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Project Summary

Study of Pathogenic Free-Living Amebas in Fresh-Water Lakes in Virginia

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Pathogenic free-living amebas may produce a fatal infection of the central nervous system of man known as Primary Amebic Meningoencephalitis (PAM). In Richmond, Virginia, 17 such cases have occurred to date, more than in any other geographic location in the world. The major objectives of this study were to examine fresh-water lakes in the vicinity of Richmond, Virginia, for the presence of pathogenic free-living amebas, particularly *Naegleria*, and to gain some understanding of their prevalence and the conditions or factors which might affect their existence or concentrations.

Nine lakes, all within a 24-mile radius but with different limnological characteristics, were selected for biweekly sampling and study. Two of the lakes were previously shown to be the source of several human infections. Techniques for isolating and studying amebas from the environment were either modified from the methods of others or newly developed. Although ameboflagellates consistent with *Naegleria* were often isolated from all lakes, pathogenic ones were found infrequently. The major reason for this finding appeared to be the temperate climate and the short periods of time (days to weeks) that sustained elevations of water temperature (>26°C) occurred, unlike the situation observed in the more tropical areas of Florida.

Soil samples proved to be more dependable sources than water from which amebas could be isolated, particularly those amebas growing at 44°C and those that were pathogenic. The techniques of soil sampling and testing were recommended as simple, reliable, and of potential usefulness to public health and environmental officials for screening for pathogenic *Naegleria*. Pathogenic amebas were isolated only from shallow bottom soil and its associated water (depth, six to ten inches), but not from deep water samples.

During biweekly attempts at isolating amebas, temperature, pH, dissolved oxygen, salinity, and conductivity of the water were measured. Of these, water temperature appeared to correlate best with the presence of amebas, and when it exceeded 30°C, pathogenic forms might be expected to be isolated. In addition, daily climatological data were assembled; and on several occasions each lake was studied extensively both chemically and biologically by the Environmental Studies Group at Virginia Polytechnic Institute and State University (VPISU). The most enriched lakes did not necessarily yield frequent or large numbers of pathogenic amebas.

The bacteriologic flora of the lakes studied was also examined. Although no quantitative (MPN) relationship of the presence of coliform bacteria to pathogenic amebas or ameboflagellates could be shown, qualitatively

there may have been a relationship. Two types of bacteria frequently isolated from lake waters, *Pseudomonas fluorescens* and *Serratia marcescens*, appeared in the laboratory to be inhibitory to pathogenic *Naegleria*; and indeed in virtually every lake from which *Ps. fluorescens* was consistently isolated, no pathogenic *Naegleria* were found.

Of the lakes studied, the North Anna Reservoir or complex containing several coolant lagoons and canals for a nuclear power plant was the most intriguing; for the majority of pathogens and the most virulent organisms were isolated from this complex of lakes. Studies of this complex suggested that if natural fresh waters are artificially heated (thermal enrichment) for a sustained period of time, pathogenic *Naegleria* may be selected or concentrated, even in a cold or hostile climate, possibly serving as a source or nidus for contaminating other nearby lakes or streams. This study represented the first of its kind in which thermally enriched waters were studied before, during, and after thermal enrichment occurred; i.e., three sites at the North Anna Reservoir were studied biweekly for over a year before thermal enrichment was initiated and for over a year during and after which thermal enrichment occurred. Pathogenic *Naegleria* were isolated only with thermal enrichment, but not before or after.

This project summary was developed by EPA's Health Effects Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Objectives

The aims of this study were to examine a number of fresh-water lakes in the vicinity of Richmond, Virginia, in an attempt to isolate and study pathogenic free-living amebas (particularly of the genus *Naegleria*) and to acquire some understanding as to the prevalence of such organisms in this locale and the condition(s) or selective force(s) which might control their appearances.

Background Information

For many years, free-living amebas, although highly ubiquitous in nature, were virtually ignored by medical scientists. They were considered harmless and more of a biologic curiosity than any possible threat to health. Usually, such organisms were encountered in the bacteriology or virology laboratories as contaminants of plate or cell cultures; or they were selected by biologists or protozoologists as the subject of a doctoral thesis.

However, in 1958, as serendipity would have it, everyone's views changed entirely concerning the potential virulence of such organisms. In what initially was suspected as an unsuitable lot of vaccine containing live infective polio virus, a free-living ameba, recently assigned the appropriate name *Acanthamoeba culbertsoni*, was discovered. The ameba proved highly pathogenic for primates, as well as for a variety of other animals, producing a fatal meningoencephalitis. From these observations and subsequent ones, it was correctly surmised that natural infections in man and other animals due to these organisms might occur.

