

Genotypic, phenotypic, biochemical, physiological and pathogenicity-based categorisation of *Acanthamoeba* strains

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Abstract. The genus *Acanthamoeba* includes more than 20 morphological species, but classification is problematical. Recently, the discovery of substantial interstrain differences in ribosomal DNA (rDNA) sequences has prompted questions about the relatedness of strains of the same species. In this study, therefore, we have investigated relationships between two isolates of *A. polyphaga*, CCAP 1501/3c and ATCC 30871, using morphological, biochemical, physiological, molecular and cytotoxicity assays. We observed that *A. polyphaga* ATCC 30871 exhibited up to six arms in endocyst while *A. polyphaga* CCAP 1501/3c exhibited a maximum of 5 arms thus indicating their position in group 2 and 3, respectively. *Acanthamoeba polyphaga* ATCC 30871 exhibited growth at 37°C and growth on 1M mannitol plates while *A. polyphaga* CCAP 1501/3c did not. In addition, both isolates exhibited differences in isoenzyme banding patterns and rDNA restriction fragment polymorphisms. More importantly, *A. polyphaga* ATCC 30871 produced cytotoxicity on corneal epithelial cells while *A. polyphaga* CCAP 1501/3c had no effects, suggesting differences in pathogenicity. Thus, all the results provide evidence for significant differences between the strains and further provided the basis for reclassification of the isolates. Implications of these results in the clinical diagnosis of pathogenic *Acanthamoeba* are discussed.

Species of the genus *Acanthamoeba* Volkonsky, 1931 are present in diverse environments including swimming pools, surface waters, thermally polluted discharges, ocean waters, soil, air and tap waters. Based on epidemiological information to date, they can be considered as waterborne pathogens (De Jonckheere 1980, 1983, Marciano-Cabral 2000, Khan et al. 2001).

Although the species of *Acanthamoeba* can cause fatal infections involving brain (Gonzalez et al. 1986, Di Gregorio et al. 1991, Friedland et al. 1992, Gordon et al. 1992), however, they are most frequently associated with eye keratitis (Auran et al. 1987, John 1993, Badenoch et al. 1994, Niederkorn et al. 1999). *Acanthamoeba* keratitis is characterised by intense pain and a slowly worsening clinical course. If not diagnosed early and treated aggressively, the infection may spread to deep stroma and to other ocular tissues (Yang et al. 1997, Niederkorn et al. 1999). At present, diagnosis of the disease is not straightforward and treatment is problematic, consisting of hourly topical application of a combination of drugs for extended periods of time. In view of the devastating nature of the disease and the problems associated with the therapy, our goals are to find means to rapidly identify *Acanthamoeba* species and to differentiate pathogenic and non-pathogenic species/isolates. We have recently shown the use of morphological characters (Khan 2001), osmotolerance,

PCR (Khan et al. 2001), and extracellular proteases (Khan et al. 2000), to differentiate pathogenic and non-pathogenic *Acanthamoeba* strains. Our results directly correlated an isolate's pathogenic potential in its ability to produce cytotoxicity on corneal epithelial cells using *in vitro* assays, suggesting the potential use of these assays in clinical diagnosis of *Acanthamoeba*.

Attempts to correlate pathogenicity with species have proven difficult due to inconsistencies in the morphological-based classification. Recently, the genus *Acanthamoeba* has been reclassified into 13 different genotypes based on rDNA sequence analyses i.e., T1–T12 and T14 (Stothard et al. 1998, Gast 2001). However, two strains of *A. polyphaga* (Puschkarew) Page, 1967, *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c, exhibited significant rDNA sequence differences between these two isolates and *A. polyphaga* ATCC 30871 was placed in T4 clade (mostly pathogens) and *A. polyphaga* CCAP 1501/3c was regrouped with *A. palestinensis* (Reich) Page, 1967 and placed in T2 clade (mostly non-pathogens). The aims of our study were to determine whether other morphological, physiological, biochemical, molecular and *in vitro* assays support the findings based on rDNA sequencing in order to differentiate these two isolates and whether these differences can be correlated with their pathogenic potential in order to develop methods for clinical diagnosis of pathogenic strains of *Acanthamoeba*.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Laboratories (St. Louis, MO) unless otherwise stated.

