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A Study of Soil Amoebae Infective to Mice

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A STUDY OF SOIL AMOEBAE

INFECTIVE TO MICE

(TITLE)

BY

PAMELA McDANIEL

THESIS

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I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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The undersigned, appointed by the Head of the Department of Zoology,

have examined a thesis entitled

A STUDY OF SOIL AMOEBAE INFECTIVE TO MICE

Presented by

PAMELA McDANIEL

a candidate for the degree of Master of Science

and hereby certify that in their opinion it is acceptable.

ABSTRACT

On January 1, 1973, 10 soil samples were collected from areas in Coles County, Illinois, in an effort to isolate soil amoebae. All 10 soil samples were cultured on bacto-nutrient agar and colonies of amoebae genera isolated were Acanthamoeba and Hartmannella. Amoeba-bacteria cultures prepared from soil isolates were injected into each of 10 adult mice which were sacrificed at the end of the nine days and examined for evidence of infection and pathogenicity. Lesions were observed in two mice and amoebae cysts and bacterial colonies were present in nine mice. Amoeba-bacteria cultures were purified using streptomycin antibiotic discs and the bacteria-free amoeba cultures were injected into each of 10 adult mice. After seven days the mice were sacrificed. No lesions were observed. However, microscopic examination of the liver, spleen, kidney, small intestine and brain revealed the presence of amoeba cysts in all the mice. Appropriate controls were used. Results suggest that local free-living soil amoebae could adapt to parasitism and may be potentially pathogenic. No deaths were recorded from the amoebae although some mice appeared moribund during the incubation period. Acanthamoeba was present in a wider range of soil samples and appeared more infective to mice than Hartmannella.

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INTRODUCTION

Many species of amoebae exhibiting a variety of morphological shapes, sizes, and nutritional modes are found in the soil. These amoebae are thought to be a factor affecting soil fertility and the presence of bacteria in the soil (Sandon, 1927, and Waksman, 1927). Under laboratory conditions some free-living forms have demonstrated the ability to develop into potential pathogens (Butt, 1966). Invasion of vertebrate tissue causing necrosis and even death have been documented in the literature (Culbertson, 1961, Culbertson et al., 1958, 1959, McCowen and Galloway, 1959, Bovee et al., 1961, Ensminger and Culbertson, 1966, Butt, 1966, Wilson et al., 1967, Cerva and Novak, 1968, and Callicott, 1968). The purpose of this study is to isolate free-living soil amoebae from a variety of local soil samples and test some genera for their ability to parasitize vertebrate tissue.

LITERATURE REVIEW

Many free-living protozoa are found in the soil with the great majority being flagellates and amoebae (Pelczar and Reid, 1965, and Waksman, 1927). Sandon (1927) listed over 250 species. Frosh in 1897 was one of the first to study amoebae with bacteria from the soil (Manwell, 1968). Martin and Lewin were the first to demonstrate the presence of trophic amoebae in the soil (Cutler, Crump and Sandon, 1922).

Some important pathogens occur among the amoeboid group of protozoa. The family Endamoebidae contains the well known pathogen Entamoeba histolytica, which causes amoebiasis and amoebic dysentery (Kudo, 1966, and Wenyon, 1965). Musgrave (1931) said amoebiasis was caused by Entamoeba sp. and involved necrosis and degenerated cells of the intestine and occasionally liver, lungs, brain, pericardium, abdominal wall, oral cavity, appendix, bladder and blood stream. Among other early researchers in the field, Geiman and Ratcliffe (1936) studied amoebae producing amoebiasis in reptiles using an Entamoeba species closely resembling Entamoeba invadens. Other species of this genus and other genera in the family are important parasites of animals.