Shortly thereafter in 1965, almost simultaneously, there appeared a report from South Australia of four fatal cases of amebic meningitis recognized retrospectively through autopsy studies, and a report from Florida of the occurrence of several fatal human cases of what appeared to be the same disease. In both reports, the responsible pathogens proved to be free-living amebas, subsequently identified as members of the genus *Naegleria*, which represented ameboflagellates different from but related to the *Acanthamoeba* previously discovered. Thus, a new disease was recognized. Investigators became concerned as to whether or not it was a new entity or an old one that had been overlooked, an issue that still has not been completely resolved.

Nevertheless, subsequent to these observations, numerous reports began to appear in the medical literature testifying to the worldwide distribution of the disease and the virulence of the responsible pathogens. It was soon discovered that two genera of free-living amebas appeared to be responsible for producing fatal meningoencephalitis in man: *Naegleria* and *Acanthamoeba*. The former organism was almost invariably acquired from intimate contact with fresh water; frequently resulted in epidemics; and produced predictably an acute, fulminant, rapidly fatal disease characterized by hemorrhagic necrosis of the brain in an otherwise healthy individual, and entering the central nervous system via the nasal passage and olfactory nerve route. The latter organism differed, however, as it was not necessarily acquired from contact with fresh water (in fact the epidemiology remains completely obscure); appeared to occur sporadically; often produced a subacute or chronic (but likewise, fatal) disease, characterized by a granulomatous as well as a neutrophilic reaction; often occurred in an immunocompromised patient; and appeared to enter the central nervous system not only by the olfactory route but perhaps more frequently via hematogenous spread from other parts of the body. The disease produced by either of these organisms became known as "Primary Amebic Meningoencephalitis" or PAM to distinguish it from secondary spread of infection to the brain due to *Entamoeba histolytica*, a parasite, poorly tolerant of oxygen, which commonly produced a primary infection of the colon known as amebiasis.

To date, PAM due to *Naegleria* has been reported from every continent except the Artic and Antarctica: from England, Ireland, Belgium, Czechoslovakia, East Africa, India, Korea, Australia, New Zealand, Venezuela, and the states of Virginia, Florida, Georgia, North Carolina, Pennsylvania, New York, Arkansas, Texas, Louisiana, Arizona, and California. From a public health and environmental standpoint, the most disturbing features of the disease due to this organism were its frequent occurrence in epidemic proportions and its acquisition from fresh water, respectively; e.g., the common source epidemics and sporadic cases in Florida were traced to several fresh-water lakes in the Orlando area used for recreation; the epidemics in Virginia to three fresh-water lakes within a five mile radius used by the public for swimming and diving; the outbreak in Czechoslovakia to a heated swimming pool in Bavaria; similarly, the outbreak in Belgium to a heated indoor pool; the cases in New Zealand and California to natural thermal pools; recent outbreaks in England to mineral baths; and, most disturbing, in Australia to contamination of the public potable water supply for the entire Spencer Gulf area. However, despite the apparent ubiquitous nature of the responsible organisms and the widespread distribution of outbreaks and sporadic cases, the disease appeared to be endemic only to certain locales.

Approach

At the time this study was undertaken, little data was available on the distribution and frequency of occurrence of pathogenic *Naegleria* in the environment. Many lakes and pools had been sampled and studied, particularly those responsible for outbreaks of PAM; however, usually only nonpathogenic *Naegleria* and/or *Acanthamoeba* were isolated. It was not until 1972 when the suggestion that temperature tolerance might be utilized as a selective force in isolating pathogenic *Naegleria*, that epidemiological and environmental studies began to make considerable headway. Pathogenic *Naegleria* survived (and multiplied) at temperatures as high as 44-45°C; whereas nonpathogenic ones did not.

In 1975, pathogenic *Naegleria* were isolated from a thermally polluted canal in Belgium, the source of a fatal infection for a boy who had been swimming there. Subsequently, reports began to filter in that pathogenic *Naegleria* could frequently be isolated from lakes in Florida.

