

REVIEW ARTICLE

Isolation, identification and increasing importance of 'free-living' amoebae causing human disease

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Amphizoic small amoebic protozoa are capable of existing both in 'free-living' and in 'parasitic' form depending on the actual conditions. Two genera (*Naegleria* and *Acanthamoeba*) have become recognised as opportunist human parasites. Since the first description in 1965 of a lethal case of primary amoebic meningoencephalitis (PAM) caused by *Naegleria*, many more (mostly lethal) cases have been reported, while granulomatous amoebic encephalitis (GAE), as well as eye (keratitis, conjunctivitis, etc.), ear, nose, skin and internal organ infections caused by *Acanthamoeba* have also occurred in rapidly increasing numbers. Both pathogenic and non-pathogenic species of *Naegleria* and *Acanthamoeba* are found worldwide in water, soil and dust, where they provide a potential source of infection. Successful differential diagnosis and appropriate (specific) therapy depends on precise laboratory identification of the 'free-living' amoebae. In most cases, isolation from the environment can be achieved, but identification and differentiation of the pathogenic and non-pathogenic strains is not easy. The methods presently available do not fulfil completely the requirements for specificity, sensitivity and reliability. Morphological criteria are inadequate, while thermophilic character, pH dependency and even virulence in infected mice, are not unambiguous features of pathogenicity of the different strains. More promising are molecular methods, such as restriction endonuclease digestion of whole-cell DNA or mitochondrial DNA, as well as iso-enzyme profile analysis after iso-electric focusing and staining for acid phosphatase and propionyl esterase activity. Use of appropriate monoclonal antibodies has also yielded promising results in the differentiation of human pathogenic and non-pathogenic strains. However, quicker, simpler, more specific and reliable methods are still highly desirable. The significance of endosymbiosis (especially with *Legionella* strains) is not well understood. The results of a systematic survey in Hungary for the isolation and identification of 'free-living' amoebae, including an investigation of the Hungarian amoebic fauna, the isolation of possibly pathogenic *Naegleria* strains and of some *Acanthamoeba* strains from eye diseases, as well as the finding of a case of endosymbiosis, are also reported here.

Introduction

Protozoa causing human diseases can be divided into those that have a life cycle with vector-mediated transmission to the human host (e.g., *Plasmodium* and *Trypanosoma* spp.), and those that are transmitted directly between human hosts (e.g., *Entamoeba histolytica* and *Giardia lamblia*). Another group of the protozoa that cause infections of man is formed by the amphizoic small amoebae belonging to the genera *Naegleria* and *Acanthamoeba*. The term 'amphizoic' was suggested by Page [1] for protozoa that are capable

of being either free-living or parasitic, regardless of which is the primary mode of existence [2]. Free-living amoebae belonging to the genera *Naegleria* and *Acanthamoeba* can be pathogenic to man in their parasitic form. Thus, the expression 'amoebosis', reserved previously for diseases caused by *E. histolytica*, has to be extended to those diseases caused by free-living amoebae. *Naegleria* is the aetiological agent of fulminant primary amoebic meningoencephalitis (PAM), whereas *Acanthamoeba* can produce chronic and acute granulomatous amoebic encephalitis (GAE) in man [3]. Furthermore, several different types of infections have been associated with *Acanthamoeba*, including some internal organ, eye, ear, skin and nasal infections [2, 4].

Both pathogenic and non-pathogenic species of *Naegleria* and *Acanthamoeba* are found worldwide in water, soil and dust [3]. Lakes used for bathing may receive thermal discharges containing pathogenic free-living amoebae, and may represent a dangerous source of infection for man. However, in practice these organisms can be found everywhere: in the most diverse forms of natural waters (lakes, rivers, brooks, brackish waters), springs (including hot springs), swimming pools (even in pools treated 'adequately'), industrial cooling water, heating ventilation and air-conditioning units, soil, dust and oceanic sediment, including soil and water from the Antarctic; they have been isolated even from domestic tap water, from bottled mineral waters and contact lens cleaning and storing liquids [5–13]. They have also been isolated from the human cornea, skin, lung and central nervous system [4–6, 8–12, 14, 15].

Identification of the free-living amoebae is not well-resolved, although a precise distinction between the pathogenic and non-pathogenic strains would be the only reasonable and acceptable base for differential diagnosis and specific treatment in the case of vision-threatening or sometimes lethal human diseases [3, 5, 6, 16–22]. Morphological criteria are inadequate for the distinction of pathogenic and non-pathogenic strains [5, 6, 20, 23]. Differences in antigenic determinants have been found between pathogenic and non-pathogenic *Naegleria* strains [7, 20], while determination of the iso-enzyme profile by iso-electric focusing and analysis of mitochondrial or whole-cell DNA profiles also seem to be promising [5, 6, 21, 22, 24–26].

In Hungary, the first two cases of human disease caused by free-living amoebae were reported by this Department in 1985 and 1988 [7, 14]. Since then, an additional case was reported in 1995 [6]. Subsequently, a systematic co-operative study was organised to isolate, identify and study the possible pathogenicity of the free-living amoebic flora in Hungary, with special reference to sites of human recreation, such as natural waters, hot springs and swimming pools. The first results and conclusions from this programme [6, 27] are also included in this review.

***Naegleria fowleri* in the environment and in human disease**

PAM is a rare, but rapidly fatal, disease of the central nervous system of man, leading to death within a week. It is caused by the free-living amoebic flagellate *N. fowleri* [28, 29]. Sparagano and co-workers state that, among the members of the genus *Naegleria*, only *N. fowleri* is pathogenic for man and produces an acute and fatal meningoencephalitis [20]. PAM is a fulminant neurological disease of healthy young persons with a recent history of contact with recreational water during

swimming, bathing, water skiing or boating in fresh or brackish waters (lakes, rivers, ponds). The entry portal of *N. fowleri* is the olfactory neuroepithelium [30]. The disease has a sudden onset and a fulminant course associated with a diffuse haemorrhagic necrotising meningoencephalitis [31]. Since 1965, when this disease was first described in Australia [32], a number of cases have been reported worldwide [3, 16, 17, 19, 20, 29, 33, 34].

