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Cultivation of Pathogenic and Opportunistic Free-Living Amebas

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INTRODUCTION

In contrast to gastroenteric infections caused by parasitic amebas as represented by *Entamoeba histolytica*, a relatively small number of infections are caused by free-living amebas, organisms normally found in soil or water. The genera included in this category are *Naegleria*, *Acanthamoeba*, and, more recently, *Balamuthia*. Representatives of these genera are pathogenic but not parasitic. They cause disease by being in the right place at the right time or by taking advantage of a host with impaired immune defenses. In other words, they are opportunistic pathogens. A number of reviews are available concerning the biology and pathogenic potential of these amebas (13, 18, 39, 40, 48, 50–52, 64, 70, 78).

Naegleria spp. as Infectious Agents

Naegleria is associated with primary amebic meningoencephalitis (PAM), a fulminating, rapidly fatal infection of the central nervous system (CNS). *Naegleria fowleri* is the causal agent of most PAM infections, but other species of *Naegleria* having pathogenic potential have been described (*Naegleria australiensis* and *Naegleria italica*). Currently, there are more than a dozen species of *Naegleria* that have been recognized based upon small subunit ribosomal DNA. The type habitat for *N. fowleri* is a natural or man-made lake, a thermally polluted body of water, or an inadequately chlorinated swimming pool where the amebas can feed upon bacteria and proliferate. With respect to humans, mostly children, teenagers, and young adults in good health are infected by swimming or washing in such waters, where amebas enter the nostrils, migrate along the olfactory nerves to the cribriform plate, and gain access to the CNS. Amebas proliferate rapidly and cause extensive damage to neural tissue (Fig. 1A). Diagnosis is difficult and is dependent upon recognition of the amebas in the cerebrospinal fluid; not infrequently, trophic amebas are dismissed as leukocytes. Because of the rapid onset, delayed diagnosis, destructive nature of the disease, and dearth of effective antimicrobial agents, death is an almost invariable consequence of infection.

Acanthamoeba spp. as Infectious Agents

Acanthamoeba spp. also infect the CNS, causing granulomatous amebic encephalitis (GAE). Much more so than *Naegleria*, *Acanthamoeba* is ubiquitous in the environment, with amebas being widely disseminated in soil and water. Unlike the healthy individuals acquiring *Naegleria* infections, persons con-

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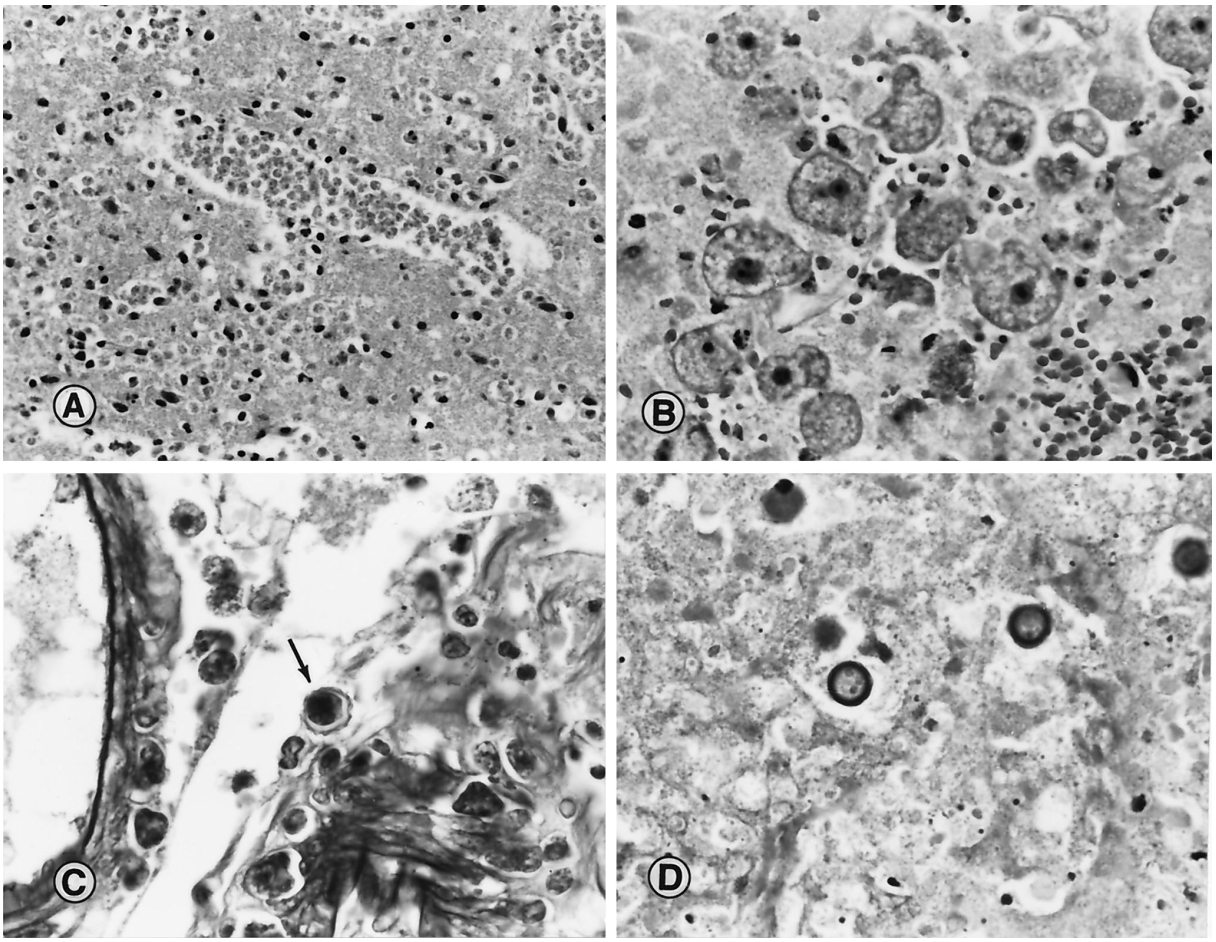

FIG. 1. (A) Section of cerebral cortex from a patient with PAM due to *N. fowleri*. A large cluster of trophic amebas is seen in the brain tissue. Magnification, ×210; original magnification, ×230. (B) Section of brain tissue from a patient with *Balamuthia* GAE. Typical trophic amebas are seen within the necrotic CNS tissue. Magnification, ×210; original magnification, ×230. (C) Tissue section showing cysts of *A. castellanii* within the wall of a blood vessel and the perivascular space. Note the double wrinkled wall seen around one of the cysts (arrow). Magnification, ×370; original magnification, ×400. (D) Cysts of *B. mandrillaris* in necrotic brain tissue from a patient with amebic encephalitis. Magnification, ×520; original magnification, ×560. (Micrographs copyright A. J. Martinez.)

tracting *Acanthamoeba* infections of the CNS are compromised hosts, suffering from concurrent diseases such as AIDS (30, 71) or other conditions such as alcoholism that predispose them to opportunistic infections. The portal of entry of ameba can vary. It may be intranasal, allowing amebas to migrate directly to the CNS, or entry can be via a break in the skin or through the respiratory tract, with subsequent spread of amebas to the CNS by a hematogenous route. The disease assumes a chronic status, leading to slow deterioration. Diagnosis is most often made by postmortem examination of brain tissue (Fig. 1C).

Another major class of infection caused by *Acanthamoeba* spp. is amebic keratitis. This condition was first noted in individuals suffering corneal trauma due to injury to the corneal surface that became infected with amebas (43, 49). More commonly, amebic keratitis occurs in contact lens users when, due to improper maintenance and poor sanitary precautions, amebas proliferate in the ophthalmic solutions or in the lens cases and are transferred to the corneal surface when the lens is inserted. These infections are localized, and amebic spread to the CNS from corneal sites has not been reported.

