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ORIGINAL PAPER

18S ribosomal DNA genotypes of *Acanthamoeba* species isolated from contact lens cases in the Philippines

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Abstract This study was carried out to document the genotypes of *Acanthamoeba* present in contact lens cases from 50 randomly selected contact lens wearers living in Quezon City, Metro Manila, Philippines. *Acanthamoeba* species were isolated from eight (16%) in 50 contact lens cases examined. We analyzed partial 18S ribosomal DNA (*Rns*) sequences of the eight isolates and found that the sequence differences were sufficient to distinguish the genotypes. After the isolates were genotyped, using the Basic Local Alignment Search Tool program, their phylogenetic positions relative to known *Acanthamoeba* isolates were determined. The model-based (GTR+Γ+I) neighbor-joining, maximum likelihood, and Bayesian inference analyses, as well as the non-model-based maximum parsimony analysis were used. Results showed that of the eight isolates, six were *Rns* genotype T5 while two were *Rns* genotype T4. This present study indicates that genotype T5 is also a common contaminant in contact lens storage cases.

Introduction

Acanthamoeba is one of the most prevalent and ubiquitous free-living amoebae found in the environment. Many strains frequently cause *Acanthamoeba* keratitis (AK), a potentially blinding infection of the cornea in non-immunocompromised individuals (Marciano-Cabral and Cabral 2003). *Acanthamoeba* has been successfully cultured from environmental samples, as well as from contact lenses, lens cases, and lens-cleansing solutions (Yu et al. 2001; Booton et al. 2002; Jeong and Yu 2005; Tzanetou et al. 2006). Although a positive culture of the lens or related paraphernalia does not confirm the diagnosis of AK, it nonetheless indicates infection with *Acanthamoeba*. Cases of AK dramatically increased from the early 1970s to the mid-1980s mostly associated with increasing use of soft contact lenses (Hammersmith 2006). Contact lens wearers comprise most of the AK cases with estimates of 80–86% (Hammersmith 2006) or even up to 93% (Niyadurupola and Illingworth 2006; Tzanetou et al. 2006).

Our present study identified the genotypes of *Acanthamoeba* species isolated from contact lens cases in the Philippines through analysis of the diagnostic region (diagnostic fragment 3) of the nuclear small subunit 18S ribosomal RNA gene (*Rns*). Previous use of *Rns* genotyping indicates that nearly all potentially pathogenic acanthamoebae are of genotype T4 (Hewett et al. 2003). The use of the diagnostic fragment 3 (DF3) of *Rns* permits the genotypic identification of the acanthamoebae in cultures that contained other microorganisms (Booton et al. 2002). The demonstrated clinical relevance of the genus *Acanthamoeba* and the relationship between the genotype and eye infection suggest that more studies on the identification of the *Rns* genotype of *Acanthamoeba* strains obtained from contact lens paraphernalia are needed (Zhang et al. 2004). Our study aimed to discriminate the *Rns* genotypes of contact lens cases from asymptomatic individuals and to analyze the correlation between our isolates with published strains from other areas.

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Table 1 *Rns* sequence similarities of *Acanthamoeba* isolates used in this study and the reference isolates using 892C sequencing primer

Sample	Similar GenBank accessed reference sequence/s	GenBank accession	Genotype	Percent similarity	Coverage (%)
A2_2	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 from a swimming pool in France	FJ815175	T5	100 (160/160)	100
A5_2	*Acanthamoeba castellani* CDC:0981:V006 from a keratitis patient in India	FJ815176	T4	98 (184/187)	100
A6_2	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815177	T5	100 (158/158)	100
A7_2	*Acanthamoeba lenticulata* strain JC-1 ATCC 50428 from a freshwater stream in New York, USA	FJ815178	T5	100 (158/158)	100
	Acanthamoeba lenticulata strain PD2S ATCC 30871 (see above)		T5	98 (155/158)	100
A9_2	*Acanthamoeba lenticulata* strain JC-1 ATCC 50428 (see above)	FJ815179	T5	100 (159/159)	100
	Acanthamoeba lenticulata strain PD2S ATCC 30871 (see above)		T5	98 (157/159)	100
A11_2	*Acanthamoeba* sp. OSU 04-023 clone 3 from an infected toucan liver tissue	FJ815180	T4	96 (184/191)	99
A20_2	*Acanthamoeba lenticulata* strain JC-1 ATCC 50428 (see above)	FJ815181	T5	100 (159/159)	100
	Acanthamoeba lenticulata strain PD2S ATCC 30871 (see above)		T5	98 (157/159)	100
TVT_2	*Acanthamoeba lenticulata* strain JC-1 ATCC 50428 (see above)	FJ815182	T5	100 (158/158)	100
	Acanthamoeba lenticulata strain PD2S ATCC 30871 (see above)		T5	98 (156/158)	100