The pathogenic potential of *Naegleria* strains has been tested in mice by nasal instillation of amoebae [3], and it has been found that virulent strains from human patients kill mice rapidly [34]. In order to cause fatal PAM, *Naegleria* must be able to multiply in the host and has to survive the fever caused by this infection. Griffin [34] reported a test showing that temperature tolerance is related to virulence. Two distinct groups of *Naegleria* strains were obtained. The strains from fatal human infections grew well above the temperature of the highest fever (as high as 42°C), while non-pathogenic amoebae did not grow above normal human temperatures. Griffin stated that, for examining the pathogenicity of *Naegleria* isolates, the temperature test was more useful than tests of virulence in mice, probably because susceptible strains of mice still differ somewhat in their susceptibility to different *Naegleria* strains. Other factors may also contribute, but their importance is unknown [34].

The pathogenic *N. fowleri* is ubiquitous, but most strains are isolated from naturally warm and artificially heated aquatic environments [3, 16, 29, 35–38]. Since almost all human infections have occurred during hot weather and after swimming in warm water, a definite relationship to thermal pollution is suggested [34]. Although elevated temperature is generally associated with increased occurrence of *N. fowleri*, the actual thermal conditions that contribute to the induction of pathogenic strains of this amoeba are not understood completely [29]. Tyndall *et al.* [33] examined a new reactor cooling lake and found that the concentrations of thermophilic amoebae and thermophilic *Naegleria* spp. increased by as much as five orders of magnitude, compared with source water and sediment samples, in samples obtained at a temperature of 40°C during periods of thermal additions. Pathogenic *N. fowleri* increased by as much as two orders of magnitude. After cessation of thermal additions, concentrations of amoebae returned to the original pre-thermal perturbation levels within 30–60 days. Concentrations of thermophilic amoebae and thermophilic *Naegleria* spp. showed a significant correlation with temperature and conductivity [33]. The data of Sykora *et al.* [16] suggest that the optimum temperature for pathogenic *Naegleria* strains in heated effluents is 27–35°C. Although their occurrence was related to elevated temperatures, no significant correlation was found for other biological parameters. However, most non-pathogenic *Naegleria*

isolates were also obtained from discharges at elevated temperatures, and the isolation and identification of all *Naegleria* strains (either pathogenic or non-pathogenic) was based on thermal separation at 45°C. De Jonckheere *et al.* [39] also found non-pathogenic *N. fowleri* in a thermally polluted canal by indirect immunofluorescence analyses of heterogeneous populations that grew at 42°C. They suggested that, under certain conditions, non-pathogenic strains may undergo physiological changes, on the basis of which they may become pathogenic. It should be mentioned that *N. australiensis* is thermophilic up to 42°C and pathogenic to experimental animals, but has not, so far, been identified in man, while *N. andersoni* (thermophilic up to 40°C) is non-pathogenic. In addition, *N. lovaniensis*, like *N. fowleri*, grows up to 45°C, but is non-pathogenic to man and experimental animals [19]. Considering these data, incubation of samples at 45°C cannot be considered as 'selective' for pathogenic *N. fowleri* from environmental samples [3]. Although this is not a strict rule, the presence of thermophilic (but, so far, non-pathogenic to man or experimental animals) *Naegleria* spp. in the water should be considered simply as an indication that the conditions are suitable for the growth of pathogenic *N. fowleri* [19]. To make the picture more complicated, pathogenic *Naegleria* strains have been found also in water at a relatively low temperature. Thus a pathogenic isolate was obtained from water at a temperature of only 16°C [40], while Wellings *et al.* [41] isolated pathogenic *N. fowleri* from bottom sediments when the water temperature was no more than 12°C. Sykora *et al.* [16] supposed that the pathogenic amoebae developed in the cooling water when the temperature was higher and somehow survived the change in the environment.

Identification of *N. fowleri*

At least five species of amoebae in the genus *Naegleria* have been isolated from aquatic habitats. Thus, it is important that pathogenic and non-pathogenic species are identified accurately in environmental surveys [29]. As Sparagano [20] states: 'For environmental monitoring in a preventive objective, we need to distinguish pathogenic *N. fowleri* from non-pathogenic *Naegleria* spp. in water samples and to be sure to identify the three forms of the amoeba (cysts, trophozoites, and flagellates) ... identification techniques have to be specific, sensitive and rapid to differentiate *N. fowleri* encephalitis from *Acanthamoeba*, viral, or bacterial encephalitis'. However, despite general agreement that pathogenic and non-pathogenic species should be identified accurately and rapidly for medical diagnosis, this goal is far from being achieved. The lack of distinguishing morphological criteria, the absence of a strict dependency on temperature, and the ineffectiveness of the pH in predicting the survival and virulence of *N. fowleri* at a range of 2.1–8.15 [16] combine to

make a great demand for the use of more fastidious techniques [42].