Wells (1911) found a form of dysentery to be caused by a motile amoebae of the "limax" type found in the soil. Intranasal and intracerebral injection of mice, monkeys and rabbits with the soil amoeba, Hartmannella castellanii, by Culbertson (1961) did not illustrate invasion of tissues by the amoebae. Wilson et al. (1967) found in their

study that the free-living soil hartmannellids injected into mice and rats did not cause death and illness, but on sacrifice, tissues were infected. Levine (1973) cited two studies one by McConnell, Garner and Kirk in 1968 and one by Patton in 1969 in which Hartmannella sp. were found in a bull which died of gangrenous pneumonia and a sheep with cervical lymphangitis respectively.

Bovee et al. (1961) used axenic cultures of Acanthamoeba which produced lesions or death in mice when injected subcutaneously or intraperitoneally. Illness and death of mice and monkeys occurred in a study by Culbertson et al. (1959) using Acanthamoeba. McCowen and Galloway (1959) used intracerebral inoculation of mice with several species of Acanthamoeba and found the average survival time to be approximately 5 days. Intracerebral and intraspinal inoculation of Acanthamoeba into monkeys produced extensive choriomeningitis and destructive encephalomylitis with death occurring in 4-7 days (Culbertson et al., 1958). Butt (1966) injected Acanthamoeba intracerebrally into mice and found focal paralysis, neural malfunctions, purulent meningitis, a granulomatous response and death.

Wilson et al. (1967) found amoeba cysts and trophozoites in the coelom, lymph nodes, kidney and lung of mice and in the same tissues plus the peritoneum, liver, spleen, brain and blood of rats when he injected Acanthamoeba sp. Extensive pneumonic changes, cerebral abscesses, hemorrhage and lesions of the lungs and other tissue damage in mice plus encephalitis in monekys and rabbits was produced by Acanthamoeba in a study by McCowen and Galloway (1959). Injections of Acanthamoeba sp. which resembled the free-living soil type but found in monkey tissue culture was injected via intracerebral, intraspinal and intra-

muscular routes with the uncentrifuged, undiluted culture fluid and the amoebae were found to migrate from the injection site and cause damage in other tissues (Culbertson et al., 1959).

Acanthamoeba castellanii was found to be a lethal invader of tissues in mice, monkeys and rabbits (Wilson et al., 1967). Intracerebral injection of Acanthamoeba in mice caused destructive encephalitis, lesions, ulceration of the nasal mucous membrane, and invaded the adjacent base of the skull involving the frontal lobes of the brain to cause death in about 4 days (Culbertson et al., 1958). Trophozoites and cysts were found in the coelomic fluid, intestinal lymph nodes, kidney and liver with abscesses and edema in rats inoculated with Acanthamoeba sp. (Bovee et al., 1961). Bovee also found that intravenous inoculation into mice of Acanthamoeba caused perivascular granulomatous lesions in the lungs, associated with severe pneumonia, extensive fibrinopurulent exudate containing polymorphonuclear leucocytes and monocytes, hemorrhage and invasion of the pulmonary veins followed by the formation of thrombi containing the amoebae.

Man is susceptible to the pathogenicity of Acanthamoeba. Cerva and Novak (1968) in a study in northern Bohemia found tissue lesions, purulent meningitis and massive infection of the central nervous system which eventually caused death in 16 cases of amoebic meningoencephalitis caused by an Acanthamoeba sp. from an indoor swimming pool.

Butt (1966) also found primary meningoencephalitis to be caused by Acanthamoeba in humans who swam in tepid lake water. He found motile amoebae in the spinal fluid, intestinal amoebiasis, respiratory paralysis, lesions in the brain, inflammatory reaction, hemorrhage, intestinal pneumonitis, headache and fever as some of the symptons caused by Acanthamoeba. Eight cases of acute fulminating meningoencephalitis

in man and symptons such as pulmonary edema, swelling and inflammation of the brain and meninges and presence of amoebae in the spinal fluid were noted by Callicott (1968) in his study of Acanthamoeba and its effect on man.