Materials and methods

Isolation and cultivation of *Acanthamoeba* isolates

Contact lens cases from 50 randomly selected contact lens wearers living in Quezon City, Metro Manila, Philippines were used for isolation of *Acanthamoeba* species. Briefly, amoebae were obtained by flushing the contact lens case with sterile phosphate-buffered saline (pH 7.4). One hundred microliters of the suspension was cultured on a non-nutrient agar plate lawned with *Escherichia coli* (Page 1988).

DNA extraction, polymerase chain reaction, and DNA sequencing

Genomic DNA was extracted in each *Acanthamoeba* isolate using phenol–chloroform–isoamyl alcohol (Rivera et al. 1996) and then used as template in the polymerase chain reaction (PCR) as described by Booton et al. (2004). Primers JDP1 and JDP2 were used to amplify the ASA. S1 region of the gene (*Rns*) coding for the amoeba's nuclear, small subunit ribosomal RNA (Schroeder et al. 2001). PCR products were purified using QIAquick® gel extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced using primers 892C and 892 producing the sequences coded _2 and _sense, respectively. These two primers amplify the highly variable DF3 region of the *Rns* gene, which can be used to distinguish between genotypes (Booton et al. 2002). DNA sequencing was performed using an ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI PRISM 3100 model; Applied Biosystems).

Table 2 *Rns* sequence similarities of *Acanthamoeba* isolates used in this study and the reference isolates using 892 sequencing primer

Sample	Reference isolate with greatest similarity	GenBank accession	Genotype	Percent similarity	Coverage (%)
A2_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 from a swimming pool in France	FJ815183	T5	100 (150/150)	100
A5_sense	*Acanthamoeba polyphaga* Page-23 CCAP1501 strain ATCC 30871 from a freshwater pond in WI, USA	FJ815184	T4	100 (147/147)	99
A6_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815185	T5	100 (150/150)	99
A7_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815186	T5	100 (127/127)	100
A9_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815187	T5	100 (150/150)	100
A11_sense	*Acanthamoeba* sp. OSU 04-023 clone 2 from infected toucan liver tissue	FJ815188	T4	99 (118/119)	100
A20_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815189	T5	100 (150/150)	100
TVT_sense	*Acanthamoeba lenticulata* strain PD2S ATCC 30871 (see above)	FJ815190	T5	100 (150/150)	99

Phylogenetic analysis

Sequences were uploaded into the Basic Local Alignment Search Tool (BLAST) program of the US National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) to search for the most similar reference sequences. These were then aligned and removed of ambiguous nucleotide positions along with reference sequences representing the other *Acanthamoeba* species genotypes. The trees, rooted to a sequence of *Balamuthia mandrillaris*, were constructed using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI), which were all based on the GTR+Γ+I 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 (Swofford 2000) was used for MP and NJ while sequences were uploaded into the website PHYML (atgc.lirmm.fr/phyml) for the ML analysis (Guindon et al. 2005). Version 3.1.2 of the MrBayes program (Ronquist and Huelsenbeck 2003) was used to compute for the posterior probabilities in the BI analysis. Clusters in the phylogenetic trees were only considered valid if their posterior probabilities for BI were >0.70 or if their bootstrap support were >50% for NJ, ML, and MP.