Balamuthia spp. as Infectious Agents

Balamuthia is another free-living ameba that causes GAE in humans and other animals (Fig. 1B and D). First described as an isolate from the brain of a pregnant mandrill baboon that died in a zoological park (76), *Balamuthia* infections have been reported in individuals with compromised health status, with AIDS patients among those diagnosed (3, 30), as well as in immunocompetent individuals. While this ameba undoubtedly occurs in nature, it has been isolated only from CNS tissue of individuals or animals that have died from the infection (see Addendum in Proof). Diagnosis in most cases has been made

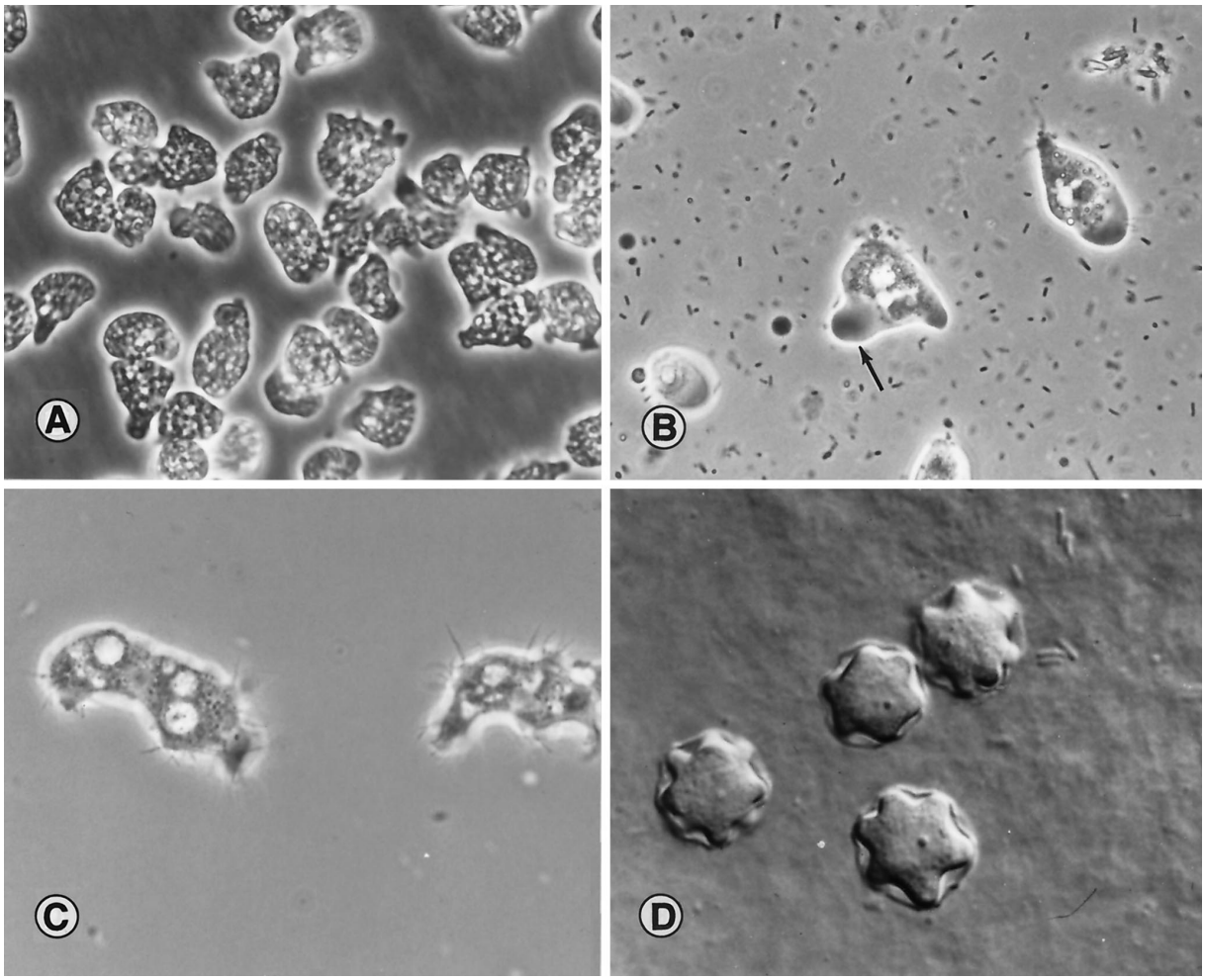

FIG. 2. (A) *N. fowleri*, from a patient with PAM, growing in axenic medium in tissue culture flasks. Amebas contain numerous fluid-filled vacuoles and appear larger than their counterparts growing on bacteria. The cultures were photographed in situ on an inverted microscope. Magnification, ×960; original magnification, ×1,000. (Micrograph copyright T. H. Dunnebacke.) (B) *N. fowleri* amebas in a wet-mount microscope slide. The prominent clear, ectoplasmic pseudopod is seen (arrow). Adjacent particles are bacteria. Magnification, ×960; original magnification, ×1,000. (C) Trophic *A. castellanii* ameba from culture. Amebas show the characteristic projecting pseudopods (acanthopodia) over the surface. Magnification, ×960; original magnification, ×1,000. (D) Interference-contrast image of cysts of *Acanthamoeba* sp. isolated from a human brain biopsy specimen. The cysts are on the surface of an agar plate. Magnification, ×1440; original magnification, ×1,500. (Micrographs B to D copyright G. S. Visvesvara.)

postmortem. The portal of entry of the ameba is not known but, as with *Acanthamoeba*, is likely to be through the nostrils or through breaks in the skin with hematogenous spread.

Aspects of *Naegleria* morphology are seen in Fig. 2A and B. Amebas growing in axenic culture fill with vacuoles containing culture medium (Fig. 2A). When feeding on bacteria, *Naegleria* amebas are more likely to exhibit the typical slug-like morphology of the genus, with a definite antero-posterior polarity (Fig. 2B). Trophic *Acanthamoeba* are recognized by the projecting pseudopods around the periphery of the ameba (Fig. 2C). Cysts of *Acanthamoeba* are surrounded by a thick wall and a stellate ameba within the cyst (Fig. 2D). The junction of the arm of the star and the cyst wall is the location of a pore, which is used when the cyst germinates, releasing the ameba. *Balamuthia* amebas, seen in Fig. 3, are less well-defined in their morphology. The shape of the amebas is dependent to a great extent on the manner of cultivation (cell-free medium or tissue culture feeder cells) and age of the culture; trophic forms show a high degree of pleomorphism.

