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Studies of Limax Amoebae in a Swimming Pool

by

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Introduction

Within the years 1962 to 1965, 16 fatal cases of primary amoebic meningoencephalitis have been reported from northern Bohemia (Červa et al. 1968). Since all persons infected had bathed in the local swimming pool shortly before the first symptoms had signalled the start of a disease the water in the pool was suspected to contain pathogenic amoebae. Nonpathogenic strains of amoebae of the species *Naegleria gruberi* – the etiological agent of this disease – and related species, generally listed to the group of the so-called *limax* amoebae, are common members of the water fauna and of the soil in nature. There is no detailed information available in the pertinent literature on their occurrence in swimming pools supplied with chlorinated drinking water. The unusual epidemiological situation called for quick action to obtain some information on the possibilities of a longlasting survival and reproduction of these microorganisms under similar conditions. This paper presents the results of investigations of the swimming pool in Ústí n./L., where all these fatal cases had been infected. These investigations lasted from Oct. 30, 1967 to Jan. 10, 1969. Detailed descriptions are given of the methods which have been found most suitable for the detection of amoebae.

Material and methods

a) Cultivation methods

aa) Cultivation on agar.

The universal medium used for cultivation was a 1—2% agar in distilled water (Bacto-Agar-Difco). A layer of 2—3 mm of medium

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was placed in petri dishes of 6—10 cm in diameter. One to two drops of suspension containing heat killed clean culture of *Aerobacter aerogenes* (see § ab) were spread with a bent glass rod over the solid surface of the agar. The plates were stored upside down in hermetically closed polyethylen bags in the refrigerator until the time of use. Condensed water was not removed from the lid before inoculation to retain the highest possible humidity during cultivation itself. Incubation of the inoculated plates was carried out at + 37°C. This thermal selection enabled the registration of such strains of amoebae only, growing at this temperature and possibly capable of potential pathogenicity. Because amoebic growth is read microscopically from the agar surface through the bottom of the dish, only new and unscratched dishes have to be used in this cultivation method.

ab) Preparation of the suspension of Aerobacter aerogenes (CULBERTSON *et al.* 1965).

Several petri dishes with bouillon agar were inoculated with a massive dose of *Aerobacter aerogenes* culture and incubated for 24—36 hrs at 37°C. The bacterial layer was removed from the agar surface with a bent glass rod and about 2 ml of sterile distilled water. This bacterial water suspension was transferred to the test tubes under strictly sterile conditions, washed twice in distilled water by centrifugation and, if necessary, thickened to a creamy consistency. Then, ampules were filled with 0.5—1 ml of suspension, sealed and heated in the ultrathermostat to 65°C for 30 min. Such suspension can be stored in the refrigerator for several months.

ac) Cultivation in fluid media

Fluid media were used for axenic cultivation of isolated strains particularly for the preparation of amoebic suspensions to be employed in infection experiments. Part of the strains, all members of the genus *Acanthamoeba*, grew readily on 2% Bacto Casitone Difco (ČERVA 1966) in distilled water. To incite growth of other strains, particularly those indicating pathogenic capabilities, (ČERVA 1969) 10% of fresh horse serum had to be added to the medium. The majority of isolates, however, did not grow on fluid media. The growth of contaminating microbes was blocked by adding 500 I.U. penicillin and 50 gamma streptomycin/l ml medium to the fluid media. The harmlessness of these concentrations of antibiotics has been confirmed empirically (ČERVA 1969).

b) Detection of free amoebae in water

ba) Cultivation of a series of decimally reduced water inocula (culture titration)

Water known to contain or, at least, suspected of containing, higher concentrations of the limax type amoebae was titrated quantitatively by direct cultivation. Water samples were collected from the pool into flasks with a silicon coated inner surface and inoculated immediately onto agar plates. When cultivating an inoculum of less than 0.1 ml, the water sample was decimally prediluted with distilled water in solicon coated test tubes. The maximum amount of inoculum on a 10 cm plate could be up to 10 ml water. One volume of inoculum was used always for 10 plates. The inoculated plates were hermetically closed in polyethylen bags and incubated with the bottom downwards at 37°C for 24 hrs to allow for sedimentation and the attachment of the amoebae and cysts on the agar surface. Then, the plates were reversed and cultivated for another 6 to 10 days for final microscopical examination of the growth (magnification approx. 100 x; objective 10 x, ocular 8—10 x).

The series of cultivations used for water examination were as follows:
10 x 10 ml, 10 x 1 ml, 10 x 0.1 ml, 10 x 0.01 ml etc.
Amoebic counts (x) per 1 liter of water were established by the formula:

$$x = \frac{100\ n}{d}$$

n = the number of plates positive after inoculum d
d = the smallest amount of inoculum in ml, in which less than 90% of plates were positive.

Under perfect cultivation techniques, all plates inoculated with a dose of 10 d should be positive while only occasional positive findings should be obtained from plates inoculated with less than 1 d.

bb) Quantitative determination by cultivation of membrane filters.