Culture of amoebae. Two strains of *Acanthamoeba*, *A. polyphaga* CCAP 1501/3c (isolated from old distilled water carboy, Culture Collection of Algae and Protozoa) and *A. polyphaga* ATCC 30871 (freshwater isolate, American Type Culture Collection) were used. Parasites were routinely grown in PYG medium [proteose peptone 0.75%(w/v), yeast extract 0.75%(w/v) and glucose 1.5%(w/v)] at 30°C.

Cytotoxicity assays using corneal epithelial cells. To determine the pathogenic potential of the *Acanthamoeba* isolates, cytotoxicity assays were performed as previously described (Khan et al. 2000). Briefly, immortalised rabbit corneal epithelial cells were grown to monolayers in 24-well plates. Both amoeba isolates (10^6 per well) were incubated with corneal epithelial cells for up to 12 h. Cytopathic effects were observed visually after haematoxylin staining. In addition, supernatants were collected and cytotoxicity determined by measuring lactate dehydrogenase (LDH) release (using Cytotoxicity detection kit, Roche, Indianapolis, IN). Percentage cytotoxicity was determined as follows: (sample value – control value / total LDH release – control value) \times 100. Corneal epithelial cells incubated alone were used as controls. Total LDH release was determined from cells treated with 1% Triton X-100 for 30 min at 37°C. Each experiment was performed in triplicates and repeated at least three times.

Analyses of cyst morphology. Cyst morphology has been used in differentiation of *Acanthamoeba* species (Pussard and Pons 1977, Visvesvara 1991). In order to determine whether cyst morphology can be used to differentiate the amoeba isolates tested in this study, cyst morphology analyses assays were performed. Briefly, both *Acanthamoeba* isolates were inoculated on non-nutrient agar plates overlaid with *Klebsiella aerogenes* as previously described (Khan et al. 2001). Plates were observed daily for growth of amoeba trophozoites (vegetative infective form), followed by transformation into cysts (dormant form). Semi-thin sections of the cysts were made and stained using haematoxylin and observed microscopically.

Osmotolerance and temperature tolerance assays. Previously we have shown that osmolarity can be used as a marker to differentiate pathogenic and non-pathogenic *Acanthamoeba* strains (Khan et al. 2001). Osmolarity assays were performed to determine whether the amoeba isolates used in this study can be differentiated based on osmolarity. Briefly, amoeba isolates were inoculated on non-nutrient agar plates overlaid with *K. aerogenes* and containing 1M mannitol (0.25 osmolar in contrast to 0.025 osmolar without mannitol). Plates were incubated at 30°C for up to 72 h. For temperature tolerance assays, amoebae were inoculated onto non-nutrient agar plates overlaid with *K. aerogenes* and incubated at 37°C for up to 72 h. Growth of the organisms was observed microscopically as well as by measuring the diameter of clearance in the bacterial lawn (Khan et al. 2001).

Isoenzyme analyses. Both *Acanthamoeba* isolates (5×10^7) were harvested by centrifugation at $800 \times g$ for 10 min.

Pelleted cells were resuspended in cold phosphate buffered saline and centrifuged again at $800 \times g$ for 10 min. This process was repeated twice. The washed cell pellet was then resuspended in an equal volume of enzyme stabiliser solution [Stock enzyme stabiliser, 1000 \times conc. (200 mM ethylene diamine tetra-acetic acid, pH 7.0 and 200 mM dithiothreitol and, once dissolved, 200 mM of ϵ -amino-caproic acid was added)]. The suspension was freeze-thawed $3 \times$ in liquid N_2 and centrifuged at $20,000 \times g$ for 20 min at 4°C. The supernatant was electrophoresed on cellulose acetate gels (76 mm \times 76 mm Titan[®] III cellulose acetate plates, Helena Laboratories, England) according to manufacturer's instructions. Electrophoresis was performed at room temperature at 200 volts for 25 min. Following electrophoresis, enzymatic activities of esterase (ES), malate dehydrogenase (MDH), phosphoenol pyruvate dehydrogenase (PEPD), isocitrate dehydrogenase (IDH), aldehyde oxidase (AO), xanthine dehydrogenase (XDH), glucosephosphoisomerase (GPI) and phosphoglucosyltransferase (PGM) were determined. The reproducibility and position of the isoenzyme patterns were confirmed with different extracts.

