JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1999, p. 2687–2693 Vol. 37, No. 8
0095-1137/99/$04.00+0

Fluorescent Oligonucleotide Probes for Clinical and Environmental Detection of *Acanthamoeba* and the T4 18S rRNA Gene Sequence Type

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Received 30 November 1998/Returned for modification 18 February 1999/Accepted 28 April 1999

The first genus- and subgenus-specific fluorescent oligonucleotide probes for in situ staining of *Acanthamoeba* are described. Sequences of these phylogeny-based probes complement the 18S rRNA and the gene encoding it (18S rDNA). The genus-specific probe (GSP) is a fluorescein-labeled 22-mer specific for *Acanthamoeba* as shown here by its hybridization to growing trophozoites of all 12 known *Acanthamoeba* 18S rDNA sequence types and by its failure to hybridize with amoebae of two other genera (*Hartmannella vermiformis* and *Balamuthia mandrillaris*), two human cell lines, and two bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*). The sequence type T4-specific probe (ST4P) is a rhodamine-labeled 30-mer specific for *Acanthamoeba* 18S rDNA sequence type T4, as shown here in hybridization tests with trophozoites of all 12 sequence types. T4 is the subgenus group associated most closely with *Acanthamoeba* keratitis (AK). GSP also was tested with corneal scrapings from 17 patients with a high index of clinical suspicion of AK plus 5 patient controls. GSP stained both trophozoites and cysts, although nonspecific cyst wall autofluorescence also was observed. Results could be obtained with GSP in 1 to 2 days, and based on results from cell culture tests, the probe correctly detected the presence or absence of *Acanthamoeba* in 21 of 24 specimens from the 22 patients. The use of GSP with cultured trophozoites and cysts from corneal scrapings has illustrated the suitability of using fluorescent oligonucleotide probes for identification of the genus *Acanthamoeba* in both environmental and clinical samples. In addition, the use of ST4P with cultured amoebae has indicated the potential of oligonucleotide probes for use in subgenus classification.

The genus *Acanthamoeba* consists of small, ubiquitous amoebae that exhibit a biphasic life cycle consisting of a vegetative trophozoite stage and a physiologically static cyst stage. These amoebae have been isolated from a variety of environmental sources (7) and are associated with human infection (17, 24–26, 37, 39). In immunocompromised patients, *Acanthamoeba* infections include granulomatous amoebic encephalitis (GAE), which is a fatal brain disease, and disseminated infections of various other tissues. In otherwise healthy individuals, the prominent disease is *Acanthamoeba* keratitis (AK), a potentially sight-threatening eye infection. This disease was discovered in 1973 (19, 27). More than 500 cases of AK had been reported in the United States by 1993 (31), and another 200 cases had been reported by 1997 (25). AK is most prevalent among contact lens (CL) wearers (34). A recent prospective population-based study estimated that the incidence of AK among soft-CL wearers in Scotland was 1 in 6,710 (32). In other countries—for example, India—AK usually is associated with non-CL-associated ocular trauma (33).

Identification of acanthamoebae in ocular and other tissues can be difficult and time-consuming, even for trained microscopists. In histological preparations, these amoebae look very similar to keratoplasts as well as neutrophils and monocytes, and this often leads to false-negative and/or false-positive results. It has been estimated that up to 70% of clinical AK cases are misdiagnosed as viral keratitis (2, 12, 18). Thus, many patients are initially treated with inappropriate drug therapies.

Early detection of *Acanthamoeba* infection is important because trophozoites, which predominate in the initial stages of infection, are more susceptible to treatment than the subsequent cysts. Calcafluor white (42) and similar fluorescent stains, which often are used to detect *Acanthamoeba*, stain cyst walls and fail to detect trophozoites. The mean time to diagnosis of AK can average 2.5 weeks longer for non-CL wearers than for CL users (6). This lag time may hamper disease resolution, since several studies have found that diagnosis and treatment within 1 month of onset results in lower morbidity and a better visual outcome (1, 2, 38). Thus, the availability of a rapid, accurate, and relatively simple diagnostic test would significantly enhance the initiation of appropriate chemotherapy.