Other Amebas as Infectious Agents

A recent report (B. B. Gelman, S. J. Rauf, R. Nader, V. Popov, J. Borkowski, G. Chaljub, H. W. Nauta, and G. S. Visvesvara, Letter, JAMA **285:**2450-2451, 2001) has identified *Sappinia diploidea*, a soil ameba, as the cause of amebic encephalitis in a human. The report suggested that the portal of entry was the respiratory tract and that, because the patient

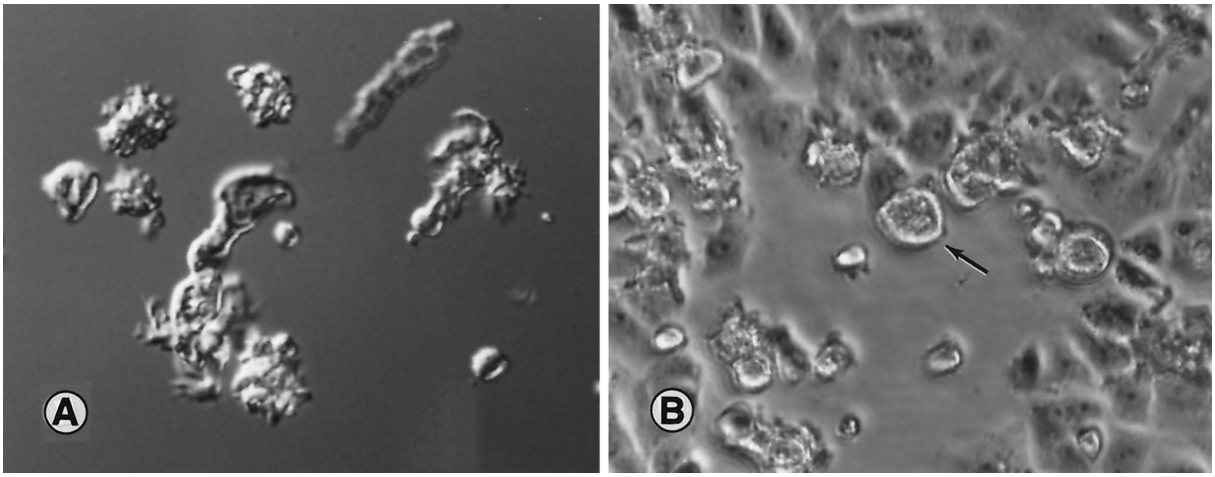

FIG. 3. (A) Interference-contrast micrograph of trophic *Balamuthia* amebas in a wet-mount preparation. The amebas are pleomorphic and are likely to show varied morphology depending on whether they are in a cell-free medium or feeding on tissue culture cells. Magnification, ×380; original magnification, ×400. (B) Light micrograph of monkey kidney cell monolayer in a tissue culture flask. The open area seen in the micrograph is where the cells have been fed upon by *Balamuthia* amebas (arrow), about 24 h after the amebas were inoculated into the flask. The photograph was taken in situ on the stage of an inverted microscope. Magnification, ×190; original magnification, ×200.

recovered, this ameba was probably less virulent than the other amebas described above. Given the increasing numbers of immunocompromised and debilitated hosts, it is likely that more of the free-living amebas will be recognized as opportunistic infectious agents.

GROWTH IN BACTERIZED OR XENIC CULTURES

General Guidelines

Naegleria spp. and *Acanthamoeba* spp. can be readily cultivated on either nonnutrient agar or agar media containing low concentrations of nutrients (e.g., peptone 0.05%, yeast extract 0.05%, glucose 0.1%) in the presence of living or killed bacteria. In general, the bacteria of choice include nonmucoid strains of *Klebsiella pneumoniae*, *Enterobacter* spp. (*Enterobacter aerogenes* and *Enterobacter cloacae*), and *Escherichia coli*. The presence of a mucoid capsule around bacteria appears to impede phagocytosis by amebas and leads to bacterial overgrowth of the ameba population. Nutrients, particularly glucose, enhance bacterial overgrowth and inhibit ameba proliferation. A number of studies have examined the suitability of different bacteria as food sources for soil amebas (see, for example, reference 80). *Balamuthia*, however, will not grow with bacteria as a food source but has been grown from brain tissue by providing tissue culture cells as a feeder layer (76). Some of the early attempts at axenic growth of the free-living amebas have made use of heat-killed bacteria as an intermediate stage between xenic and axenic cultivation (56).

Acanthamoeba and Endosymbionts

For reasons that are not known, *Acanthamoeba*, more so than other soil amebas, is often likely to harbor endosymbiotic bacteria. While their presence is not strictly related to cultivation of amebas, bacterial endosymbionts that have been detected in *Acanthamoeba* isolates are of considerable interest (24). These bacteria may have a role in promoting virulence of amebas (16, 26). *Acanthamoeba* has been implicated as a potential host for *Legionella* spp., the causal agent of legionellosis and Pontiac fever; *Legionella* sp. has been recently isolated from *Acanthamoeba* taken from soil samples (59). Other pathogenic or potentially pathogenic bacteria that have been described in associations with *Acanthamoeba* spp. are *Mycobacterium avium* (72), *Afipia felis* (B. La Scola and D. Raoult, Letter, Lancet **353:**1330, 1999), *Listeria monocytogenes* (47), *Burkholderia pseudomallei* (37), *E. coli* serotype O157 (8), *Chlamydia* spp. and *Chlamydia*-like bacteria (23, 25), and *Vibrio cholerae* (74).

GROWTH IN AXENIC CULTURES

General Guidelines

To various degrees, free-living amebas can be readily established in axenic culture from initially bacterized cultures by providing an enriched nutrient medium with antibiotics (penicillin-streptomycin and gentamicin) added to kill off contaminating bacteria. The basic nutrient medium that is used for *Acanthamoeba* typically contains peptone, yeast extract, and glucose, in concentrations generally higher than those used for growth in bacterized cultures (e.g., peptone 2.0%, yeast extract 0.5%, glucose 0.5%). For nonpathogenic *Naegleria* spp., the nutrient medium contains peptone and yeast extract, with or without glucose, with liver extract and calf serum added as additional supplements. *Balamuthia* is a more fastidious organism and requires a heavily supplemented basic medium (66). The three genera of amebas can be grown axenically in the presence of tissue culture monolayers. In each of these instances, the tissue culture cells provide a feeder layer for the actively phagocytic amebas—in reality a predator-prey relationship. It is worth noting that potentially pathogenic *Acan-*

TABLE 1. Undefined medium SCGYEM for cultivation of *N. fowleri*[a]

Component	Amt or concn
Organic	
Isoelectric casein	10.0 g
Glucose	2.5 g
Yeast extract	5.0 g
Fetal calf serum	100.0 ml
Penicillin-streptomycin	200.0 μg/ml (each)
Distilled water	900.0 ml
Buffering	
Na_2HPO_4	1.325 g
KH_2PO_4	0.8 g

[a] Medium SCGYEM was described previously by Chang (13) and De Jonckheere (21).

thamoeba organisms have been isolated from tissue cultures, where they are present as contaminants. In the course of growing poliomyelitis virus in monkey kidney cell cultures for vaccine preparation, Culbertson et al. (18, 19) observed areas of tissue culture monolayer destruction and subsequently isolated an ameba (later named *Acanthamoeba culbertsoni*) from dead and dying mice inoculated with culture supernatant.

Naegleria spp.

