The Influence of Seawater Media on Growth and Encystment of *Acanthamoeba polyphaga*

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ABSTRACT: A new isolate of *Acanthamoeba polyphaga* was established in culture from cysts of the amoeba discovered in nonsterile distilled water. In vitro cultivation on distilled water-agar and seawater-agar media showed that the amoeba would grow in both freshwater and marine environments. Direct subculture from freshwater medium to full strength high salinity seawater (35%) delayed growth and encystment, and cyst morphology was abnormal. Although *A. polyphaga* adapted and grew on media designed to approximate the marine environment, the cysts were not characteristic of the genus *Acanthamoeba*. Rapid growth was restored after amoebae were maintained on seawater-agar through 4–5 generations, and when they were returned to distilled water-agar. Abnormal cyst morphology was retained on seawater-agar, but returned to normal upon subculture to distilled water-agar. The new strain, designated OX-1, was not pathogenic for mice after intranasal inoculation of 100–5,000 amoebae per animal.

Small free-living amoebae of the genus *Acanthamoeba* are well-known inhabitants of freshwater and soil, and their specific taxonomic characters have been carefully reviewed by Page (1967a). The discovery of a new species, *A. gigantea*, in seawater (Schmoller, 1964) showed that the habitat of these highly adaptive amoebae also includes the marine environment. Of the known species of *Acanthamoeba*, *A. polyphaga* Puschkarew, 1913, probably is the most universally distributed and the most frequently isolated (Page, 1967a). A new strain of *A. polyphaga*, herein designated OX-1, was isolated in our laboratory from a carboy of distilled water. The presence of *A. polyphaga* as a laboratory contaminant prompted a study to determine whether they would grow on culture media used to maintain amoebae from marine shellfish and seawater. Earlier studies (Chatton, 1913; Hollande, 1921; Wolff, 1927) showed that some species of freeliving freshwater amoebae reversibly or irreversibly lost their capacity to produce cysts on media containing high concentrations of sodium chloride. The present study was designed to test the influence of salt concentration on the growth response of *A. polyphaga*, and to determine whether or not significant changes in morphology could lead to incorrect species identifications. The importance of standardized culture methods for growing and identifying different species of *Acanthamoeba* has already been stressed by Culbertson, et al. (1965), and by Page (1967b). Further evidence for the influence of culture conditions on cyst morphology is documented in this report.

Strain OX-1 was tested for pathogenicity by intranasal inoculation into mice because of the known pathogenicity of some strains of *Acanthamoeba* in mammalian tissue culture cells (Jahnes, et al., 1957), and in experimental animals (Culbertson, et al., 1959).

Materials and Methods

*Acanthamoeba polyphaga* (OX-1) was isolated from nonsterile distilled water. Samples of 5 ml of the water were transferred to 60 mm plastic dishes, *Pseudomonas fluorescens* was added as a food source, and incubation was at room temperature (22–25 C). Actively dividing amoebae were transferred with a wire loop to 60 mm dishes containing 1.5% nonnutrient agar (Difco) in distilled water. Stock cultures were maintained by subculturing small blocks of agar from parent cultures on fresh plates streaked with bacteria. After 3 months of maintenance a low-nutrient agar was substituted for the non-nutrient agar, and *Aerobacter aerogenes* was substituted for *P. fluorescens*. The low-nutrient agar (MYA) contained 0.1 g maltose extract,

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1 Trade names do not imply endorsement of commercial products.
0.1 g yeast extract, and 15 g Difco agar in 1.0 liter of distilled water.

Preparation of experimental media: The low-nutrient agar described above was made in distilled water (DWA), low salinity seawater (LSA), and high salinity seawater (HSA). Low salinity seawater (10–13%) was obtained from the Tred Avon River, Oxford, Maryland, and high salinity seawater (32–35%) was obtained from Chincoteague Bay, Franklin City, Virginia. Salinities were calculated from specific gravity readings. Distilled water and seawater were filtered through 0.45 μM Millipore filters and final media were sterilized by autoclaving. Other media of known salt concentrations were prepared with 1.0, 2.0 and 3.5% sodium chloride dissolved in water containing maltose extract, yeast extract, and agar (MYA). Bacteria-free axenic cultures of *A. polyphaga* were established in Neff’s liquid medium (Neff, 1957) by washing agar blocks from stock cultures in three changes of sterile distilled water containing 500 units penicillin and 250 μg streptomycin sulfate per ml of water. After three washings by centrifugation at 80 g, one drop of the sediment containing the amoebae was added to Neff medium in screw-capped test tubes. Cultures were maintained in an upright position at room temperature.

