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Development of an *Acanthamoeba*-specific Reverse Dot-Blot and the Discovery of a New Ribotype

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ABSTRACT. *Acanthamoeba* is a genus of free-living amoebae, of which some species have been found to cause opportunistic infections in humans. The identification of these amoebae in natural and disease samples is based primarily upon morphological features. While these features are more than adequate for identification to the genus level, they are not useful for species-level identification. This not only leads to difficulty in the diagnosis of infections, but it makes an accurate assessment of the natural distribution of acanthamoebae very difficult to achieve. To improve this situation, a detection method was developed that utilizes both selective polymerase chain reaction amplification and the reverse dot-blot. Oligonucleotides were designed to be specific for the described ribosomal groups (or ribotypes) of *Acanthamoeba*, as well as one specific for the genus itself. When this method was used to analyze a series of *Acanthamoeba* cultures from Pakistan, a new ribotype was identified in addition to the detection of the ubiquitously distributed T4 type.

Key Words. Colorimetric detection, PCR amplification, 18S rDNA.

MEMBERS of the genus *Acanthamoeba* are free-living amoebae that can be isolated from almost any type of environment (e.g. brackish water, fresh water, soil) (De Jonckheere 1991). Some isolates have been found to cause opportunistic infections of humans, including *Acanthamoeba* keratitis, a painful and sight-threatening corneal infection, a fatal brain infection (granulomatous amebic encephalitis: GAE) (John 1993), and the infection of internal organs and the skin (Sison et al. 1995; Szénási et al. 1998). In addition, acanthamoebae can harbor pathogenic (or potentially pathogenic) microbes, such as *Chlamydia* spp., *Legionella* spp., *Vibrio* spp., and *Listeria* spp. (Amann et al. 1990; Cirillo et al. 1999; Field 1991; Ly and Muller 1990; Thom et al. 1992). Detection of acanthamoebae is essential for appropriate and timely treatment, and studies of their distribution in natural samples is necessary to understand their potential role as vectors in the dissemination of other diseases.

Identification of acanthamoebae is based primarily on the morphological characteristics of the amoeba (active) or cyst (dormant) stage after the organisms have been grown in culture. Identification of the genus is relatively easy, but a large degree of variability in cyst structure, even within a clonal culture, can lead to unreliable identification at the species level (Stratford and Griffiths 1978). Several methods have been used in attempts to overcome ambiguities in identification. Isoenzyme patterns and mitochondrial restriction fragment length polymorphisms were used to define and relate isolates and species of *Acanthamoeba* (Bogler et al. 1983; Costas and Griffiths 1986; Daggett et al. 1983; De Jonckheere 1983; Yagita and Endo 1990). These analyses have been very informative, but a significant drawback is their need to be applied to cultured isolates. This introduces the probability of culture bias, with the organisms that grew not being representative of the ones originally dominant in the sample.

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More recently, monoclonal antibodies (Hiwatashi et al. 1997) and fluorescently labeled oligonucleotide probes (Stothard et al. 1999) were developed. Although the monoclonal antibodies show great promise in utility for the detection of keratitis isolates, they were not able to distinguish different isolates (or species) of acanthamoebae. Fluorescent oligonucleotide probes were used to detect members of the *Acanthamoeba* genus and the T4 ribotype in clinical samples. These probes function extremely well for the direct detection of amoebae in tissues. Even though both these methods have been shown to work well, they are limited in the number of different targets (or species) that can be visualized in a single sample. This is primarily due to the limited number of different fluorescent label colors that can currently be detected.

Polymerase chain reaction amplification (PCR) is often used for the detection of organisms due to its ability 1) to be used without prior culture of the organism of interest, 2) to be useful at various levels of specificity, and 3) to detect small numbers of cells. Although primers have been designed for the specific amplification of acanthamoebae at the genus level (Gast and Byers 1995; Lehman et al. 1998; Vodkin et al. 1992), the identification of the different *Acanthamoeba* species, or groups of species, has not been attained. Therefore, the goal of my current project has been to develop a method that would allow both the detection and identification of multiple types of acanthamoebae from a single sample, either clinical or environmental. Oligonucleotides specific for the ribotypes described in Stothard et al. (1999) were developed and applied in a reverse dot-blot format (i.e. oligonucleotides bound to the membrane). *Acanthamoeba*-selective amplification primers were also designed for the small subunit ribosomal RNA gene (18S rDNA) to generate probes for use in the hybridization. Protocols such as this have been used previously for the specific detection of mutations or microorganisms (Fiss et al. 1992; Kawasaki et al. 1993; Levesque et al. 1998; Robinson et al. 1996). The *Acanthamoeba* reverse dot-blot was found to be specific for each of the ribotypes, permitted the detection of multiple ribotypes from a single sample, and allowed the detection of new ribotypes.