Long known as a nonpathogenic species with a life cycle comprised of a trophic ameboid stage, a nonfeeding and nondividing flagellate stage, and a dormant cyst stage, *Naegleria gruberi* amebas were established axenically in an enriched medium consisting of peptone or Proteose Peptone, yeast extract, liver concentrate (4), and additional supplements. These supplements included calf serum as Balamuth medium (4), killed bacteria (55, 60, 61, 63), chicken embryo extract (65), folic acid (46, 54), or hemin (7). It was somewhat surprising, therefore, to find that the pathogenic isolates, once they were recognized as such, required a simpler nutrient medium based on peptone and calf serum (10, 13, 57). Ultimately, pathogenic isolates were determined to be members of a new species, *N. fowleri*, with less demanding nutritional requirements for growth (Fig. 2A). For example, Cerva (10, 11) grew his isolates in a medium made up of Casitone and serum, a medium that would not support growth of *N. gruberi*. Willaert (85) used Cerva's original formulation and one supplemented with folic acid and biotin for growth of *Naegleria* for immunoelectrophoretic studies. A medium (Table 1) developed by Chang (13–15) was originally formulated for growth of nematodes, and while it supported growth of pathogenic isolates, it was not successful in growing the nonpathogenic *N. gruberi*. The medium consisted of casein, glucose, fetal calf serum, and fresh yeast extract (14). This medium was used successfully by De Jonckheere (21) for cultivation of *Naegleria* spp., pathogenic as well as nonpathogenic strains. Chang (13) also experimented with sheep blood and liver extract as additives to this basal medium. Another medium that was successful in supporting growth of pathogenic *Naegleria* was developed by Nelson (cited in references 83 and 86) and consisted of glucose (1.0 g), liver infusion or liver digest (2.0 g), fetal calf serum (50.0 ml), and distilled water (450.0 ml) (pH ≈ 6.5). (The medium was modified from its original formulation because some of the components are unavailable.) Haight and John (32) later compared growth of *N. fowleri* in Balamuth, Chang, Cerva, and Nelson media in agitated and unagitated cultures, concluding that optimal growth of amebas occurred with agitated cultures in Nelson medium. They also found variation in cell yields among 10 different isolates of *N. fowleri* growing in Nelson medium (32). John (40) reported cell yields of 3×10^9 amebas/ml were achieved with a generation time of 5.5 h. Cline et al. (17) developed a modification of Balamuth and Nelson media (40, 84, 86) that would support growth of both *N. gruberi* and *N. fowleri*, giving cell yields of about 10^6 amebas/ml. This medium contained Proteose Peptone, yeast extract, liver digest, and glucose and was supplemented with both calf serum and hemin (17, 50).

A partially defined medium based on Willaert's medium (84) was formulated by Cursons et al. (20). This medium contained Casitone, glucose, rutin, L-methionine, L-histidine, folic acid, thiamine·HCl, vitamin B_{12}, and hemin; generation times of both pathogenic and nonpathogenic isolates of *Naegleria* were about 15 h in this medium (20). Nerad et al. (58) developed a chemically defined medium (Table 2) that would support the growth of *N. fowleri*, *Naegleria lovaniensis*, and, with a 10-fold increase in the metals and several other changes, the more nutritionally exacting *N. australiensis*. They obtained cell yields of better than 10^6 amebas/ml with generation times of about 20 h.

Haight and John (33) found quantitative differences in growth of *N. fowleri* with 17 different types of sera added to Nelson medium. Calf, pig, monkey, newborn calf, and dialyzed calf sera gave growth of ca. 10^6 amebas/ml, while human and fetal calf sera, cerebrospinal fluid, and hemin gave less growth of 5×10^4 to 1×10^5 amebas/ml. John (40) suggested that the presence of iron in lakes and streams may be a factor in enhancing growth of *N. fowleri*. Enrichment of the medium with various lipid components at 100 μg/ml improved growth of amebas over the calf serum control (33).

Several studies have examined the relationship between in vitro cultivation and loss of virulence of *N. fowleri*. Hu et al. (34) found that loss of virulence correlated with a loss of pathogenic protein synthesis patterns in axenically grown amebas. Induction of gene activity correlating with virulence occurred when amebas fed upon tissue culture cells but not when they fed upon bacteria or when they were grown in axenic culture (35). Cultivation of amebas in the presence of cholesterol (100 μg/ml) over a 6-month period resulted in a loss of virulence (42). John (40) noted that virulence of *N. fowleri* is affected by growth temperature (30 to 37°C produced more virulent amebas), growth phase (late logarithmic to early stationary phase amebas were more virulent for mice), and strain.

Although *N. gruberi* is not a pathogenic ameba, it is of interest to contrast its nutritional requirements with those of pathogenic isolates. Growth parameters for *N. gruberi* growing in rotary cultures in an enriched medium were defined by Weik and John (84). They reported a biphasic pattern of logarithmic growth, with generation times of 7 and 19 h during the two phases. Cell yields were 5×10^6 amebas/ml.

Fulton and coworkers (27) prepared a semidefined medium and, later (28), a chemically defined medium that would support the growth of a variant strain of *N. gruberi* with a doubling

TABLE 2. Defined medium for pathogenic *Naegleria* spp.[a]

Component	Concn (mg/liter)
Amino acids	
L-Alanine	460.0
L-Asparagine	600.0
L-Aspartic acid	1,170.0
L-Arginine	940.0
L-Cysteine · HCl	40.0
L-Glutamic acid	1,865.0
L-Glutamine	600.0
Glycine	2,705.0
L-Histidine	200.0
L-Isoleucine	525.0
L-Leucine	760.0
L-Lysine · HCl	880.0
L-Methionine	970.0
L-Phenylalanine	480.0
L-Proline	1,200.0
DL-Serine	600.0
L-Threonine	530.0
L-Tryptophan	140.0
L-Tyrosine	180.0
L-Valine	700.0
Nucleic acid precursors	
Adenine	10.0
Adenosine 3′,2′-monophosphoric acid	15.0
Guanosine	10.0
Guanosine 3′,2′-monophosphoric acid	10.0
Hypoxanthine	10.0
Cytosine · H_2O	10.0
Cytidine 3′,2′-monophosphoric acid	25.0
Thymine	10.0
Thymidine	16.0
Uracil	10.0
Uridine 3′,2′-monophosphoric acid	15.0
Vitamins	
p-Aminobenzoic acid	1.0
Biopterin	0.01
D-Biotin	0.01
Choline chloride	1.0
Folic acid	1.0
Hemin	10.0
i-Inositol	1.0
Nicotinamide	1.0
Ca pantothenate	1.0
Pyridoxal · HCl	1.0
Pyridoxamine · diHCl	1.0
Riboflavin	1.0
Thiamine	1.0
DL-Thioctic acid	0.5
B_{12}	0.001
Salts and metals	
$CaCl_2$	40.0
$MgSO_4 \cdot 7H_2O$	800.0
KH_2PO_4	362.0
Na_2HPO_4	500.0
$FeCl_2 \cdot 4H_2O$	0.0105
$FeSO_4 \cdot 7H_2O$	0.0105
$MnSO_4 \cdot H_2O$	0.0095
$ZnSO_4 \cdot 7H_2O$	0.0220
$(NH_4)Mo_7O_{24} \cdot 4H_2O$	0.0036
$CuSO_4 \cdot 5H_2O$	0.0016
NH_4VO_3	0.00046
$CoSO_4 \cdot 7H_2O$	0.00048
H_3BO_3	0.00057
$NiSO_4 \cdot 6H_2O$	0.00045
$CrK(SO_4) \cdot 12H_2O$	0.00096
$BaCl_2 \cdot 2H_2O$	0.00050
Other	
D-Glucose	8,000.0
Na citrate	100.0
L-Ornithine.HCl	40.0
Putrescine.diHCl	1.0
Orotic acid	20.0
DL-Citruline	8.0
5-Sulfosalicylic acid	1.0

[a] This defined medium was described previously by Nerad et al. (58).

time of 8 to 19 h. The medium, however, would not support growth of the parent stock of the variant. Initially, peptone was replaced by L-methionine and a serum fraction was required. The defined medium contained 14 amino acids of which 11 were essential, six vitamins, glucose, hematin, guanosine, uracil, glycerol, and sodium pyruvate. This medium was pared down to eliminate components that were not essential for growth, with a consequent drop in yield from 5×10^6 to about 10^6 amebas/ml and a doubling time of 12 to 15 h (28).

Acanthamoeba spp.