Several RNA- and/or DNA-based methods are being developed to aid in the detection and accurate identification of *Acanthamoeba* in clinical and environmental settings. These include PCR with and without DNA sequencing (16, 23, 35, 40), restriction fragment length polymorphism analysis (5, 36), and nucleic acid blotting methods (9). Although these techniques are important diagnostic aids, they involve indirect evaluation of samples. They are unable to distinguish different developmental stages of *Acanthamoeba* spp. and lack precision in the localization of the organisms in situ. These methods cannot determine whether the majority of amoebae in a sample are cysts or trophozoites; they also cannot discern the density of the infection or the depth of corneal penetration of

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TABLE 1. *Acanthamoeba* strains used in testing the genus- and T4-specific probes[a]

Strain	Source	18S rDNA sequence type
Acanthamoeba species strain V006[a]	CDC0981:V006	T1
A. palestinensis GE 3a[a]	ATCC 50252	T2
A. palestinensis 1501/3c[a]	CCAP 1501/3c	T2
A. griffini S7	ATCC 30731	T3
A. griffini Panola Mt.[a]	ATCC 30487	T3
A. castellanii Neff	ATCC 50373	T4
A. castellanii V042	ATCC 50493	T4
A. castellanii V014	ATCC 50492	T4
A. castellanii Ma	ATCC 50370	T4
Acanthamoeba species strain Diamond[a]	CDC Diamond	T4
A. polyphaga V029	CDC0884:V029	T4
A. rhysodes Singh	ATCC 30976	T4
Acanthamoeba species strain 88-2-37	ATCC 50497	T4
A. lenticulata PD_2S	ATCC 30841	T5
A. palestinensis 2802	ATCC 50708	T6
A. astronyxis Ray & Hayes	ATCC 30137	T7
A. tubiashi OC-15c	ATCC 30867	T8
A. terricola FS	F. Schuster	T9
A. culbertsoni Lilly A1	ATCC 30137	T10
A. hatchetti BH-2	T. Sawyer	T11
A. heayli V013	CDC1283:V013	T12

[a] Species name has been revised according to the nomenclature of Stothard et al. (35).

the amoebae. Traditional histological techniques, in trained hands, often can identify amoebae in situ, but this depends on morphology or immunoreactivity. Furthermore, structural characteristics for identification of acanthamoebae at the subgenus level are unreliable.

The most specific and sensitive technique devised to date for in situ staining is phylogenetic staining, first used by DeLong and coworkers (8) for identification of single cells in samples of mixed species. This technique uses oligonucleotide probes complementary to phylogenetically informative segments of rRNA. Different probes can provide accurate identification of an organism at various levels of taxonomic organization. In addition, specific probes labeled with different chromophores can be used in fluorescent in situ hybridization (FISH) to differentiate the different species or other taxonomic levels in a mixed sample.

The present article reports the development and use of two fluorescent probes. The first identifies all members of the genus *Acanthamoeba* (genus-specific probe [GSP]); the second is specific for the subgenus group that is most commonly identified in AK infections (sequence type T4-specific probe [ST4P]). We have used these probes for identification of *Acanthamoeba* in laboratory cultures and in human corneal scrapings from patients presumed to have AK based on traditional methods of microscopy and follow-up cultures.

MATERIALS AND METHODS

Cell cultures and corneal scrapings. The in situ hybridization of the modified universal probe (MUP), GSP, and ST4P described below was tested with cultured trophozoites from 21 strains (Table 1) representing the 12 different *Acanthamoeba* 18S rDNA sequence types previously described (35). Cultures were maintained at Ohio State University (OSU) as described previously (4, 10). GSP also was tested with 24 human corneal scraping specimens obtained in a study of microbial keratitis in Scotland (32). *Acanthamoeba terricola* was a gift from Frederick Schuster, Brooklyn College, Brooklyn, N.Y. Human U937 lymphocytes and human HeLa cells were provided by the OSU laboratories of Ing-ming Chiu and Mark Muller, respectively. The amoeba *Balamuthia mandrillaris* and the *Acanthamoeba* strains prefixed by CDC in Table 1 were gifts from Govinda S. Visvesvara of the U.S. Public Health Service's Centers for Disease Control and Prevention, Atlanta, Ga. *Hartmannella vermiformis* and *Acanthamoeba palestinensis*, originally from the Cambridge Collection of Algae and Protozoa (see Table 2), were provided by Peter H. H. Weekers, University of Nijmegen, Nijmegen, The Netherlands. The remainder of the *Acanthamoeba* cultures (see Table 2) were obtained from Thomas Nerad at the American Type Culture Collection, Manassas, Va. Bacterial specimens were from laboratory cultures at OSU.

The corneal scrapings examined here were isolated by two of us (J.H. and D.V.S.) during a population-based longitudinal study of microbial keratitis in western Scotland (32). Scrapings were placed in sterile isotonic saline after being obtained from the eye by an ophthalmologist. Unstained aliquots were examined by bright-field or phase-contrast microscopy. This was followed by culture of samples for *Acanthamoeba* in a medium described previously (15). One to 19 months after the initial specimen collection, portions of the original scraping samples were delivered to OSU, where they were retested for growth in culture and analyzed with the GSP probe described in this paper.