Restriction endonuclease digestion of whole-cell DNA

Characterisation of *Naegleria* spp. by the use of restriction endonuclease digestion of whole-cell DNA deserves special attention as a possible reliable method for distinction [29, 43, 44]. In the study by Huizinga *et al.* [29], a newly created cooling reservoir (Clinton Lake, IL, USA) was surveyed for *Naegleria* spp. before and after thermal additions from a nuclear power plant. It was found that the DNA of *N. fowleri* isolated from Clinton Lake and digested with *Bgl*II, *Eco*RI, and *Hind*III restriction endonucleases, showed DNA restriction fragment length profiles (RFLPs) that were homologous with *N. fowleri* reference strain Lee isolated from a human case of PAM acquired in West Virginia, but showed major differences compared with the patterns of the other reference strains, *N. gruberi* and *N. australiensis*. This provided strong evidence for the genetic homology of the two *N. fowleri* strains isolated, one from the southern and one from the northern areas of the USA [29]. In contrast, De Jonckheere [18] found a difference between strains from Australia and Europe with restriction enzyme analysis of whole-cell DNA. On the basis of RFLPs, a subtype of the Australian type has been found in New Zealand, whereas different types, related to either the European type or the Australian type, have been detected in the USA. Recently, the isolation of *N. fowleri* from the environment in Japan was reported by De Jonckheere *et al.* [19]. When the RFLPs of the Japanese isolates were compared with those of *N. fowleri* strains from other geographic areas, the RFLPs of the Japanese isolates were found to be related most closely to the strains found in Oceania and most distantly to the European strains. This finding serves as additional evidence of the gradual differentiation of *N. fowleri* with increasing geographical distance. However, it does not settle the question as to whether the *N. fowleri* species originated from the American continent, as proposed originally by De Jonckheere and colleagues [17, 18]. Nevertheless, the RFLP assay seems to provide an additional useful method for the characterisation of *N. fowleri* isolates. Together with other biological and morphological identification methods, it may allow more precise epidemiological investigations to be performed whenever cases of PAM occur.

Agarose iso-electric focusing and staining for acid phosphatase and propionyl esterase activity

For the specific identification of water-soluble protein extracts from axenically growing *Naegleria* spp., De Jonckheere *et al.* [19, 45] separated the proteins (obtained from isolates from different geothermal and industrial waters) by agarose iso-electric focusing on

pH 3.5–10 gradient gels and stained for acid phosphatase (AP) and propionyl esterase (PE) activity. The banding patterns were compared with those obtained for *Naegleria* reference strains run on the same gel. The AP patterns were identical for *N. fowleri* isolates of different geographical origin, and were only slightly different from the *N. lovaniensis* pattern. However, with the PE patterns, *N. lovaniensis* was differentiated easily from *N. fowleri*, while only small differences in banding were seen among *N. fowleri* isolates of different geographical origin. The PE iso-enzyme pattern of the Japanese *N. fowleri* isolates corresponds to the *N. fowleri* reference strains from Australia [19]. Tyndall *et al.* [33] used iso-enzyme profiles as an additional measure for speciating *Naegleria* isolates. After electrophoresis on an acrylamide 7.5% gel, the iso-enzyme patterns of pathogenic *Naegleria* spp. isolated from a new reactor cooling lake in Georgia, USA, were identical to the patterns of *N. fowleri* isolates from other sites in the USA and Belgium, but differed from those of *N. lovaniensis* [33]. Thus, it seems that no (or only very slight) differences in PE banding patterns between *N. fowleri* isolates from different continents can be discovered when the proteins are separated by iso-electric focusing [19].

Use of monoclonal antibodies

An immunological approach with monoclonal antibodies (MAbs) appears to be a promising tool for identification of *Naegleria* spp. Preliminary results for trophozoite forms have been reported [31, 46]. Visvesvara *et al.* [46] described the successful production of MAbs to *N. fowleri*. The MAbs reacted intensely with strains of *N. fowleri* originating from different geographical areas, but showed no reactivity with four other species of *Naegleria*, i.e., *N. gruberi*, *N. jadini*, *N. lovaniensis* and *N. australiensis*, or a strain of *Acanthamoeba castellanii*. It was also shown that these MAbs could be used successfully to identify trophozoites of *N. fowleri* in brain sections of patients who died of PAM, and also in the brain sections of mice that were infected experimentally with the HBWS-1 strain of *N. fowleri*. However, these MAbs did not cross-react with amoebae in brain sections from patients who died of GAE caused by *A. castellanii*. Because of their specificity, these MAbs can be used successfully for the differential diagnosis of PAM infections retrospectively [46]. Other results have also supported the diagnostic potential of *N. fowleri*-specific MAbs and demonstrated their ability to differentiate at post-mortem examination between *Acanthamoeba* and *Naegleria* spp. in a case of granulomatous amoebic encephalitis [31]. Sparagano *et al.* [20] produced two MAbs that reacted with *N. fowleri* trophozoite, cysts, or flagellate forms, but not with *N. lovaniensis* or other *Naegleria* spp. MAbs can be powerful tools, not only for clinical diagnosis, but also for environment control [20].

An important argument for the use of MAbs has been highlighted by Visvesvara *et al.* [46], who state that, in the absence of any clear-cut morphological differences between *N. fowleri* and other *Naegleria* spp., iso-enzyme analysis or animal pathogenicity tests should be performed to differentiate pathogenic *N. fowleri* from other *Naegleria* spp. Both of these procedures require the growth of large numbers of organisms, which is not only time-consuming but expensive. However, with species-specific MAbs that react only with *N. fowleri*, even very small numbers of *N. fowleri* amoebae can be identified quickly.