Unlike the situation with *Naegleria* amebas, where there appears to be a difference in nutritional requirements between pathogens and nonpathogens, sharp distinctions are not evident with *Acanthamoeba* spp. For the most part, pathogenic and nonpathogenic species of *Acanthamoeba* grow well in the same media.

Acanthamoeba are better able to tolerate a range of growth conditions than *Naegleria* spp. They readily survive over a wide range of osmolarities, both in vivo and in vitro, having been isolated from marine and fresh waters, from tissue culture media where they occur as contaminants, and from soil. They appear to be nutritionally less exacting than *Naegleria* amebas, in that they readily go from bacterized to axenic cultures without the prolonged adaptation or selection that often occurs with *Naegleria*.

A basic medium that supports growth of *Acanthamoeba* spp. consists of Proteose Peptone or peptone, yeast extract, and glucose (PPYG or PYG, respectively). Neff (56) isolated a widely used strain of *Acanthamoeba castellanii* and grew it axenically in Proteose Peptone (0.75%), yeast extract (0.75%), and glucose (1.5%). What varies in most formulations for growth of *Acanthamoeba* are the concentrations of these components. Jensen et al. (38) used the basic medium components with rotary agitation to obtain cell yields of *A. castellanii* of 3×10^7 amebas/ml, with a 6-h generation time.

Defined media have been devised for several species. Adam (1) prepared a medium for the Neff strain containing 18 amino acids, acetate as a carbon source, and the vitamins B_{12} and thiamine. Working with several species, including the Neff strain, Band (5) later demonstrated a need for biotin. Subsequently, Band (6) formulated a medium with seven amino acids; glucose or sodium acetate as a carbon source; and the vitamins B_{12}, thiamine, and biotin for *Hartmannella* (*Acanthamoeba*) *rhysodes*. Adam and Blewett (2) compared carbohydrate utilization of different strains of *A. castellanii* in a basal medium containing 5 (arginine, leucine, isoleucine, methionine, and valine) of the 10 essential amino acids and found variations in use of sucrose, melibiose, mannitol, and raffinose as carbon energy sources. These defined media supported growth, but often with extended generation times from 40 to >60 h. In order to produce a higher growth rate, Byers et al. (9) formulated a defined medium (Table 3) based on these earlier studies. Their media, DGM-21A and DGM-21B, gave generation times of about 13 and 16 h, respectively, and cell yields of 2×10^6 to 3×10^6 amebas/ml. The two media differed in vitamin content and salts, with DGM-21B lacking four vitamins (ascorbic, folic, and thioctic acids and riboflavin) and four salts present in DGM-21A. They reported that glucose was

TABLE 3. Defined medium DGM-21A for *Acanthamoeba* spp.[a]

Component	Concn
Amino acids (g/liter)	
L-Alanine	0.2
L-Asparagine	0.5
L-Aspartic acid	0.3
L-Arginine.HCl	1.0
L-Cysteine	0.2
L-Cystine	0.1
L-Glutamic acid	0.5
L-Glutamine	0.5
Glycine	1.5
L-Histidine.HCl	0.2
L-Isoleucine	0.6
L-Leucine	0.9
L-Lysine · HCl	1.0
L-Methionine	0.3
DL-Phenylalanine	0.9
L-Proline	0.8
L-Serine	0.2
L-Threonine	0.5
L-Tryptophan	0.2
L-Tyrosine	0.2
L-Valine	0.7
Vitamins (mg/liter)	
Biotin	0.3
Folic acid	0.2
Ascorbic acid	2.0
Riboflavin	1.0
Thiamine · HCl	0.01
Thioctic (lipoic) acid	0.4
B_{12}	0.01
Salts (g/liter)	
$CaCl_2 \cdot 2H_2O$	0.0074
$MgSO_4 \cdot 7H_2O$	0.25
KH_2PO_4	0.27
$NaHCO_3$[b]	0.25
NH_4Cl	0.0005
NH_4 formate	0.0006
$FeSO_4 \cdot 7H_2O$	0.009
Trace elements (mg/liter)	
$ZnSO_4 \cdot 7H_2O$	1.00
$MnCl_2 \cdot 4H_2O$	2.30
$(NH_4)Mo_7O_{24}.4H_2O$	0.40
$CoCl_2$	0.017
$CuSO_4$	0.0033
H_3BO_3	0.10
Na_2EDTA	0.01
Other components (g/liter)	
Glucose	15.0
Na acetate	2.5
Ethanol[b]	0.5

[a] Medium DGM-21A was described previously by Byers et al. (9). Individual groupings of components were prepared as stock solutions. pH before autoclaving, 6.5.
[b] Introduced from vitamin stock solution.

necessary for enhancing growth rate, while acetate, which was present in the two media, had a minor affect on growth rate. Omission of the carbon sources induced encystment of the amebas (9). (Griffiths and Hughes [31], among others, have explored methods to induce encystment, for example, by suspending trophic amebas in a $MgCl_2$ solution.) The medium of Byers et al. (9) has been used for growing a variety of *Acanthamoeba* species and strains for development of a genus- and subgenus-specific fluorescent oligonucleotide probe (73). Ingalls and Brent (36) formulated a defined medium for *Acanthamoeba polyphaga* containing 11 amino acids; the vitamins B_{12}, biotin, and thiamine; and glucose. Acetate could not substitute for glucose as a carbon source.

Weekers et al. (82) used laboratory fermentors with aeration to scale up growth of *A. castellanii*, in a Proteose Peptone and glucose medium (10.5 liters). Growth was monitored over a time period of 20 to 30 days, with generation times during the exponential phase varying from 67 to 90 h, depending on the degree of buffering in the medium. Cell yields were about 4×10^5 amebas/ml, or 3 g of cells (wet weight). They found a buildup of ammonia and a concomitant pH increase (from 6.5 to >7), although neither the increasing ammonia concentration (to ca. 5 mM) nor glucose depletion (12 to 19%) was responsible for terminating exponential growth. They invoked Pigon's hypothesis (62) of a growth-inhibitory exudate produced in *Acanthamoeba* cultures to explain termination of exponential growth and onset of the death phase in cultures. Weekers and Vogels (81) used a chemostat for axenic cultivation of *A. castellanii* with cell yields of about 3×10^6 amebas/ml with a generation time of about 25 h.

In recent years, a large number of strains and species of *Acanthamoeba* have been isolated from clinical samples. Many of these can be grown on the basic PPYG medium used for the free-living isolates. De Jonckheere (22) used the ability of *Acanthamoeba* spp. to grow in a medium of casein, glucose, and yeast extract at 37°C as an indicator of virulence. Some strains and possibly new species, however, required a richer medium consisting of the PPYG base supplemented with calf serum and a vitamin mixture (67). Even at that, growth of these strains did not match that of the soil and water isolates of nonpathogenic *Acanthamoeba*. Although isolated from human hosts, a number of these amebas grew better at 30°C than at 37°C. Generation times ranged from about 10 to >40 h (67).

Shukla et al. (68), working with *A. culbertsoni*, a pathogenic species isolated from tissue culture, tested a variety of peptones, Proteose Peptone, protein hydrolysates, and vitamin supplements, reporting yields of 1×10^7 to 2×10^7 amebas/ml with Proteose Peptone, yeast extract, and glucose. In a later paper, Shukla et al. (69) presented a defined medium for *A. culbertsoni* that gave approximately the same cell yields as their earlier media. The optimal medium (Table 4) contained 11 amino acids; the vitamins biotin, B_{12}, and thiamine; and glucose and sodium citrate. Generation time in this medium was about 10 h, with cell yields of 2×10^7 to 3×10^7 amebas/ml. Another variation contained seven amino acids (omitting histidine, lysine, threonine, and tryptophan); cell yield was somewhat lower, and the generation time extended to about 23 h.