Pretreatment and fixation of amoebae and other cells for FISH. All solutions, with the exception of culture media, were prepared with diethylpyrocarbonate-treated double-distilled water (DEPC water). Portions of the human corneal scrapings (see Table 2) that had been suspended in sterile saline and stored at 4°C for 1 to 19 months were fixed. One milliliter of a laboratory cell culture or 100 μl of a scraping specimen was spun in a Microcentrifuge (Costar model 10) for 15 s to loosely pellet the cells. The medium was pipetted off, and the cells were resuspended by gentle shaking in 1× phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO4 in DEPC water; pH 7.2). The cells were then loosely pelleted again by centrifugation for 15 s. The wash buffer was pipetted off, and the cells were gently resuspended in 1 ml of cold (4°C) 12% freshly depolymerized paraformaldehyde (PFA). The PFA was made by dissolving 6 g of paraformaldehyde in 40 ml of 1× PBS for 10 min at 60°C with stirring. Then 10 ml of 0.1 M NaOH in DEPC water was added to clear the solution, and the mixture was chilled before being used. The cells were fixed at 4°C for at least 5 h and to a maximum of overnight. After being fixed, the cells were loosely pelleted by a 15-s centrifugation. The PFA was pipetted off, and the cells were washed in 1 ml of DEPC water with gentle shaking. The cells were loosely pelleted again by a 15-s centrifugation. The DEPC water was pipetted off, and the cells from cultures or corneal scrapings were resuspended in ~100 or ~30 μl, respectively, of 70% ethanol and stored at 4°C indefinitely.

Probes. Three oligonucleotide probes containing a fluorochrome at the 5′ end were synthesized by Amitof Biotech, Inc. (Boston, Mass.). The 21-base MUP (5′-rhodamine-GWATTACCGCGGCTGCTGGCA-3′) was complementary to positions 653 to 633 in the 18S rRNA of *Acanthamoeba castellanii* Neff (13). The 22-base GSP (5-fluorescein-TTCACGGTAAACGATCTGGGCC-3′) was complementary to positions 957 to 936 in the Neff strain rRNA. The 30-base ST4P (5′-rhodamine-GCTGCCAAAACCAACTGAAAATAGGAGGAC-3′) was complementary to positions 1066 to 1037 in the Neff strain rRNA. MUP is based on the universal probe designed by Giovannoni and coworkers (11), with three modifications: base 14 was changed from K to T, a G between bases 16 and 17 was removed, and a GCA triplet was added to the 3′ end. Probes were resuspended in DEPC water to a final concentration of 1 μg/ml and stored at −20°C. Immediately before hybridization, probes were diluted to 30 ng/ml in DEPC water.

Whole-cell hybridization. Cells fixed in suspension were pipetted onto poly(L-lysine)-coated microscope slides (Sigma, St. Louis, Mo.) in 2- to 5-μl aliquots and air dried. The slides were then incubated in a methanol-formalin mixture (9:1) at room temperature for 20 min. The slides were then rinsed briefly in DEPC water and allowed to air dry. Thirty microliters of a hybridization mixture containing 60 ng of each probe in hybridization buffer (0.9 M NaCl, 20 mM Tris [pH 7.8], 5 mM EDTA, 0.01% sodium dodecyl sulfate) was pipetted onto each dry slide and covered with a 24- by 50-mm coverslip. The slides were immediately transferred to a dark chamber humidified by a sponge soaked in DEPC water and incubated at 50 to 52°C for no more than 2 h of hybridization. Temperatures above 52°C led to a reduction in the fluorescence signal for all probes used.

After hybridization, the coverslips were floated off in DEPC water at room temperature and the slides were washed in wash buffer (30 mM NaCl, 4 mM Tris [pH 7.8], 1 mM EDTA) at 50°C for 20 min in the dark. The slides were rinsed quickly in DEPC water at room temperature and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) for 10 min at room temperature in the dark. The slides were next washed briefly in DEPC water and allowed to air dry in the dark. Coverslips were mounted on dry slides by using 1 drop of Antifade Light mounting medium (Molecular Probes, Eugene, Oreg.) and permanently sealed with nail polish. The slides were stored indefinitely in the dark at 4°C. The preparations were examined with an inverted microscope (Zeiss Axioscope, Oberkochen, Germany) fitted for epifluorescence detection with a high-pressure mercury bulb and filter sets CZ902 (for DAPI), CZ 915 (for rhodamine), and CZ 909 (for fluorescein).