Water samples with a small concentration of amoebae (tapwater etc.) cultivated after spontaneous sedimentation or centrifugation offered most unsatisfactory and incomparable results. Excellent results were obtained by filtrating the water through a coarse membrane filter used there as inoculum (30 mm disk filters Sartorius 11002 with pores of 2—3 μ). Filtration was performed in Seitz filtration casings. In this way the amount of cultivated fluid could be increased to 1 and even 2 1. Good results were obtained

also by using dense analytical paper filters (blue ribbon). After completing filtration the filters were removed from the casing with sterile pincers and placed upside down on agar plates. Incubation proceeded routinely at 37°C for 7—10 days. Positive cultures were distinctly visible upon microscopical examination of the free uncovered agar surface. A simple approximate estimation of amoebic concentration in the water sample under consideration may be derived from the least possible amount of filtered water still giving a positive culture.

bc) Direct microscopic examination of water with the aid of membrane filters

Membrane filters are most satisfactory for giving ready information on the biocenosis in the water examined. Good results have been obtained with Sartorius 10002 or other filters of the same parameters. A known volume of water is prefiltered in the same way as that used for the quantitative determination of filter cultures. If the water samples are relatively clean the amount of water can be 1—2 l. After completing filtration, the filters are removed from the casing and fixed upside down in sublimate alcohol. The least possible time is 5 min, but they can be stored in this liquid for longer periods. Then, the filters are transferred from sublimate alcohol to 80% alcohol. There, the filters can stay for any length of time or, if necessary, be removed after several minutes. The filters are washed in distilled water and then stained with one of the haematoxylin stains. The filter foil is cut into a convenient size, transferred to 96% alcohol, carbolated xylol and xylol and embedded in Canada Balsam, caedax or similar media.

The total number of amoebae (x) in the prefiltered amount of water is determined by the formula

$$x = \frac{p \cdot f}{z}$$

p = average number of amoebae in one field of view of the microscop (immersion objective)
f = total area of the membrane filter passed by the filtered water (without covered margins of the filter)
z = area of one field of view of the microscop (immersion objective)

c) Detection of sessile amoebic populations

ca) Cultivation

Discs of a certain size (e.g. 1 cm^2) were cut from the filtration paper and sterilized with hot air in petri dishes. A moistened sterile disc was placed over the area under examination, slightly pressed

on to it with a rubber stopper of the appropriate size and the surface of the examined area taken off by rotating movements (tiles, metal fittings in the pool), stairs, ladders etc.). Then, the paper discs were transferred with pincers to the surface of the cultivation medium. The results obtained with this cultivation had to be corrected with regard to the concentration of amoebae in the water of the pool. Sometimes – in very clean water – this method may give an approximate picture of amoebic incidence on the various areas moistened by water.

cb) Amoebic accretions on various submerged subjects

Various subjects such as membrane filter leaves, stripes of cellophane, microscopical slides etc. were placed on the bottom of the pool, on its walls and on various fittings inside the pool. These subjects were removed in weekly intervals and the attached microorganism fixed in sublimate alcohol. The cellophane stripes and membranous filters were stained with haematoxylin only (the background stains intensively with acidophilic dyes) in the same way as described in the section of microscopic examination of water on membrane filters. Preparations on slides can be stained with various methods (Masson's trichrome gives favourable results).

This method of biological examination has been found excellent not only for quantitative and qualitative studies of accretions of the *limax* amoebae, but also for a relatively exact determination of the density and species composition of complete populations of sessile protozoans forming naturally at certain sites of the pool. In our opinion this method is extremely valuable.

d) Isolation of the clones

The strains of clones used in these experiments were obtained either from cysts or from trophozoites according to the requirements of the situation. Since the natural accretion of amoebae on the agar plates is too dense and thus unsuitable for mechanical cutting out of the individual microorganisms, the initial cultures were washed off the agar surface with PBS and with the aid of a bent glass rod. Cysts washed in a larger amount of PBS excysted successfully in the new medium (Jensen, Dubes 1962). About 0.1 ml of diluted amoebic suspension or cysts was placed on a special agar plate (3% agar in distilled water, thickness of layer about 1 mm) and distributed over the whole area with a glass rod to separate the individual organism at distances of several mm. After sedimentation, the sites occupied by the amoebae and their cysts were marked off under the microscope on the bottom of the petri dish with small dots. With a bacteriological loop (about 1 mm in diameter) bent at a right angle to the

wire handle the marked sites were cut out of the agar and transferred to clean agar plates. This procedure is considerably more productive and safer against secondary contamination than the monosporic isolator Meopta (CULBERTSON et al. 1965).

e) Identification of the amoebae

The criteria given by PAGE (1967a, b, 1968) were used for basic classification and arrangement of the individual isolates into genera and species. Most of the isolates could be identified by this system after their properties had been studied in detail. Doubtful results were obtained when assessing to identify the amoeba species from microscopical slides only. However, to our knowledge, there are no better methods available for identifying amoebae of the *limax* group.

f) Pathogenicity tests

The pathogenic capability of the cleaned clone isolates of amoebae growing at 37°C was tested in mice. Suspensions of inoculated amoebae were prepared either by centrifugation of the axenic cultures from liquid media or by removing the amoebae from the surface of the agar cultures. Massive concentrations of amoebae were taken off the agar plates before encystment with about 2 ml PBS using a bent glass rod; the suspension was transferred to sterile silicon coated test tubes. According to the number of amoebae counted in Bürker's chamber, the density of this suspension was arranged either by centrifugation or dilution to 100 to 1000 specimens per 0.01 ml of suspension, and used for inoculating intracerebrally 10 mice of 10—13 g/b.w.; the mice were inspected every day for a fortnight. Thus, it was possible to exclude strains with no lethal effect on the mice after intracerebral inoculation as nonpathogenic. Cultivation of the brains of the surviving animals gave information on the capability of the strain to encyst and survive in the brain tissue.