Amplified ribosomal DNA restriction analyses (ARDRA). Restriction enzyme analyses were performed as described previously (Kong and Chung 1996). Briefly, PCR was performed using primers 5'-TTTGAATTCGCTCCA ATAGCGTATATTAA and 5'-TTTGAATTCAGAAAGAGC TATCAATCTGT in a volume of 50 μ l containing 1.25 U Taq polymerase (Amersham, La Jolla, CA), 0.1–1.0 ng DNA (measured using Gene Quant, Amersham), 200 μ M dNTPs, 4 mM $MgCl_2$ and 0.5 μ M primer. The PCR reactions were performed at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 30 cycles and final elongation step of 10 min at 72°C. Amplified DNA was electrophoresed on a 2% agarose gel prepared in 1 \times TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA). Gels were stained using 0.5 μ g/ml of ethidium bromide and visualised under UV illumination.

For restriction enzyme analyses, amplified DNA was extracted from agarose gels using a rapid gel extraction system (Invitrogen, Carlsbad, CA). All procedures were performed according to the manufacturer's instructions using *AluI*, *CfoI*, and *HinfI* restriction enzymes.

RESULTS

Acanthamoeba polyphaga strain ATCC 30871 but not CCAP 1501/3c produced corneal epithelial cell cytotoxicity

To determine pathogenic potential of *A. polyphaga* ATCC 30871 and CCAP 1501/3c, cytotoxicity assays were performed using corneal epithelial cells. We determined that *A. polyphaga* ATCC 30871 produced cytopathic effects within 12 h as observed visually (Fig. 1A), as well as by measuring LDH release (Fig. 1B). In contrast, *A. polyphaga* CCAP 1501/3c did not exhibit cytotoxicity on corneal epithelial cells (Fig. 1A, B). Based on these data, *A. polyphaga* ATCC 30871 was considered as pathogenic while *A. polyphaga* CCAP 1501/3c as non-pathogenic.

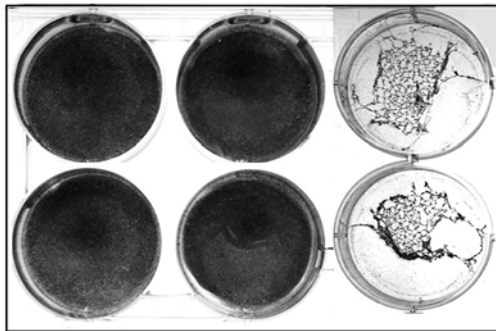
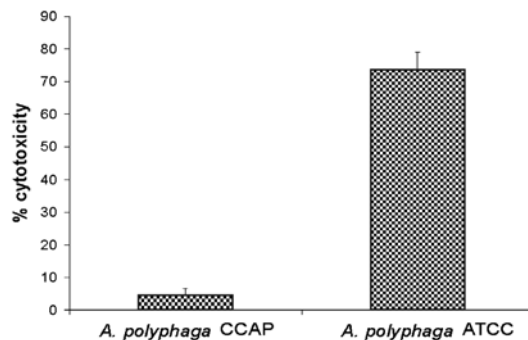
ACells alone *A. polyphaga* CCAP *A. polyphaga* ATCC**B**

Fig. 1. Pathogenic *Acanthamoeba* strains produced cytotoxicity on corneal epithelial cells. **A** – Representative effects of *A. polyphaga* on corneal epithelial cells after 12 h. Amoebae (10^6) were incubated with epithelial cells in 24-well plates in serum free media as described in “Materials and Methods”. Plates were incubated at 37°C in a 5% CO₂ incubator and cytotoxicity assessed by haematoxylin staining. Note that epithelial cells incubated with *A. polyphaga* ATCC 30871 exhibited disrupted monolayers while epithelial cells incubated with *A. polyphaga* CCAP 1501/3c remained intact. **B** – Following 12 h incubation as described above, supernatants were collected and LDH release was determined and converted to percentage cytotoxicity as described in “Materials and Methods”. Note that *A. polyphaga* ATCC 30871 produced cytotoxicity on epithelial cells while *A. polyphaga* CCAP 1501/3c did not. Results represent mean of three independent experiments and each experiment was performed in triplicate. Error bars represent standard deviation.