RESULTS

Use of MUP with trophozoites. MUP was used here as a control that would stain all organisms tested in the FISH analyses. It was derived from the universal probe, designed by Giovannoni et al. (11), which originally was made to hybridize

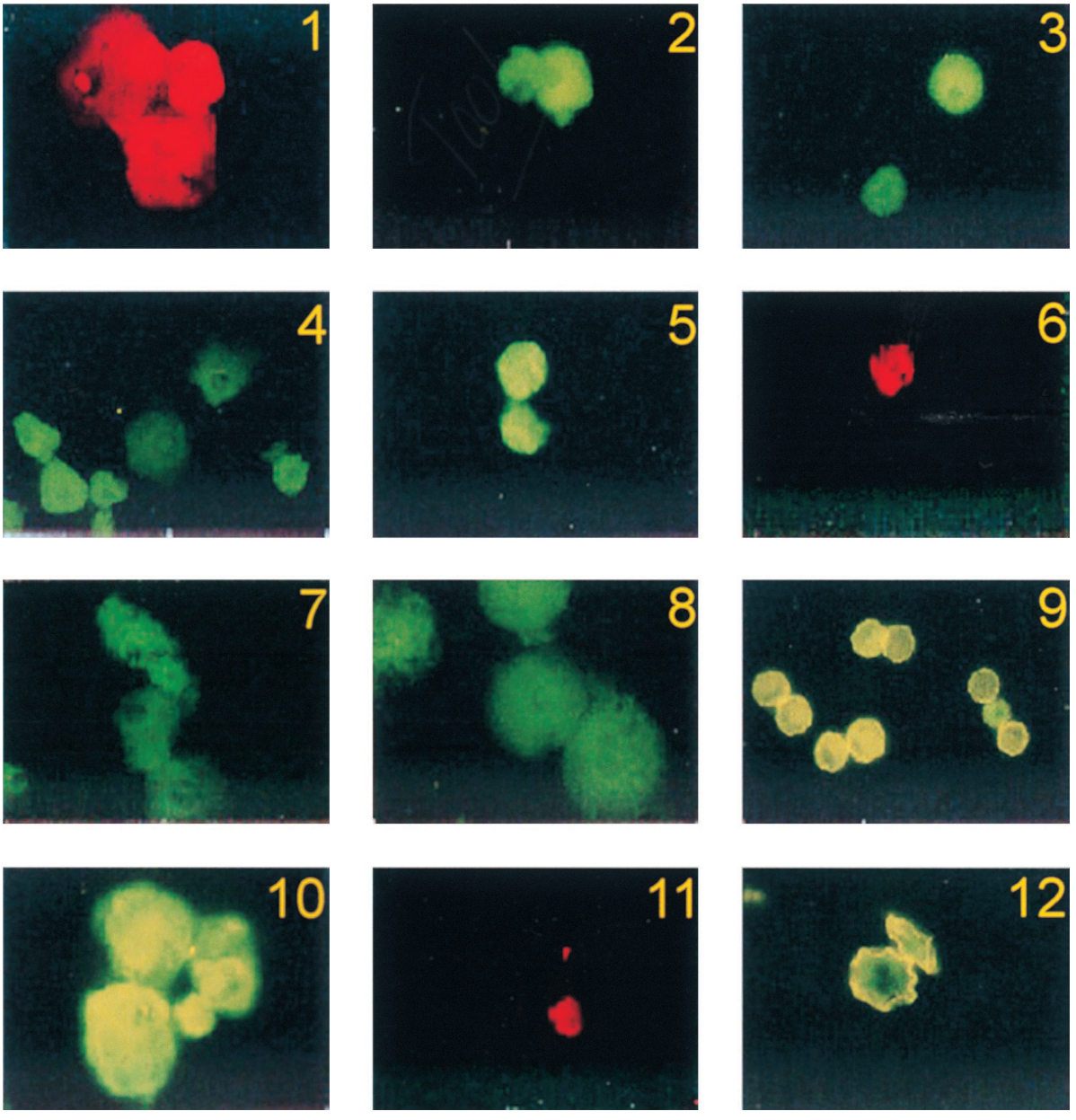

FIG. 1–12. In situ hybridization of MUP, GSP, and ST4P with seven sequence types of *Acanthamoeba* and with its closest relative, the genus *Balamuthia*.
FIG. 1. One amoeba of *A. castellanii* V014 (T4) (upper right) and two amoebae of the larger *B. mandrillaris* V139 are stained red by MUP.
FIG. 2. Same field as Fig. 1, but only *A. castellanii* V014 is stained green with GSP.
FIG. 3. GSP stained amoebae of *Acanthamoeba* species strain V006 (T1) green.
FIG. 4. *A. palestinensis* 1501/3c (T2) amoebae were stained green by GSP.
FIG. 5. GSP stained amoebae of *A. griffini* Panola Mt (T3) (bottom amoeba) and *A. castellanii* V014 (T4) (top amoeba) green.
FIG. 6. Same field as Fig. 5, but only *A. castellanii* V014 (T4) is stained with ST4P. Amoebae stained with this probe appear red because it has the same fluorochrome as MUP.
FIG. 7. GSP stained *A. lenticulata* PD_2S (T5) amoebae.
FIG. 8. *A. tubiashi* OV-15c (T8) amoebae were stained by GSP.
FIG. 9. GSP stained *Acanthamoeba* species cysts from a corneal scraping.
FIG. 10. Amoebae of *A. terricola* FS (T9) (the three large amoebae) and *A. castellanii* V014 (T4) (the single smaller amoeba) were stained by GSP.
FIG. 11. Same field as Fig. 10, but only *A. castellanii* V014 (T4) is stained red with ST4P.
FIG. 12. Two sectioned cysts of *A. castellanii* stained with GSP and showing green amoebae within yellow cyst walls.

to all three domains of life: archaebacteria, eubacteria, and eukaryotes. In the present study, however, the probe of Giovannoni and coworkers did not provide a satisfactory signal due to the higher-stringency conditions used. Modification of two sites to be more consistent with the eubacteria and eukaryotes used in the present study and addition of 3 bases at the 3′ end led to a 100% match between MUP and targets and an unambiguously positive red fluorescent signal from all cells tested. For example, hybridization of MUP with *B. mandrillaris* and *A. castellanii* is illustrated by the red stain in Fig. 1.