When death of the mice was accompanied by symptoms signalling a damage of the CNS, the pertinent amoebic strain was submitted to further testing. A suspension similar to that used in the first phase of the test, but of a higher amoebic concentration (10—100 000 amoebae per 0.01 ml) was prepared. A dose of 0.02 ml of this suspension each was dropped into the nostrils of 10 mice (10—13 g/b.w.) narcotized with ether. The specific death following the intranasal instillation of the amoebae was preceeded mostly by typical symptoms (movements in constantly diminishing circles with a sideways bent head, disturbed equilibrium and loss of motility coordination). The brains of the mice dying spontaneously within 14 days after inoculation, were cultivated on agar plates and

examined histologically. The mice surviving for another 10 to 14 days were dissected and the frontal lobes of the brain cultivated on agar plates. According to the results obtained in animal experiments and in control cultivation, the possible pathogenic properties of the isolated strain can be divided into 6 groups (Table I).

TABLE I

	Degree of pathogenicity						
	Non-pathogenic		Moderately pathogenic			Highly pathogenic	
	I.	II.	III.	IV.	V.	VI.	
Capability of encystment and longlasting survival in the brain of mice after intracerebral inoculation	0	+	+	+	+	0	
Capability of killing the mice after intracerebral inoculation	0	0	+	+	+	+	
Capability of penetrating the brain of mice after intranasal instillation, followed by encystment and longlasting survival of the cysts	0	0	0	+	+	0	
Capability of killing the mice after intranasal instillation	0	0	0	0	+	+	

RESULTS

a) Description of the swimming pool

A brief description of the swimming pool in the recreation centre "Lázne Dr. Vrbenského" in Ústí nad Labem.

The swimming pool has been in public use since 1931. It measures 25 x 12 m, its water volume is 530 m³. The recirculating system of the pool consists of a sedimentation tank (90 m³) in which the water is chlorinated and supplied with ammonium sulphate, of a sand filter, of a sump fitted for dosing soda to the water, of a heating system and of a system for the additional supply of gaseous chlorine. The complete recirculation system contains approximately another 120 m³ of water. The pool is filled with tap water every 7—8 weeks, the daily exchange of water is 60 m³. The sand filters are washed

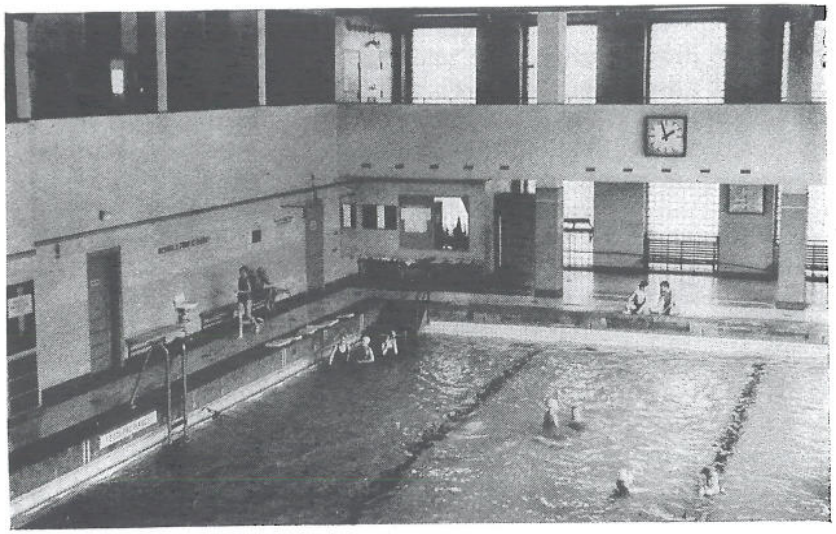


Fig. 1. The inside of the hall with the swimming pool (recreation centre Dr. Vrbensky).

twice a week with another 90—120 m³ of fresh water. The recirculation speed is 20 l of water/per sec. In this way the water is changed about 3 times a day. One third of the water-supply flows into the pool through openings in the frontal wall (depth 0.5 m) and two thirds through horizontal slits at the bottom of the deep part of the pool (depth 3.5 m), returning to the recirculation through troughs along the side walls of the pool. The pH of the pool is kept within 7.4 to 7.6 at levels of 0.3 mg/l of free chlorine in the pool. The temperature of the water is 22 –24°C. Each morning before opening hours, the sediment is scrapped off mechanically from the bottom of the pool and discharged through the bottom outlet. Especially in recent years great attention has been given to the general cleanliness of the place.