Culture methods: Survival, growth, and morphologic appearance of *A. polyphaga* were recorded from cultures on DWA, LSA, and HSA. Amoebae were established on LSA by transferring small blocks of agar from DWA to LSA, and on HSA by transferring them from DWA or LSA to HSA. Permanent stocks were maintained on all three types of media, and short-term or one generation experiments were performed with agar containing known amounts of sodium chloride. Observations of all cultures were made with a Leitz inverted microscope, and the number of days required for new populations to grow and encyst was recorded. Cysts from experimental cultures were photographed to document changes in their morphology, and abnormal cysts from HSA were re-cultured on DWA to test the stability of the change and the viability of amoebae. Cysts from DWA and HSA were transferred to glass cover-slips and stained by the hematoxylin procedure of Mitchell (1966) for measurement with an ocular micrometer. Dehydration experiments with cysts were made by allowing DWA cultures to evaporate slowly to dryness at room temperature and rehydrating them with distilled water after 5 to 67 weeks (Table 1).

Photomicrographic techniques: Phase contrast photomicrographs of living trophozoites and cysts were made with a Zeiss photomicroscope and Kodak Plux X film. Small blocks of agar were transferred to clean glass slides and covered with 11 × 22 mm coverslips moistened with a small drop of distilled water or seawater, and photographed at 400× magnification. Amoebae grown in liquid Neff’s medium were transferred to 22 × 40 mm cover-glasses, placed in a moist chamber for 30 min to allow them to attach, and photographed on a glass depression slide after sealing the cover-glass with melted paraffin-vaseline.

Identification and characterization of *A. polyphaga*: To confirm the specific identification of the amoebae used in the present study, pure cultures of related species, *A. castellanii* (Neff strain), *A. palestinensis*, and *A. astronijxis* were obtained in Neff’s medium and on agar plates. The three known species (numbers 1501/1, 1547/1, and 1534/1 of the Culture Collection of Algae and Protozoa, The Botany School, Cambridge, England) were supplied through the courtesy of Dr. Joe L. Griffin, Armed Forces Institute of Pathology, Washington, D. C. The morphology of the three known species was compared with that of *A. polyphaga* under ordinary bright field and phase contrast microscopy, and nuclear morphology was studied after staining with hematoxylin (Mitchell, 1966).

Experiments to test for pathogenicity of the new strain in laboratory mice were conducted by Dr. Clyde G. Culbertson and Mr. Paul Ensminger, Eli Lilly Laboratories, Indianapolis, Indiana. Laboratory mice were inoculated intranasally with 100, 200, 500, 1,000, or 5,000 amoebae per animal, and observations were made for symptoms of disease. Mice were sacrific-

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<th>Table 1. Time required for excystment of <em>Acanthamoeba polyphaga</em> after rehydration of dried cultures.</th>
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<td><strong>Number weeks dehydrated</strong></td>
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<td>5</td>
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ficed 6 weeks after inoculation and examined histologically for lesions in the nose, brain, and lung.

Results
Growth characteristics of *A. polyphaga* in liquid medium and on distilled water-agar

In Neff's liquid medium and on agar made with distilled water (DWA), the amoebae grew as previously reported by Neff (1957) for *A. castellanii*, and by Page (1967a) for *A. castellanii*, *A. polyphaga*, *A. palestinensis*, and *A. astronyxis*. Taxonomic criteria for trophozoites and cysts of *A. palestinensis* and *A. astronyxis* as reviewed by Page (1967a) were adequate to distinguish them from *A. castellanii* and *A. polyphaga*. Trophozoites of *A. polyphaga* and *A. castellanii* usually were triangular and slightly longer than broad (Fig. 1). Cysts of both species were polygonal orstellate and their membranes were wrinkled (Figs. 2, 3), but these two features were distinct and more pronounced in *A. castellanii* (Fig. 3) than in *A. polyphaga* (Fig. 2). Cysts of both species were nearly identical after fixation and staining, largely because of protoplasmic contraction and pseudoinflation of cyst membranes. Encystment of the two species was complete in five to seven days on agar media, but much slower and less synchro-

