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Comparison of Axenic and Monoxenic Media for Isolation of *Acanthamoeba*

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***Acanthamoeba* is a genus of ubiquitous, free-living amebae that can be difficult to isolate by standard microbiologic techniques. We retrospectively reviewed the laboratory records of patients with ocular acanthamoebic infection for the period from January 1973 to June 1996 and found that *Acanthamoeba* isolates were recovered from 73, 71, and 70% of clinical specimens inoculated onto buffered charcoal-yeast extract agar (BCYE), nonnutrient agar with live or dead *Escherichia coli*, and tryptic soy agar (TSA) with horse or sheep blood, respectively. We then prospectively compared the recovery of a corneal isolate of *Acanthamoeba* on commercial media from Remel and BBL (TSA with 5% sheep blood, TSA with 5% horse blood, TSA with 5% rabbit blood, V agar, chocolate agar, BCYE, and selective BCYE with polymyxin B, anisomycin, and vancomycin) and on axenic and monoxenic media prepared with live or dead bacteria (*Enterobacter aerogenes*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia*). Good recovery of trophozoites was obtained on BCYE, TSA with rabbit blood, TSA with horse blood, and Remel TSA with sheep blood. BBL TSA with horse blood or rabbit blood provided good recovery of cysts. All species of live or dead bacteria yielded good recovery of trophozoites; however, only nonnutrient agar with live *P. aeruginosa*, live *E. aerogenes*, or live *S. maltophilia* gave good recovery of cysts. TSA with either rabbit blood or horse blood, BCYE, and nonnutrient agar prepared with live *P. aeruginosa*, *E. aerogenes*, or *S. maltophilia* offer optimal recovery of *Acanthamoeba*.**

Acanthamoeba is a genus of ubiquitous, free-living protozoa that causes keratitis, encephalitis, and cutaneous and other lesions. Acanthamoebae are often grown on nonnutrient agar spread with *Escherichia coli* or *Enterobacter aerogenes* (14); the trophozoites move over the agar surface, ingest the bacteria, and leave characteristic trails (23). These free-living amebae also survive on other bacteria, yeasts, and mammalian cells at optimal cultivation temperatures of 23 to 37°C (8). Although *Acanthamoeba* seems to prefer certain genera of bacteria as food sources, previous studies comparing these preferences are inconsistent (1, 5, 11, 19, 20).

Bacteria-free media have been developed for axenic culture and include peptone-yeast extract-glucose and chemically defined media (7, 14, 18, 22). Acanthamoebic trails have been detected on blood agar (9), and *Acanthamoeba* has been shown to preferentially phagocytize erythrocytes of different animal species (17). The relative efficacies of these various media for the isolation of *Acanthamoeba* have not been determined.

We performed a retrospective review of our laboratory records of patients with ocular acanthamoebic infections to compare the recovery of *Acanthamoeba* from clinical specimens on various media. We then prospectively evaluated the recovery of a corneal isolate of *Acanthamoeba* on commercially available media that are routinely used in clinical microbiology laboratories. Since in our clinical experience we noted differences in recovery on media from two different manufacturers, we included media from both sources. Recovery on axenic and monoxenic nonnutrient agars that incorporated various bacterial genera was also evaluated.

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MATERIALS AND METHODS

Clinical record review. We reviewed our laboratory records for the period from January 1973 to June 1996 for all ocular specimens that yielded growth of *Acanthamoeba*. We studied only patients with culture-positive specimens and included repeat cultures for these patients only if cysts were seen on a paired smear or if at least one medium was positive for *Acanthamoeba*. The percentage of positive cultures detected on a particular medium was calculated by the following equation: (number of positive cultures divided by the number of cultures tested on that medium) × 100.

Clinical isolate. A strain of *Acanthamoeba* isolated in our laboratory was used to compare recovery on various media. The patient was a 15-year-old female who rubbed her eye while wearing a rigid gas-permeable contact lens 6 weeks prior to diagnosis and was initially misdiagnosed as having herpetic keratitis. Cysts were seen on a smear of corneal scrapings stained with calcofluor white (Polysciences, Inc., Warrington, Pa.), and acanthamoebae were recovered within 3 days on tryptic soy agar (TSA) with horse blood with GCHI (a supplement containing hemin and NAD) and on buffered charcoal-yeast extract agar (BCYE; Remel, Lenexa, Kans.). The strain was identified as *Acanthamoeba* by using a genus-specific oligonucleotide probe and was further characterized as a small-subunit ribosomal DNA sequence type 4 strain that belongs in group II of the Pussard and Pons morphologic classification schema of *Acanthamoeba* which includes *A. castellanii*, *A. polyphaga*, *A. lugdunensis*, and *A. rhysodes* (2, 3).

Preparation of inoculum. The isolate was grown in modified Neff's (8) peptone-yeast extract-glucose broth in tissue culture flasks (Corning Costar, Corning, N.Y.) at 35°C in an ambient atmosphere for approximately 2 weeks to yield suspensions containing at least 95% trophozoites. These flasks were then incubated approximately 3 weeks longer at room temperature in atmospheres with increased CO_2 levels to yield suspensions containing at least 90% cysts. Hemacytometer counts were performed, and the suspensions were diluted to yield 1.0×10^3 to 1.2×10^3 organisms per ml. Six 25-μl aliquots containing 25 to 30 organisms each were placed onto two plates of each type of agar.