Balamuthia mandrillaris

The ameba *B. mandrillaris* was only recently described (76), the type species having been isolated from the brain of a mandrill baboon (79). Additional isolates have been obtained postmortem from humans in compromised health (a chronic alcoholic suffering seizures and an amputee with skin abscesses, both elderly), as well as from apparently immunocompetent humans (mostly young children). Another isolate in culture was from a horse (44). Attempts to culture these ame-

TABLE 4. Defined medium M-11 for *A. culbertsoni*[a]

Component	Concn
Amino acids (mg/liter)	
L-Arginine · HCl	825.0
Glycine	1,500.0
L-Histidine · HCl	160.0
L-Isoleucine	600.0
L-Leucine	900.0
L-Lysine · HCl	1,250.0
L-Methionine	300.0
L-Phenylalanine	900.0
L-Threonine	500.0
L-Tryptophan	200.0
L-Valine	700.0
Other (g/liter)	
Glucose	1.8
Na citrate	1.0
Vitamins (mg/liter)	
Biotin	0.25
B_{12}	0.00125
Thiamine · HCl	1.25
Salts (g/liter)	
$CaCl_2 \cdot 2H_2O$	0.0588
$MgSO_4 \cdot 7H_2O$	0.985
KH_2PO_4	0.340
$Na_2HPO_4 \cdot 2H_2O$	0.445
$(NH_4)_2SO_4 \cdot FeSO_4.6H_2O$	0.0196
Trace elements (mg/liter)	
$MnCl_2 \cdot 4H_2O$	2.3
$ZnSO_4 \cdot 7H_2O$	1.0
$(NH_4)\ Mo_7O_{24}.4H_2O$	0.4
$CuSO_4 \cdot 5H_2O$	0.0033
$CoCl_2$	0.017
H_3BO_3	0.1
Na_2EDTA	0.01

[a] Medium M-11 was described previously by Shukla et al. (69). Initial pH, 6.5.

bas on bacteria, either living or dead, were unsuccessful. The amebas were isolated from samples of brain tissue using monolayers of African green monkey kidney cells, upon which the amebas fed and proliferated (Fig. 3B). Schuster and Visvesvara (66) established these strains, including the baboon isolate, in an enriched axenic medium (BM-3) containing Biosate peptone, *Torula* RNA, and yeast and liver extracts supplemented with calf serum, hemin, lipid mixture, vitamin mixture, nonessential amino acids, taurine, and glucose (Table 5; Fig. 3A). Generation times were 20 to 28 h and yields were about 10^6 amebas/ml, with differences between strains. Michel and Janitschke (53) developed cell-free cultures of the baboon isolate using a modified Chang medium (13, 21) for *Naegleria*. The medium was prepared at a relatively high salt concentration (8‰) using sea salt. Their generation times were 32 to 36 h, with cell yields of about 8×10^5 amebas/ml.

Unsuccessful attempts have been made to isolate *Balamuthia* from environmental water and soil samples. The ameba grows slowly in culture (generation time of ≥25 h), and probably does not compete effectively against other soil amebas, soil fungi, or accompanying bacteria. John and Howard (41) have isolated pathogenic (for mice) leptomyxid amebas from pond water using nonnutrient agar and *E. coli* as a food source. Although they have a superficial similarity to *Balamuthia* amebas, the leptomyxids were different from *Balamuthia* as indicated by immunofluorescence staining patterns. Thus, isolation and identification of *Balamuthia* from environmental samples remains a challenge to an understanding of the ecological niche and mode of infection of this newest protozoal agent of amebic meningoencephalitis.

TABLE 5. Complex growth medium BM-3 for *B. mandrillaris*[a]

Component	Amt
Basal medium	
Biosate peptone	2.0 g
Yeast extract	2.0 g
Torula yeast RNA	0.5 g
Double-distilled water	345.0 ml
Supplements	
Hanks' balanced salts, 10×	34.0 ml
5% Liver digest in Hanks' salts	100.0 ml
MEM vitamin mixture, 100×	5.0 ml
Lipid mixture, 1,000× (Gibco)	0.5 ml
MEM nonessential amino acids, 100×	5.0 ml
10% Glucose	5.0 ml
Hemin, 2 mg/ml	0.5 ml
0.5% Taurine	5.0 ml
Calf serum	50.0 ml

[a] Medium BM-3 was described previously in reference 66. pH adjusted to 7.2.

CULTURING AMEBAS AS A DIAGNOSTIC TOOL

This section deals with techniques for isolating amebas from clinical or environmental samples. This is often the most readily available procedure for confirming an amebic infection and identifying the ameba that caused it. Cerva (12) has summarized methods and materials needed for isolation and cultivation of *Naegleria* amebas from clinical specimens, and Visvesvara (75) has dealt with techniques applicable to all three genera of opportunistic free-living amebas. A general flowchart for isolating amebas from clinical and environmental samples is presented in Fig. 4.

Clinical Samples

N. fowleri, the cause of PAM, is most readily isolated from CSF of the patient. It can also be isolated from brain tissue, particularly the olfactory lobes in the case of biopsy or autopsy. *Acanthamoeba* is more likely to be isolated from brain tissue or skin lesions (tissue or swab specimens). Strains that cause amebic keratitis are isolated from corneal scrapings. *Balamuthia* has been isolated from brain tissue, upon either biopsy or, more typically, autopsy. *Acanthamoeba* is generally not found in CSF, and *Balamuthia* has not been recovered from CSF.

The procedure for growing *Naegleria* and *Acanthamoeba* from clinical specimens is the use of a nonnutrient agar spread with *E. coli* or some other nonmucoid bacteria (40, 75). Amebas begin feeding on bacteria and soon grow to cover the agar surface in 1 to 2 days at 37°C. The presence of the amebas can be ascertained by examining the agar surface using an inverted microscope or with a conventional microscope by inverting the plate on the stage and focusing through the agar using a 10× objective.

Naegleria amebas, as seen in a wet-mount preparation on a

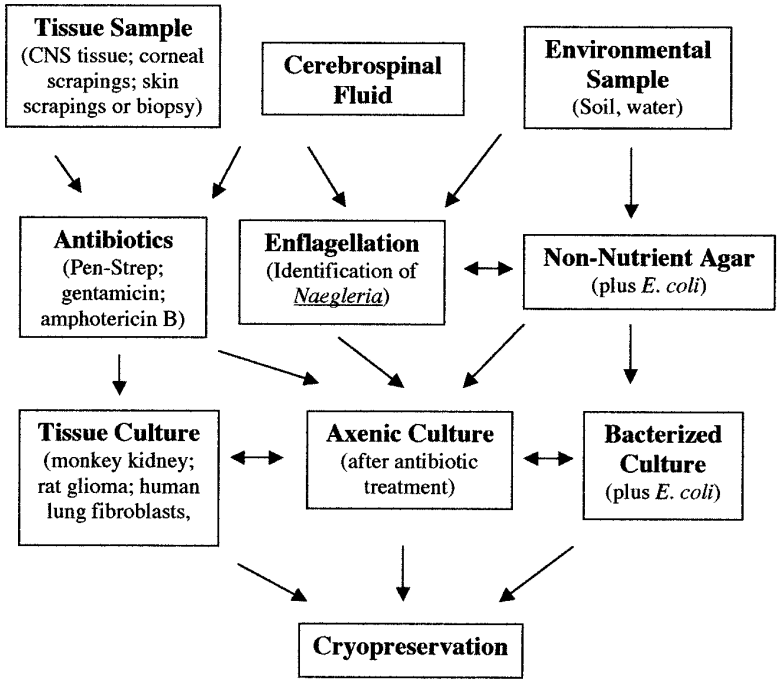

FIG. 4. The chart presents a general scheme for isolation of free-living pathogenic amebas from clinical samples or from environmental sources. The goal is to establish the amebas either in an axenic culture (cell-free growth medium), with a tissue culture feeder layer, or in a xenic culture with a suitable bacterial strain as a food source. Although two different tissue culture types are noted (monkey kidney or rat glioma cells), other cell types (e.g., human lung fibroblasts) are suitable as feeder layers. When possible, cryopreservation of ameba strains is recommended as an alternative to regular subculturing and as a backup in the event of loss of a culture.

