LABORATORY DIAGNOSIS OF PRIMARY AMOEBIC MENINGO-ENCEPHALITIS AND METHODS FOR THE DETECTION OF LIMAX AMOEBAE IN THE ENVIRONMENT

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Abstract. Laboratory examination methods and processes recommended for the diagnosis of primary amoebic meningo-encephalitis and detection of Naegleria fowleri in the environment are summarized. The most simple methods suitable for diagnostic laboratories in the sphere of medical parasitology have been chosen.

The laboratory detection of Naegleria fowleri, etiological agent of primary amoebic meningo-encephalitis, in the clinical material and in outer environment is a specialized examination. The methods used differ considerably from the conventional protozoological laboratory techniques. This examination is demanded in various situations. First of all it is a prerequisite for the timely application of a specific therapy with patients suffering from primary amoebic meningo-encephalitis, since every minute of delay may cause fatal effects. Even the additional demonstration of amoebic etiology of the disease based on the examination of postmortem material is of great importance not only for the final diagnosis but also for further epidemiological investigation and measures. In the last years, great attention has been paid to ecological studies in free nature, particularly in waters heated and contaminated by industrial effluents or heated for recreational and sport purposes.

The laboratories dealing with the diagnosis of Naegleria fowleri apply various methods according to their technical equipment and working experiences. Some of the examination schemes have been published by Chang (1971), Cursons and Brown (1976), Griffin (1973), Molet and Kremer (1976), O'Dell (1977) and others. In our opinion, these recommended procedures include some operations which are not quite rational and adequate for the given purpose due to their laboriousness and requirements of the material, as application of complicated culture media, plaquing on bacterial layers, application of tissue cultures as substrate etc.

The present paper summarizes the diagnostic and detection methods successfully applied in our laboratory. Only the most simple methods giving reliable results have been included. The form and structure of the survey was adapted to the needs of diagnostic laboratories in medical parasitology.

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1. Description of the causative agent of PAME

The etiological agent of PAME are virulent strains of amoebae of the species *Naegleria fowleri*. These mononuclear amoebae measure 10—20 μm in movement and form wide lobopodia in a characteristic eruptive manner. The plasma contains one or more contractile vacuoles. The nucleus is spherical, with a large endosome. Only the vegetative stages of amoebae divide, while the nuclear membrane remains preserved (= promitosis) and the endosome divides into so-called polar masses. The so-called interzonal body is formed between the separating layers of chromosomes in the anaphase and telophase. A special feature of the genus *Naegleria* is the ability to change the shape of body from amoeboid to ovoid and to produce two flagella on the anterior end of body. The flagellar stages are formed in a diluted water environment. The changes of the body shape and mode of movement can be very rapid and they are reversible. The cysts of amoebae are usually mononuclear, lentil-shaped, of almost rounded ground-plan and smooth in outline. There are three to five pores in the cyst wall through which the amoeba leaves the membrane during excystation. The cyst wall is rather thin and double-layered.

The pathogenic *N. fowleri* cannot be differentiated from the nonpathogenic *N. gruberi* on the basis of their morphology. A suitable criterion for the differentiation of these two species is the fact that the nonpathogenic species is mostly inhibited by the temperature of 35 °C and higher, whereas *N. fowleri* can reproduce successfully even at the temperature of 46 °C (Griffith 1972).

In histological preparations the amoebae appear like circular plasmatic structures of almost the same size as leukocytes, i.e., about 7 μm. A characteristic feature is the relatively small nucleus with a large and conspicuous endosome. Flagellar and cystic stages do not occur in the tissues. The species determination of amoebae in histological preparations cannot be made on the basis of their morphology.

2. Detection of *Naegleria fowleri* in clinical and postmortem material

2.1 Indication of laboratory examination of PAME

A special examination of clinical and postmortem material for the presence of the agent of PAME is indicated in all cases of purulent meningitis or meningo-encephalitis showing at least some of the following characteristics:

a) a sudden onset of the disease from a full health,
b) bacterial etiology has not been determined with certainty,
c) application of antibodies and sulphonamides produces a low or no effect,
d) bathing a short time before the onset of the disease can be traced in the anamnesis of the patient,
e) the course of the disease is extraordinarily acute (five days on the average).
2.2 Material suitable for the examination
a) fresh cerebrospinal fluid
b) cotton swabs from various organs
c) postmortem material — unfixed for culture examination
   — fixed for histological methods