Media. Commercially available media were obtained from BBL (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and Remel and included TSA with 5% sheep blood, TSA with 5% horse blood, TSA with 5% rabbit blood, V agar (5% human blood), chocolate agar, BCYE, and selective BCYE with polymyxin B, anisomycin, and vancomycin (BCYE/PAV). *E. aerogenes* ATCC 13048, *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Serratia marcescens* B1523 (corneal isolate), *Staphylococcus aureus* ATCC 25923, and *Stenotrophomonas maltophilia* ATCC 13637 were used

TABLE 1. Percent recovery of *Acanthamoeba* from clinical specimens inoculated onto various media

Medium[a]	No. positive/ total no.	% Positive
BCYE	16/22	73
Nonnutrient agar with:	34/48	71
Dead *E. coli*	28/41	68
Live *E. coli*	6/7	86
Blood agar[b]	55/79	70
Horse blood agar	50/68	74
Sheep blood agar	4/10	40
Lowenstein-Jensen agar	2/4	50
Sabouraud agar with gentamicin	12/27	44
Trypticase soy broth with gentamicin	3/7	43
Anaerobic blood agar	6/16	38
Chocolate agar	13/39	33
Brain heart infusion broth with gentamicin	2/17	12
Thiol or thioglycolate broth	3/28	11

[a] Specimens were corneal scrapings inoculated directly onto media or corneal buttons or biopsy specimens processed in a tissue grinder and inoculated onto media. One specimen was inoculated into tissue culture medium, which was negative for *Acanthamoeba* at 6 weeks. *Acanthamoeba* was also recovered from two specimens inoculated onto cornmeal agar.

[b] One specimen was inoculated onto rabbit blood agar, which was positive for *Acanthamoeba* at 3 days.

to prepare axenic and monoxenic nonnutrient agar plates. The bacteria were suspended in saline and autoclaved at 121°C for 15 min for the axenic media; monoxenic media were prepared by using suspensions of live bacteria. The densities of the autoclaved and live suspensions were adjusted spectrophotometrically to approximately 1.5×10^9 cells per ml. Agar plates were prepared by adding 0.5 ml of the standardized bacterial suspension to 20 ml of 1.5% tap water agar with methylene blue and pouring this mixture into a sterile plastic petri dish (15 by 100 mm; Fisher Scientific Co., Pittsburgh, Pa.). Axenic and monoxenic media were inoculated on the day of preparation.

Incubation and observation of media. The plates were incubated at 35°C in an ambient atmosphere and were observed for amebic trails under a Bausch & Lomb stereomicroscope with an American Optical illuminator (Leica, Deerfield, Ill.). The commercial media were observed every 12 h for 3 days and then daily for 11 days, and when cysts were studied, the plates were observed again at the end of an additional week of incubation. The axenic and monoxenic nonnutrient media were observed daily for 7 days for trophozoites and 14 days for cysts and at the end of an additional week of incubation for both.

Statistical analysis. The number of trophozoites that migrated outside the inoculum edge was counted; the total number of acanthamoebae that produced trails was counted when cysts were inoculated onto commercial media because no inoculum edge was visible. The daily recovery was defined as the number of trophozoites producing trails at each day of observation divided by the original inoculum number. Good recovery was arbitrarily defined as 50% of the original inoculum number by day 7 for trophozoites and by day 14 for cysts. The number of inoculation sites that became too difficult to count due to the length and crisscrossing of the trails was noted. Counting of trails was not performed after the number was twice the original inoculum or greater. An analysis of variance (ANOVA) with repeated measures over time and a *t* test (unpaired) were performed to compare recovery rates on axenic and monoxenic media. ANOVA results were used to rank order the media, and significant differences were determined by Fisher's protected least significant difference for time.

RESULTS

Clinical record review. Ninety cultures of specimens from 40 patients with culture-proven keratitis ($n = 39$) or conjunctivitis ($n = 1$) were included in our review (Table 1). Acanthamoebae were recovered from 16 (73%) of 22 specimens inoculated onto BCYE. No statistically significant difference was found among BCYE, nonnutrient agar with live or dead *E. coli*, and blood agars. There was no significant difference between nonnutrient agar with live or dead *E. coli* ($P = 0.7$), and recovery was slightly better on horse blood agar than sheep blood agar ($P = 0.06$).