Table 1. Isolates of *Acanthamoeba* species used to test reverse dot blot.

RIBO-TYPE	ISOLATE[a]	ATCC NUMBER and SOURCE
T1	*A. castellanii* CDC:0981:V006	50494 Brain, USA
T2	*A. pustulosa* GE 3a	50252 Water, France
T3	*A. pearcei* 205-1	50435 Marine sewage dump, USA
T4	*A. hatchetii* 2-1-AX	Uncatalogued Marine sewage dump, USA
T5	*A. lenticulata* PD2S	30841 Swimming pool, France
T6	*A. palestinensis* 2802	50708 Swimming pool, France
T7, 8, 9	*A. tubiashi* OC-15C	30867 Freshwater, USA
T10	*A. culbertsoni* Lilly A-1	30171 Human cell culture, USA
T11	*A. hatchetii* FDA-97B-AX	50673 Marine sewage dump, USA
T12	*A. healyi* CDC:1283:V013	Uncatalogued Brain, Barbados

[a] All 18S rDNA sequences are available from GenBank, except FDA-97B-AX.

MATERIALS AND METHODS

Cultures. The cultures of acanthamoebae used to test the reverse dot-blot method are listed in Table 1. All were obtained from ATCC, except *Acanthamoeba castellanii* V006, *Acanthamoeba lenticulata* PD2S, *Acanthamoeba tubiashi*, and *Acanthamoeba healyi*, which were received from Dr. T. Byers at the Ohio State University. These amoeba cultures were grown axenically in 25-cm² Costar flasks on 712 PYG medium (ATCC Catalogue) at 26 °C. Sixteen cultures of acanthamoebae isolated from Pakistani eye and stool samples were obtained from Dr. Viquar Zaman. These cultures were maintained at 26 °C in 25-cm² Costar flasks on 997 medium (ATCC Catalogue) supplemented with 10 μl of autoclaved *Escherichia coli* in 1× PBS.

DNA isolation. *Cultures.* Amoebae were collected for DNA isolation by vigorous agitation of the media when cells had formed a confluent layer on the bottom of the flask (~ 1×10^6 amoebae; 10^4 amoebae for Pakistani cultures). Amoebae were pelleted at 1,000 *g* for 10–15 min and the resulting cell pellets were lysed by the UNSET method (Gast et al. 1996) or by hot detergent and bead mill homogenization (Kuske et al. 1998). The hot detergent/bead mill homogenization method was monitored for the lysis of both amoebae and cysts by visual inspection of the lysate after each incubation and homogenization step. Very few cysts were detectable at the end of the procedure. DNA was precipitated using ethanol, and the air-dried pellet was resuspended in 100 μl of sterile distilled water (20 μl for Pakistani cultures) and stored at −20 °C.

Mixed ribotype analysis. Six grams of sediment that was negative for acanthamoebae by the reverse dot-blot method were spiked with three different cultures of amoebae. These cultures were *Acanthamoeba hatchetii* 2–1-AX (T4), *Acanthamoeba hatchetii* FDA-97B-AX (T11), and *Acanthamoeba pearcei* 205–1 (T3). The sediment was then processed using the hot detergent/bead mill homogenization method and recovered DNA was further purified using a Wizard DNA Clean-Up Kit (Promega, Madison, WI).

Oligonucleotide design. All oligonucleotide sequences are shown in Table 2. A and B primers are specific for eukaryotic small subunit ribosomal genes, and their use has been previously described (Gast et al. 1996; Medlin et al. 1988; Stothard et al. 1998). The *Acanthamoeba*-selective primers and genus- and ribotype-specific oligonucleotides were designed based upon conserved regions in the alignment of 44 *Acanthamoeba* 18S rDNAs. The amplification primers were designed to target a region of the gene between positions 989 and 1778 (in reference to *Acanthamoeba castellanii* Castellani 18S rDNA sequence). The amplified fragment contains three variable regions, which were used for design of the ribotype-specific oligonucleotides.