2.3 Examination scheme

a) liquor — microscopic preparation of fresh CSF
   — cultivation on NN agar
   — cultivation in BCS medium
   — cultivation in tissue culture
   — animal experiment

b) cotton swabs
   — cultivation on NN agar
   — cultivation on BCS medium (sterile material)

2.4 Detailed instructions for processing the examined material

2.4.1 Direct microscopic demonstration of amoebae in the liquor

The microscopic detection of live naegleriæ in the cerebrospinal fluid is very important, since it is still the only method for the confirmation of amoebic etiology in the cases of acute CNS disorders in man at the time when a therapy is still possible. The feasibility of the direct microscopic detection of amoebae in the liquor of patients was demonstrated by several authors, in spite of a large number of leukocytes present in the purulent liquor. According to the data available in the world literature, the number of amoebae in the liquor was somewhere between $10^3$—$10^7$ mm$^3$.

After the material was transferred to the slide the amoebae are in a resting, spherical form. At this stage they cannot be reliably differentiated from the cells of leukocytes either on the basis of their size or of the plasma structure, even if phase contrast microscope is used. Only after some minutes of rest the amoebae attach to the slide. This process can be speeded up by heating moderately the lower part of the slide up to 45°C. The bodies of amoebae flatten dorsoventrally and the nuclei are well visible particularly if phase contrast is used. At the same time, pseudopodia begin to arise in a typical eruptive manner even at the room temperature. The bodies of amoebae, still of irregular shape, elongate in the direction of movement. This direction is always more or less apparent. The character of movement distinctly differentiates the amoebae from leukocytes and enables their identification even in such a material where a single amoeba is surrounded by hundreds of leukocytes.

Fixed and stained preparations are usually made from dry smears of liquor for bacteriological examinations. There is no hope to identify the amoebae in preparations stained by Gram and even in those stained by Giemsa's method, usually applied in protozoological diagnosis, the morphology of amoebae is poorly distinguishable. The stained plasma of amoebae appears like a blush of indefinite shape, with a red nucleus inside. There is no typical morphological character so that even an experienced microscopist can only suspect the presence of amoebae in the dry smears.

2.4.2 Culture isolation of amoebae

2.4.2.1 Cerebrospinal fluid

It holds true about the liquors that the transparent samples without leukocytes do not contain any amoeba. If PAME is really concerned, the liquor is always very turbid and contains a mixture of leukocytes and amoebae. The examined sample of liquor is inoculated on NN agar (see 4.1) in the amount of 1—2 drops in the middle of the plate. The centrifugation of the sample before inoculation is unnecessary and does not increase the number of isolated strains. Moreover, it may result in a me-
chanical damage of the amoebae. Since the naegleriae attach to the walls of the tubes, it is necessary to shake their contents before taking the inoculum. A positive cultivation can be obtained also from the sample of liquor kept for three days at the temperatures of 2—37 °C, but the material for cultivation is damaged by freezing.

If the sample of cerebrospinal fluid was taken under strict aseptic conditions, the naegleriae can be isolated directly in an axenic culture by inoculation of BCS medium (see 4.3) (1—2 drops of liquor per 5 ml of medium).

A direct isolation from the sterile material can also be carried out if the tissue cultures are used. They are now available in all larger virological laboratories.

According to our experience, all species of mammalian cells growing at 37 °C can be easily infected, provided that streptomycin at the concentration higher than 50 gamma/ml or even trace quantities of mycostatic preparations (e.g., amphotericin B, Nystatin) were not present in the medium. Virulent strains of naegleriae evidently injure or completely destroy the cell layer during 24 to 48 hours after inoculation. The beginning changes in the cultures are best visible in the lines of epithelial cells. After several days the amoebae form almost confluent layers on the walls of the culture flasks and replace the original cell culture.

2.4.2.2 Postmortem material

The postmortem material, even if removed under sterile conditions, should be regarded and processed always as a contaminated material. In suspected PAME cases it is most suitable to examine the material from the sites of the cortex and subcortex of cerebrum and cerebellum in which haemorrhages are visible, samples from the cerebral base, olfactory nerve, nasal mucous membrane in the region of lamina cribiformis and also liquor. The agar plates can be inoculated by an inoculum of 1—5 mm³, if possible of flat shape to increase the contiguous area of the inoculated material. The examined material can be stored for 24 hours at the temperatures higher than 2 °C without any effect on the viability of amoebae. However, even a short-term freezing completely destroys the vegetative stages of naegleriae.