Comparison of commercially available media. Trophozoites demonstrated maximal growth on Remel BCYE and BBL TSA with rabbit blood, with final mean recoveries of 91 and 87%, respectively ($P = 0.87$). Good recovery of trophozoites was obtained on Remel TSA with horse blood, Remel TSA with horse blood with GCHI, Remel BCYE/PAV, BBL TSA with horse blood, Remel TSA with sheep blood, and Remel TSA with rabbit blood (Fig. 1 and 2). Although recovery was less

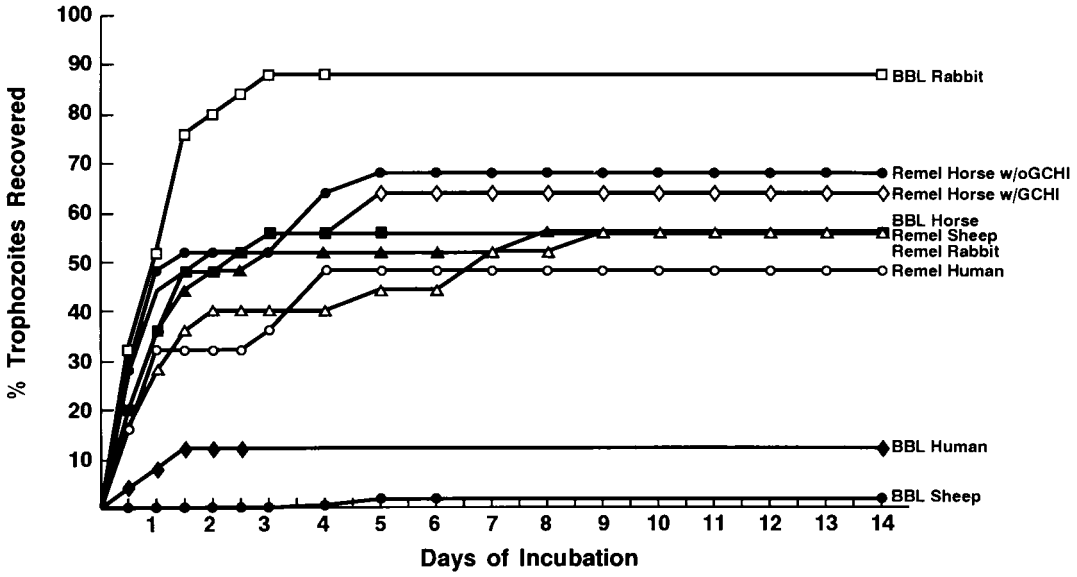

FIG. 1. Percentage of trophozoites recovered on blood agars over 14 days.

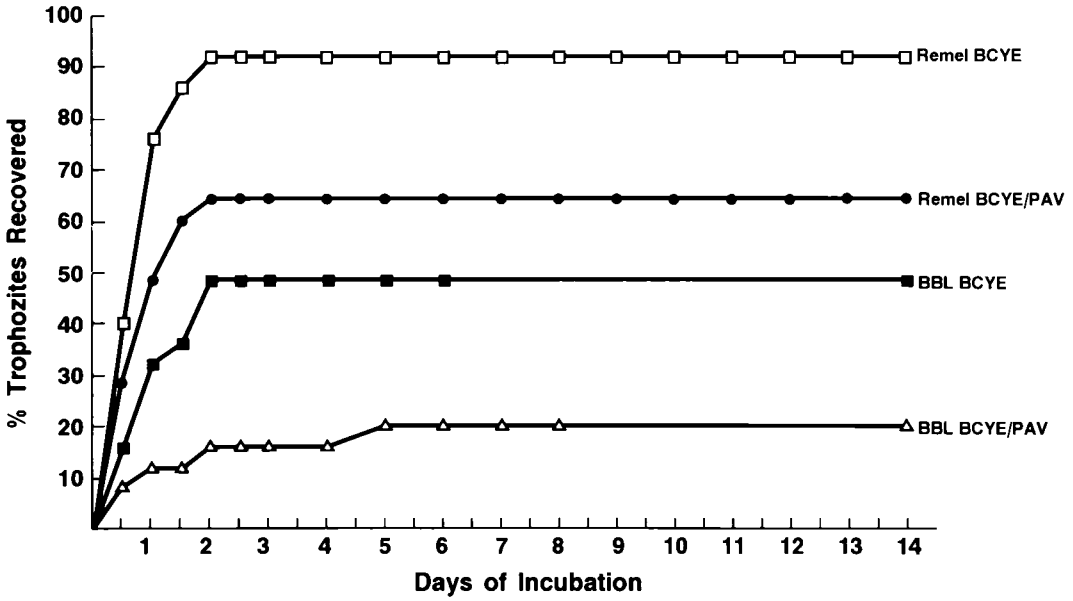

FIG. 2. Percentage of trophozoites recovered on charcoal agars over 14 days.

than 50% on BBL BCYE, all six inoculation sites became too difficult to count by day 7. Growth was poorest on BBL V agar and BBL TSA with sheep blood. The rank order of the commercially available media for the recovery of trophozoites is presented in Table 2.

Cysts demonstrated good recovery only on BBL TSA with horse blood and BBL TSA with rabbit blood (Fig. 3 and 4). Thirteen (43%) of 30 sites inoculated onto Remel TSA with sheep blood, Remel TSA with rabbit blood, Remel BCYE, Remel BCYE/PAV, and BBL BCYE became too difficult to count by day 21. Growth was poorest on BBL V agar and BBL TSA with sheep blood. The rank order of the commercially available media for the recovery of cysts is presented in Table 2.