b) Findings of free amoebae in the water

Preliminary attempts performed in order to obtain amoebae from the water of the pool by cultivating either the sediment from 50 cm high glass columns with hydrophobic walls or sediment obtained by centrifugation gave mostly negative results, whereby no reasonable relationship could be established among the occasional positive results. A systematic confirmation of the presence of amoebae in the water of the pool was obtained by cultivation of the membrane filters. By continously reducing of the amount of water passing through the filters such high concentration of amoebae was revealed

that the agar plates could be inoculated directly with this water. The results obtained from the quantitative cultivation on the agar plates showed that the number of amoebae in one liter water ranged from 10^2—10^3 and exceptionally up to 10^4. Therefore, positive results were obtained regularly in series of inocula of a volume of 1 ml and above; occasionally also from only 0.1 ml. Regular microscopical inspection of the agar plates showed quite clearly that the amoebae are growing on the agar surface from one or several centres, forming gradually increasing and fairly regular circular patterns with the main zone of growth located on the periphery. In some strains these zones of growth could be viewed macroscopically in slanting light. Generally, it is necessary to view the whole surface of the plate at a low magnification of the microscop (about x 100). Also the bacterial film on the agar surface is visible at this low magnification. Sometimes, the morphological differences of the individual amoebic isolates are clearly visible in primary cultures especially when cysts are formed. The most frequent contaminating microorganisms were bacterial forms resembling *Proteus*; fungal contamination was very rare and flagellates or infusorians were found temporarily only in primary culture on several of the plates. Generally speaking, this mode of cultivation seems to be almost selective for amoebae of the *limax* group. We have been able to receive by culture titration amoebic clones from the smallest doses of inocula.

The species predominant in isolates from free water were *Hartmanella vermiformis* and *H. limacoides*; less frequent was *H. exudans*. Of the genus *Acanthamoeba* the most frequent species was *A. polyphaga*; *A. castellanii* was isolated only occasionally from free water. Amoebae of the genera *Vahlkampfia* and *Naegleria*, *Flabellula* and *Hyalodiscus* were isolated only occasionally from free water of this swimming pool and that from samples taken from the bottom corners of the deepest part of the pool.

The quantitative cultivation from water does not offer any information on the stage (vegetative or cysts) of the amoebae in the samples. This, however, is of greatest importance for estimating the hygienic importance of the concentration of microorganisms, for detecting the sources of protozoan contamination of the water and also for estimating the epidemiological significance of the finding of pathogenic strains of amoebae. The infectivity of the cyst is considerably lower than that of the trophozoites. Therefore, the preparations obtained from the filtration of 1 liter of water by membrane filters had to be examined microscopically to obtain the necessary information. These examinations revealed that the ratio of cysts to vegetative stages of *limax* amoebae – without distin-

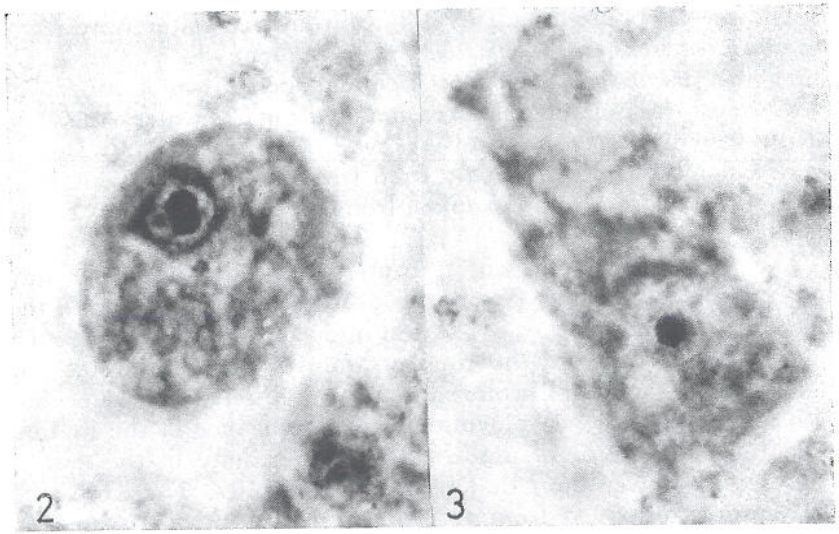
guishing the various species – was relatively stable in the pool under consideration, being close to 2 : 3. The total number of amoebae in a certain amount of water was slightly lower than that obtained with culture titration. This difference may be caused by the fact that part of the protozoans on the membrane filters escape identification in the dense clusters of organic material

The differences in the concentration of amoebae and their cysts in free water were studied by the water cultivation method. Series of samples were taken from 5 sites of the pool.

1. From the surface in the centre
2. From the bottom in the centre
3. From one bottom corner in the deepest part (3.5 m)
4. From the surface of a corner above the deepest part
5. From a corner of the shallow part (0.5 m)

The results showed that the highest concentrations of amoebae in the water were constantly present in the bottom corners of the deepest part of the pool. There, these concentrations are only minimally influenced by the cycle of water exchange. A relatively stable concentration was observed also in the corners of the shallow part. The greatest changes in amoebic numbers were found to occur in all samples taken from the surface layer in the centre of the pool.