microscope slide, have a characteristic limacine (slug-like) pattern of locomotion (Fig. 2B), with a clear, ectoplasmic pseudopod at the anterior end of the ameba. This characteristic appearance is of help in identifying *Naegleria* in CSF but is difficult to observe in amebas on agar plates. As an additional aid in identification, *Naegleria* amebas transform into flagellates when suspended in distilled water (75). To induce enflagellation, CSF containing presumptive *Naegleria* amebas can be diluted directly and suspended in 1 ml of distilled water. Flagellates, usually with two anterior flagella, should appear in <1 h. Not all amebas transform and, once transformed, revert to the ameboid stage. Visvesvara (75) estimates that 30 to 50% transformation occurs with isolates of *N. fowleri*. John (40), in reviewing published reports, gave a range of 0 to 55% enflagellation for different isolates of *N. fowleri*. If dealing with amebas on an agar surface, the amebas can be scraped off the agar with a bacteriological loop and transferred to 1 ml of distilled water to induce the same type of enflagellation response.

Balamuthia does not feed on bacteria, requiring the use of a tissue culture monolayer (such as monkey kidney [ATCC CRL 1586] or rat glioma [ATCC CCL 107] cells) as a feeder layer (Fig. 3). Small macerated pieces of brain tissue are introduced directly into the culture flasks, and the flasks are checked for evidence of ameba growth on the cell layer using an inverted microscope. Unlike the other amebas, *Balamuthia* is slow to adapt to culture conditions, and several weeks may be required before the amebas begin proliferating (76). Once adapted, however, *Balamuthia* grows well on the tissue culture monolayers. It is usually necessary to add antibiotics to the flask to prevent bacterial and fungal contamination, either from the brain specimen itself or from the laboratory environment. Penicillin-streptomycin (at 100 U/ml and 100 μg/ml, respectively) or gentamicin (100 μg/ml) is commonly used to prevent bacterial growth, and amphotericin B (Fungizone) is used to prevent fungal growth. Except for amphotericin B, these antimicrobials do not adversely affect ameba growth, and higher concentrations may be used as needed to eliminate persistent contaminants. *Naegleria* and, to a lesser extent, *Balamuthia* are sensitive to amphotericin B, which should be used sparingly (<1 μg/ml) or not at all in these cultures. The technique outlined for *Balamuthia* can also be used for *Naegleria* and *Acanthamoeba*, which, like *Balamuthia*, actively feed upon tissue culture cells.

Cultures of *Naegleria* and *Acanthamoeba* can be maintained with bacteria as a food source (xenic cultures) or, by addition of antibiotics to destroy bacteria, can be established in cell-free (axenic) cultures (Fig. 2A and B). Appropriate media for axenic cultivation are described above in the section on axenic cultivation. *Balamuthia* can be maintained on tissue culture cells or can be established in a cell-free medium (Fig. 3).

In attempting isolation of amebas from clinical materials, it is helpful to have positive control cultures in parallel as a check on the methodology and ability to recognize amebas in the samples. Table 6 lists several strains of free-living amebas available from the American Type Culture Collection (ATCC) that can be used as controls. Some of these are nonpathogenic

TABLE 6. Selected species of free-living pathogenic and nonpathogenic amebas available from the ATCC[a]

Species	ATCC accession no.	Comments	Pathogenicity
A. castellanii	30010	Classic strain (Neff isolate)	Nonpathogenic
A. castellanii	30011	Classic strain (Castellani isolate)	Nonpathogenic
A. culbertsoni	30171	Tissue culture contaminant	Pathogenic
A. polyphaga	30461	Corneal isolate (Jones et al.)	Pathogenic
N. fowleri	30174	Isolated from human CSF (HB, strain)	Class III pathogen
N. gruberi	30133	Soil isolate (EG)	Nonpathogenic
B. mandrillaris	50209	Original isolate from mandrill baboon	Class III pathogen

[a] The ATCC can be accessed via the Internet at www.atcc.org.

isolates (*A. castellanii*, the Castellani and Neff strains; *N. gruberi* EG strain), while others are isolates from patients with amebic encephalitis or amebic keratitis.

Subculturing Schedules

Table 7 presents suggested time intervals for subculturing amebas. Culture schedules can vary with temperature, bacterial growth, and the presence of cysts in the culture. The time intervals described in the table are for stock cultures. Cultures being used in experiments would require more-frequent transfers (days instead of weeks) as, for example, the use of logarithmic phase cultures in growth experiments, etc. Amebas, once they encyst, tend to survive over longer culture intervals. *Naegleria* cultures, however, appear more sensitive than *Acanthamoeba* and *Balamuthia* to culture conditions (pH changes and waste accumulation), and if not transferred regularly, cultures may abruptly "crash." Some cultured strains lose their ability to form cysts after prolonged subculturing. Pathogenic amebas, which grow optimally at 37°C, will also grow at 30°C and will even grow at room temperature, but at a lower rate. Keeping cultures at ~20°C will prolong the intervals at which cultures have to be transferred.

Balamuthia amebas will remain viable in cell-free cultures for weeks, but it is best to transfer them at ~2-week intervals. For cultures maintained on tissue culture cell monolayers, cultures have to be fed or transferred at approximately weekly intervals since the amebas will rapidly consume the available food supply.

TABLE 7. Recommended subculturing schedules for stock cultures of free-living amebas

Ameba culture	Type of culture	Culture schedule (wk) at:			
		~20°C	Room temperature (~25°C)	30°C	37°C
Acanthamoeba spp.	Bacterized	4	3–4	1–2	1
	Axenic	4	3–4	2–3	2
Naegleria spp.	Bacterized	4	3–4	2	1–2
	Axenic	2	1–2	1–2	1
B. mandrillaris	Axenic	3	2–3	2–3	2
	With tissue culture cells			1	1

Cryopreservation of Strains

In lieu of maintaining cultures by routine subculturing, storage of cultures by freezing is an alternative. Harvested amebas (approximately 2×10^6/ml) in growth medium are mixed 1:1 with cell-culture-grade 20% dimethyl sulfoxide (DMSO) to give a final concentration of DMSO of 10% (G. S. Visvesvara, personal communication). This mixture is frozen and subsequently stored in liquid nitrogen. John (40) used a mixture of 12% DMSO, 20% heat-inactivated serum, 10% glucose, growth medium, and 10^6 amebas/ml in the exponential growth phase for cryopreservation of *N. fowleri*. He reported that viability of frozen cultures was 38% after 6 months, down from 64% at 1 month.