Use of GSP with trophozoites. Trophozoites double stained with MUP and GSP were used in tests for determining the specificity of GSP. Representatives from all 12 previously identified sequence types of *Acanthamoeba* (35) (Table 1), two amoebae relatively closely related to the genus *Acanthamoeba* (*H. vermiformis* [41] and *B. mandrillaris*), bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*), and human cells (U937 and HeLa) were tested for hybridization with these two probes. As indicated above, MUP hybridized with *Acanthamoeba* and all control cells. GSP hybridized with all acanthamoebae tested but with no control organisms. The differential staining of MUP and GSP is illustrated in Fig. 1 and 2, which include a mixture of one relatively small acanthamoeba of sequence type T4 and two larger *Balamuthia* amoebae. With the rhodamine filter (Fig. 1), all three amoebae are red, indicating hybridization with MUP. With the fluorescein filter (Fig. 2), only the smaller amoeba is green, indicating that GSP hybridized specifically to *A. castellanii*. Evidence that GSP hybridizes with divergent sequence types of *Acanthamoeba* is illustrated by the green staining of amoebae of seven sequence types that had been hybridized with this probe, as shown in Fig. 3 (T1), 4 (T2), 5 (T3 and T4), 7 (T5), 8 (T8), and 10 (T9 and T4). See below for identification of T4 amoebae in Fig. 6 and 11.

Use of ST4P with trophozoites. The specificity of ST4P was tested by using cells doubly stained with GSP and ST4P. Amoebae from each of the 12 described 18S rRNA gene (rDNA) sequence types of *Acanthamoeba* (Table 1) plus the bacteria, human cells, and other amoebae described above were used. ST4P successfully hybridized with all acanthamoebae of the T4 sequence type, but not with acanthamoebae of any of the other sequence types or with control cells. The degree of specificity is best observed in Fig. 5 and 6, which illustrate differential staining of the two most closely related sequence types, T3 (*A. griffini* Panola Mt.) and T4 (*A. castellanii* V014). In Fig. 5, amoebae of both sequence types appear green with the fluorescein filter, indicating that both types have hybridized with GSP. In Fig. 6, however, only the amoeba of sequence type T4 appears red with the rhodamine filter, indicating that it alone has hybridized with ST4P. The specificity of ST4P also is seen in Fig. 10 and 11, in which staining with this probe and with GSP is compared for T4 (*A. castellanii* V014) and a representative of a more distantly related sequence type, T9 (*A. terricola* FS). Both amoebae are green in Fig. 10, indicating that both hybridized with GSP, but only the smaller *A. castellanii* is red in Fig. 11 and, thus, was able to hybridize with ST4P.