***Acanthamoeba polyphaga* strains ATCC 30871 and CCAP 1501/3c can be differentiated based on cyst morphology**

Previously, *Acanthamoeba* isolates have been divided into three groups, based on cyst morphology (Pussard and Pons 1977, Visvesvara 1991). In this study, we determined whether *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c can be differentiated based on cyst morphology. We observed that cysts of *A. polyphaga* ATCC 30871 and CCAP 1501/3c

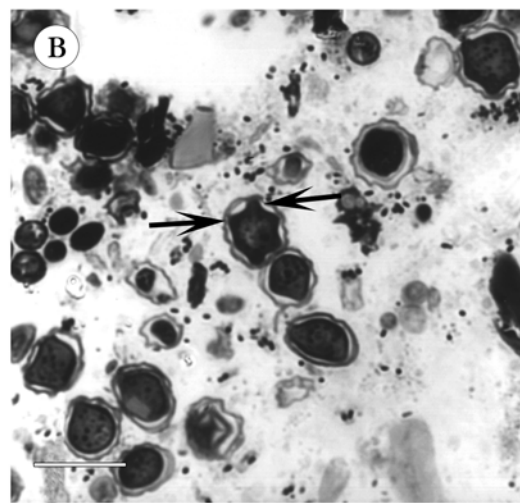
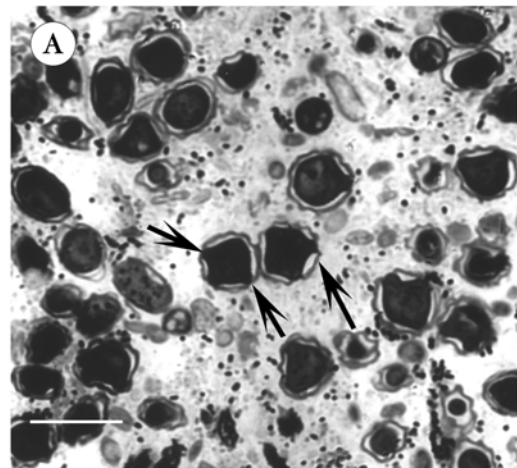


Fig. 2. *Acanthamoeba polyphaga* ATCC 30871 and CCAP 1501/3c are distinct based on cyst morphology. Cysts of *A. polyphaga* CCAP 1501/3c (A) or *A. polyphaga* ATCC 30871 (B) were prepared and stained as described in “Materials and Methods”. Note that *A. polyphaga* ATCC 30871 showed a maximum of 6 arms (group 2 isolates characteristic) while *A. polyphaga* CCAP 1501/3c exhibited up to 5 arms (group 3 isolates characteristic). Results are representative of three independent experiments. Scale bar = 15 µm.

are distinct, clearly indicating that both strains belong to distinct species (Fig. 2). *Acanthamoeba polyphaga* ATCC 30871 cysts exhibited a smooth outer cyst wall (ectocyst) that was separated from the inner cyst wall (endocyst). The endocyst was connected with the ectocyst at a certain number of extensions (known as arms or rays). The maximum number of arms determined in *A. polyphaga* CCAP 1501/3c was five (Fig. 2A), thus compatible with Group 3 isolates (Pussard and Pons 1977, Visvesvara 1991). In contrast the number of arms in *A. polyphaga* ATCC 30871 was six (Fig. 2B) indicating its position in Group 2 (Pussard and Pons 1977, Visvesvara 1991). All results are representative of three independent experiments.