***Acanthamoeba* in the environment and in human disease**

Members of the genus *Acanthamoeba* are the commonest amoebae in fresh water and soil. Dry cysts can survive for several years and are isolated regularly from natural waters, inadequately (and adequately!) treated swimming pools, soil, sewage, freshwater fish, brackish water, ocean sediments, dust, air and even bottled mineral water, or contact lens cleaning and soaking solutions [6–13, 15, 23, 27, 47–62]. This normally ‘free-living’ organism occurs occasionally as an opportunist parasite of man [63]. *Acanthamoeba* may occur as a commensal in the nasopharynx of apparently healthy normal individuals [64] and in patients with possible virus infection, and is found not infrequently in tissue cultures used for virus isolation from nasal swabs [64]. Their significance seems to be increasing rapidly: *Acanthamoeba* spp. have been reported from keratitis, corneal ulcers, the central nervous system (CNS), the genitourinary tract and many other sites of the human body [8–11, 23, 51–54, 63, 65–67]. One of the aetiological agents of GAE is *Acanthamoeba* spp. [30]. The neurological infection is usually manifested with a subacute-to-chronic illness, with signs of increased intracranial pressure and focal neurological deficit because of granulomatous brain lesions [31]. The penetration of the amoebae into the CNS is probably by haematogenous spread from a primary focus in the lower respiratory tract, skin or open wounds, but the amoebic trophozoites or cysts can reach the CNS directly through the olfactory neuroepithelium [30, 42]. GAE should be differentiated from brain abscesses produced by *E. histolytica*, and from PAM produced by *N. fowleri* and characterised by an acute, fulminant neurological disease mostly in healthy young individuals [30]. In contrast, various *Acanthamoeba* spp. cause a subacute-to-chronic GAE, usually in immunocompromised patients including those with AIDS [42, 68], with ‘not-yet-understood immunological deficiencies’ [30], or ‘debilitated individuals with no exposure to contaminated water’ [42, 68]. Tuberculosis, fungal infection or cerebral cysticercosis should be considered in the differential diagnosis [30, 42]. A number of studies emphasise the need to

consider acanthamoebic infection in the differential diagnosis of eye infections that fail to respond to antibacterial, antifungal or antiviral therapy (especially in those patients who wear contact lenses) [3, 6, 12, 16–19, 29, 34, 39–41, 43–45, 52, 55, 56, 69, 70]. These infections are often a result of direct eye exposure to contaminated materials or solutions (e.g., contact lens soaking and cleaning fluids); however, commensal bacteria on the eyelids, conjunctiva and tear film may have an additional role in the pathogenesis of *Acanthamoeba* keratitis [13, 15, 51, 55]. The clarification of the pathogenic role of *Acanthamoeba* spp. (considered until now as simple commensals) in some other diseases waits for further urgently required detailed studies.

Identification of *Acanthamoeba*

The taxonomy of the small amoeba species is not well established. Consequently, little is known about the genetic relationships between pathogenic and non-pathogenic strains of *Acanthamoeba*. Several studies report on the analysis of mitochondrial DNA (mtDNA) variation in members of the genus *Acanthamoeba* as an aid in taxonomy [21, 24–26, 71]. This method has proved to be a useful approach to studying evolutionary relationships among closely related organisms [72, 73].

The methods used for the isolation and generic identification of *Acanthamoeba* spp. have become standard. The validity of the identification of the 18 or more described species is somewhat more problematic and uncertain [74, 75]; however, species identification is essential for epidemiological studies [22]. The taxonomic classification of the members of the genus *Acanthamoeba* is based on morphological observations of the trophozoite and cyst forms [59, 76]. This classification defines the genus clearly, but the variation in cyst morphology seen even within cultured strains makes the identification of many described species a subjective process [59]. Bogler and colleagues [77] consider that a single species in the genus can comprise a mixture of pathogenic and non-pathogenic strains, and that problems of identification and determination of pathogenicity are compounded by attenuation of virulence during laboratory culture. Pathogenicity is probably opportunist and it is possible that all strains have a pathogenic potential, but this is uncertain and it is equally possible that pathogenic and non-pathogenic strains are equivalent to distinct species [77]. Further investigations by several methods are needed for the differentiation of acanthamoebae. Restriction endonuclease digestion of whole-cell DNA [5, 59, 77] or mtDNA [5, 6, 21, 22, 59, 77], and iso-enzyme electrophoresis [58, 59] have proved most useful in this respect. Inter-strain variations within species, similarities between strains of separate species, and inadequacies of the present taxonomic classification of

the acanthamoebae have been demonstrated by these methods [59].

Restriction endonuclease digestion of whole-cell DNA

The conventional method for diagnosis of *Acanthamoeba* keratitis is by culture of the corneal biopsy material on non-nutrient agar seeded with a lawn of *Escherichia coli* (NNA-*E. coli*) [6, 27]. *Acanthamoeba* spp. can be identified readily by the morphological appearance of the trophozoite and cyst forms [6, 21, 27, 76]. The trophozoites can be adapted to axenic (bacteria-free) growth in liquid media. Differentiation can be achieved by restriction endonuclease digestion of whole-cell DNA to detect RFLPs following agarose gel electrophoresis [5]. This technique is highly specific for differentiating morphologically identical *Acanthamoeba* strains isolated from keratitis cases and the environment. Kilvington *et al.* [5] demonstrated that isolates from a patient's cornea, contact lens container, saline rinsing solution and kitchen cold-water tap shared identical RFLPs; thus, the study implicated, for the first time, domestic tap water as the source of *Acanthamoeba* in keratitis [5].

The relationship between 33 morphologically identical *Acanthamoeba* isolates (30 isolates from patients with keratitis, two isolates from contact lens storage containers and one isolate from soil) was investigated by restriction endonuclease digestion of whole-cell DNA by Kilvington *et al.* [59]. The 33 strains formed cysts typical of group II *Acanthamoeba* spp. and resembled *A. polyphaga*, or possibly *A. castellanii* [78]. Restriction endonuclease digestion of *Acanthamoeba* whole-cell DNA and RFLPs differentiated these isolates into seven multiple-isolate and three single-isolate groups following analysis on agarose gels. In the largest group, containing nine isolates, eight isolates were from keratitis cases in various locations. This group may, therefore, indicate the type most frequently associated with keratitis. In this study, of three consecutive isolates from the same patient over a 7-month period, only one was resistant to chemotherapeutic agents, while the other two were susceptible. As all three isolates showed identical RFLPs, this result indicates that resistance was acquired by the original infecting strain during therapy [59].