2.4.2.3 Swabs

The swabs carried out by means of a cotton tampon from nasal mucous membrane or from the surface of other objects can be broken off the skewer with a sterile Pasteur’s forceps or forceps and pressed into the surface of the agar plate. This way is not quite ideal, since only a part of the tampon surface gets into the contact with the culture medium, but it is sometimes necessary to use it. Another alternative is to use instead of tampons bands or discs of filter paper which are then put on the agar surface. This method enables minimum loss in amoebae isolations.

2.4.3 Isolation of N. fowleri from experimentally infected animals

In case that neither a special culture medium nor tissue cultures are available, laboratory animals, particularly white mice, rats or guinea pigs can be applied. The liquor need not be thickened by centrifugation. The infection develops rapidly after intracerebral inoculation of the infective material. After this inoculation the white mice die on days 2—7 after infection. The mice infected by intranasal instillation of infectious material, for which a higher dose of agent is necessary, die by two days later on the average. An advantage of this way of inoculation is the elimination of the contaminating microflora.

Other methods of inoculation of laboratory animals, besides the intracerebral and intranasal inoculation, do not result in the development of a typical amoebic meningo-encephalitis under normal conditions. Consequently, they cannot be applied in the diagnosis of PAME.

2.4.4 Histological examination

Tissue samples for histological examination need not be processed in a special manner. Formalin fixation is quite suitable for the detection of amoebae. The microscopist acquainted with the morphology of amoebae is capable of identifying them in the sections stained by any of the conventional histological methods. The polychromatic methods staining distinctly RNA of the large nucleus of amoebae, as green trichrome after Masson, can be recommended. The nucleus shows an intensive red colour on the grey-green background of plasma and karyoplasm.
The distribution of amoebae in the cortical and subcortical layer of amoebae can be very uneven. The predisposed places of massive occurrence are mainly in the basal parts of the brain. Before a negative protozoological diagnosis is made it is always necessary to examine microscopically a larger number of samples of brain tissue.

The identification of amoebae may be difficult in the tissues with a strong infiltration of leukocytes. They are best visible in the perivascular spaces of the subcortical layer at the sites where no tissue reaction occurs.

3. Detection of *N. fowleri* in water and in soil

3.1 Indication of laboratory examination of water and soil for the presence of *N. fowleri*

The laboratory detection of *N. fowleri* in water and soil requires detailed consequent identification tests. This examination is therefore very laborious and a large amount of material is necessary. Moreover, the probability of a positive finding is much lower than in postmortem or clinical material from human cases of PAME.

The examination of water can be indicated in the following situations:

a) It is necessary to verify or exclude the source of infective agent of already proved cases of PAME in man.

b) The surface water used for sport or recreation swimming is contaminated by warm industrial effluents.

c) It is necessary to verify the effectiveness of the recirculation and disinfection system of a swimming pool.

3.2 Demonstration of the presence of *N. fowleri* in water

Three types of material can be used for the detection of *N. fowleri* in a certain water reservoir or running water:

a) free water,

b) natural accretions from walls and different objects submerged in water,

c) material from the surface layer of bottom (sand, mud, sediment).

3.2.1 Cultivation from free water

Since the amoebae prefer the sessile way of living, the isolation of naegleriæ from free water is least probable. However, this examination is often very important if the epidemiological significance of the situation is to be estimated. The representative samples of water are taken into sterile flasks with a hydrophobic surface (polyethylene or silicon-coated glass) to prevent the amoebae from attaching to the walls of the flasks. The cultivation from membrane filters is then used for further processing. Most suitable are the filters with greater pores, e.g., 30 mm disc filters Sartorius 11002 (Sympor No. 2), pores measuring 2—3 μm in diameter. The filtration is carried out in Seitz filter bushes by means of a vacuum pump. The volume of filtered water may be even two liters per one filter in pure samples. As soon as the filtration is finished the membrane is removed from the bush by a sterile forceps and put upside down on the surface of NNA agar. The sample is then incubated at 42 °C for three to five days. Approximate concentration of amoebae in the tested sample can be simply determined on the basis of the smallest volume giving a positive result of cultivation of membrane filter. For example, 10, 100 and 1,000 ml of water from the same sample can be gradually filtrated.

3.2.1.1 Quantitative cultivation from free water

If the presence of naegleriæ in free water has already been proved, their number can be determined by culture titration.
Immediately after sampling water is inoculated by sterile silicon-coated pipettes on NN agar. The following inoculation scheme is recommended for standard examinations:

10 plates à 10 ml,
10 plates à 1 ml,
10 plates à 0.1 ml.