Comparison of nonnutrient agars with bacteria. Trophozoites produced trails on all nonnutrient media incorporating the various genera of live and dead bacteria, and the number of trails on media with live *P. aeruginosa* and live *E. aerogenes* was equal to 100% or more of the original inoculum within 24 h of inoculation. By day 2, the mean recovery was greater than 50% on all media. Although media with live *P. aeruginosa*, *E. aerogenes*, *S. maltophilia*, and *S. marcescens* all demonstrated a final recovery of 100% or greater, maximal growth occurred on

TABLE 2. Comparison of commercial media for recovering trophozoites and cysts over 14 days

Medium (manufacturer)	Trophozoites			Cysts		
	Rank[a]	Mean ± SD[b]	% Recovery ± SD[c]	Rank	Mean ± SD	% Recovery ± SD
BCYE (Remel)	1	22 ± 6	91 ± 25	3	11 ± 4	43 ± 11
BCYE (BBL)	9	12 ± 3	49 ± 11	8	9 ± 3	36 ± 9
BCYE/PAV (Remel)	3	16 ± 6	65 ± 24	9	9 ± 2	35 ± 7
BCYE/PAV (BBL)	12	5 ± 2	20 ± 11	11	4 ± 2	13 ± 5
Rabbit blood (Remel)	7	13 ± 3	55 ± 12	7	10 ± 4	36 ± 15
Rabbit blood (BBL)	2	21 ± 9	87 ± 40	1	15 ± 3	53 ± 4
Horse blood (Remel)	4	16 ± 4	67 ± 17	6	10 ± 2	36 ± 7
Horse blood (Remel) with GCHI	5	15 ± 2	63 ± 7	5	10 ± 4	36 ± 12
Horse blood (BBL)	6	14 ± 8	57 ± 32	2	13 ± 3	53 ± 9
Sheep blood (Remel)	10-11	12 ± 3	54 ± 13	4	10 ± 4	41 ± 12
Sheep blood (BBL)	15	1 ± 1	2 ± 3	15	0 ± 0	0
Human blood (Remel)	10-11	12 ± 3	49 ± 9	10	7 ± 2	26 ± 9
Human blood (BBL)	14	3 ± 4	11 ± 18	14	<1 ± 1	<1 ± <1
Chocolate (Remel)	8	13 ± 4	60 ± 15	12	3 ± 3	27 ± 7
Chocolate (BBL)	13	4 ± 5	18 ± 22	13	1 ± 2	10 ± 9

[a] The rank was determined by an ANOVA over time on the basis of the number of trails per inoculum site each day.
[b] The mean represents the weighted daily average of the number of trails per inoculum site each day.
[c] The percent recovery is the mean percentage of the original inoculum isolated (counted) on day 14 for trophozoites and day 21 for cysts.

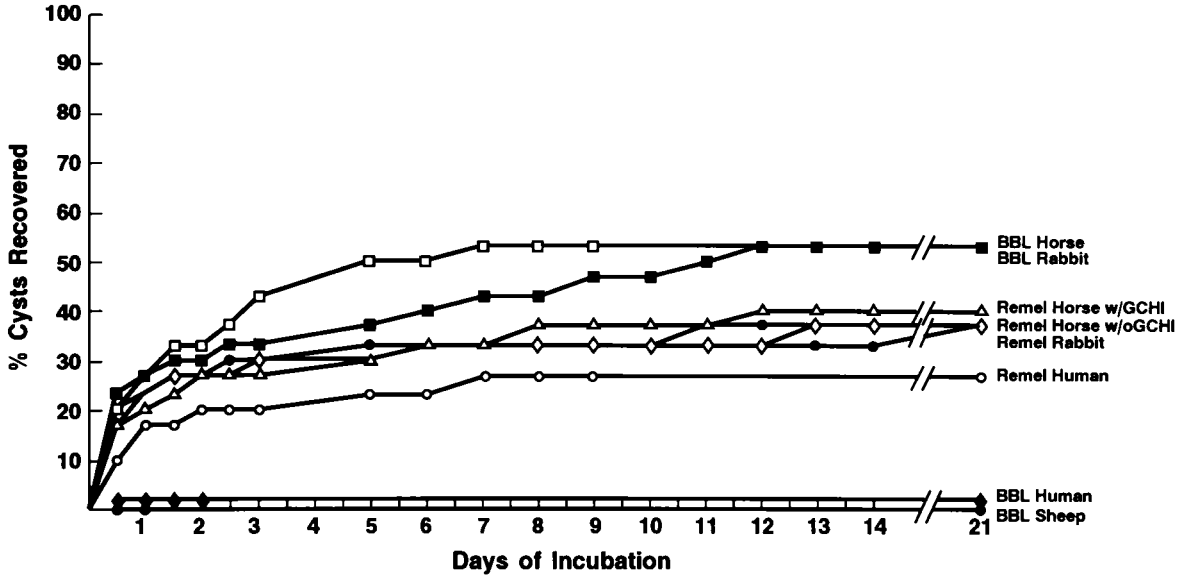

FIG. 3. Percentage of cysts recovered on blood agars over 21 days.