RFLP profiles may also be useful in identifying pathogenic *Acanthamoeba* strains. The virulence of acanthamoebae has been shown to attenuate during axenic culture [79] and may account for the description of both pathogenic and non-pathogenic strains within the same species [80].

It appears that restriction endonuclease digestion of whole-cell DNA is a potent technique for differentiating morphologically identical *Acanthamoeba* strains by the detection of mtDNA RFLPs. However,

presently, it is unclear whether *Acanthamoeba* mtDNA RFLPs indicate intra- or inter-species differences [59].

Restriction endonuclease digestion of mtDNA

The method is based on electrophoresis of DNA fragments obtained by restriction enzyme digestion of mtDNA. It has been used to study the intra- and inter-species relationships in a variety of organisms. This type of analysis has proved useful in clarifying phylogenetic relationships among closely related organisms [21, 72, 73].

Bogler *et al.* [77] tested this approach with amoebae by examining the relationships among 15 strains from four species; pathogenic and non-pathogenic strains were included in the study [77]. DNA fragment size polymorphisms were used to estimate nucleotide sequence polymorphisms, in the expectation that the latter would correlate with the overall genetic relatedness of the various strains [81]. Ten distinct families of electrophoretic patterns (digestion genotypes) were observed [77]. Seven genotypes were found for seven strains considered non-pathogenic or of unknown pathogenicity. Three genotypes were associated with pathogenic strains. One of these genotypes included a single pathogenic strain, a second included one pathogen and one strain of unknown pathogenicity, and the third included five pathogenic strains. The latter five strains were of widespread geographical origin and had been assigned previously to two different species. The results suggested that extensive nucleotide sequence diversity occurs among strains from a single species of *Acanthamoeba*, but that subgroups of strains with similar sequences also occur. Thus, restriction enzyme analysis can identify clusters of strains and may be a useful approach to classification in the genus. However, it is clear that pathogenicity is not associated with a single subgroup. Bogler *et al.* concluded that improvements in classification should help clarify relationships among pathogenic and non-pathogenic strains [77].

Yagita and Endo [21] used RFLP analysis to type *Acanthamoeba* isolates from human eye infections, contact lens containers and soil in Japan. Four distinct mtDNA RFLP genotypes were discovered for eight strains. The discovery of four distinct RFLP genotypes for various strains of *A. castellanii* is evidence for DNA sequence diversity within a species [21, 77]. Three strains of *A. polyphaga* from different sources and one strain of *A. castellanii* shared one RFLP genotype identical to the RFLP genotype of the Ma strain of *A. castellanii*. Although there might be an argument about the morphological classification reported by Yagita and Endo, the results cannot be attributed simply to the difficulties in distinguishing these species of amoeba. Assuming that mtDNA in *Acanthamoeba* behaves similarly to that in other organisms, i.e., as a clonal molecule, it would not

be surprising to find most of the variability in *A. castellanii* and very little in *A. polyphaga*. This would be particularly so if *A. polyphaga* had diverged from *A. castellanii* recently [21]. Similar results have been observed in man [82], mice [83] and fungi [84].

Bogler *et al.* [77] noted the close relationship between mtDNA of the Ma and Castellani strains; the Ma strain was isolated from human eye infection and the Castellani strain from yeast culture. Consequently, they highlighted the need to test the pathogenicity of the Castellani strain, which is the type strain for *A. castellanii*. One of the strains from a human eye infection had an RFLP pattern identical to that of the Castellani strain. In addition, the mtDNA genotype of a strain isolated from a patient's contact lens container, identified as *A. polyphaga*, was identical to the pathogenic Ma strain and was also found among various other isolates. Based on the above, the mtDNA genotypes may be a useful aid in the taxonomy of free-living amoeba species and in the recognition of the pathogenic potential [21].

Few epidemiological studies have included environmental isolates originating from the same geographical area in which the clinical cases occurred. Gautom *et al.* [22] undertook an investigation to determine the usefulness of mtDNA fingerprinting as an epidemiological tool for identifying potential reservoirs of infection. The inclusion of environmental isolates demonstrated that the most common clinical isolates do have counterparts that are isolated readily from the surrounding environment, and that some of these counterparts appear to be geographically widespread. This study confirmed the usefulness of mtDNA fingerprinting in the analysis of *Acanthamoeba* epidemiology and systematics [22].

Iso-enzyme pattern analysis

For the specific identification of the water-soluble protein extracts of an axenically growing *Acanthamoeba* isolate (obtained from a patient with keratitis), Matias *et al.* [58] subjected the extracts to iso-enzyme studies. Iso-electric focusing was performed by PAGE with a pH gradient 3–10. The iso-enzymes investigated included acid phosphatase (AP), glucose-6-phosphate dehydrogenase (G-6-PDH), β -hydroxybutyric dehydrogenase (β -HBDH), alcohol dehydrogenase (ADH) and esterase. Based on the iso-enzyme patterns for G-6-PDH, β -HBDH, ADH and esterase, the isolate was related more closely to *A. quina*–*A. lugdunensis* than to other *Acanthamoeba* spp. This apparent close relationship to *A. quina*–*A. lugdunensis*, coupled with the lethality of the former in BALB/c mice, underlined the potential pathogenicity of this isolate [58]. Kong *et al.* [85] observed inter-strain polymorphisms of iso-enzyme profiles and mtDNA fingerprints among seven strains of *Acanthamoeba* isolated from different sources and assigned morphologically to *A. polyphaga*.