The inoculated plates are left to stay for 12–24 hours to enable the sedimentation and attaching of amoebae to agar, then the plates are turned upside down. Large water inocula may be (but need not be) poured off. The plates enclosed in polyethylene bags are then incubated at 42°C for three to five days. The number of amoebae in 1 liter of water \( (x) \) can be determined after the following formula:

\[
x = \frac{100n}{d}
\]

\( n = \) number of plates positive after inoculum \( d \),
\( d = \) smallest volume of inoculum in ml giving less than 90 % of positive plates.

Under faultless culture conditions all plates inoculated with the dose of 10 \( d \) are positive. If doses lower than 1 \( d \) are used, positive plates occur only exceptionally. The formula for the calculation of amoebae concentration in the sample of water does not give the number of individual amoebae or cysts, but the number of particles which may contain one or more viable amoebae. In fact, most frequently flakes of biological materials consisting of clusters of bacteria, protozoa etc. are involved, often attached to an object from various organic detritus. However, this information is sufficient and decisive in epidemiological situations.

3.2.2 Cultivation from natural accretions

Natural biological accretions from the surface of various objects submerged in the examined water or wetted by it can be collected for culture examination either by means of strips of sterile filter paper (3 x 1 cm) wiping off the accretion under a slight pressure and then putting it on the surface of NN agar or by means of sterile cotton swabs on a skewer (2.4.2.3). For a long-term investigation of a certain water reservoir microscopic slides, cellophane bands of foils of membrane filters can be placed in various places and the formation of accretions can be then followed at various intervals by cultivation (or even microscopically).

3.2.3 Cultivation from bottom material

For this examination, a 5 mm thick layer of the bottom cover is taken to form about one half of the sample in the tube with water. The tube is thoroughly shaken, larger particles are left to settle and the supernatant is examined by cultivation, similarly as that from free water (3.2.1).

3.3 Demonstration of the presence of \( N. fowleri \) in soil and dust

Soil samples often contain a large amount of amoebae of various species. For a simple demonstration of naegleriæ a sample of about 1 mm³ can be put directly on the surface of NN agar. Quantitative data, at least approximate, can be obtained after shaking a known amount of soil with a known volume of sterile distilled water, using glass pearls if necessary. After sedimentation of heavier soil (or dust) particles the sample can be processed like at quantitative cultivation of water (3.2.1.1).
3.4 Identification of *N. fowleri* isolates

At incubation temperatures of 42 to 45 °C besides the virulent strains of *N. fowleri* also the nonvirulent strains of this amoeba, some strains of Hartmannella and Acanthamoeba and exceptionally also thermo-tolerant strains of other species, as *N. gruberi*, can grow in the primocultures from water and soil (Singh and Hammahiah 1977). Most of these contaminants disappear spontaneously during the second or third passage on NN agar at the temperature of 42 °C. In spite of this, the final isolates must be subjected to identification tests and tests of virulence. The following characters may be recommended for an identification of *N. fowleri*:

a) *N. fowleri* can be passaged repeatedly on NN agar at 42 °C,

b) the flagellar test at 42 °C is positive.

This test can be carried out directly on agar plates in the following way. Several drops of distilled sterile water are dropped on a previously marked place with amoeba outgrowth. The plate is then rapidly turned upside down to form a hanging drop in this place. At a microscopical control through the bottom of the plate the change of amoebae in flagellates can be well observed already after several minutes of incubation.

In case of larger series of cultures it is better to wash the surface of plates by means of bent glass sticks into about 3 ml of distilled water and to transfer the suspension by a pipette into blood tubes. The contents of stoppered tubes can be then controlled under a slant or inverse microscope at intervals of 15 min. The flagellar stages are moving rapidly and they are well visible in water, whereas the amoebae attach to the walls of the tubes. The attempts to carry out the flagellar test using strains passaged for a long time in the laboratory are often unsuccessful.

c) The cysts of the genus *Naegleria* have a characteristic inner structure.

Most of the fresh amoeba isolates form numerous groups of cystic stages on the agar surface. The outlines of the nucleus and the nucleolus are well visible inside the cysts of *Naegleria* amoebae using a phase contrast immerse objective. Clusters of granular structures are situated perinuclearly.

d) *N. fowleri* (both virulent and avirulent strains) grows readily in axenic conditions in BCS medium. *N. fowleri* can be reliably separated from thermo-tolerant strains of *N. gruberi* if the isolates of *Naegleria* amoebae are transferred to axenic culture on BCS medium.

e) Serological identity of the new isolate with collection strains of *N. fowleri* can be verified by an indirect immunofluorescent method or other reactions if available.