Use of GSP with cysts from corneal scrapings. Hybridization of GSP with intact cysts from a corneal scraping is illustrated by the green stain in Fig. 9. In addition, *Acanthamoeba* cyst walls autofluoresced either in the presence or in the absence of hybridization conditions. Attempts to quench the fluorescence with a number of different agents failed. Nevertheless, the fluorescein filter set did distinguish between the yellow autofluorescence of the cyst walls and the green fluorescence attributable to hybridization of the fluorescein-labeled GSP to the rRNA and rDNA of the encysted amoebae. This distinction is best seen in sectioned cysts from the human cornea (Fig. 12).

To test the usefulness of GSP with corneal scrapings, we employed 24 specimens obtained from 22 patients, 17 with either confirmed AK or a high index of suspicion of AK and 5 with non-*Acanthamoeba* keratitis or conjunctivitis (Table 2). Bright-field and phase-contrast microscopy were used with wet films to evaluate the specimens. Fifteen scrapings produced positive *Acanthamoeba* cultures at Tennent Institute of Ophthalmology (TIO), OSU, or both institutions. It was observed, however, that wet-film microscopy detected acanthamoebae in only 6 (40%) of the 15 culture-positive specimens.

Microscopy using FISH with GSP was then used to evaluate the corneal scraping specimens. Acanthamoebae were detected in 12 (80%) of the 15 culture-positive samples. Overall, FISH results were consistent with the positive or negative culture results for 21 (88%) of the 24 specimens. The only discrepancies were three FISH-negative specimens (no. 1a, 17, and 23) that were considered culture positive even though positive results were obtained at only one of the two institutions (see Discussion).

DISCUSSION

The major advantage of using fluorescent oligonucleotide probes and FISH for identification of *Acanthamoeba* is that such probes have the potential to simultaneously detect and classify amoebae in situ. FISH provides a means of rapid, unequivocal identification of *Acanthamoeba* in cases of AK for which clinical diagnosis is putative and the appropriate expertise for identification by bright-field and/or phase-contrast microscopy is unavailable. As we have seen here, even when that expertise is available, the number of false negatives can be relatively high in the absence of FISH unless the observations are backed up by tests for culture growth. However, culture may take a week or more to yield satisfactory division of amoeba, especially if the cornea has been exposed to a range of antimicrobial or antiviral drugs prior to the procurement of the scraping specimen.

Although the examples of GSP and ST4P staining described in this paper have used amoebae growing in culture or in corneal scrapings, we also have been able to detect *Acanthamoeba* in sectioned human cornea (Fig. 12) (20).

In these studies, successful culture of a specimen at both TIO and OSU provided the highest level of confidence that viable acanthamoebae were present in an original scraping. However, successful culture at either one of the two institutions also was considered strong evidence that amoebae were originally present in the scraping. This is because the amoebae in the scrapings we examined were encysted. In our experience, negative culture results due to failure of excystment are relatively common whereas positive culture results due to accidental contamination with acanthamoebae are relatively rare. Thus, in the present study, the observance of an occasional failure of culture growth for scrapings that included cysts was not surprising. However, obtaining positive cultures from scrapings that did not originally include amoebae was highly unlikely. Therefore, in our analysis, all specimens giving rise to cultures at TIO, OSU, or both institutions were considered to have included acanthamoebae in the original scrapings. In some cases, positive cultures might have been obtained from samples with very few amoebae. Because FISH was performed on the original samples, small numbers of cells could have resulted in negative FISH results. On this basis, FISH with GSP detected *Acanthamoeba* in 80% of the culture-positive samples, and FISH and culture results were consistent for 88% of all samples. The comparable values are slightly higher if the FISH results, which were all obtained at OSU, are separately compared with culture results from each of the two laboratories. In this case, FISH detected amoebae in 86% (12 of 14) of culture-positive samples, and FISH and culture results were consistent for 92% (22 of 24) of all samples tested at OSU. At TIO, the values were 92% (11 of 12) and 92% (22 of 24), respectively. If comparisons of FISH and cell culture results are limited to the 20 specimens for which positive or negative culture results were identical at TIO and OSU, there was 100%

TABLE 2. Comparison of bright-field and phase-contrast microscopy, culture growth, and FISH evaluations of corneal scrapings as follow-up to clinical presumption of AK