PCR amplification. *Probes to test the reverse dot blot.* Probes were generated by PCR amplification (Saiki et al. 1988) of an internal 18S rDNA fragment using primers Ac1000C and Ac1982 (Table 2). Products were labeled by the incorporation of alkali stable digoxigenin-11-dUTP (DIG-dUTP; Roche/Boehringer Mannheim, Indianapolis, IN) using a PCR nucleo-

Table 2. Oligonucleotides and primers used for reverse dot blot detection and PCR amplification of *Acanthamoeba* species.

Oligonucleotide	Sequence (5′–3′)[a]	Position
Ac1000C	TAATGGAATARGACCYTG	989–1006[d]
Ac1982	RAGTCCCTCTAAGAAGC[b]	1762–1778[d]
AcGenus	CCTAATCGCTGGTCGGCA[b]	1226–1243[d]
AcT1A	GAAGTGGAGGCGGTCTC	1273–1289[e]
AcT2B	CTTCAGTTGGTTAACTTGTA	1002–1021[e]
AcT3A	TTTGCCGCGAGGACCAG	1020–1036[e]
AcT4A	AGTTGGTTTTGGCAGCGC	1020–1037[d]
AcT5B	CCGCTGTTAATCCTTTCGGGG	1337–1357[e]
AcT6C	TTCACTGCATATTAATGT	1676–1694[e]
AcT7, 8, 9,B	GCATGGTTTYGTCTAAAGGAG	1246–1266[e]
AcT10A	GGGAACGATTCGTCCTG	1314–1330[e]
AcT11D	GGTCCGCTGCAGGACAG	1667–1683[f]
AcT12B	GAAGGAACGATTTGTCTTAAT	1330–1350[e]
A	AACCTGGTTGATCCTGCCAGT[c]	5′ end
B	GATCCTTCTGCAGGTTCACCTAC[b,c]	3′ end

[a] Oligonucleotides synthesized as written.
[b] Complementary to RNA sequence.
[c] Modified from A and B primers of Medlin et al. 1988.
[d] In reference to nucleotide positions in *A. castellanii* Castellani 18S rDNA sequence.
[e] In reference to nucleotide positions in 18S rDNA from the isolate used to test the oligo (see Table 1).
[f] In reference to nucleotide positions in *A. hatchetti* BH-2 rDNA.

tide mixture with a 1:19 ratio of DIG-dUTP:dTTP. Each amplification reaction was carried out in a total vol. of 50 μl with the following mixture of components; 1 μl of DNA, 1× reaction buffer, 2 mM $MgCl_2$, 100 ng each primer, 200 μM nucleotides, and 2.5 units of Taq polymerase (Promega). Amplification cycles were preceded by denaturation of the samples for 5 min at 95 °C, followed by 35 cycles of 45 sec at 94 °C, 45 sec at 50 °C, and 2 min at 72 °C. PCR products were checked on 1% agarose gels stained with ethidium bromide to confirm amplification.

Mixed ribotype amplification. To detect acanthamoebae in the spiked sediment sample, A and B primers were used in an initial amplification, followed by reamplification with primers A and Ac1982. These amplification reactions were standard (Gast et al. 1996) and consisted of 35 cycles of 45 sec at 94 °C, 1 min at 42 °C (1 min at 50 °C for A/Ac1982), and 3 min at 72 °C. Reamplification reactions to generate the labeled probe were accomplished with the same cycling protocol as described above. One μl of template DNA, or of the previous PCR reaction, was used in each reaction.

Sequence analysis. The 18s rDNAs from three Pakistani samples that gave hybridization results that were not T4 were fully sequenced. The gene was amplified in two pieces using primer pairs A/Ac1982 and Ac1000C/B. Three PCR reactions were combined and precipitated, then resuspended in 20 μl of water. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and plasmids were sequenced using ABI Big Dye™ terminators (PE Applied Biosystems, Foster City, CA). Samples were run on an ABI 377 automated sequencer and analyzed using Sequencer 3.1.1 (©Gene Codes Corp., Ann Arbor, MI). At least 85% of the 18S rDNA molecule was sequenced on both strands and the final sequences were deposited in GenBank under the accession numbers AF333607 (*Acanthamoeba* sp. PN15), AF333608 (*Acanthamoeba* sp. PN14), and AF333609 (*Acanthamoeba* sp. PN13).