Therefore, this site has been selected for longlasting investigations to reveal any dependence of the amoebic incidence on the bacterio-



Figs. 2 and 3. Various forms of amoebae from preparations obtained by water filtration through membrane filters. Heidenhain's ferric haematoxylin, phase contrast (× 3,000).

logical findings, on the chemical state of the water and on the cycles of regular water exchange. Regular bacteriological and chemical examinations were performed by the pertinent laboratories of the District Hygienic Centre in Ústí n/.L. The results of these examinations performed within the period of one year (Jan. 4 1968—Jan. 15 1969) showed that the chemical composition of the water in the pool never surpassed the qualitative limits of drinking water. Bacteriological examination gave no evidence of the occurrence of *Escherichia coli*; the number of mesophilic germs per 1 liter of water ranged from 0—22; in one instance only, 372 germs were found in 1 ml.

Simultaneously, the concentration of amoebae was examined with the method of culture titration; the results ranged from 20—5,000 specimens in one liter of water. No direct relationship could be established between the number of amoebae in the water and the values obtained from chemical and bacteriological examinations.

Therefore, we tried to obtain information on the influence of the 7—8 weeks cycle of refilling the pool with fresh water on amoebic concentration. Fig. 4 illustrates the development of amoebic populations in the water of the pool within two 8 weeks cycles. For these examinations we employed the routine method of culture titration of the surface layer of water in the centre of the pool. The course of both curves is very similar indicating a speedy increase of amoebic concentration in the water during the first two weeks following the exchange of water with a maximum in the 7th till 8th week. At this time we found about 1,000 specimens per liter. During the whole time of examination we observed repeatedly considerable deviations from the course of the curve in both directions, which lasted only a short time and may have been caused by minute irregularities in the regime of circulation and water conditioning (failure in the chlori-

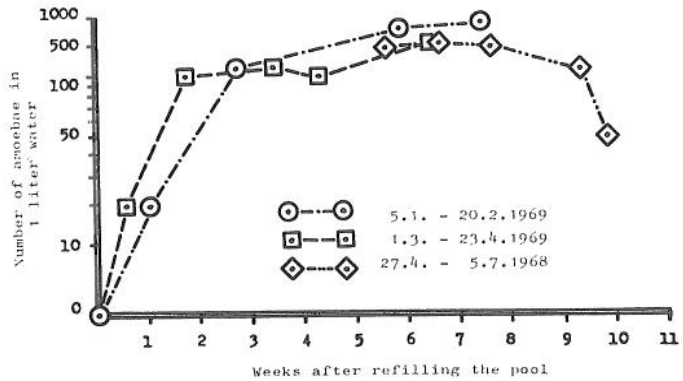


Fig. 4. Quantitative changes in the findings of amoebae from the superficial layer of water in the centre of the pool after the exchange of water.

nation equipment, in the pumps of the recirculation system, insufficient mechanical cleaning of the bottom of the pool etc.). These exceptional, shortlasting conditions account for an occasional finding of high amoebic concentrations in this pool reaching values of up to 10^4 per liter.

Since the water in the pool is regularly exchanged, the influence of further changes of the chemical quality of the water on the development of amoebic populations could not be traced. It seems possible, however, that the natural accumulation of salts in the water may have an inhibiting influence on amoebic growth. This is suggested by the declining final portion of the third curve of the figure illustrating the state of the water during an exceptional prolongation of the period of regular water exchange.

c) Sessile amoebic populations

These populations have been studied on the walls and bottom of the pool and on various other parts of its internal fittings and equipment. Originally, the pool had been equipped with two rows of wide stairs in the corners of the shallow part of the pool and with four ladders one at each side of the central part and one in each corner of the deepest part. The skeleton of these ladders was made of chrome plated metal tubes, the steps of hardwood, which was greatly macerated. The initial preliminary quantitative examination performed with the aid of smears on filter paper showed that these wooden surfaces harboured the highest concentrations of *limax* amoebae of the most various species. In order to reduce the danger of the presence of pathogenic strains of amoebae, these steps were replaced upon recommandation by steps made of nonporous desks of hard PVC; unfortunately it was impossible to assess the influence of this exchange on the general quality of the water in the pool. Upon later examination, amoebic concentration on the surface of these desks made of artificial material was similar to that observed on the surface of the tiles covering the pool.

Fig. 5 demonstrates the results of a single cultivation examination of the bottom of the pool performed in the 8th week after refilling the pool with fresh water. Each of the 16 points marks the site from which smears were taken on filter paper from an area of 1 cm^2.

The distribution of the sites with positive findings indicates that the density of amoebae attached to the bottom of the pool was higher in the deeper half of the pool. Similar results were obtained by cultivation of the smears taken from the walls of the pool. In the deeper half of the pool, dense amoebic populations were found also near the surface. These findings suggest that the height of the water layer per se does not play a decisive role in creating suitable conditions for amoebic growth. It seems more likely that some influence is exerted by the slower speed of water recirculation in the high layer of water in the deep part of the pool. There should be a marked difference in the rate of fresh water inflow to the shallow part and deep part of the pool.