Since it was well known that the Richmond, Virginia, area was highly endemic for PAM due to *Naegleria*, accounting for 17 cases, and since these cases had occurred over a time span of 10 years, it was believed that a study of lakes in this region of the country might aid in understanding the unpredictable appearance of PAM and the factor(s) or force(s) that might be responsible for the presence of pathogenic amebas. In addition, since the source for all previous cases of PAM in Richmond had been clearly traced to one of three fresh-water lakes located within a five-mile radius, and since numerous other lakes in the locale had never been incriminated as a source of PAM, even though frequently used for swimming by the public, it was believed that a careful study of the "Non-PAM-related" lakes might yield valuable information if compared to data obtained from "PAM-related" lakes. Thus, nine lakes were selected for study for a variety of reasons. Included in the study were two lakes previously identified by patient histories as a source of PAM, namely, Moore's Lake and Lake Manchester. (The third lake, Lake Chester, associated with PAM in the locale could not be included since it had been closed and drained several years before by court order). In addition, two lakes (Lake Pocohontas and Pahlisades Lake) were selected because pathogenic *Naegleria* had been previously recovered from their waters. Four lakes (Lake Salisbury, Swift Creek Reservoir, Falling Creek Reservoir, and Overhill Lake) were chosen because they had not been associated with the occurrence of PAM; and finally, one lake (Lake Anna) was chosen because it was soon to become thermally enriched by the cooling effluent of a nuclear power plant (VEPCO North Anna Station).

Techniques of isolation and sampling for amebas were: temperature tolerance of pathogenic *Naegleria*; i.e., samples of water and bottom soil were to be incubated at 44° - 45°C, with the belief that non-pathogenic amebas would be suppressed while pathogenic ones would survive. The methods of acquiring the samples would be filtering a large volume of lake water through a sterile sand column (with the belief that amebas would adhere to the sand particles), subsequently eluting amebas from the sand by a gentle detergent (beef broth), and finally harvesting the eluted amebas; and simultaneously collecting a small sample of soil from the surface of the bottom. Along with these protozoological studies, an analysis of the physicochemical (pH, dissolved oxygen, salinity, conductivity, and temperature) parameters of each lake would be studied biweekly, as well as the concurrent climatological aspects of the locale. If possible, it was hoped that more extensive chemical analyses, as well as limnological studies, of the lakes could be obtained, perhaps through two nearby environmental groups at Virginia Polytechnic Institute and State University. In other words, every effort would be made to identify, characterize, and monitor the chemical and biologic properties of every lake under study with a particular effort to identify the differences and similarities which might serve as selective forces for the appearance or disappearance of pathogenic free-living amebas in nature.

Conclusions

A 33-month study of the isolation of free-living amebas, especially pathogenic ones, from nine fresh-water lakes located in and around Richmond, Virginia, an area endemic for Primary Amebic Meningoencephalitis (PAM), and the relationship(s) of their isolation to a variety of physicochemical, bacteriological, and climatological factors revealed the following:

1. That ameboflagellates tentatively identified as *Naegleria* which could grow and survive at 44°C were present to varying degrees in every lake studied. Of a total of 1,311 samples of lake water and bottom soil tested, 16.2 percent were positive for such ameboflagellates.
2. That such ameboflagellates were isolated more frequently in the summer (the warmer months) than at any other time of year. Isolation of such ameboflagellates also varied widely from lake to lake (five percent for Lake Overhill to 33.8 percent for Lake Anna at its number I site). These findings were consistent with the seasonal (summer) appearance of PAM.
3. That free-living amebas which grew at 44°C but did not flagellate were isolated from 53.9 percent of the total 1,311 samples of water and bottom soil tested. The pathogenicity of these organisms was not tested and remained unknown.
4. That pathogenic *Naegleria* (i.e., *N. fowleri*) were present, but apparently in concentrations so small that their presence, especially in natural lakes, was detected infrequently. Of the 1,311 samples of water and soil examined, only 13 (one percent) contained pathogenic amebas, only five of which were from natural, nonthermally enriched lakes. These findings were consistent with the infrequent occurrence or rarity of the disease PAM in the local population.
5. That pathogenic *Naegleria* were isolated more readily from thermally enriched impoundments or lakes than from natural, nonthermally enriched lakes. Such impoundments or lakes through their artificially elevated water temperatures could serve as potential "reservoirs" for perpetuation and possible spread of such organisms to other lakes in temperatue zones where such amebas are either nonexistent or of little consequence. These findings might suggest that these lakes will probably be a source of PAM in the future if man continues to use them for water sports or bathing.