Phylogenetic analysis. Twenty-six of the available *Acanthamoeba* 18S rDNA sequences were used to reconstruct a molecular phylogeny that also included the three Pakistani *Acanthamoeba* sequences reported in this work. Sequences representative of each previously identified ribotype were selected from GenBank, and the accession numbers are shown on the tree. Alignments were generated in SeqLab (GCG version 10, Wisconsin Package™) and were based upon conserved secondary structures as well as primary sequence similarity. Regions of ambiguous alignment (e.g. hypervariable regions) were excluded from the analyzed dataset, and alignments are available from RJG upon request. The phylogeny was reconstructed using the maximum likelihood algorithm in PAUP 4.0b7 (Swofford 1998). Nucleotide frequencies were empirically determined and transition/transversion ratios were estimated by the program. The distribution of rates at variable sites was set to follow a gamma distribution. Starting trees were obtained via neighbor-joining addition and branch-swapping was accomplished by tree bisection-reconnection. The 18S rDNA sequence from the T9 ribotype was defined as an outgroup, but the resulting trees are unrooted.

Reverse dot-blot assembly. Oligonucleotides were synthesized commercially and tailed with dTTP in the laboratory using terminal transferase (Promega). The tailing reaction consisted of 100 pmole oligonucleotide, 2.5 mM dTTP, 1× reaction buffer, and 1.5 μl terminal transferase (Promega) in a total vol. of 100 μl. This was incubated at 37 °C for 45 min. An additional 1 μl of enzyme was added, and the incubation continued for another 45 min to further increase tail length (Fiss et al. 1992). One μl of each tailed oligonucleotide was spotted onto a 3.5 × 1 cm piece of Hybond-NX nylon membrane (Amersham), starting with the genus-specific oligonucleotide and progressing through the T1–T12 oligonucleotides. The membranes were air dried, baked at 80 °C for 1 h, and then either stored between pieces of Whatmann paper in a sealed bag or used immediately for hybridization.

Hybridization and detection. Membranes were prehybridized for 1 h at 35 °C in 5 ml hybridization solution (5× SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent; Roche/Boehringer Mannheim). An entire digoxigenin PCR reaction (~ 50 μl) was added to an equal vol. of 0.4 M NaOH and denatured at 100 °C for 5 min. The denatured probe mixture was added directly to the prehybridization solution, and hybridization was allowed to proceed overnight at 35 °C with gentle agitation. Blot wash conditions were varied by changing either sodium citrate buffer (SSC) concentration or the temperature to determine those conditions optimal for obtaining specific detection of the desired ribotype without cross-reactivity (see Results). Visualization of positive hybridization was accomplished through colorimetric detection following Genius kit protocols (Roche/Boehringer Mannheim).

RESULTS

Oligonucleotide design. Ribotype-specific oligonucleotides were selected so that they had one or no mismatches with any of the other sequences in that ribotype. To reduce false positives and cross-hybridization, oligonucleotides should have more than two mismatches with any of the other ribotype sequences. Potential oligonucleotides and primers were subjected to BLAST searches at GenBank (Bilofsky and Burks 1988) to confirm that the sequence did not significantly match anything else in the database (more than two mismatches).

Oligonucleotides were successfully designed for each ribotype, except for T7, 8, and 9, which are represented by a single oligonucleotide. It was decided that distinction between these was not currently necessary because species from these three groups are very rarely isolated and are not considered potentially pathogenic. It was difficult to design ribotype-specific oligonucleotides with more than one mismatch for the T11, T3, and T4 ribotypes because they are all relatively closely related (Fig. 1). In both the T3 and T4 oligonucleotides, the mismatch with the T11 probe sequence was an insertion/deletion (indel) event. The mismatch between the T3 oligonucleotide and the T11 probe was located 4 bases from the 5′ end of the oligonucleotide, whereas the indel between the T11 probe and the T4 oligonucleotide was 4 bases from the 3′ end of that oligonucleotide.