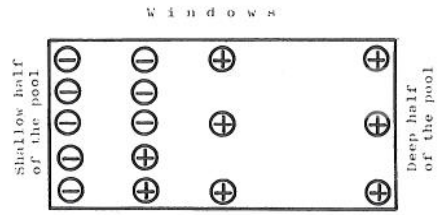


Fig. 5. Distribution of positive and negative sites at the bottom of the pool obtained by cultivating the smears on filtration paper from an area of 1 cm^2.

The development of the density of sessile amoebic populations has been studied in detail on slides, cellophane foils and membrane filters by microscopical inspection of the growing populations. These studies revealed that amoebic concentration is highest on the floor and on the walls near the floor in the deepest part of the pool. Slides placed into the water at these sites became covered with various species of *limax* amoebae already within the first days; the density of this cover was several thousand amoebae on one cm^2. Within the following days there was no increase of amoebic concentration, but there were considerable changes in the ratio of vegetative stages to cysts, being greatly in favour of the cysts. The coating covering the substrates examined contained also various species of infusorians, flagellates, yeasts and bacteria.

Amoebic growth, density or composition of the species has not been influenced significantly either by deviations in the bacteriological and chemical quality of the water in the pool or by the time of examination between two refillings of the pool.

d) Amoebae in the recirculation system

Our investigation included also the examination of the water in the two main water tanks of the recirculation system – the sedimentation tank and the sand filter. The method employed was that of quantitative culture titration.

The sedimentation tank holding about 90 m^3 is fed by approximately 20 l water/sec. from the discharged troughs of the pool. An automatic doser supplies the required amount of chlorine and ammonium sulphate. Chlorine levels have to be considerably higher

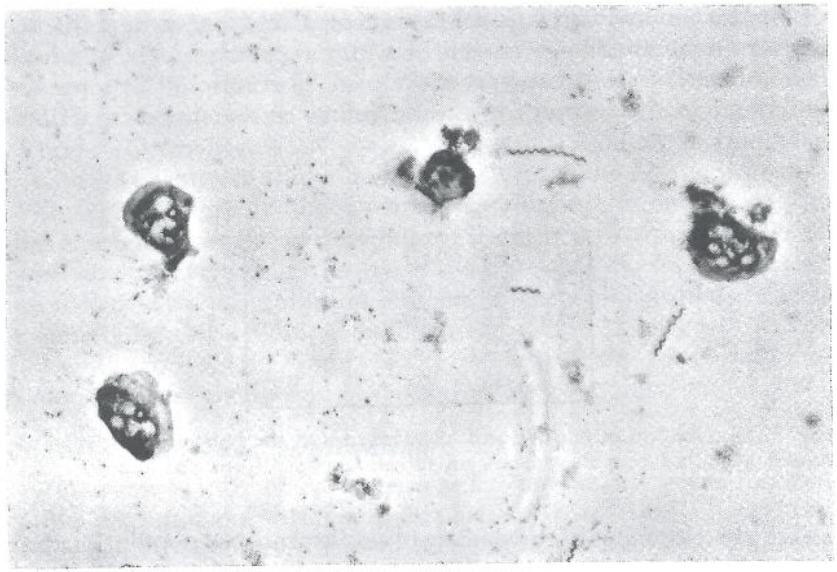


Fig. 6. Natural accretion of amoebae on a slide placed at the bottom of the pool. Trichrome, phase contrast (× 700).

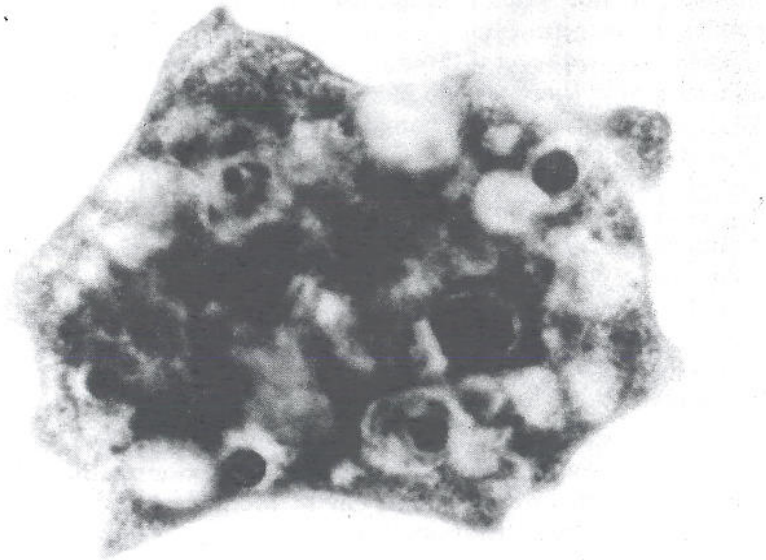


Fig. 7. Large form of amoeba from an accretion on the slide placed at the bottom of the pool. Trichrome, phase contrast (× 3,000).

in the sedimentation tank than in the water of the pool, allowing for the loss of free chlorine during the passage of the water through the other parts of the recirculation system and in order to obviate additional dosage of chlorine to the water before it enters the pool. Samples of water for a quantitative cultivation were taken from the surface of the water in the sedimentation tank and from the bottom of the tank. The concentration of amoebae was the same in both instances (10^2 per liter).