Table 3 The reference *Acanthamoeba* isolates included in the analyses

Genotype	Name of isolate/strain	GenBank accession	Sample source
T1	*Acanthamoeba* sp. CDC V548	DQ339096	Granulomatous amoebic encephalitis, human brain, USA
T1	*Acanthamoeba castellani* CDC:0981:V006	U07400	Granulomatous amoebic encephalitis, human brain, Georgia, USA
T2	*Acanthamoeba palestinensis* Reich ATCC 30870	U07411	Soil, Israel
T3	*Acanthamoeba 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	*Acanthamoeba 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	*Acanthamoeba castellani* CDC:0981:V006	U07401	Keratitis, India
T4	*Acanthamoeba* sp. ATCC 50497 strain BCM:0288:37	U07410	Keratitis, Texas, USA
T5	*Acanthamoeba lenticulata* strain 45 ATCC 50703	U94730	Nasal mucosa, Germany
T5	*Acanthamoeba lenticulata* strain JC-1 ATCC 50428	U94739	Freshwater stream, New York, USA
T5	*Acanthamoeba lenticulata* strain PD2S ATCC 30871	U94741	Swimming pool, France
T6	*Acanthamoeba 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 comandoni* strain Comandon & de Fonbrune	AF019066	Soil, France
T10	*Acanthamoeba culbertsoni* Lilly A-1 strain ATCC 30171	AF019067	Human cell culture, Indiana, USA
T11	*Acanthamoabe hatchetti* BH-2	AF019068	Brackish water, Maryland, USA
T11	*Acanthamoeba* sp. PN15	AF333607	Clinical sample, Pakistan
T12	*Acanthamoeba healyi* strain CDC 1283:V013	AF019070	Granulomatous amoebic encephalitis, Barbados
T13	*Acanthamoeba* sp. UWC9	AF132134	Contact lens case
T13	*Acanthamoeba* sp. UWET39	AF132136	Soil, USA
T14	*Acanthamoeba* sp. PN15	AF333607	Clinical sample, Pakistan
T15	*Acanthamoeba jacobsi* AC080	AY262361	Water supply, Pakistan
T15	*Acanthamoeba jacobsi* AC194	AY262362	Freshwater sediment, UK
T15	*Acanthamoeba jacobsi* AC304	AY262364	Untreated water, Australia
–	*Balamuthia mandrillaris* strain CDC:V039	AF019071	No source given

GenBank references

The 892C sequences determined in this study were deposited in GenBank as accessions FJ815175–FJ815182 (Table 1) while the 892 sequences were deposited as GenBank accessions FJ815183–FJ815190 (Table 2). The reference sequences used are listed in Table 3.

Results

Only two *Rns* genotypes were identified from eight contact lens case isolates. Six of the eight isolates were genotype T5 while the remaining two were genotype T4. The BLAST results of the DF3 sequences of isolates A2, A6, A7, A9, A20, and TVT indicate that these belong to

