side represents one change per ten nucleotide positions. The brackets show the clustering of the study isolates to reference genotypes.

GenBank as accessions KJ652982-KJ652990 (Table 2). The reference sequences used are listed in Table 3.

3. Results

A total of 12 isolates were cultivated, 8 (4.44%) from human samples and 4 from soil samples (100%). The high-exposure group had 7 positive samples (6.36%) while the low-exposure group had 1 (1.43%) (Table 1). DNA was successfully extracted, amplified, and sequenced in 9 of the 12 samples.

Three *Rns* genotypes were identified from the 9 nasal swabs and soil samples. Five of the 9 samples were genotype T5, where 4 were from human nasal swabs and 1 from a soil sample. Three were genotype T4, where 2 were from human nasal swabs and 1 from a soil sample. The last sample from soil was identified as genotype T11. Table 2 summarizes the *Rns* sequence similarities of *Acanthamoeba* isolates used in this study and reference isolates. Moreover, the isolates also clustered with the reference strains (Table 3). The isolates are most similar to the reference strains in at least 2 of the 3 phylogenetic tree analyses used in this study with bootstrap support of 50 and above for NJ, ML, or MP. Figure 1 shows a consensus tree based on the ML tree summarizing the results of ML, NJ, and MP.

4. Discussion

Acanthamoeba colonization of the nasal cavity is mostly associated with its ability to disperse through the air in the form of resistant cysts[6,7]. As dust and soil particles are carried by the wind, *Acanthamoeba* cysts are transmitted to the nasal epithelium, transformed to trophozoites and are able to colonize it due to several factors like their presence of acanthopodia, binding proteins, and neuraminidase[2,5,13]. Infection by *Acanthamoeba* is also affected by its host characteristics. Moreover, infection of humans may also depend on its susceptibility and hygiene[5].

Results of this study show that street sweepers and garbage collectors are susceptible to *Acanthamoeba* infection. This is due to the nature of their work. They are constantly exposed to dust and soil and garbage, respectively, for several hours a day that may be contaminated with *Acanthamoeba*. Lower rate of infection was observed in students that can be attributed to less contact with contaminated soil or water. The percentage of isolation in this study is small (4.44%), compared to other studies (28.4%) that are similar in nature[14]. However, the risk for acquiring AGE is still high due to the ability of the cysts to disperse through wind.

In this study, genotype T5 is more commonly isolated than genotype T4, contrary to previous studies that had T4 as the most prevalent genotype in nasal cavity colonization[5,14]. T4 has been identified as the major genotype associated with keratitis and non-keratitis infections such as AGE mostly due to its greater virulence and/or properties that enhance its transmissibility[15]. T5 is the more commonly isolated genotype in nasal mucosa infections, possibly making it a more common contaminant of soil, dust and solid waste in the Philippines. In contrast, most studies isolated T4 and T5 or isolated more T4 followed by the T5[14,16].

The T5 isolates were closely related to reference strains, *Acanthamoeba lenticulata* PD2S ATCC 30871 (GenBank Accession U94741) (*A. lenticulata*) and *A. lenticulata* JC-1 ATCC 50428 (GenBank Accession U94739) isolated from freshwater stream and swimming pool, respectively. It can also be noted that in the NJ tree, the T5 isolates clustered with *A. lenticulata* strain 45 ATCC 50703 (GenBank Accession U94730) which was isolated from nasal mucosa. In contrast to a previous study in the Philippines[17], all T4 isolates in this study were more similar to the reference *Acanthamoeba castellani* CDC:0981:V006 (GenBank Accession U07401) (*A. castellani*) isolated from a keratitis case rather than *Acanthamoeba* sp. OSU 4-023 clones 2 and 3 (GenBank Accession DQ41162 and DQ41163, respectively) from infected toucan liver tissue.

Like T4, genotype T5 was also identified to have high levels of pathogenicity and resistance in addition to being prevalent in the environment[16,18]. Infection by *Acanthamoeba* sp., especially pathogenic genotypes such as T4 and T5 may have low rates but as the number of susceptible host increases, the risk of infection also increases.

Soil genotypes from AS1 and AS2 were found to be T4 and T5, respectively. The same genotypes were common in the environment from a previous study in the Philippines[19], making these two become the most probable contaminants in the environment in the Philippines. These soil genotypes were similar to the genotypes of the isolates from the nasal swabs of the laborers. The phylogenetic trees corroborate the similarities of these isolates. However, genotype T11 was isolated in another soil sample. The T11 sample was isolated from an organic fertilizer made out of rice husk and was most similar to the T11 reference *Acanthamoeba hatchetti* BH-2 (GenBank Accession AF019068) (*A. hatchetti*) obtained from brackish water. To the best of our knowledge, this is the first report of isolation of *Acanthamoeba* genotype T11 from the environment in the Philippines. The organic

fertilizer is characterized by being very loose and easily carried by the wind. This characteristic of the soil would increase the transmissibility of *Acanthamoeba* to infect nasal cavities[6,7], however, no T11 genotype was observed in any human isolate. T11 is mainly associated with keratitis cases[18,20]. Nevertheless, a new area of study would be the selective infection and susceptibility of the human corneal membrane to *Acanthamoeba* genotype T11.

Genotype T11 is a possible pathogenic strain and both T4 and T5 can be pathogenic to humans, hence, healthy provisions, especially for high exposure groups, should be given more attention and reevaluated, especially to those more exposed to this organism.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thank Davin Edric V. Adao for the technical assistance. This work was supported by funds from the Natural Sciences Research Institute, University of the Philippines Diliman (Grant No. BIO-12-2-02).

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