An oligonucleotide that would recognize all members of the genus (Ac genus) was also designed. It could serve as a positive control for hybridization, and the assumption was made that new ribotypes could be detected through hybridization to that probe. The Ac genus oligonucleotide designed for this project is not the same as the genus oligonucleotide (GSP) designed by Stothard et al (1999). A new genus-specific oligonucleotide was designed because the GSP oligonucleotide target lies outside of the fragment being amplified for use as a probe in this project.

PCR amplification. Amplification of acanthamoebae with the *Acanthamoeba*-selective primers Ac1000C and Ac1982 generated PCR products of the expected size. The size of the ribosomal fragment from *Acanthamoeba* is ~ 750 basepairs, about 250 basepairs larger than typical 18S rDNAs due to the presence of expansion segments and variable regions (Gast et al. 1996; Gunderson and Sogin 1986). The product obtained from *A. lenticulata* has a size that is about 1,000 basepairs due to the presence of a group I intron (Gast et al. 1994; Schroeder-Diedrich et al. 1998). Consequently, the size of the PCR prod-

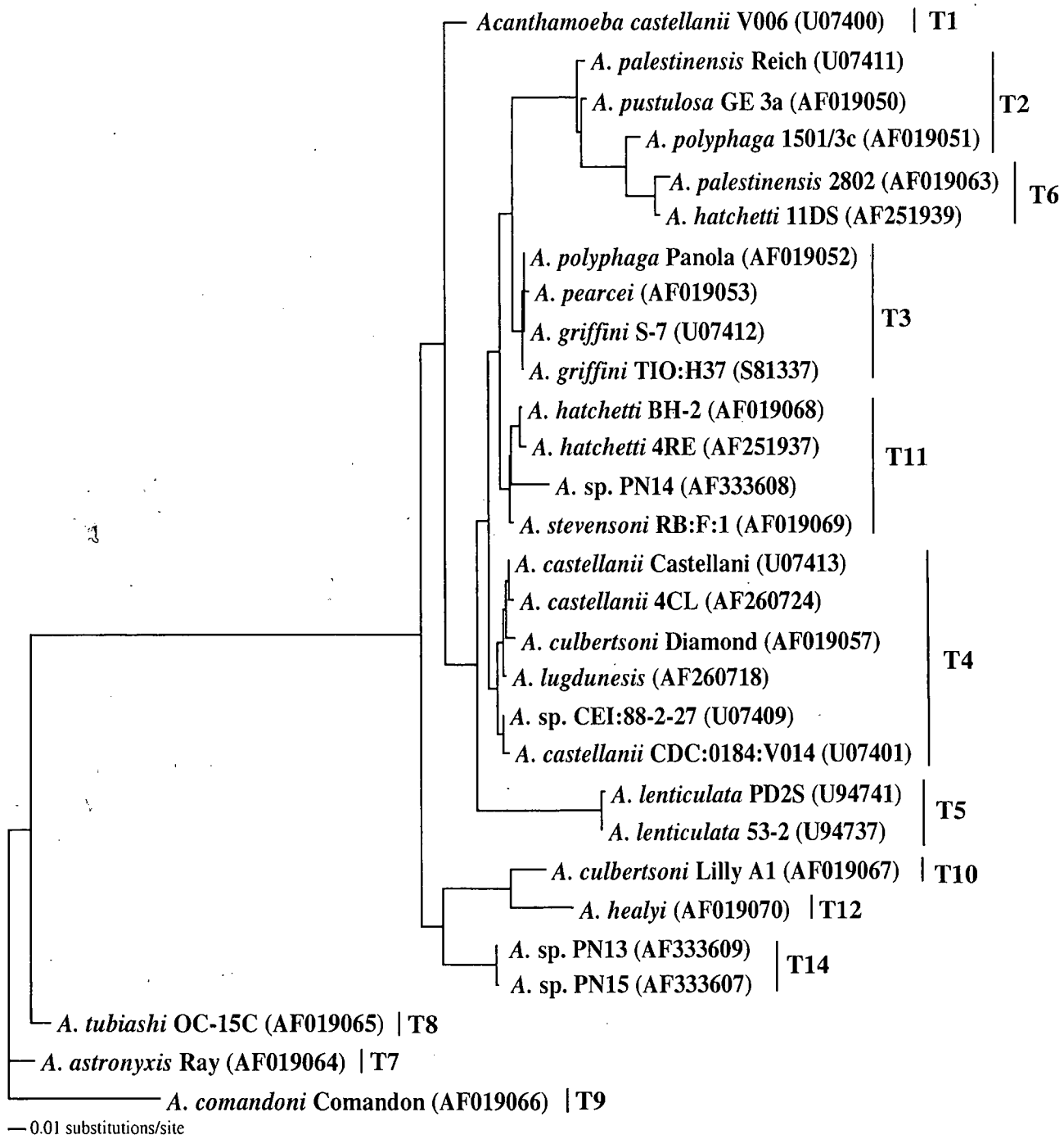

Fig. 1. Phylogenetic reconstruction based on 18S rDNA by maximum likelihood for 26 *Acanthamoeba* isolates. Ribotype groupings are indicated at the right of the tree. Accession numbers of the isolates included in the analysis are listed on the tree.

uct can also be diagnostic for the presence of acanthamoebae in the sample.

Reverse dot-blot detection. The specificity of the oligonucleotides designed in this project was determined by hybridization with probes generated from representatives of each ribotype (Stothard et al. 1998), The results of these hybridizations are shown for the T4 and T11 ribotypes (Fig. 2). Several representatives from the T4 ribotype group were used to test the specificity of that oligonucleotide, and because the results were all the same, only one has been shown.