While no amoebae could be cultivated from water taken off the surface of the sand filter, amoebic concentration in water samples taken off the bottom of the sand filter was the same as that found in the swimming pool, i.e. 10^3 per liter. This shows that, although increased chlorine levels and the effect of sedimentation itself reduced the incidence of amoebae in the sedimentation tank, the filtration of water through the sand filter offered convenient conditions for a rise of concentration to initial values. Also the extensive inner surface of the sand filter is suitable for amoebic growth and, hence, the washing of the filter with hot water twice a week is not effective enough.

e) Findings of amoebae in the garden attached to the swimming pool

During the summer months, the large glass panels along one side of the hall in which the swimming pool is situated, are thrown open and the visitors can pass from the pool into the garden, in which there are several small pools and showers. Since we suspected that *limax* amoebae may be transferred from this external environment to the water in the pool, we collected various samples for examination. These included: samples of soil from the lawns and flowerbeds (particles of this soil samples were placed directly on agar), water samples from the smaller pools in the garden and from the troughs along the entrance to the hall, which have to be passed by all visitors before returning to the hall.

All these samples were positive containing mostly several amoebic species on each plate. This indicates that during the summer months, new species of amoebae are transferred to the main pool in the hall not only by air, but also directly by the persons coming in from the garden. Some of these strains may form longlasting and constant populations in the main pool.

f) The species composition of the amoebic populations

Within the course of our studies of the swimming pool we succeeded in isolating 100 pure clone strains of amoebae which were

TABLE II

Species	Water and walls at the surface	Water, walls and bottom in the depth	Smears from the wooden stairs	Sedimentation tank	Sand-filter	Garden
Acanthamoeba castellanii (DOUGLAS 1930)	—	1	2	—	1	2
Acanthamoeba polyphaga (PUSCHKAREW 1913)	1	2	5	1	1	2
Hartmannella limacoides (PAGE 1967)	7	4	3	—	—	2
Hartmannella vermiformis (PAGE 1967)	4	1	9	1	2	2
Hartmannella exudans (PAGE 1967)	3	—	3	—	1	1
Naegleria gruberi (SCHARDINGER 1899)	—	1	2	1	—	2
Vahlkampfia inornata (PAGE 1967)	—	1	—	—	1	1
Vahlkampfia avara (PAGE 1967)	—	—	1	—	—	—
Vahlkampfia jugosa (PAGE 1967)	—	1	—	—	—	—
Flabellula platypodia (GLÄSER 1912)	1	2	1	—	—	—
Hyalodiscus actinophorus (AUERBACH 1855)	1	1	—	—	—	—
Unidentified species	5	3	9	1	—	4
Total	22	17	35	4	6	16

subjected to laboratory tests. Until the present we have been able to identify 78 strains belonging to well-described species, while the position of the remaining 22 strains could not be solved. Probably, several of these will have to be described as new species. The identified species are listed in Table II.

g) Results of the tests on pathogenicity and of histological examinations

The pathogenic capability of all isolated clones has been tested on white mice. The results of these tests and of the following histological examinations are given in Table III.

TABLE III

Species	Degree of pathogenicity I.	II.	III.	IV.	VI	VI.
Acanthamoeba castellanii	1	4	—	—	1	—
Acanthamoeba polyphaga	3	5	1	1	2	—
Hartmannella limacoides	16	—	—	—	—	—
Hartmannella vermiformis	—	19	—	—	—	—
Hartmannella exudans	—	8	—	—	—	—
Naegleria gruberi	6	—	—	—	—	—
Vahlkampfia inornata	3	—	—	—	—	—
Vahlkampfia avara	1	—	—	—	—	—
Vahlkampfia jugosa	1	—	—	—	—	—
Flabellula platypodia	4	—	—	—	—	—
Hyalodiscus actinophorus	2		—	—	—	—
Unidentified species	22	—	—	—	—	—

Highly pathogenic strains were found only among the members of the genus *Acanthamoeba*. Isolations of pathogenic strains of the species *Acanthamoeba polyphaga* are among the first in the world and will be discussed in a separate paper.

The cysts of the cyst-forming species of the genus *Hartmannella* – although nonpathogenic – are most viable and can survive for a long time in the cerebral tissue of mice. By contrast, amoebae of the genera *Naegleria* and *Vahlkampfia* have never been confirmed by cultivation in the brain of surviving mice.

DISCUSSION

In spite of extensive investigations performed in the whole area covered by the recreation centre "Dr. Vrbensky" in Ústí n./L. we were unable to assess all factors participating in the development of pathogenic and nonpathogenic populations of amoebae of the *limax* type in swimming pools in general. Intensive teamwork of various

professions extended over several years may bring the desired results and give a more complete picture. Our work devoted to the study of conditions in one of the swimming pools only may offer a certain methodological and factical basis not only for our further investigations, but also for studies in the fields of hydrobiology, water hygiene, water technology, sport medicine, swimming pool designing etc.; sooner or later, all these professions will have to become engaged in these problems.