Table 2. Response of *Acanthamoeba polyphaga* to growth on distilled water, seawater, and sodium chloride-agar media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th>Appearance of cyst wall</th>
<th>Comment</th>
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<tr>
<td>Distilled water</td>
<td>+ Normal-Wrinkled</td>
<td>Typical morphology</td>
<td></td>
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<tr>
<td>Seawater (10–13%)</td>
<td>+ Normal-Wrinkled</td>
<td>Marked polymorphism</td>
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<tr>
<td>Seawater (32–35%)</td>
<td>+ Abnormal-Smooth</td>
<td>Oval, vermiform amoeba</td>
<td></td>
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<tr>
<td>1.0% NaCl</td>
<td>+ Normal-Wrinkled</td>
<td>Marked polymorphism</td>
<td></td>
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<tr>
<td>2.0% NaCl</td>
<td>± Abnormal-Smooth</td>
<td>Oval, vacuolated</td>
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<td>3.5% NaCl</td>
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<td>Degenerate</td>
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* 1.5% agar media streaked with *Aerobacter aerogenes*.

Table 2. Response of *Acanthamoeba polyphaga* to growth on distilled water, seawater, and sodium chloride-agar media.

Amoebae grew on agar media containing the nutrients provided naturally in low salinity seawater (LSA) and on agar made with one per cent sodium chloride (Table 2). Although amoebae and cysts were of essentially the same morphology on DWA and LSA, encystment was slower on LSA and small numbers of amoebae were present for up to 10 days. Cyst morphology of *A. polyphaga* was more variable with one per cent sodium chloride than with LSA of comparable salt concentrations (Fig. 4), but the specific taxonomic features were unmistakable.

Amoebae transferred directly from DWA to HSA did not show obvious growth until 7–10 days after subculture, and the period required for encystment of the entire population was

Figure 1. Living trophozoite of *Acanthamoeba polyphaga* in Neff's liquid medium. Phase contrast, x560.
extended to 20–25 days. Because of the lag period uncontrolled bacterial growth occasionally inhibited the growth of amoebae. Although *A. polyphaga* adjusted to HSA (approx. 35%), growth did not occur when medium was made in 3.5% sodium chloride solution. In HSA medium, cysts were spherical or oval and some contained a vacuole (Figs. 5, 6). The typical
stellite endocyst and wrinkled cyst wall was markedly reduced or absent on HSA medium, and the abnormal endocysts were comma-shaped (Fig. 6). Although growth did not occur on 3.5% sodium chloride medium, moderate growth was obtained with 2.0% sodium chloride. Cysts produced on the lower concentration were large, smooth, and vacuolated (Fig. 7), and did not resemble cysts from any of the other media. Cysts that were produced on HSA or 2.0% sodium chloride agar medium did not resemble cysts of any recognized species of Acanthamoeba.

The significance of the extended period of time required for amoebae to grow and encyst upon transfer to HSA was tested in two ways: (1) maintaining amoebae through several successive generations on HSA; and (2) subculturing amoebae from LSA to HSA to reduce the drastic change in tonicity between DWA and HSA. Growth and encystment occurred without a lag period under both of these conditions. Thus, the extended period required for growth and encystment of A. polyphaga on HSA was temporary and did not persist after the initial period of adjustment. Other experiments showed that a lag period did not occur when amoebae were transferred back from HSA to DWA, and that normal cyst morphology was restored on DWA. The abnormal morphology of cysts grown on HSA remained constant upon serial cultivation in this medium.

Resistance of cysts of A. polyphaga to dehydration

Agar cultures of A. polyphaga on DWA that were dried at room temperature for periods up to 67 weeks produced new populations of amoebae after rehydration with sterile distilled water (Table 1). The new populations appeared within 24–48 hr in cultures that had been dried for less than one month, and after five days in older cultures. The viability of cysts that were dried for over one year before rehydration might explain the frequent recovery of A. polyphaga as a laboratory contaminant.

Influence of culture media and age on cyst measurements

The largest living cysts on DWA or HSA media usually were immature (prechysts), and showed residual protoplasmic streaming or cystoclasis. The smallest were produced by amoebae that had undergone a second period of encystment on the same culture plate. The double encystment cycle was observed more often on HSA than on LSA or DWA.