Initial wash conditions (2× SSC, 0.1% SDS; 15 min at 45 °C) were not very stringent, and cross-hybridization of the T11 probe with the T3 and T4 ribotype oligonucleotides was observed (Fig. 2, T11 low). The single indels near the ends of the oligonucleotide/probe hybrid did not destabilize the association enough to discourage cross-reactivity under those wash conditions. Washes that varied temperature and/or salt concentration were tested with the T11 probe until cross-reactivity was eliminated (0.2× SSC, 0.1% SDS; 15 min at 45 °C; Fig. 2, T11 high). The higher stringency wash was then tested to determine

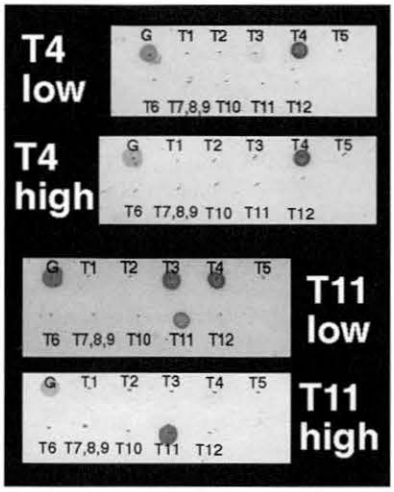

Fig. 2. Oligonucleotide/probe specificity for *Acanthamoeba* T4 and T11 ribotypes. Oligonucleotide order is as follows: Top row, Acgenus, T1, T2, T3, T4, T5; Bottom row, T6, T7–8–9, T10, T11 and T12.

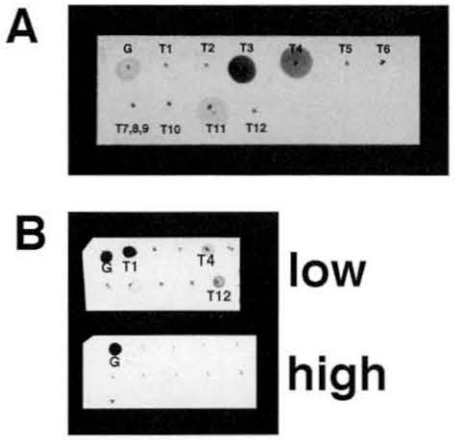

Fig. 3. Detection of *Acanthamoeba* ribotypes in sediment samples spiked with acanthamoebae. **A.** Detection of multiple ribotypes in spiked sediment samples. The reverse dot-blot reveals the presence of T3, T4, and T11 (very faint spot) ribotypes, as well as hybridization with the genus-specific oligonucleotide (G). **B.** Hybridization patterns for the pseudo T1/T12 ribotype. Loss of hybridization is evident at high wash stringencies. The identification of each oligonucleotide spot is indicated on the blots.

whether the other correct probe/oligonucleotide hybrids were stable under those conditions. Only T6 hybridization was unstable at the higher stringency, but attempts to redesign the T6 oligonucleotide with an increased calculated melting temperature were unsuccessful. Therefore, the current scheme is to hybridize blots in duplicate and wash one at high and one at low stringency. Overall time and work was not significantly increased by the dual wash strategy, and it can aid in detecting novel ribotypes by the instability of hybridization at stringent conditions (see Identification of new ribotypes). Efforts to stabilize the T6 hybridization will continue.