media with live *P. aeruginosa*, with a final recovery of more than double the original inoculum. Recovery on media with live *E. coli*, the species widely recommended for use in the isolation of *Acanthamoeba*, was significantly lower than those on media with all other live bacterial species ($P \leq 0.03$) except *K. pneumoniae* (Fig. 5). Recovery was significantly higher on media with live or dead *S. aureus* than live or dead *E. coli* ($P \leq 0.03$). When recoveries on media with live or dead bacteria of the same species were compared, significantly higher numbers of trophozoites were recovered on media with live *P. aeruginosa* ($P < 0.0001$), live *E. aerogenes* ($P < 0.0001$), live *S. maltophilia* ($P < 0.0001$), and live *S. marcescens* ($P < 0.0001$); the differences in recovery between media containing live or dead *E. coli*, *K. pneumoniae*, and *S. aureus* were not significant ($P > 0.9$) (Fig. 6). The rank order of nonnutrient media with live or dead bacteria for the recovery of trophozoites is presented in Table 3.

Cysts demonstrated good recovery only on nonnutrient agar with live *P. aeruginosa*, *E. aerogenes*, *S. maltophilia*, and *S. marcescens*, and the difference among all four media was significant ($P < 0.0001$) (Fig. 7). Maximal growth occurred on media with live *P. aeruginosa*, with a final recovery of more than 200% by day 3; the number of trails on media with live *E. aerogenes* was equal to 100% of the original inoculum by day 4. Recovery of cysts on nonnutrient agar with heat-killed bacteria ranged from 2% on media with dead *S. aureus* to 8% on media with dead *S. maltophilia* (Fig. 8). When media with live or dead bacteria of the same species were compared, significantly higher numbers of cysts were recovered on media with live *P. aeruginosa* ($P < 0.0001$), live *E. aerogenes* ($P < 0.0001$), live *S.*

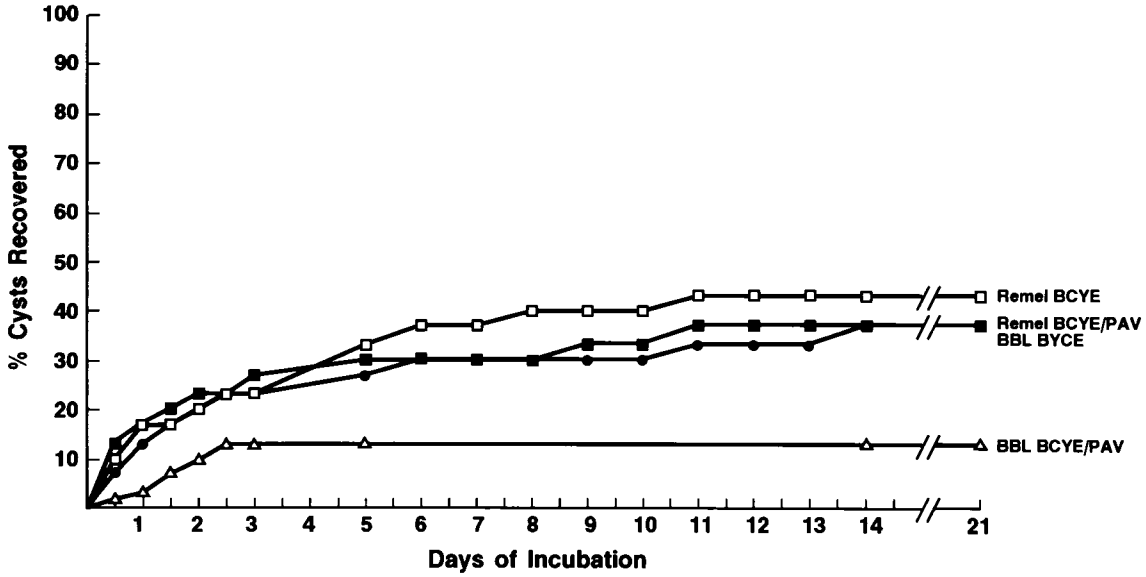

FIG. 4. Percentage of cysts recovered on charcoal agars over 21 days.