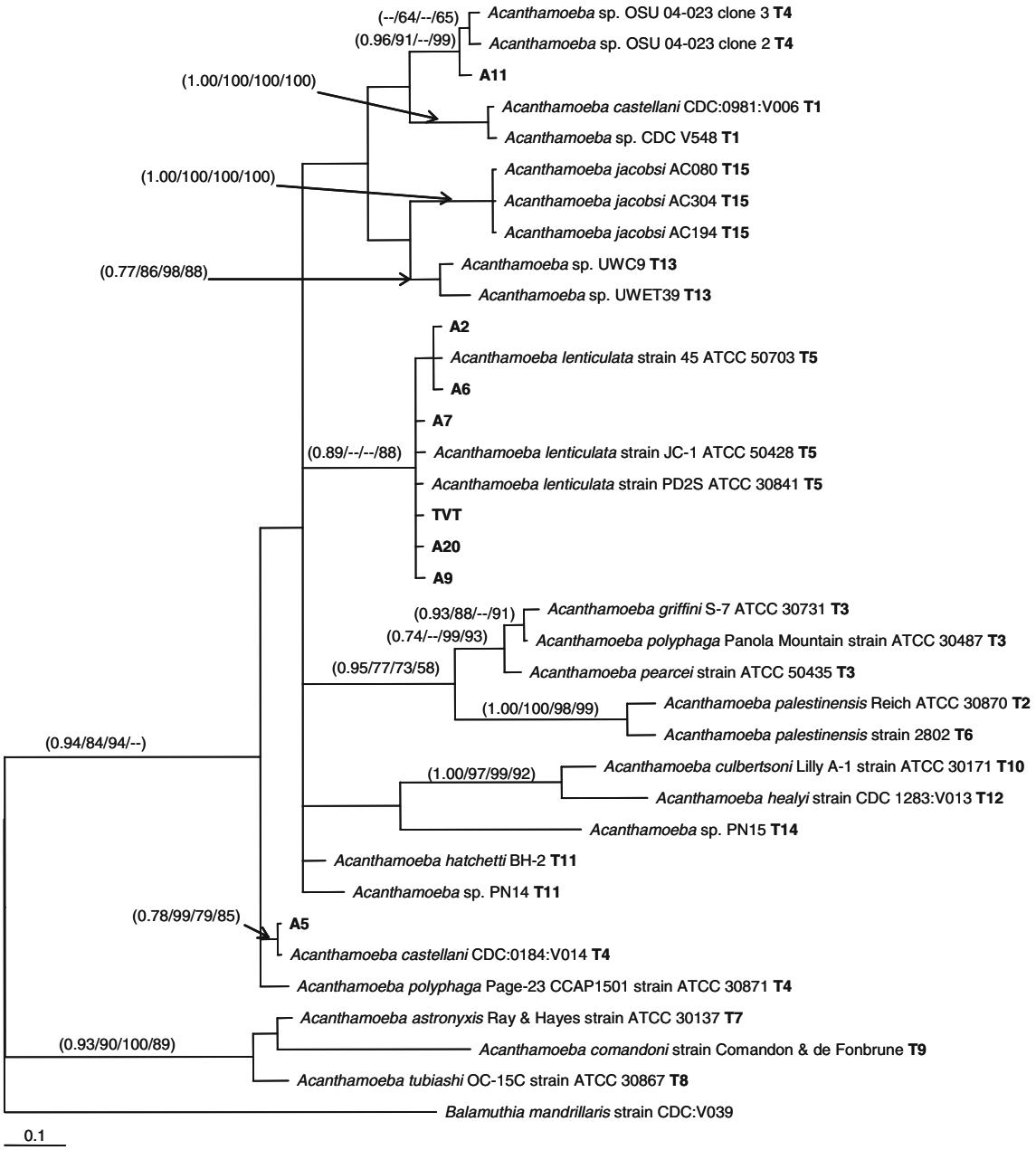

Fig. 1 Phylogenetic tree of the contact lens case isolates from this study and reference *Acanthamoeba* sp. isolates, based on 261 concatenated unambiguously aligned nucleotide base pairs of the DF3 region of the *Rns* gene. The tree, constructed using the Bayesian inference analysis, was rooted on *Balamuthia mandrillaris*. The four values at each node represent the posterior probability from BI and the bootstrap support from neighbor-joining, maximum likelihood, and maximum parsimony analyses, respectively. Posterior probability values <0.70 and bootstrap support <50% are not shown. The scale bar on the lower left side represents one change per ten nucleotide positions

genotype T5. Similarly, the BLAST results of the DF3 sequences of isolates A5 and A11 both show that these isolates belong to genotype T4. Tables 1 and 2 show the BLAST results for the 892C and 892 sequences, respectively. Moreover, these isolates also clustered with the reference sequences most similar to them in at least two of the four phylogenetic tree analyses used in this study with bootstrap supports of 50 and above for NJ, ML, or MP and/or posterior probabilities of 0.70 and above for BI. Figure 1 shows a consensus tree based on the Bayesian inference tree summarizing the results of NJ, ML, MP, and BI.