Detection of mixed ribotypes. Oceanic sediments were spiked with cultures of acanthamoebae to answer two questions. First, is it possible to amplify and detect more than one ribotype in a sample? Second, are the primers and probes specific for acanthamoebae in the presence of other organisms from natural samples? Sediment samples were utilized because there is usually a complex mixture of other microorganisms present, and future research directions are aimed at the use of this detection method on marine sediments. The sediments were first analyzed by reverse dot-blot to determine that acanthamoebae were not detected, then cultures were added to the sediment and DNA was extracted. T3, T4, and T11 ribotype cultures were selected for this experiment because they exhibit the most similarity to each other in primary sequence and would potentially cross-react in a hybridization mixture.

The detection of more than one ribotype in a probe mixture was possible, and the results are shown in Fig. 3A. Hybridization with the T3 and T4 oligonucleotides is very easily visualized, but spots resulting from hybridization with the genus and T11 oligonucleotides are faint. The detection of color is currently considered a positive result, as long as it is present on both low and high stringency washed blots. Variability in color strength could be the result of several different factors, including the amount of probe produced in the PCR reaction, the amount of digoxigenin incorporated into the probe, the amount of oligonucleotide actually present on the membrane, and the efficiency of the hybridization. Variability in signal is not taken to represent different levels of starting material, or to be quantitative in any way.

Amplification of spiked sediment used for this study with primers Ac1000C and Ac1982 produced only products of the correct size. The amplification of extracts from other sediments (spiked and not spiked) has occasionally resulted in spurious products of a smaller size in addition to the band from acanthamoebae. Sequences obtained from three of the spurious bands (from different samples) were found to be similar to a fungus, a dinoflagellate, and a diatom. Despite comparing the primers sequences to everything present in the databases, unidentified organisms in the environment were amplified. Fortunately, when these smaller products were used as probes on the reverse dot blot, they did not hybridize with any of the oligonucleotides, even at the low stringency wash. Primers Ac1000C and Ac1982 are accordingly considered to be only selective for acanthamoebae, not specific.

Level of detection. In preliminary experiments to determine the detection limit of this method in relation to natural samples, *Acanthamoeba*-negative sediments were spiked with cultures and DNA was extracted. The number of added cells ranged from 10^6/gm down to zero/gm in 10-fold increments. At least 10,000 amoebae/gm of sediment were needed in the original sample in order for a signal to be recovered. Because sediment samples are well known to be inhibitory to PCR, it is expected that the level of sensitivity would be much better in clinical or water samples, but this has not been determined empirically. Efforts are currently directed towards improving the detection level.

Identification of new ribotypes. The reverse dot blot was used to determine the ribotypes of 16 cultures of acanthamoebae from Pakistan. Two of the samples did not yield amplification products, and amoebae were not detectable in the cultures when inspected visually. Five of the cultures were identified as belonging to the T4 ribotype. Two of these were isolated from cases of keratitis, whereas the other three were from stool samples. Two other stool sample cultures were identified as T11 ribotypes. The remaining 7 stool sample cultures gave hybridization results that were temporarily named "pseudo T1/T12" (Fig. 3). Cysts from one of the pseudo T1/T12 cultures are shown in Fig. 4B, and they appear to be a group III type.

The 18S rDNA from representatives of the Pakistani T11 and pseudo T1/T12 ribotypes was sequenced and compared with other *Acanthamoeba* ribosomal genes. The pseudo T1/T12 ribotype is described by two sequences (PN13 and PN15) that

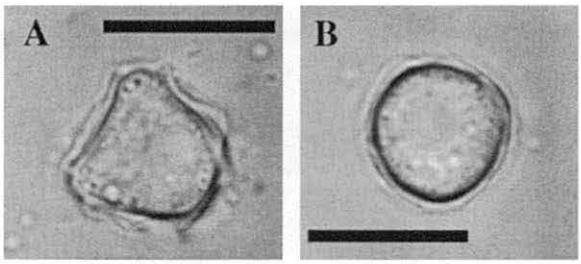

Fig. 4. Cysts from Pakistani cultures. **A.** Cyst from PN14 culture (T11 ribotype); **B.** Cyst from PN15 culture (T14 ribotype). Scale bar in image is approximately 10 μm, DIC microscopy.