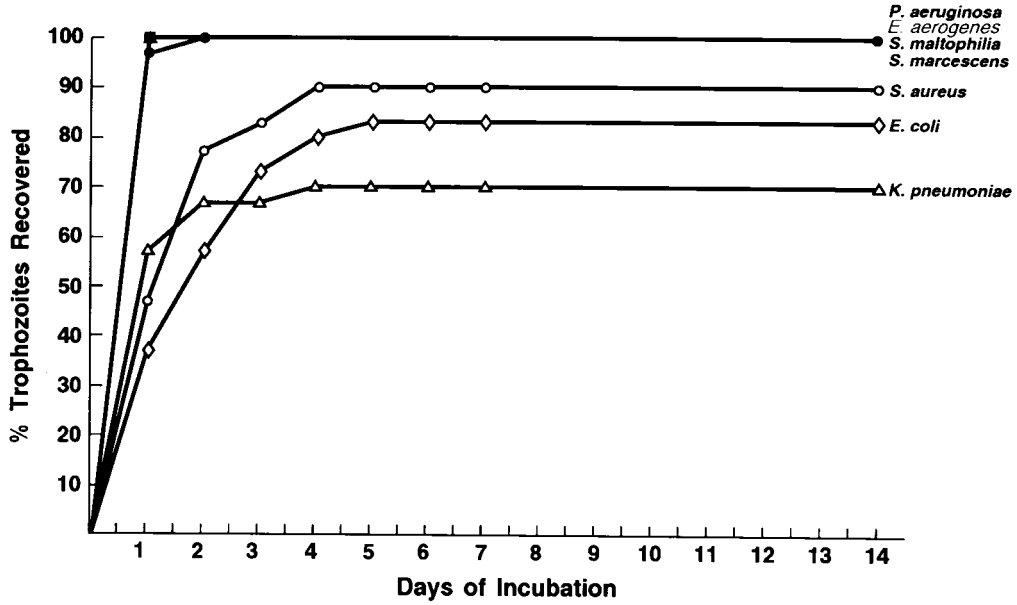

FIG. 5. Percentage of strain 1 trophozoites recovered on nonnutrient agar with live bacteria over 14 days. The maximum recovery illustrated is 100%.

maltophilia ($P < 0.0001$), live *S. marcescens* ($P < 0.0001$), and live *E. coli* ($P < 0.0001$); the differences in recovery between media containing live or dead *K. pneumoniae* ($P = 0.14$) and live or dead *S. aureus* ($P = 0.84$) were not significant. The rank order of nonnutrient media with live or dead bacteria for the recovery of cysts is presented in Table 3.

DISCUSSION

Isolation of *Acanthamoeba* from ocular and other specimens can be problematic. Laboratory confirmation of *Acanthamoeba* keratitis is important because acanthamoebic infection can resemble other causes of corneal disease and because treatment involves multiple drugs that are often used for an extended period and are potentially toxic. Isolation also allows identification and susceptibility testing to be performed; axenic isolation facilitates these procedures by eliminating the need to make a monoxenic culture axenic.

TSA with rabbit or horse blood offered good recovery of *Acanthamoeba* trophozoites and cysts. Trophozoites have been shown to preferentially phagocytize erythrocytes from different mammalian species. On the basis of the number of ingested erythrocytes, horse erythrocytes were most preferred, followed by guinea pig, rat, mouse, sheep, and human erythrocytes (17). Our data also indicated that sheep or human blood agar was not good for recovery of *Acanthamoeba*, especially with a cyst inoculum. Similar results were obtained with another corneal isolate that grew more slowly (15).

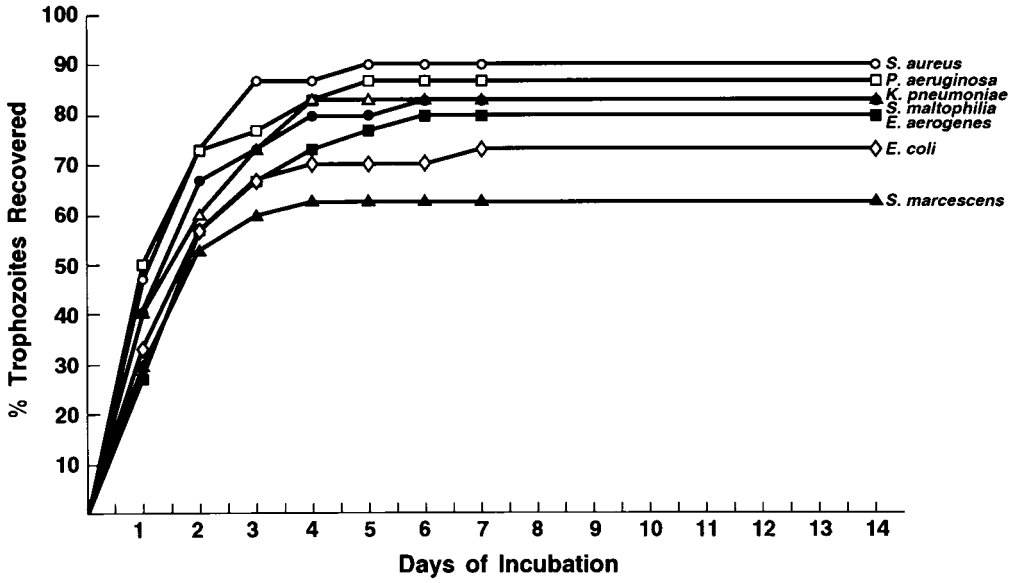

FIG. 6. Percentage of strain 1 trophozoites recovered on nonnutrient agar with dead bacteria over 14 days.