Naegleria gruberi, a common soil amoeba related to Hartmannella and Acanthamoeba has been proven to be pathogenic to chick embryos (Dunnebacke and Schuster, 1971). This genus was found to be a cause of death to man and also to be pathogenic to mice and guinea pigs (Cerva, Zimak and Novak, 1969).

MATERIALS AND METHODS

Materials required for this study followed those recommended by Bovee (1970).

Ten soil samples were collected from various locations in Coles County, Illinois. Three ounces each of soil was collected at the following sites: a well-sodded vacant lot at the southeast corner of Ashby Drive and University Drive about 100 feet due southeast from the street; a prairie loam soil from the Campus Arboretum at Eastern Illinois University, about 500 feet east of the 1800 block on University Drive; an ooze from along the east shore of the Teacher's College Lake on the Eastern Illinois University campus; forest litter soil from under leaves at Lakeview Park in Coles County; a well-sodded grass soil from outside the Stanford Building, Regency Apartments, S. 9th Street; a dark black fertilized field at 2 miles south of Charleston on Illinois Route 130 and then 1 mile west; a combination barnyard and field in which cows grazed, located 3 miles north on E. Harrison Street Road; a barnyard manure soil from a lot 2 miles east on Route 316; another barnyard soil from a lot 2 miles west on the first road before the Embarrass River bridge on Route 130 where ponies were kept; and an artificial acid soil from the Life Science Building under the first bald cypress tree across from the Annex entrance. Collections were aimed at getting a variety of soil types that may yield a variety of different kinds of protozoa.

Each soil sample was wetted with 5-10 ml. of Neff's amoeba saline, diluted 50% with sterile water, mixed and allowed to stand 5 minutes. Five ml. of this material were transferred by sterile pipette to Petri dishes containing a layer of nutrient bacto-agar. This preparation was immediately overlain with 1.0 ml. of Neff's amoeba saline and the dish marked by date, source of inoculum and accession number. Cultures were incubated at room temperature (21-23°C) for 48-72 hours. Development of amoeba trophozoites and cysts was usually sufficient at 48 hours; however, some of the cultures required 72 hours for adequate growth to occur. Only those cultures were used in which an average of three trophozoites or three cysts per 10 fields could be seen using a microscope at 430x magnification. Cultures of Acanthamoeba castellanii, purchased from the American Type Culture Collection and used here as a control, were prepared and treated as described above.

Amoeba isolated from the soil were identified using Kudo (1966) and Levine (1973). Hartmannella trophozoites are 9-17μ in diameter, have a well-developed ectoplasm, a single contractile vacuole and a single vesicular nucleus with a large endosome. The cysts are smooth, spherical and 10-15μ in diameter. Acanthamoeba trophozoites are 12-40μ in diameter when rounded, do have a well-developed ectoplasm and form tapering hyaline acanthopods. The cysts are wrinkled, have two membranes with the inner one positive for cellulose. They are from 12-25μ in diameter.

Two ml. of active culture were aspirated into a sterile hypodermic syringe and inoculated subcutaneously into the back of an adult laboratory mouse. Cultures obtained from each of the ten soil samples were used. A culture of Acanthamoeba castellanii and a control of

sterile saline only were also utilized. Each mouse was placed in a separate gallon jar marked by date and source of inoculum and observed for 9 days. Food and water were available. At the end of 9 days mice were killed by etherization and autopsied for signs of tissue necrosis and presence of bacteria and amoebae. Cover glass smears were made of the liver, small intestine, kidney, spleen and brain by pressing a clean cover glass to the freshly cut surface of the organ, fixing in methyl alcohol and staining in Giemsa. Records were made of sites of apparent necrosis and the presence of bacteria and amoebae.