The inter-strain polymorphisms for AP, lactate dehydrogenase and G-6-PDH were associated with similarity for glucose phosphate isomerase, leucine aminopeptidase and malate dehydrogenase. Daggett *et al.* [86] also used iso-enzyme electrophoresis of three different enzyme systems to compare 71 strains assigned to the 15 *Acanthamoeba* spp. that are recognised currently. A phylogenetic (cladistic) analysis of the zymograms arranged the strains in 15 distinguishable lineages, not all of which corresponded to current taxonomic assignments. The analysis also made it possible to place strains which had been identified previously only to the genus level. Therefore, these results suggest that previous criteria used to classify *Acanthamoeba* are not adequate for fully resolving taxa to the species level [86].

Naturally occurring bacterial endosymbionts

The presence of endosymbionts has been demonstrated in many protozoan species [57, 87–90]. The symbionts may either occur 'naturally' or may represent more recently phagocytosed organisms that have adapted to the intracellular environment. Phagocytosed bacteria may grow and reproduce within the protozoan host and, in some cases, have been shown to eventually become symbionts [91]. The process itself, and its pathogenic significance, is not well known; hardly any data can be found on the mechanism by which it develops and by which it supports or at least allows the survival of both organisms.

Small, free-living amoebae such as *Naegleria* and *Acanthamoeba* have been found to have bacterial endosymbionts [87, 88]; indeed, Fritsche and Gautom [57, 89] have suggested that endosymbiosis occurs commonly among members of the family Acanthamoebidae. Several studies have speculated that endosymbionts are potential virulence factors [87, 89, 90]. Fritsche *et al.* [57] demonstrated intracellular bacteria in 24% of axenically grown *Acanthamoeba* isolates. These micro-organisms were gram-negative and non-acid fast. They could not be cultured by routine methodologies, although electron microscopy revealed multiplication within the amoebic cytoplasm. Examination for *Legionella* spp. with culture and nucleic acid probes was unsuccessful. The bacteria appear to be endosymbionts that multiply within their amoebic hosts. Rod-shaped bacteria were identified in five of 23 clinical *Acanthamoeba* isolates, four of 25 environmental isolates, and two of nine *Acanthamoeba* isolates from the ATCC that were previously unrecognised as having endosymbionts. Coccus-shaped bacteria were also present in one clinical isolate and two environmental isolates. No statistical difference was found between the numbers of endosymbiont strains originating from clinical and environmental amoebic isolates. They were found in different geographical areas, demonstrating their widespread occurrence in

nature. These findings suggest that endosymbiosis occurs commonly among members of the family Acanthamoebidae. The role of such endosymbionts in pathogenesis remains unknown, but they have been implicated in the development of amoebic keratitis [57].

Experimental transmission of two bacterial endosymbionts to symbiont-free isolates of *Acanthamoeba* spp. was studied by Gautom and Fritsche [89] to determine the specificity of the host–symbiont relationship. Both symbionts originated from amoebic isolates displaying an identical mtDNA *EcoRI* fingerprint. Symbiosis was established easily in one amoebic isolate with a homologous mtDNA fingerprint. Exposure of a heterologous amoebic isolate to the two symbionts resulted in cell death without the establishment of symbiosis. These studies suggest that there is a specific recognition system between particular isolates of *Acanthamoeba* and their symbionts, and that the appearance of a killer phenotype is related to contact between mismatched, although recognised, pairs.

Since the first report by Rowbotham [92], the association of the amoebae *Naegleria* and *Acanthamoeba* with the symbiont *Legionella pneumophila*, the causative agent of Legionnaires' disease, has been a subject of interest [91, 93]. *Acanthamoeba*, *Naegleria*, *Hartmannella*, *Vahlkampfia* and *Echinamoeba* have been shown to support the growth of legionellas [94], and environmental growth of legionellas in the absence of protozoa has not been documented. It is thought likely that the protozoa are the primary means of proliferation of these bacteria under natural conditions [95, 96]. This inter-relationship within the ecosystem can modify the virulence of *Legionella* [97]; it may also be involved in the observed phenomenon that *L. pneumophila* can be viable but non-detectable by cultivation on BCYE agar-based systems [98]. Hay *et al.* have proposed that the latter observation may have profound implications with regard to surveillance of water systems for *Legionella* [99], especially with respect to prevention of outbreaks of nosocomial Legionnaires' disease [100].

Isolation and identification of *N. fowleri* and *Acanthamoeba* in Hungary

Cases of PAM have been described in many countries, but no confirmed cases have been reported in Hungary. However, until recently, no trials had been performed to investigate the occurrence and possible pathogenic significance of 'free-living' amoebae. If *N. fowleri* was isolated, *N. fowleri* might then be considered as a possible causative agent when cases of meningoencephalitis are diagnosed in Hungary.

Naegleria spp. are found invariably in warm waters, at temperatures up to 45°C. Therefore, it seemed worth-

while to investigate geothermal pools for pathogenic *N. fowleri*. Thus, in 1994, a systematic study was started in Hungary to analyse the amoebic fauna of some natural and geothermal waters, as well as swimming pools and bathing pools fed by natural (geothermal) water [6, 27]. The results obtained are summarised in Table 1. Of particular interest was a thermotolerant *Naegleria* spp. found in a Szeged swimming pool fed by geothermal water and containing chlorine for disinfection. This isolate did not adapt to axenic growth in serum-casein-glucose-yeast-extract-medium (SCGYEM) as easily as reported previously for other *Naegleria* spp. [19, 67]. However, the iso-enzyme profile of this *Naegleria* isolate growing axenically was highly similar to that of an *N. philippinarum* (tentative name) isolate from the brain aspirate of a 12-year-old boy with PAM in Manila (unpublished results). This finding supports a role as a possible human pathogen for the *Naegleria* isolate from Szeged, Hungary. The pathogenicity of this isolate in mice is the subject of current investigations although, according to Griffin [34], temperature tests may prove more useful than virulence tests in mice in identifying *Naegleria* which are pathogenic for man. The strains of mice generally used are selected to be susceptible, but differ considerably in their degree of susceptibility [101, 102]. As an example, amoeba strains HN-3 and A5 of Culbertson both killed the mice used by Singh and Das, whereas only strain HN-3 killed the mice used by Culbertson *et al.* [101, 102].