TABLE 3. Comparison of nonnutrient agar with live or dead bacteria for recovering trophozoites and cysts over 21 days

Nonnutrient agar with bacteria	Trophozoites			Cysts		
	Rank[a]	Mean ± SD[b]	% Recovery ± SD[c]	Rank	Mean ± SD	% Recovery ± SD
P. aeruginosa (live)	1	56 ± 11	>200 ± 0	1	53 ± 18	>200 ± 0
P. aeruginosa (dead)	7	24 ± 6	88 ± 21	9	2 ± 1	7 ± 5
E. aerogenes (live)	2	43 ± 11	145 ± 36	2	30 ± 26	119 ± 91
E. aerogenes (dead)	11	21 ± 7	80 ± 19	10	2 ± 2	6 ± 8
S. maltophilia (live)	3	34 ± 5	116 ± 15	3	21 ± 7	82 ± 21
S. maltophilia (dead)	8	22 ± 7	82 ± 20	7	2 ± 1	8 ± 4
S. marcescens (live)	4	30 ± 1	101 ± 3	4	13 ± 6	56 ± 17
S. marcescens (dead)	14	17 ± 7	64 ± 25	8	2 ± 1	7 ± 4
E. coli (live)	10	22 ± 9	84 ± 26	5	4 ± 5	17 ± 22
E. coli (dead)	13	20 ± 7	73 ± 24	12	1 ± <1	3 ± 1
K. pneumoniae (live)	12	20 ± 3	70 ± 12	6	3 ± 3	11 ± 11
K. pneumoniae (dead)	9	22 ± 6	83 ± 16	11	1 ± 1	3 ± 2
S. aureus (live)	6	25 ± 9	90 ± 27	13	1 ± 1	3 ± 4
S. aureus (dead)	5	25 ± 7	91 ± 18	14	<1 ± 1	2 ± 3

[a] The rank is determined by an ANOVA over time on the basis of the number of trails per inoculum site each day.
[b] The mean represents the weighted daily average of the number of trails per inoculum site each day.
[c] The percent recovery is the mean percentage of the original inoculum isolated (counted) on day 14 for trophozoites and on day 21 for cysts.

A serendipitous finding in our laboratory led to the use of BCYE for isolation of acanthamoebae. When a conjunctival biopsy specimen from a human immunodeficiency virus-infected child with granulomatous conjunctivitis was ground and inoculated onto various media, numerous trails appeared rapidly on BCYE (15). Our experimental study confirmed that BCYE gives excellent recovery and facilitates detection of *Acanthamoeba*. Most of the inoculum sites on BCYE became too difficult to count due to the length and crisscrossing of the trails.

The presence of particulate matter has been shown to increase the growth rate of *Acanthamoeba*. Erythrocytes and charcoal particles might stimulate phagocytosis and enhance trophozoite growth and cell division (6, 17). Media containing charcoal have been used to isolate *Entamoeba histolytica* from stool specimens; however, the function of the charcoal was believed to be the adsorption of oxygen to provide anaerobiosis and the adsorption of toxic bacterial metabolites rather than the direct stimulation of diapedesis and growth (12, 21). Our laboratory experience suggests that particulate-containing media such as BCYE are useful in isolating *Acanthamoeba* from clinical specimens.

All species of live or dead bacteria used in this study supported the growth of *Acanthamoeba* trophozoites on nonnutrient agar, but only live *P. aeruginosa*, *E. aerogenes*, *S. maltophilia*, and *S. marcescens* yielded good recovery of cysts. Live bacteria were, in general, better than heat-killed ones; none of the species of heat-killed bacteria offered good recovery of

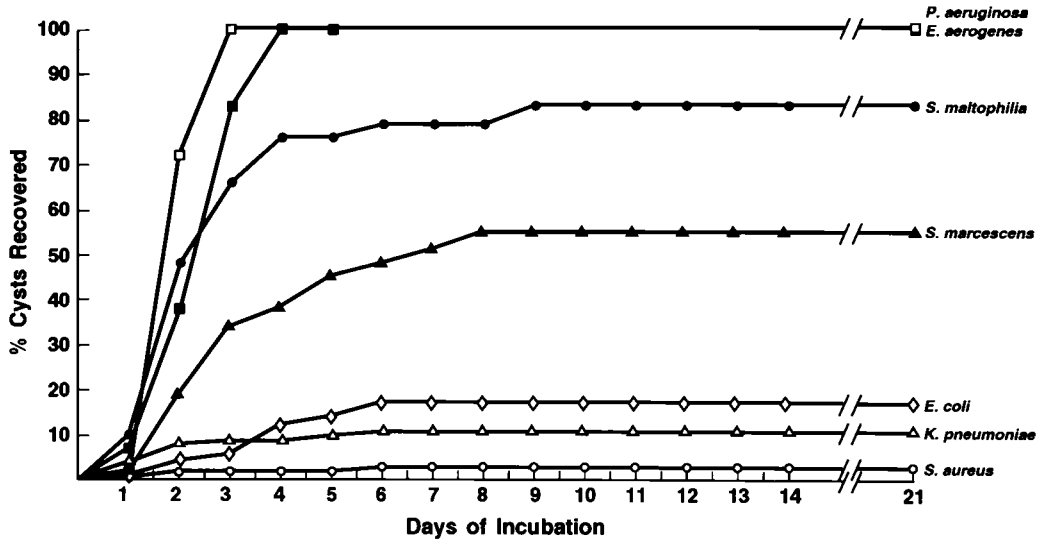

FIG. 7. Percentage of cysts recovered on nonnutrient agar with live bacteria over 14 days. The maximum recovery illustrated is 100%.