Discussion

Acanthamoeba contamination of contact lens cases are mainly associated with improper care of contact lens and lens storage cases. Contamination is mostly associated with rinsing of contact lenses and lens storage cases with tap water (Seal et al. 1999; Booton et al. 2002; Kilvington et al. 2004; Jeong et al. 2007) most especially if the source were storage tanks which promote growth of microorganisms (Kilvington et al. 2004; Jeong and Yu 2005). Thus, *Acanthamoeba* has been isolated from contact lens storage cases (Lee et al. 1997; Yu et al. 2001; Booton et al. 2002; Jeong and Yu 2005; Jeong et al. 2007) and even from tap water from the houses of these lens storage case owners (Seal et al. 1999; Booton et al. 2002; Jeong and Yu 2005; Jeong et al. 2007). These lens case and tap water isolates were identified either as genotype T3 (Booton et al. 2002) or T4 (Booton et al. 2002; Jeong et al. 2007). In our present study, the two genotypes identified from contact lens storage cases were T4 and T5, the most commonly isolated (Gast et al. 1996; Stothard et al. 1998; Walochnik et al. 2000; Booton et al. 2004, 2005; Zhang et al. 2004; Köhsler et al. 2006; Lorenzo-Morales et al. 2006) and second most commonly isolated (Booton et al. 2005) genotypes, respectively. These two genotypes have also been isolated previously from samples from both environmental and contact lens storage cases in the Philippines where genotype T5 was more commonly isolated than T4 (Rivera and Adao 2008). Similarly, most of the isolates in this present study were T5, indicating that this genotype is possibly the more common contaminant in contact lens cases in the Philippines. In contrast, previous studies in other countries usually isolated T4 and T3 genotypes as contaminants of contact lens cases and contact lens paraphernalia (Booton et al. 2002; Jeong et al. 2007). Moreover, all the T5 isolates and one T4 isolate were most similar to the same reference strains of *Acanthamoeba* species as most of the previous isolates from the Philippines (Rivera and Adao 2008). These reference strains were the *Acanthamoeba lenticulata* strain PD2S ATCC 30871 (GenBank accession number: U94741) and *A. lenticulata* strain JC-1 ATCC 50428 (GenBank accession number: U94739) for the T5 isolates and *Acanthamoeba* sp. OSU 04-023 clones 2 and 3 (GenBank accession numbers: DQ451162 and DQ451163, respectively) from an infected toucan liver tissue for the T4 isolate A11.

Virulent strains of *Acanthamoeba* species as well as those found in contact lens cases and paraphernalia are usually identified as T4 (Gast et al. 1996; Stothard et al. 1998; Gast 2001; Booton et al. 2002; Zhang et al. 2004). The two T4 isolates from this study, A5 and A11, are most similar to potentially pathogenic T4 isolates. The 892C sequence of A5 is most similar to an *Acanthamoeba* keratitis isolate from India (Table 1) while the entire DF3 sequence of A11 are most similar to *Acanthamoeba* species from an infected toucan liver tissue (Tables 1 and 2). Isolates of T5 are usually free-living although there are cases where this genotype has been isolated in patients with *Acanthamoeba* keratitis (Spanakos et al. 2006) and disseminated acanthamoebiasis (Barete et al. 2007). However, further tests are necessary to fully determine the pathogenicity of these isolates.

The presence of *Acanthamoeba* contamination of contact lens and lens storage cases can also be attributed to other improper ways of cleaning them besides rinsing with tap water. One commonly associated factor is the use of ineffective amoebicidal disinfectants, i.e., lens care solutions and chlorine tablets (Silvany et al. 1990; Seal et al. 1999; Tzanetou et al. 2006; Joslin et al. 2007) or lack of use of disinfectant (Seal et al. 1999). Other factors such as not rubbing lenses while cleaning and reusing lens care solutions (Joslin et al. 2007) or even simply leaving the lens storage cases wet (Seal et al. 1999) as well as taking a shower or swimming while wearing contact lenses (Kaji et al. 2005; Wynter-Allison et al. 2005; Joslin et al. 2007) have been linked to *Acanthamoeba* contamination and even AK. Thus, the isolates in this study are also most likely from improper lens cleaning practices.

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