6. That pathogenic *Naegleria* may not be present in deep waters but only in shallow waters (depending on the sensitivity of the test). Five pathogens were isolated from shallow water and eight from shallow bottom soil samples, but none from deep water or deep bottom soil (as opposed to observations in Florida where deep sites were important and frequently positive).
7. That detection of pathogenic *Naegleria* increased significantly when water temperatures equaled or exceeded 30°C, especially when such water temperatures were sustained with little fluctuation over a period of 30 days or more. These findings appeared to explain, at least in part, the occurrence of PAM in this locale only in the warm months, particularly in the latter part of the summer.
8. That such pathogenic *Naegleria* were generally highly virulent, but occasionally identical forms of lesser virulence could be isolated, the detection of which occurred only after subcultures of primary cultures of infected mouse brains were performed.
9. That often free-living amebas and/or ameboflagellates growing in Chang's liquid axenic media, a supposedly selective medium for pathogenic forms, were not pathogenic as determined by mouse virulence tests. Such growth, although initially good, could not be sustained, and such amebas eventually were lost in subculturing.
10. That pH, dissolved oxygen, salinity, and conductivity of lake waters appeared to differ little from lake to lake within the area under study, and furthermore within the ranges observed did not appear to be important determinants of whether or not pathogenic *Naegleria* could be isolated. However, pathogenic forms were notably absent from two swimming lakes (Overhill and Moore's) to which frequently were added large quantities of chlorine, potassium carbonate, and aluminum sulfate. Nevertheless, in the past one of these lakes (Moore's) has been associated with PAM (as judged from patient histories.)
11. That isolation of pathogenic *Naegleria* from natural, nonthermally enriched lakes appeared to be less during the year 1979 following two severely harsh winters than during the years before (1977 and 1978), but this was not significant statistically. As expected, water temperatures of natural lakes varied directly with the ambient temperatures of the locale. These findings were operational in past epidemic years in which the disease PAM occurred in the resident population at risk, and supported the thesis that sustained elevations of water temperature were essential for detectable concentrations of pathogens to occur.
12. That the bacterial flora of the lakes studied may well have been important in determining the population of pathogenic *Naegleria* present, and that such determinations appeared to depend on both qualitative as well as quantitative aspects. Several species, *Serratia marcescens* and *Pseudomonas fluorescens*, which were isolated from lake waters in large numbers, appeared to inhibit the growth of pathogenic *Naegleria* as determined by in vitro testing, and in some instances were toxic for them. Such findings may have explained the infrequent isolation of pathogenic forms.
13. That special extensive qualitative studies of the water and soil of the lakes under study indicated that highly enriched lakes (e.g., Lake Manchester) might not be associated necessarily with detectable levels of pathogenic *Naegleria*.
14. That these same special studies indicated that Lakes Anna I, II, and III, sites which were thermally enriched and which were associated with the greatest number of isolates of pathogenic *Naegleria*, contained high concentrations of iron and manganese, especially in their bottom soil. The significance of this was obscure, although it is known that pathogenic *Naegleria* are avid consumers of red blood cells (RBC), which contain iron in the form of hemoglobin, and their pathogenicity frequently can be revived by addition of RBC's to the culture medium.
15. That the strains of pathogenic *Naegleria* isolated were susceptible *in vitro* to the same agents and compounds and in the same concentrations as those which inhibit strains of *N. fowleri* isolated from humans dying from PAM. The polyenes were predictably amebicidal against environmental isolates of pathogenic *Naegleria*.
16. That culturing small samples of soil (about 500 gms) for pathogenic *Naegleria* by the methods outlined was easier, more economical, and more sensitive than culturing large volumes of water (72 L) and thus could be of practical use in future screening studies of fresh-water lakes. By the water sampling techniques employed, in order to isolate amebas, ameboflagellates, or pathogens required that at least one trophozoite per ten ml water be present. In undisturbed waters this concentration may be infrequent.
17. That prolonged incubation (up to 300 to 500 hrs.) of samples at 44°C appeared to enhance the isolation of pathogenic *Naegleria*, thus enhancing the sensitivity of the testing procedure and minimizing fears of transportation and storage losses.
18. That plankton nets were not acceptable for sampling lake waters for free-living amebas.

In summary, these studies demonstrate that although the presence of pathogenic *Naegleria* in natural fresh-water lakes may depend on a variety of factors, water temperature, nutrients and bacterial flora appear to be the most important of the major determinants. High concentrations of iron, and perhaps other metals or minerals, may also be important, but this remains to be proven. For natural fresh-water lakes, extremes of pH and excessive salinity (>0.5% NaCl) and/or conductivity adversely affect pathogenic *Naegleria*, but depressed levels of dissolved oxygen may not be as critical as once believed despite the aerobic nature of these organisms.