Bacteria-free cultures of amoebae were obtained by streaking Petri dishes of nutrient bacto-agar, containing two streptomycin treated filter paper discs (10 mcg/disc), with cultures of amoebae previously prepared. Transfers were made with a sterile bacteriological wire loop. Cultures were incubated at 21-23°C and growth observed at 48 and 72 hour intervals. A microscope was used to isolate cysts at bacteria-free areas in the culture and these cysts were transferred to a second series of antibiotic treated agar plates. Three transfers were made to insure purity of the cultures. After the third series of plates was observed for growth, amoeba cysts were transferred to test tubes of sterile Neff's nutrient amoeba medium. These amoeba cultures were allowed to incubate at room temperature for at least 48 hours. Some cultures required a longer time period than others for demonstration of sufficient growth as previously determined. Cover glass smears of the preceeding named tissues were also made. Cultures were also checked before inoculation to determine the genus being injected and that no cultures of mixed genera were used.

RESULTS

Two genera of amoebae were isolated from the soil samples (Table 1). Acanthamoeba was present in a wider range of soil samples than was Hartmannella. At least one genus of amoebae was present in all 10 samples cultured in Petri dishes of bacto-nutrient agar. Cyst stages were the most prevelent with trophozoite stages rarely seen.

Autopsies of the ten mice injected with the amoeba-bacteria cultures revealed lesions in two mice, #3 and #9 (Table 2). Cover glass impressions of tissue from infected mice showed amoeba cysts (Table 2) and bacterial colonies in all except one. Mouse #3 was negative.

Controls revealed the results shown in Table 2. Amoeba cysts were found in the tissues of the mouse injected with Acanthamoeba castellanii but no lesions. Bacteria colonies were observed in the mouse infected with amoeba-free bacteria culture but no amoeba cysts or lesions. The mouse inoculated with saline control showed no cysts, bacteria, or lesions.

Amoeba cultures of the 10 soil samples developed bacteria-free in Petri dishes containing streptomycin antibiotic discs.

All 10 soil samples of axenic amoeba cultures inoculated into mice from test tubes of Neff's nutrient medium demonstrated the presence of cysts in the tissues observed (Table 3). Cysts were found in the brain tissue of all the specimens used. Mice #5, #9, and #10 appeared moribund in the late stages of the test.

Amoeba cysts of bacteria-free Acanthamoeba castellanii culture and lesions were found in all examined tissues of the mice injected with this inoculum. No cysts or lesions were seen in the tissues of the mouse infected with sterile culture medium (Table 3).

Table 1. Genera of Sarcodina found in soil samples in Coles County, Illinois - January 1973 (Each sample = 5 ml.)

	Site	Genus
1.	Vacant lot - Ashby Drive	Acanthamoeba sp.
2.	Campus Arboretum	Hartmannella sp.
3.	Campus Pond	Hartmannella sp.
4.	Lakeview Park	Hartmannella sp.
5.	Regency Apartments	Acanthamoeba sp.
6.	Fertilized Field - Route 130	Acanthamoeba sp.
7.	Barnyard - E. Harrison Street Road	Acanthamoeba sp.
8.	Barnyard - Route 316	Acanthamoeba sp.
9.	Barnyard - Embarrass River	Acanthamoeba sp.
10.	Life Science Building	Acanthamoeba sp.

Table 2. Observations of amoeba cysts in tissues of mice 9 days after inoculation with soil amoeba-bacteria cultures, plus *Acanthamoeba castellanii*, saline and bacteria only as controls.

Soil sample number, mouse number*	Lesions#	Liver#	Small intestine	Kidney	Spleen	Brain
1	0	+	0	+	+	+
2	0	0	0	+	+	0
3	+	+	0	+	0	0
4	0	+	0	+	0	0
5	0	0	0	0	0	0
6	0	+	0	0	+	+
7	0	+	+	0	+	0
8	0	0	0	+	0	0
9	+	+	+	+	+	0
10	0	+	0	+	0	+
Acanthamoeba castellanii	0	0	0	+	+	0
Saline	0	0	0	0	0	0
Bacteria	0	0	0	0	0	0

*See Table 1.

#Indicates positive for lesions or presence of amoeba cysts.