The most important information obtained in our work is the fact that the constant presence of numerous populations of amoebae of the *limax* group cannot be prevented even under the strictest observation of all routine safety measures applied to the water system of the pool. This indicates the theoretical possibility of acquiring further infections with amoebic meningoencephalitis through bathing.

Thus, nowadays system of bacteriological and chemical inspection of water in swimming pools shows to be ineffective. It does not offer even an approximate information on the state of populations of amoebae in a certain object. Therefore, at least a minimum of protozoological cultivation tests should be included to the routine examination.

The regular exchange of water in the pool resulted only in a shortlasting decrease of amoebic populations and it will be necessary to confirm by further studies that the disturbance of the equilibrium in a more or less closed water system may be rather harmful from the hygienic point of view. Conditions for the formation of dense amoebic populations were found to be exceptionally favourable on the surface of porous material (e.g. wood), on large surfaces (e.g. sand filters), in the deep part of the swimming pool with relatively stagnant water. The biological coating on the inner spaces of the pool and recirculation system protects the amoebae even against higher chlorine concentrations (above 1 mg/l) and against a high pH (above 9). The sand filter seems to be the site of speedy reproduction of the amoebae and a source of water contamination. In addition to these internal routes of contamination, various amoebae species may be transported by man to the pool from the adjoining garden and its pools.

Increased attention should be paid to the incidence of the species *Naegleria gruberii*. Pathogenic strains of this species may cause acute amoebic meningoencephalitis of man. Attention should also be paid to the species *Acanthamoeba castellanii* and *A. polyphaga* because their pathogenicity has been proved experimentally in laboratory animals. It has not been possible to make a definitive evaluation of the hygienic importance of all other species concerned.

During our investigations we have been unable to isolate any of the pathogenic strains of the species *Naegleria gruberi* by cultivation. It appears that the existence of such a strain which probably had been present in the swimming pool during the years 1962—1965 and had caused the infection, must have been interfered with by the various adaptation activities performed after the occurrence of the last cases of infection from 1965 till January 1967.

The results of these investigations suggested various measures which may restrict the populations of *limax* amoebae in the water and reduce the danger of infection with primary amoebic meningoencephalitis. These are:

The routine treatment of the water with chlorine is, even under strict observation of all rules, not satisfactory and cannot prevent the reproduction of *limax* amoebae in the water. At present, no other more effective treatment of water is applied. This shows the necessity of developing other measures which would successfully control amoebic reproduction at the most dangerous sites.

In our opinion all porous material used for the equipment inside the pool should be removed (wood, textile, cork etc.) and replaced by plastic material with an unporous surface. The bottom and the walls of the pool should be rigorously cleaned every day with a convenient mechanical device to remove all deposits and to avoid the formation of biological associations uncontrollable by the chemical and physical state of the water and not detectable by routine biological inspection. The sand filter should be cleaned daily with vapour or hot water of at least + 70°C in all layers of the filter. This is the only way to avoid recontamination of the pool through the filter which until now served as an amoebic reservoir. The frequency of the regular complete exchange of water should be reduced to a minimum and performed only when the chemical parameters become irreparable (a breakdown of some parts of the cleaning system) or when necessary for some other organisatory reasons.

Most of these measures should be introduced to similar centres. However, they will have to be operated for some time before their actual value will be known. The control of the occurrence of amoebae in swimming pools is only one of the problems associated with the research of primary amoebic meningoencephalitis.

Summary

Longlasting investigations have been performed in a swimming pool, which has been the source of a pathogenic agent responsible for 16 fatal cases of primary amoebic meningoencephalitis. A

description is given of the methods employed for confirming, by cultivation and microscopy, the presence of potential pathogenic strains of amoebae of the *limax* type in the water and in all areas of the swimming pool moistened by the water from the pool and also of methods employed for testing the pathogenicity of the isolated strains.

Quantitative cultivation examination revealed that the concentration of amoebae capable of growth at 37°C in water treated with the prescribed amount of free chlorine and answering to all bacteriological and chemical norms, ranged from 10^2—10^3 specimens in one liter of water. The principle site of amoebic reproduction are the walls and the floor of the pool mainly in its deeper parts, where the density of protozoan populations may reach several thousands per 1 cm^2. Also the large inner surface of the sand filter offers most favourable conditions for amoebic reproduction. No direct relationship has been observed between the occurrence of amoebae and the bacteriological and chemical state of the water. The presence of 11 species of amoebae of the *limax* group has been identified in the pool including several strains of the species *Naegleria gruberi*. The moderate pathogenic capability of only 5 strains of the species *Acanthamoeba castellanii* and *A. polyphaga* has been confirmed in experimental infection of mice. Pathogenic strains of the species *Naegleria gruberi*, the etiologic agent of primary amoebic meningoencephalitis of man, has not been found either in the pool itself or in the adjoining area. Various control measures have been suggested which may reduce the density of amoebae of the *limax* type in the pool and, hence, reduce the danger of infection with primary amoebic meningoencephalitis acquired through bathing.

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