Testing for Pathogenic Potential

If an ameba isolate is from a clinical specimen, it is clearly pathogenic (brain tissue) or potentially pathogenic (corneal isolates). Environmental samples of amebas can be either pathogenic or nonpathogenic. Ability of these isolates to survive and grow at 37°C or higher temperatures is an indication that the strain could also grow at mammalian body temperature. Mouse inoculation, usually intranasal with a suspension of amebas to be tested, is a relatively reliable way of determining pathogenicity of an isolate (Fig. 5). Young mice are more likely to develop infections than older mice (40). Intracerebral inoculation is another route (12). Death of mice can occur within <1 to 4 weeks, depending upon the ameba, the dose, and the virulence of a particular strain (77). Subculturing of a strain can lead to loss of virulence for mice; conversely, virulence can be restored by mouse passage or even subculturing on tissue culture cells.

Specimen Handling for Cultivation

Freezing of clinical specimens should be avoided, particularly with samples (CSF and brain tissue) that might contain *Naegleria*, and specimens should be processed as quickly as possible. All three amebas encyst, but *Naegleria* cysts are more fragile than those of *Acanthamoeba* and *Balamuthia. Acanthamoeba* and *Balamuthia*, however, have been isolated from frozen brain tissue (G. S. Visvesvara, personal communication). The latter two amebas encyst within brain tissue, but *Naegleria* does not (Fig. 1C and D).

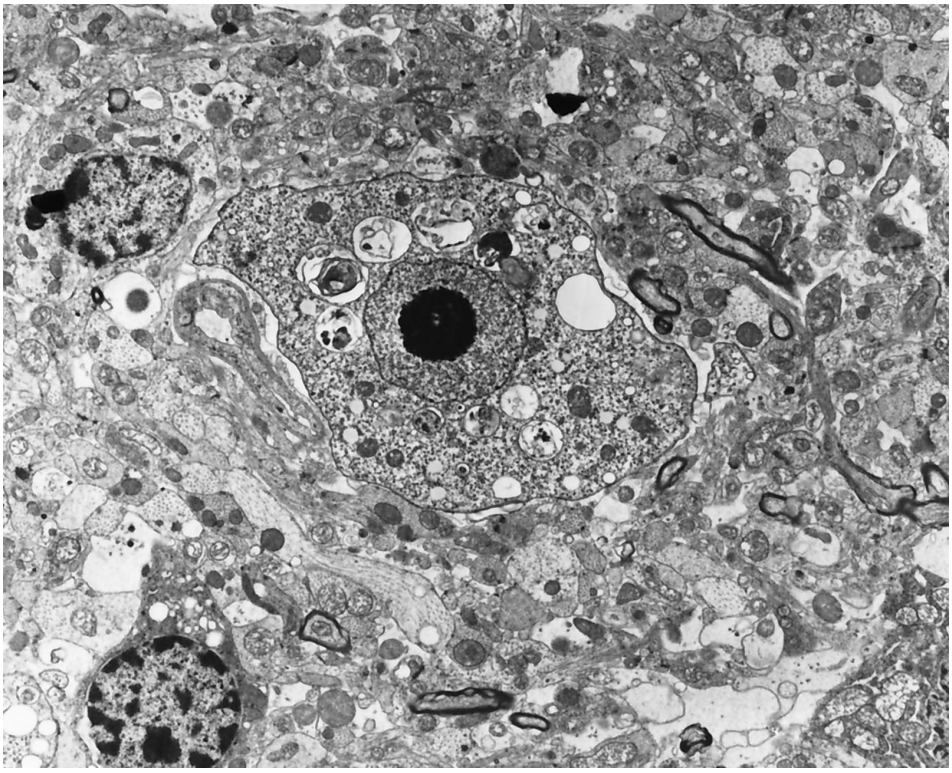

FIG. 5. Transmission electron micrograph of *N. fowleri* in brain tissue of a mouse that had been experimentally inoculated with a suspension of the amebas. The characteristic nuclear morphology of the ameba can be seen as a large nucleus with a centrally located nucleolus (vesicular nucleus). Original magnification, ×7,500. (Micrograph copyright A. J. Martinez.)

Laboratory Safety

Another consideration in handling samples and cultures of pathogenic free-living amebas is the safety of laboratory personnel working with the cultures. There have been no reported infections with free-living amebas of laboratory workers, either from specimen samples or from cultures of organisms. Except for extreme carelessness, it is unlikely that aerosols would be produced within the laboratory during routine handling of materials and cultures. Care should be taken by personnel to avoid getting culture materials on the skin or in open cuts or abrasions. Gloves should be worn when handling materials, and surgical masks may be used, particularly when performing animal inoculation studies. If available, it is advisable to handle cultures in a biological safety cabinet. This not only protects personnel from chance contact with amebas but also minimizes the chance of contamination of cultures by airborne molds, bacteria, and yeasts. Laboratory personnel wearing contact lenses should be instructed about precautions in working with *Acanthamoeba* spp., since corneal infections can be caused by any number of different species, even those without a history of pathogenesis.

Environmental Samples

In attempting to establish the source of an amebic infection, it is often necessary to screen environmental samples for the presence of amebas. The most commonly examined samples are soil (garden soils or soils in flower pots) or water (from lakes, ponds, home aquaria, hot tubs, humidifiers, heating and ventilating air conditioning units, and eye wash irrigation stations, etc.). For instances in which *Acanthamoeba* keratitis may be related to contact lens wear, the lens case may be examined and cultured for amebas. Unlike clinical specimens, these samples will almost certainly contain bacteria and fungi, other protozoa, and, perhaps, metazoa (nematode worms or aquatic arthropods). The difficulty in working with such samples is to encourage growth of the amebas but inhibit the growth of the other soil organisms.

The basic procedure is the use of nonnutrient agar with *E. coli* as a food source, as described for clinical specimens. For environmental samples, use of enriched media suitable for axenic cultures should be avoided as they will stimulate heavy growth of contaminating bacteria and fungi and prevent ameba growth. Once the amebas are established in a bacterized culture, antibiotics can be added to kill off bacteria and the amebas can be transferred to the appropriate axenic medium.

Both *Naegleria* and *Acanthamoeba* have been isolated from a variety of environmental samples by employing the techniques described. *Balamuthia*, however, has yet to be isolated from the environment, and its niche in nature remains to be defined.

CONCLUSIONS

Free-living amebas are recognized as opportunistic agents of disease. Cultures of these amebas are useful in defining their basic nutritional requirements, testing efficacy of antimicrobial agents for therapeutic value, understanding their phylogenetic relationships, and perfecting diagnostic techniques for rapid identification of isolates. *Naegleria* and *Acanthamoeba* are readily isolated from environmental samples (soil and water), but the isolation of *Balamuthia* remains an important goal. Its slow growth, its apparent inability to feed on bacteria, and the presence of highly competitive soil fauna (fungi, bacteria, other protozoa, and metazoa) have complicated the task. Though it is generally regarded as free living, this cannot be know for certain until the organism is isolated from soil or water samples.

It is likely that, on a global scale, many cases of amebic encephalitis go undiagnosed and unreported. Development of techniques to improve on isolation and cultivation of these amebas will help in obtaining a more accurate assessment of the extent of these infections.

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ADDENDUM IN PROOF

A *Balamuthia* ameba associated with a fatal case of amebic encephalitis in northern California has been isolated from soil of a potted plant in the home of the patient (F. L. Schuster, T. H. Dunnebacke, C. Glaser, D. Vugia, A. Bakardjiev, P. Azimi, M. Maddux-Gonzalez, G. S. Visvesvara, 54th Annu. Meet. Soc. Protozool., 2002). The ameba is morphologically similar to the ameba isolated postmortem from the patient, gave positive immunofluorescence reactions with serum from the patient and with rabbit anti-*Balamuthia* serum, and had a similar antimicrobial sensitivity profile. The environmental isolate is in culture on tissue culture cells and in a cell-free medium. This represents the first isolation of *Balamuthia* from the environment.

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