3.5 Differentiation of virulent and avirulent strains of *N. fowleri*

A reliable differentiation can be made only using experimental animals. For an orientation test of the virulence of a new isolate a group of five mice weighing 13—15 g is quite sufficient. The animals are inoculated intranasally with naegleriæ from an axenic culture. At least 10,000 amoebæ in the volume of 0.01—0.02 ml are dropped into the nostrils of mice at a deep aether narcosis. The mice die on days 3—14 after inoculation with a virulent strain showing the symptoms of encephalitis.

After intracerebral inoculation with even the smallest doses of the virulent strain the mice die usually on days 2—7.

In special cases, when the nature of isolates must be determined rapidly, also amoebæ washed off the surface of agar plates can be used for intranasal inoculation of mice.

It is possible that in the nearest future the virulent and avirulent isolates of *N. fowleri* will be differentiated by relatively simple methods at cultivation in vitro. This is suggested by the results of some experimental works of the recent years (Cursons 1978, de Jonckheere 1977).
4. Culture technique

4.1 NNA (Non Nutrient Agar)

A mixture of 15 g of Bacto Agar Difco with 1,000 ml of distilled water is sterilized in an autoclave, well mixed and poured on Petri dishes (10 cm in diameter) in doses of 20 ml per dish. After solidification 1—2 drops of killed suspension of Aerobacter aerogenes (4.2) are put on the surface of the agar plate by means of a pipette and spread on the whole surface by a bent glass stick. For storage and incubation the plates are placed in polyethylene bags upside down. They can be stored in a refrigerator even for 14 days. All procedures are carried out under strictly sterile conditions, since the material may be contaminated by moulds.

Subcultivation of strains grown on agar. A place with an accretion of vegetative stages of amoeboae or cysts is chosen and marked on the bottom of Petri dish under the microscope. This part is then removed from the agar by a sterile lancet or other suitable instrument, transferred to another agar plate upside down and shifted on its surface for several millimeters to release the amoeboae from the accretion.

4.2 Suspension of Aerobacter aerogenes

Petri dishes with a nutrient agar suitable for the growth of Aerobacter are inoculated on the whole surface with a culture of A. aerogenes or Escherichia coli. After incubation at 37 °C for 24—36 hours, 3 ml of sterile distilled water are pipetted on each dish and the colonies of bacteria are washed off by means of a bent glass stick. The suspension of bacteria is then collected, mixed and transferred to ampules (0.5 ml to each ampule) under sterile conditions. Sealed ampules are heated in water bath (in ultrathermostat) at 65 °C for 30 min. The suspension prepared in this way can be stored in a refrigerator for several months.

4.3 BCS medium (Bacto-Casitone-Serum Medium) and axenisation

20 g of Bacto-Casitone Difco are solved in 1,000 ml of distilled water and sterilized in an autoclave. After cooling, 10 % (100 ml) of fresh equine, ovine, calf or rabbit serum without any conservatives, collected under sterile conditions or sterilized by filtration, are added. The medium is then filled into culture tubes in a layer not higher than 4 cm.

This medium is suitable only for an axenic culture or for isolation experiments from sterile materials (without bacteria). The contaminating bacterial flora can be suppressed, e.g., in axenisation experiments with as much as 10,000 units of penicillin, 50 gamma of streptomycin and 100 gamma of polymyxin B added into 1 ml of medium.

Subcultivation is carried out by transferring 5—10 drops of the sediment of a well grown culture (without centrifugation) to a fresh medium by means of a Pasteur’s pipette.

4.4 Incubation

In isolation experiments on agar, the optimal temperature for N. fowleri isolation is 42 °C or slightly higher. The maximal tolerated temperature of 46 °C is unsuitable for the isolation. The optimal temperature for direct isolation experiments on BCS medium (sterile materials) or axenisation is 37 °C.
4.5 Microscopic control of cultures

The growth of amoebae on NN agar is followed in closed dishes put upside down on the microscope stage. A lower magnification (e.g., 10×) is used. The working distance of this optical system enables us to focus on the surface of the agar layer through the bottom of the dish. The whole surface of the agar is always examined.

The amoebae in tubes grow on the inner walls from the bottom upwards. The growth can be followed under the microscope at low magnification in slant tubes.

4.6 Safety of work

All cultures and other biological material in which *N. fowleri* was detected or suspected should be processed according to safety rules valid for the work with highly virulent infections.

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