Table 3. Observations of amoeba cysts in tissues of mice 7 days after inoculation with bacteria-free soil amoeba cultures, plus *Acanthamoeba castellanii* and sterile culture medium as controls.

Soil sample number, mouse number*	Liver#	Small intestine	Kidney	Spleen	Brain
1	+	0	0	+	+
2	0	0	+	0	+
3	+	0	0	+	+
4	+	0	0	+	+
5	+	0	+	0	+
6	+	0	0	0	+
7	+	+	+	+	+
8	+	0	0	+	+
9	0	0	0	0	+
10	+	0	+	0	+
Acanthamoeba castellanii culture	+	+	+	+	+
medium	0	0	0	0	0

*See Table 1.

#Indicates positive for amoeba cysts.

DISCUSSION

Results of this study demonstrated that the free-living soil amoeba isolated locally could be infective and pathogenic to adult mice. The implication is that these organisms may also be pathogenic to other mammals including man. Butt (1966), Wilson et al. (1967), Callicott (1968), and Cerva and Novak (1968) have reached the same conclusion in studies of Acanthamoeba, Naegleria, Hartmannella and other related free-living sarcodines. The group of naked amoebae most involved in infections are the small slug-like forms called "limax types." Hartmannella and Acanthamoeba are mentioned specifically by Ensminger and Culbertson (1966) and the amoebae isolated in this study were identified as being of these two genera.

It was expected that Naegleria gruberi, usually one of the more common forms (Kuhnelt, 1961) would be found, but such was not the case. The fact that the soil samples were collected in early January may have had some influence on the presence of Naegleria in that Levine (1973) suggested that this soil protozoan is much more abundant during the summer months. Also J. N. Farmer (direct communication) has noted that the numbers of Naegleria increase by more than a thousand fold in the mud of streams whose water temperature has been raised 15°C or more above normal by prolonged thermal pollution. It seems that Naegleria populations respond positively to elevated temperature.

Vahlkampfia, another common amoeba of the "limax" group, is also a soil inhabitant (Martin, 1913, Wilson, 1915, and Sandon, 1927). This

genus is sometimes parasitic in mammals (Culbertson, 1961, and Kudo, 1966) and may be found as an inquilinic form in reptiles (Wilson et al., 1967). Vahlkampfia was not found in this study perhaps because it is usually found in cucumber soil, fresh water and fecal samples from invertebrates and vertebrates (Martin and Lewin, 1914, and Levine, 1973) none of which were sampled in this study.

Acanthamoeba was more prevalent than Hartmannella. It is widely distributed in the soil being free-living, coprozoic, or feeding on fungi, bacteria or other organic material (Callicott, 1968). The sampling was restricted and not really intended to provide data on the distribution of soil protozoa, but the results are in keeping with the findings of Sandon (1927) and Levine (1973) who noted that Acanthamoeba was widespread, in fact cosmopolitan. Actually it may be assumed that the ten soil samples utilized here showed a moderate difference in their amoeboid faunae.

As only two kinds of amoeba were isolated, the problem revolved around which of the two was more or less pathogenic. Ensminger and Culbertson (1966) demonstrated invasiveness of hartmannellid amoebae by infecting mice intranasally with a strain of Hartmannella which produced cysts in brain tissue and proved to be pathogenic. No deaths were recorded in that study nor in the present study. Autopsy of the mice infected with Hartmannella revealed lesions in only one mouse, #3, and this was with the associated bacteria also injected. Amoeba cysts were found in the liver, kidney, spleen and brain. Other studies indicated that Hartmannella does not cause death in mice, monkeys or rabbits infected (Culbertson, 1961, Wilson et al., 1967, and Levine, 1973).