In this Department, *A. castellanii* was isolated from the cerebrospinal fluid (CSF) of a 15-year-old

girl with lymphocytic meningoencephalitis [14]. In addition to the meningeal excitation symptoms, the clinical picture included focal neurological changes as well as alterations in the electroencephalogram. Negative results in bacteriological and virological examinations and the inefficiency of the initial therapy raised the possibility of protozoal infection. Following repeated laboratory examinations, the presence of *A. castellanii* in the CSF was demonstrated. Also in this Department, *A. polyphaga* was isolated from the contact lens storage solution of a patient with corneal ulcer [7].

The growing use of contact lenses increases the number of persons at risk of *Acanthamoeba* keratitis. This is an important aspect in Hungary where, because of some special economic circumstances, there has been a rapid expansion of contact lens usage since 1990. *Acanthamoeba* spp. (strain Cl) has been isolated from a commercial contact lens cleaning and disinfecting solution, from the corneal scrapings of a patient with severe eye soreness, and from the environment (strains Mo and Dun) [6, 27]. Morphologically, the human isolate (Cl) was indistinguishable from the *Acanthamoeba* strains isolated from a moss (Mo) or from the River Danube (Dun) (Fig. 1) [6, 27]. When RFLP analysis [21] was used to compare these isolates with human isolates from other countries [6], the RFLP phenotype of strain Cl was similar to that of strain JAC/E4 from six different countries [21]. Strain Dun was comparable to strain Ma from nine countries, and strain Mo was comparable to strain NZAU from New Zealand [21]. Thus, RFLP analysis of mtDNA may be a useful tool for the taxonomic classification

Table 1. Free-living amoebae isolated from sources in Hungary during the survey period 23 Sept. 1994–14 Oct. 1994

Source	<i>Acanthamoeba</i>	<i>Hartmannella</i>	Vahlkampfiid amoeba	<i>Vannella</i>	<i>Vexillifera</i>	<i>Cochliopodia</i>	<i>Filamoeba</i>	Non-cyst-forming amoeba
Szeged								
Pond Vér	+
Pond Sziksós	...	+	...	+
River Tisza	...	+
Budapest								
River Duna	+
Balatonyörök								
Lake Balaton	+
Keszthely								
Lake Balaton	...	+	...	+	+
Hévíz								
Hot spring pond	+	...	+
Szeged								
Anna spring	+	+	+	...
Drinking basin
Bath tub water								
30°C	+
36°C	...	+	+
40°C	...	+
Swimming pool	+
Moss	+
Soil	+
Human corneal scraping	+

* *Willertia magna*.

† *Naegleria* spp.

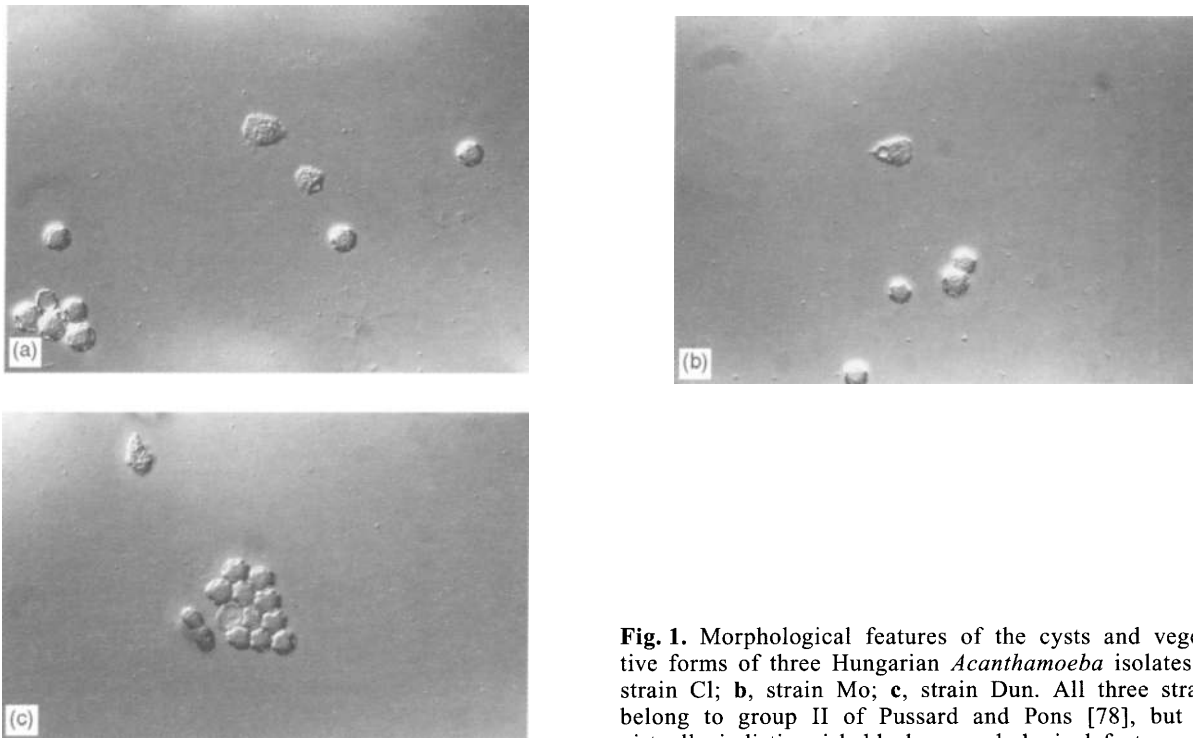


Fig. 1. Morphological features of the cysts and vegetative forms of three Hungarian *Acanthamoeba* isolates: **a**, strain Cl; **b**, strain Mo; **c**, strain Dun. All three strains belong to group II of Pussard and Pons [78], but are virtually indistinguishable by morphological features.