Since isolation of pathogenic *Naegleria* from fresh-water lakes, along with determination of factors affecting the appearance of pathogens were the objectives of this study, the area under study proved to be excellent. It had been associated frequently with human PAM, and patho-

genic *Naegleria* were present only periodically and not in all lakes. So, when pathogens were isolated, their presence could be studied and correlated with variations in physicochemical or biological changes or pressures.

Recommendations

Based on the data obtained in this study and that reported by others the following recommendations are made:

1. That all fresh-water lakes whose waters may reach or exceed 30°C and are utilized for public swimming should be assessed for their content of pathogenic *Naegleria* and for their ability to support such organisms. This may be accomplished by utilizing the methods of analyzing bottom soil described in this report, which are simple, economical, and reasonably reliable. The water itself need not be cultured.
2. That if pathogenic *Naegleria* are isolated from a fresh-water body of water used for swimming, the lake should not be opened to the public when water temperatures exceed 30°C. Authorities should be made cognizant of the fact that with each day such water temperatures exceed 30°C, the concentrations of pathogenic *Naegleria* may be increasing. If man is exposed to such waters in any intimate way (e.g., swimming), the risks of developing PAM may be heightened.
3. That if pathogenic *Naegleria* are isolated from a lake, further studies should be undertaken to determine the ease with which this can be accomplished (e.g., sequential sampling and, if possible, quantitative studies to determine the concentration of such pathogens, perhaps employing dilutional or MPN methods). Thus, the risks in terms of exposure to pathogenic *Naegleria* should be assessed.
4. That thermal enrichment of lakes containing pathogenic *Naegleria* should be discouraged, limited, or controlled, and public swimming in such lakes or impoundments should not be permitted.
5. That the Lake Anna system receive continual surveillance for pathogenic *Naegleria*, quantitatively as well as qualitatively, and residents in that locale be made aware of the threat that might exist if bathing or swimming is pursued while thermal enrichment exists.
6. That the addition of chemicals (e.g., NaC1, aluminum sulfate, and halides) to fresh-water lakes be studied as to their effect on pathogenic *Naegleria*, as well as on the competitive protozoan flora and other microbes. It is possible that such chemicals may be detrimental, as well as beneficial.
7. That potable water used for consumption and bathing and obtained from reservoirs be particularly scrutinized for pathogenic *Naegleria*, and that filters, piping systems, and the end product itself be studied for the presence of such forms.
8. That epidemiologic studies on human populations using fresh-water lakes for recreation be undertaken to determine the attack rates of pathogenic *Naegleria* and to determine if subclinical or nonfatal infections, meningitis or otherwise (e.g., respiratory) are occurring. This may be done by studying human sera and measuring antibodies against the resident pathogenic *Naegleria* isolated from lakes in the locale.
9. That bacteria (and possibly fungi and protozoa) be more carefully studied for their effect (interference or enhancement) on the appearance of pathogenic, as well as on nonpathogenic amebas (especially *Naegleria*).
10. That the interrelationships between pathogenic *Naegleria* and other protozoan species (competitors and predators) be more carefully explored than has been done in the past.
11. That the effect of iron and other metals or minerals on the appearance of pathogenic *Naegleria* be studied.
12. That microcosm studies be performed in model systems in which variables may be separated and individually tested in the laboratory to quantitate their effect and to study their interrelationships.
13. That pursuit of possible factors which might induce nonpathogenic *Naegleria* to become pathogenic forms continue to be explored.
14. That other selective methods for pathogenic *Naegleria* (e.g., media containing inhibitory agents for non-pathogenic amebas) be sought, as the use of elevated temperatures (44°C) appears to be too nonspecific and growth in Chang's liquid media not always dependable.
15. That free-living amebas growing at 44°C which are nonflagellating be further studied for pathogenicity, as there is an increasing number of fatal cases of PAM being reported from species other than *N. fowleri*.
16. That the natural spread or dissemination of pathogenic *Naegleria* or the methods whereby lakes may be seeded with such forms should be studied.

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The complete report, entitled "Study of Pathogenic Free-Living Amebas in Fresh-Water Lakes in Virginia," (Order No. PB 126 [redacted] Cost: $[redacted]; subject to change) will be available only from: 4.50 12.50
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