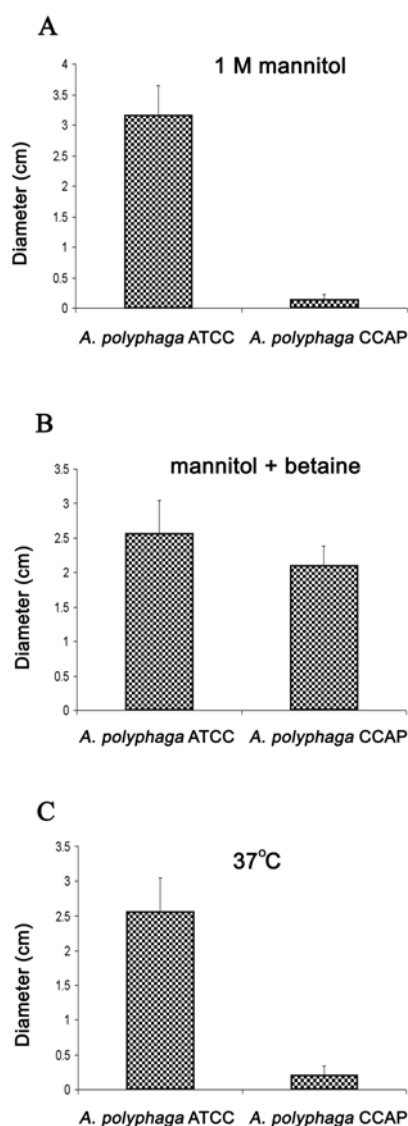


Fig. 3. Representative effects of high osmolarity and high temperature on the growth of *Acanthamoeba polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c. **A** – Both *Acanthamoeba* isolates (approx. 1000 cells) were inoculated on non-nutrient agar plates overlaid with *Klebsiella aerogenes*, containing 1M mannitol at 30°C for up to 72 h. Growth was observed microscopically as well as by measuring increase in cleared lawn of bacteria as described in “Materials and Methods”. Note that plates inoculated with *A. polyphaga* ATCC 30871 exhibited clearance in bacterial lawn while *A. polyphaga* CCAP 1501/3c was not able to grow. **B** – Assays as described in (A) were performed in the presence of 1mM betaine (osmoprotectant). Note that both *A. polyphaga* ATCC and *A. polyphaga* CCAP 1501/3c were able to grow equally well. **C** – *A. polyphaga* ATCC 30871 are temperature tolerant as compared to *A. polyphaga* CCAP 1501/3c. Both *Acanthamoeba* isolates were inoculated on non-nutrient agar plates overlaid with *K. aerogenes* and plates were incubated at 37°C for up to 72 h. Results represent mean from three independent experiments. Each assay was performed in triplicates (n = 3), error bars represent standard deviation.

***Acanthamoeba polyphaga* ATCC 30871 but not CCAP 1501/3c is temperature- and osmotolerant**

Previously we have shown that pathogenic *Acanthamoeba* strains are more temperature- and osmotolerant as compared to non-pathogens (Khan et al. 2001). To determine whether *Acanthamoeba* isolates used in this study can be differentiated based on osmolarity, assays were performed in the presence of 1M mannitol. *Acanthamoeba polyphaga* ATCC 30871 showed growth (thus clearance of bacterial lawns) in the presence of 1M mannitol while growth of *A. polyphaga* CCAP 1501/3c was inhibited (Fig. 3A). To test whether *A. polyphaga* CCAP 1501/3c growth inhibition was due to higher osmolarity, non-nutrient agar plates containing 1M mannitol were prepared in the presence of 1 mM betaine (osmoprotectant). We observed that both amoeba isolates were able to grow on mannitol plates containing betaine thus confirming an osmotic effect (Fig. 3B). In temperature tolerance assays, we observed that *A. polyphaga* ATCC 30871 grew at 37°C while *A. polyphaga* CCAP 1501/3c did not (Fig. 3C).

Isoenzyme analyses

The isoenzyme patterns for esterase (ES), malate dehydrogenase (MDH), phosphoenol pyruvate dehydrogenase (PEPD) and isocitrate dehydrogenase (IDH) were distinct consistently between *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c (Fig. 4). Differences were observed in the mobilities of individual enzymes except in the case of MDH, which showed differences both in the number and mobility of MDH. Results are representative of at least three independent experiments. Only small differences were observed for the other isoenzymes tested (data not shown).

***Acanthamoeba polyphaga* ATCC 30871 and CCAP 1501/3c can be differentiated based on ARDRA**

To determine the utility of ARDRA as a method for differentiating *Acanthamoeba* species, PCR-amplified rDNA was digested with the following enzymes: *AluI*, *CfoI*, and *HinfI*. Restriction patterns for each enzyme were distinct between *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c (Fig. 5), thus correlating well with other methods employed.

DISCUSSION

Methods for microbial species differentiation have evolved over the last few decades from simple culturing and microscopy to molecular methods such as rDNA sequencing. In this study we used morphological, biochemical, physiological and molecular methods to differentiate *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c. We observed that although rDNA sequencing provided more characters for differentiation, all other methods employed were consistently effective in the differentiation of *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c.

Previously, morphological methods using microscopy have been used as a tool for the identification and