The variability in size of the cysts of A. polyphaga grown on DWA and HSA media was reduced by measuring stained cysts that were fixed and dehydrated. Smooth spherical or oval cysts from HSA cultures yielded more uniform measurements than did wrinkled polymorphic cysts from DWA cultures, and were several microns smaller than DWA cysts of the same age (Table 3). The influence of age on cyst measurements was shown by the standard error of mean values (Table 3); 8-day-old cysts from HSA cultures had a larger S.E. than 13-day-old cysts grown on HSA. Measurements of A. polyphaga (OX-1) grown on DWA were in the same size range reported by Page (1967a) for this species.

Response of mice to infection with A. polyphaga (OX-1)

Laboratory mice inoculated intranasally with 100 to 5,000 amoebae per animal did not show overt signs of disease, and pathologic lesions were not found in tissue sections of nose, brain, or lung. The new strain of A. polyphaga was not pathogenic for mice under the conditions tested.

Discussion

The finding of A. polyphaga as a contaminant in the laboratory was consistent with earlier discoveries of A. polyphaga and A. castellanii in dust (Wells, 1911), rainwater (Pusch-
karew, 1913), cultures of soil yeasts (Castellani, 1930), and cultures of soil bacteria (Shinn and Hadley, 1936; Hewitt, 1937). A. polyphaga is morphologically similar to A. castellanii Douglas 1930, but differs from it in several significant features (Page, 1967a). The present study documents the ability of A. polyphaga to grow in freshwater and seawater media, and illustrates the loss of critical diagnostic features when grown on seawater or sodium chloride-agar media. Furthermore, better growth and more uniform cyst morphology was obtained on seawater media than on simple sodium chloride media. This suggests that media prepared in complex salt solutions as used by Neff (1957), and Page (1967b), are preferable to media prepared in sodium chloride solution. The study clearly demonstrates the requirement for a comprehensive and standardized approach to the in vitro cultivation of freshwater and marine amoebae. The classical taxonomic study by Schaeffer (1926), which utilized conditions in the ability of free-living amoebae to adjust to concentrated or diluted seawater, unfortunately, did not include data on cyst stages. Descriptions of new species in the future, especially marine species, should be based on results of a broad experimental approach. The marine species, A. gigantea, should be re-studied in freshwater media to confirm its identity.

Although the cyst morphology of A. polyphaga was altered after cultivation on HSA media, it was normal after subculture to LSA or DWA media. Hollande (1921) on the other hand, described an irreversable loss of cyst forming ability by Vahlkampfia cruciata cultured on agar media containing 3–4% sodium chloride. Wolff (1927) studied free-living hartmannellid amoebae under the same conditions and found that the loss of cyst forming ability was restored after subculture on low chloride media. In contrast to vahlkampfidi and hartmannellid amoebae, A. polyphaga encysted regularly on experimental seawater and sodium chloride-agar media.

Further studies of the marine environment should extend the growing list of habitats of the acanthamoebae, i.e., tissue culture cells (Jahnes et al., 1957); experimental animals (Culbertson et al., 1959); plant sap (Troll, 1965); seawater (Schmoller, 1964; Griffin, personal communication); soil and water (Page, 1967a); and domestic animals (McConnell, et al., 1968). The universal distribution of these amoebae in nature suggests that they may also have unrecognized host-parasite relationships in marine plants and animals. The role of amoebae as serious pathogens in marine hosts has already been established, i.e., diatoms parasitized by Ameoba bidulphi (Zuelzer, 1927), foraminifers by Vahlkampfia discorn (Le Calvez, 1940), and blue crabs by Paramoeba perniciosa (Sprague, et al., 1969; Sawyer, 1969).

New studies on the influence of natural environment and experimental culture media on morphology should answer several important questions: (1) Are some of the recognized species of marine amoebae actually well-known freshwater forms whose morphologic features have been altered by their ionic and nutritional environment? (2) Are some of the marine amoebae for which cyst stages are unknown capable of producing cysts when natural waters are diluted by rainfall during wet seasons? (3) Are the experimentally induced effects of modified culture media merely temporary physiological changes which do not persist after long-term growth and maintenance in vitro? The increasing interest on the influence of nutrition and environment on growth and morphogenesis should provide new information on the stability of critical taxonomic features among freshwater and marine protozoa.

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Hollande, A. 1921. Culture pure mixte et lev-


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**Report of the Brayton H. Ransom Memorial Trust Fund**

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**Receipts:** Interest rec’d in 1969 ............................................. 134.13

**Disbursements:** Grant to Helminthological Society of Washington ............................................. 10.00

**Balance on Hand, 31 December 1969** ......................................... 2888.02

A. O. Foster
Secretary-Treasurer

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