Of the two genera studied, Acanthamoeba sp. from the soil appeared to be the more pathogenic. In the mice injected with this genus, lesions were apparent and amoeba cysts were demonstrated in the liver, kidney, spleen, small intestine and brain or all the tissues observed. Although no deaths were recorded from the soil species of Acanthamoeba, some of the infected mice showed signs of illness during the incubation period. Acanthamoeba castellanii (Neff's strain) which was used as a control caused lesions, and amoeba cysts were present in all the preceeding named tissues.

Many researchers have reported death and serious tissue damage due to Acanthamoeba. Indications of Acanthamoeba infection in mice, monkeys, rats and rabbits are cysts and trophozoites in the coelom, lymph nodes, kidney, lungs, peritoneum, liver, spleen, intestine, blood and brain, extensive pneumonic changes, cerebral abscesses, hemorrhage, encephalitis, focal paralysis, neural malfunctioning, purulent meningitis, granulomatous response, lesions in the tissues, amoebae in the spinal fluid, ulceration of the nasal mucous membrane, extensive choriomeningitis, perivascular granulomatous lesions, severe pneumonia and repeated damage to tissues and organs (Culbertson et al., 1958, 1959, Culbertson, 1961, McCowen and Galloway, 1959, Bovee et al., 1961, Butt, 1966, Wilson et al., 1967).

Acanthamoeba has been proven to be virulent to man causing severe tissue damage and death. Primary meningoencephalitis, pulmonary edema, swelling and inflammation of the brain and meninges, amoebae in the spinal fluid, fever, headache, hemorrhage, intestinal amoebiasis, pneumonitis and tissue lesions result from Acanthamoeba infection and death usually follows (Butt, 1966, Callicott, 1968, Cerva and

Novak, 1968, and Levine, 1973). No such data exists for Hartmannella so studies support the conclusion that Acanthamoeba is more virulent.

The theoretical significance of pathogenicity of free-living soil amoeba is evident. Amoeba can live in many different environments because of their high adaptability and are found in almost every niche. Manwell (1968) says as a biological group, Sarcodina have excelled in adapting to varying habitats. Encystment during unfavorable conditions helps protect and extend the amoeba's life cycle. Soil amoeba are cosmopolitan species. Many parasitic forms have close counterparts in free-living species. Therefore with the great abundance of these free-living soil amoeba and the potential pathogenicity of such organisms to higher animals, these amoebae should be considered as possible health hazards. Soil amoebae have been known to occur in sewage and water sources. Thermal pollution increases the number of amoebae in the substrate and streams being indicators of polysaprobic water (Farmer, direct communication).

Another practical aspect of the knowledge that free-living amoebae can be pathogenic is to review the biology of known parasitic forms. Is it possible that some of these may be able to exist in a trophic state outside their hosts?

Further investigation is needed to reinforce findings of pathogenicity of free-living soil amoebae in animals. The determination of the exact species would be beneficial. An important step would be to test different antibiotics and drugs on the pathogenic amoebae isolated.

SUMMARY AND CONCLUSIONS

This study has shown that free-living soil amoebae can be isolated and cultured on agar in the laboratory. The genera isolated were Hartmannella and Acanthamoeba, with Acanthamoeba more prevalent in the soil samples. Mice injected with the amoebae and their associated bacteria produced lesions in the tissues observed. Amoeba cysts and bacterial colonies were also present in the liver, small intestine, kidney, spleen and brain.

The amoeba-bacteria cultures were purified to obtain axenic cultures of the soil amoeba. Antibiotic filter-paper discs soaked in streptomycin were used on Petri dishes containing amoeba-bacteria inocula. The amoebae were transferred to tubes of Neff's amoeba nutrient medium and from these tubes injected into the mice. The mice appeared ill, although no lesions were observed. Amoeba cysts were demonstrated in the preceeding mentioned tissues.

From the data in this study one may conclude that free-living soil amoebae become parasites in mammalian tissue, specifically mice, and Acanthamoeba is more invasive than Hartmannella. Since these amoeba types are found in many soil sources they can be easily acquired and may represent a potential health hazard to humans.

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