are basically identical to each other. There is no doubt that this sequence represents a new ribotype (called T14), based upon its molecular phylogeny (Fig. 1, T14) and the fact that it is 11% different from the most similar sequences in the dataset (*Acanthamoeba castellanii* Ma, *Acanthamoeba polyphaga* Jac/S2 and *Acanthamoeba polyphaga* V029 within the T4 ribotype). The T11 ribotype (PN14) recovered from the Pakistani stool samples was confirmed by pairwise comparisons with the available T11 sequences. It was determined that the *Acanthamoeba* spp. PN14 sequence was 3.3% different from *Acanthamoeba stevensoni* R:B:F1, 4% different from *Acanthamoeba hatchetti* BH-2 and 4.3% different from *Acanthamoeba hatchetti* 4RE. This is less than the previously defined value of 5% difference for designating different ribotypes, (Stothard et al. 1998), but more than the difference between R:B:F1 and BH-2 (2.1%). The variable regions of the *Acanthamoeba* spp. PN14 sequence show some unique patterns of substitution, but overall are still more similar to *A. stevensoni*.

DISCUSSION

Ribotype classification. Ribosomal sequence comparison and ribosomally-based phylogenies are some of our best indicators of genetic relatedness for the *Acanthamoeba* genus. The presence of two or more different *Acanthamoeba* ''species'' in a designated ribotype, or the separation of strains of the same ''species'' into different ribotypes, is an indication that classical methods of identification are potentially inadequate, not that the ribosomal method is necessarily flawed. Ribosomal analyses make it possible to design probes that are genetically informative, but not ones that are necessarily clinically informative. This is due, in part, to the inability to reliably establish pathogenic potential in isolates not recovered from infections. It will remain difficult to design probes that are relevant both genetically and clinically until reliable indicators of pathogenicity are found.

Multiple alleles and introns. The presence of multiple alleles and group I introns, which both occur in acanthamoebal 18S rDNAs, could potentially cause problems for the reverse dot-blot method. Introns within the region of interest might be disruptive for either amplification or hybridization. To date, only two species, *A. griffini* (T3) and *A. lenticulata* (T5), have been found to contain group I introns within their small subunit ribosomal gene (Gast et al. 1994; Schroeder-Diedrich et al. 1998). No interference was found in either the amplification of the desired fragment or in the detection of the ribotype by hybridization when the intron-containing *A. lenticulata* PD2S isolate was used in this study.

Multiple alleles of the 18S rDNA within a single isolate of *Acanthamoeba* exhibited between 2–8 changes (Stothard et al. 1998). Although some of these changes fell within the variable regions where the ribotype-specific oligonucleotides were designed, none was in the target region for that particular ribotype. If more than two sequence changes did fall within the oligonucleotide target, loss of hybridization with that particular allele would be expected, but due to the presence of a second allele (or more), correct hybridization for the ribotype should still be obtained. The case where a sequence was changed so that it matched a different ribotype oligonucleotide could result in a false positive. The assumption is that these would be very rare events. It currently appears that when multiple alleles do occur, they are still very similar to each other overall, and would likely not produce false positive results.

Future directions. This method is potentially very useful for the study of the natural distribution *Acanthamoeba*, and addressing the issue of low sensitivity in future modifications of the protocol will enhance its utility. The reverse dot blot method will allow researchers to avoid the problem of culture bias, permitting evaluation of the naturally predominant ribotypes. It has been shown that microbes recovered through laboratory culture techniques are not necessarily those prevalent in the environment (Lim et al. 1999). One hypothesis about the pathogenicity of acanthamoebae has been that all isolates can potentially cause infections, but members of the T4 ribotype are more prevalent in the environment and are therefore encountered more frequently. Culture enrichment methods indicate that the *Acanthamoeba* T4 ribotype is ubiquitously distributed, but whether it is actually dominant in the environment remains unknown. Currently the reverse dot-blot is being used to analyze sediment samples from coastal marine environments over a seasonal cycle in order to determine how the distributions of acanthamoebae change throughout the year. A comparison of the ribotypes before and after culture enrichment is also being accomplished to directly assess the bias introduced by enrichment culture.

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