of free-living amoeba species, and perhaps also for the determination of their pathogenic potential.

A further interesting observation identified intracellular bacteria in an axenically grown *Acanthamoeba* spp. isolated from the basin of a natural hot-water

spring [6]. Electron microscopy revealed evidence for multiplication of rod-shaped bacteria within the amoebic cytoplasm (Fig. 2). The possible role of the endosymbiont in any pathogenic process is unknown, but its identification and characterisation is the subject of further investigations.

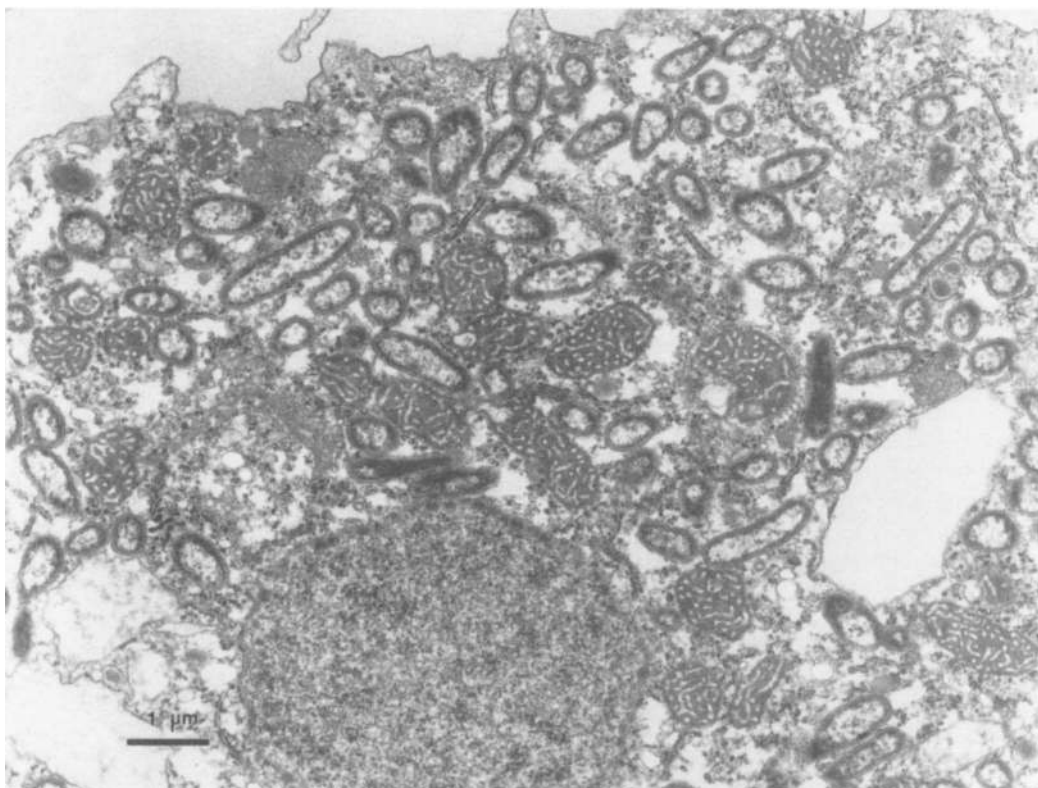


Fig. 2. Intracellular bacteria observed in an axenically grown *Acanthamoeba* spp. isolated from the basin of a natural hot-water spring.

Conclusions

'Free-living' amoebae can be found worldwide, under practically every kind of living conditions. Some (e.g., *Naegleria* and *Acanthamoeba*) are opportunist human parasites. Their pathogenic role is far more important than was thought previously. Since the first report in 1965 by Fowler and Carter [32], many lethal cases of PAM caused by *Naegleria* have been reported. In addition, a significant percentage of cases of keratitis and corneal ulcers in wearers of contact lenses, as well as many cases of swimming pool conjunctivitis (and some diseases of other organs), have been proved to be evoked by *Acanthamoeba*. Precise differentiation and identification of the pathogenic and non-pathogenic species and strains would be of primary importance from a therapeutic point of view. However, morphological markers, survival in different temperatures, pH dependency and virulence in infected animals (mice), all fail to fulfil the requirements for specificity, sensitivity and reliability. The newer molecular methods, such as restriction endonuclease digestion of whole-cell DNA, restriction endonuclease digestion of mtDNA, agarose iso-electric focusing and staining for acid phosphatase and propionyl esterase activity, and immunological analysis of fine differences by MAbs, have produced promising results with respect to the identification of species and strains, with special reference to their human pathogenicity, but quicker and more reliable methods would still be desirable in view of the clinical significance of the problem. The significance of the phenomenon of endosymbiosis is still practically unknown. A systematic, detailed analytic survey in Hungary for the isolation and identification of 'free-living' amoebae has isolated possibly pathogenic strains of *Naegleria* and *Acanthamoeba*, and has observed cases of endosymbiosis.

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