Scraping code no.[a]	Date of scraping (mo/day/yr)	Clinical diagnosis[b]	Acanthamoebae detected by microscopy of wet films[c]	Acanthamoebae cultured from scrapings[d]	Acanthamoebae detected by GSP
1a[e]	06/06/95	Typical AK	−	−/+	−
1b[e]	06/07/95	Typical AK	+	+/+	+
6	06/27/95	Typical AK	+	+/+	+
4	07/06/95	Typical AK	−	+/+	+
12	07/10/95	Typical AK	+	+/+	+
2	09/13/95	Typical AK	−	+/+	+[p]
13	10/02/95	Typical AK	+	+/+	+
9	10/17/95	Typical AK	+	+/+	+
10	02/05/96	Typical AK[f]	−	+/+	+[q]
19	?	Atypical AK[g]	−	+/+	+
18	12/03/94	Atypical AK[h]	−	+/+	+
8	09/18/95	Atypical AK[i]	−	−/+	+[p]
5	10/12/95	Atypical AK	−	+/+	+
15[a]	08/12/95	Typical AK[j]	ND	−/−	−
7	09/07/95	Typical AK[k]	−	−/−	−
16[a]	09/22/95	Typical AK[k]	ND	−/−	−
23	10/23/95	Typical AK	−	+/−	−
3a[k,l]	10/25/95	Typical AK	−	−/−	−
3b[k,l]	10/25/95	Typical AK	−	−/−	−
21	05/09/95	Non-AK BKN[m]	−	−/−	−
22	10/03/95	Non-AK MK[n]	−	−/−	−
14	12/14/95	Non-AK C[i]	−	−/−	−
11	01/24/96	Non-AK MK	ND	−/−	−
17	05/24/96	Non-AK BKS[o]	+	−/+	−

[a] All samples except no. 15 and 16 were fresh corneal scrapings transferred to sterile isotonic saline. The exceptions are scrapings originally spread on non-nutrient agar plates and then recovered from plate washings.
[b] C, conjunctivitis; MK, presumed microbial keratitis of bacterial origin; BKN, bacterial keratitis due to *Neisseria meningitidis*; BKS, bacterial keratitis due to *Staphylococcus aureus*.
[c] Bright-field or phase-contrast microscopy of original wet films. +, acanthamoebae detected; −, acanthamoebae not detected; ND, not done.
[d] Culture results at TIO/OSU.
[e] Scrapings 1a and b were from the same patient.
[f] Severe AK after 3 weeks on chlorhexidine therapy.
[g] Trachoma and acanthamoebae in a non-CL wearer (30).
[h] Large epithelial defect, sporadic diffuse corneal endotheliitis thought to be due to herpes simplex virus in a non-CL wearer.
[i] CL-associated conjunctival inflammation without keratitis.
[j] Patient was on Brolene and neomycin therapy at time of scraping; acanthamoebae found in CL storage case.
[k] Patient was on topical 0.02% chlorhexidine therapy at time of scraping; acanthamoebae found in CL storage case.
[l] Both eyes of same patient; acanthamoebae found in CL storage case.
[m] Basement membrane dystrophy.
[n] Red eye, pain, and photophobia in a CL wearer.
[o] Peripheral ulcer in a CL wearer due to *S. aureus*, with transient acanthamoebae in the scraping sample (28).
[p] One cyst seen.
[q] Two cysts seen.

agreement. Thus, FISH results were most consistent with culture results when there was no ambiguity in the latter.

The data indicate that FISH with GSP clearly improves the accuracy of microscopy for detection of *Acanthamoeba*. This method produced no false positives and only three false-negative results among the 24 scrapings. The most likely explanation for the false negatives is that viable amoebae, although present, were too sparse to be picked up by FISH. This problem probably could have been overcome by the use of a more effective method of cell concentration. The FISH result for specimen 1a (Table 2) appears to be a false negative since amoebae were successfully cultured at OSU and because specimen 1b, obtained from the same eye on the next day, was culture positive by all methods used, including FISH.

The FISH results for scraping specimens 17 and 23 also appear to be false negatives because amoebae were cultured successfully at either TIO or OSU and because PCR results to be discussed elsewhere (31a) indicated the presence of acanthamoebae in both scrapings. Specimen 17 is a special case. The clinical diagnosis was bacterial keratitis, and the acanthamoebae detected by wet-film microcopy and cell culture were identified as transients present in the scraping. This result emphasizes that detection of *Acanthamoeba* should not be the only criterion used for a diagnosis of AK. Finally, the FISH result for scraping specimen 8 appears to be a true positive because the presence of acanthamoebae also is supported by positive cell culture and PCR results at OSU.

Two important advantages of FISH are the unambiguous identification of the organisms and the relative rapidity with which results can be obtained. Identification with FISH can be accomplished in 1 to 2 days, whereas cell culture time is variable and can take up to several weeks. It is recommended, however, that all scrapings, especially those that give negative FISH results, be cultured. Any amoebae that grow can then be tested by FISH with GSP to determine whether they are acanthamoebae.