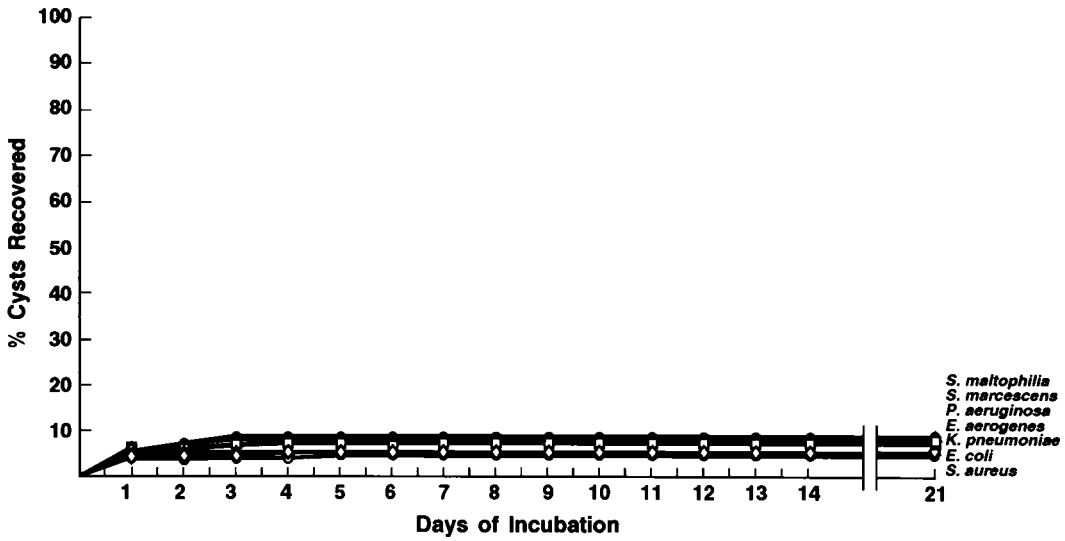

FIG. 8. Percentage of cysts recovered on nonnutrient agar with dead bacteria over 14 days.

cysts. Nonnutrient agar made with dead bacteria may offer adequate recovery of trophozoites, but it does not appear to stimulate excystment into the trophozoite stage.

Singh (20) found that amoebae prefer certain species of bacteria as a food source but noted that those producing toxic pigments, such as *P. aeruginosa* and *S. marcescens*, can be lethal to amoebae. In cocultivation studies with *Acanthamoeba* and several genera of bacteria, Bottone et al. (1) demonstrated that although *S. maltophilia* and *P. aeruginosa* adhered to trophozoites more readily than *E. coli*, only *S. maltophilia* was phagocytized; *P. aeruginosa* was rapidly amoebicidal. A sterile filtrate of a *P. aeruginosa* broth culture was also toxic (16). Although *P. aeruginosa* produces several exoenzymes that may be lethal to cells, pyocyanin, a blue pigment produced by some strains, is believed to play a major role in the toxin-mediated killing of *Acanthamoeba* (16, 20).

Contrary to the previous studies (1, 16, 20), we found that *P. aeruginosa* and *S. marcescens* permit excellent recovery of *Acanthamoeba*. The strain of *P. aeruginosa* used in this study is the one recommended for quality control for susceptibility testing and produced pyocyanin when it was tested in our laboratory. Another pyocyanin-producing strain of *P. aeruginosa* also afforded good recovery of trophozoites (15). The *S. marcescens* strain used in the study was a clinical isolate and it produced prodigiosin, the red pigment believed to be toxic to amoebae (20). Perhaps the amount of exotoxin produced on nonnutrient agar is insufficient to inhibit *Acanthamoeba*.

Some studies have suggested that amoebae prefer gram-negative bacteria, and more *Acanthamoeba* bind to contact lenses to which *P. aeruginosa* is attached than to lenses to which *Staphylococcus epidermidis* is attached (5); however, Singh (19) and Larkin and Easty (11) found no such preference. There was no significant difference between recovery of trophozoites on media with *E. coli* and *S. aureus* in the present study, but *S. aureus* did not induce excystment. Although nonnutrient agar with an *E. coli* overlay is an adequate method for the recovery of *Acanthamoeba*, *E. coli* inhabits a narrow ecological niche as part of the intestinal flora of humans and other animals (10). Other genera of the family *Enterobacteriaceae* are more widely distributed in the environment (10), and members of the family *Pseudomonadaceae* are usually associated with soil and water (4). An agar that incorporates a species of bacteria more closely associated with *Acanthamoeba* in nature may enhance recovery.

The results of the prospective study indicate that the optimal commercially available media for the recovery of *Acanthamoeba* from clinical specimens are TSA with rabbit blood, TSA with horse blood, and BCYE. This correlates with the results of the retrospective study of our laboratory experience in the isolation of *Acanthamoeba*. Although TSA with sheep blood is commonly used for routine cultures, many clinical microbiology laboratories have BCYE on hand for the isolation of *Legionella*, and this could be provided as a supplemental medium for specimen inoculation when *Acanthamoeba* infection is suspected. Excellent recovery of *Acanthamoeba* may be obtained with commercially available media, but in cases in which the amount of specimen is small or for previously treated patients, inoculation onto a nonnutrient medium with live gram-negative bacteria offers the best possibility of recovering *Acanthamoeba*.

ACKNOWLEDGMENTS

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