The availability of a large number of DNA sequences for the nuclear small-subunit rRNA gene (35) has made it possible to design cytological stains that are specific for the genus *Acanthamoeba* and for separate lineages within this genus. We plan to develop a set of probes that could be used to identify the amoebae at the subgenus level. ST4P, which identifies 18S

rDNA sequence type T4, is the first of these probes to become available. The specificity of this probe has been demonstrated for trophozoites in this study but remains to be demonstrated for cysts. We focused on this sequence type for the design of a probe mostly because of its apparent importance in AK. Our previous sequence studies (35) plus 18S rDNA sequences obtained for corneal scrapings used in the present study (31a) indicate that 29 of the 30 AK isolates we have examined to date have T4 sequences. The only exception is a T3 isolate (22), and this sequence type is very closely related to T4. Another reason for concentrating on the T4 probe is because we have 18S rDNA sequence information for many more T4 strains than for any other sequence type. The greater amount of information available about sequence variation for this lineage permitted the design of a more robust probe. ST4P hybridizes to a region of the 18S rRNA gene that differs among the sequence types. Thus, this region may be a good target for other probes designed to be specific for other sequence types. However, more information on sequence variation in most of these other types is required before a complete set of robust sequence type-specific probes can be developed.

The FISH protocol was designed to be both specific and sensitive. The cells tested included axenic cultures of trophozoites and corneal scraping specimens that mostly included cysts. GSP worked very well with either developmental stage, but ST4P was used only with multiplying amoebae. Amoebae actively growing in cultures gave the brightest signal, most likely because of a high rRNA content in the cytoplasm. However, amoebae that have been stored in isotonic saline for 1 to 19 months, as was the case for some of the corneal scraping specimens, also were detectable. The fact that this technique works on stored samples is important because there can often be some delay between the collection of a scraping and its analysis in a specialized laboratory. The success of FISH with older stored samples is not surprising because the amoebae were present as cysts. It previously has been shown that encysted acanthamoebae can remain viable in CL saline for 14 to 90 days (3). In the present study, however, cysts remained viable during refrigeration in isotonic saline for at least 19 months, as shown, for example, by scraping sample 18 (Table 2), which was collected 3 December 1994 and still produced a culture at OSU in early July 1996.

The probes were designed to hybridize with 18S rRNA in addition to the large number of rDNA copies in the nucleolus in order to maximize the number of intracellular targets. Probe specificity at the level of sequence type was tested only with trophozoites because interpretation of results from cysts might be complicated by the autofluorescence of the cyst walls. It should be possible to alleviate this problem by using colorimetric detection of a nonfluorescent tag such as digoxigenin, which has been used with probes for *Acanthamoeba* 26S rRNA (21).

The probes have a number of potential applications in addition to those tested in the present study. For example, they can detect amoebae in deparaffinized tissue sections (Fig. 12). Also, Paillasson and coworkers (29) have recently developed a procedure for in situ hybridization of FISH probes to RNA in living human cells. The cells were permeabilized with streptolysin O and probed without fixation. They then were detected with a fluorescence-activated cell sorter. We recently proved a linkage between a patient with AK, the CL storage case contents, and the patient's home water supply (22). Thus, it might be possible to use FISH probes in a fluorescence-activated cell sorter format for prophylactic studies of water from treatment plants, home water supplies, or other sources.

Although this report has focused on AK, *Acanthamoeba* also is responsible for GAE. Two of the specimens tested in this study, *Acanthamoeba healyi* V013 and *A. castellanii* V006, are isolates from patients with GAE. This disease has been increasing in incidence due to its association with AIDS (14). Hawley et al. (14) recently reported the ability to identify *Acanthamoeba* in cerebrospinal fluid via culturing. Nearly all reported cases of GAE have been fatal, but early diagnosis, possibly using FISH on cerebrospinal fluid, might improve the odds of successful treatment.

The level of specificity required in probes will depend on their use. In some cases, it may only be necessary to distinguish between organisms that are known to cause a particular infection and those that do not. For studies of biodiversity, a more varied set of probes might be needed. The large collection of 18S rDNA sequences now available at GenBank plus the sequence alignments available on the Internet (3a) should be consulted by those wishing to design other probes. In addition, since cysts can be the major component of clinical samples, methods of suppressing the autofluorescence of cyst walls or the use of nonfluorescent markers for the genus- and sequence type-specific probes will be explored.

ACKNOWLEDGMENTS

The molecular biology studies described in this report were funded by Public Health Service grant EY09073 from the National Eye Institute.

We thank our colleague Gregory Booton for helpful discussions and assistance with the figures.

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