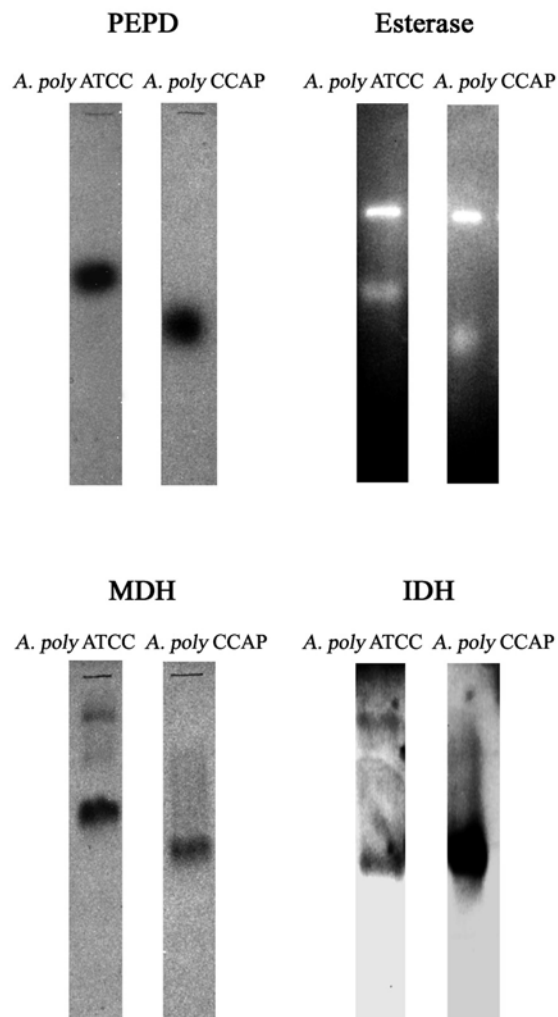


Fig. 4. *Acanthamoeba polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c exhibited differences using isoenzyme analyses. Both *Acanthamoeba* isolates were tested for isoenzyme analyses as described in "Materials and Methods" and exhibited differences using phosphoenol pyruvate dehydrogenase (PEPD), esterase (ES), malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH). Results are representative of three independent experiments.

differentiation of *Acanthamoeba* based on cyst morphology (Pussard and Pons 1977, Visvesvara 1991). *Acanthamoeba* cysts are double-walled, consisting of an outer ectocyst and an inner endocyst. The endocyst is folded into arms or rays that protrude out from the cell body and contact the ectocyst at their ends. Based on cysts morphological analyses, the genus *Acanthamoeba* has been divided into three groups. Group 1 isolates of *Acanthamoeba* are characterised by large cysts with an average diameter of $>18 \mu\text{m}$. In contrast, cyst isolates in groups 2 and 3 are $<18 \mu\text{m}$. However, group 3 isolates possess three to five arms while number of arms in group 2 isolates is six or more. We determined that

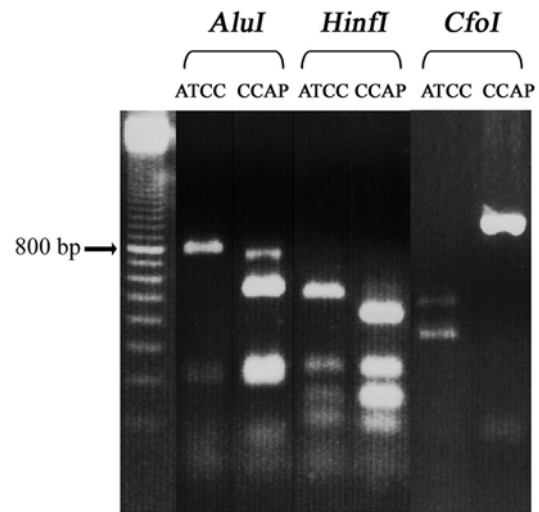


Fig. 5. Restriction analyses exhibited different banding patterns between *Acanthamoeba polyphaga* ATCC and *A. polyphaga* CCAP 1501/3c. A PCR-amplified 18S rDNA *Acanthamoeba*-specific product was obtained from both *A. polyphaga* ATCC 30871 and CCAP 1501/3c. Restriction analyses were performed using *AluI*, *CfoI*, and *HinfI*. Note that *A. polyphaga* ATCC 30871 and *A. polyphaga* CCAP 1501/3c can be differentiated with all restriction enzymes tested. Results are representative of three independent experiments.

cysts from both isolates of *A. polyphaga* ATCC 30871 and CCAP 1501/3c are $<18 \mu\text{m}$ in size, suggesting that both isolates belong to either group 2 or 3. To further determine grouping of these isolates, cysts were analysed for their number of arms. We observed that *A. polyphaga* ATCC 30871 exhibited up to six arms while *A. polyphaga* CCAP 1501/3c exhibited a maximum of five arms thus indicating their position in group 2 and 3, respectively. Overall, we were able to differentiate both isolates of *Acanthamoeba* based on cyst morphology. However, microscopic identification is based solely on morphological characteristics, thus skill and use of robust keys for identification is required. In addition, correlating pathogenicity with particular species using morphology alone is not always reliable and this presents a problem for clinical diagnosis of pathogenic *Acanthamoeba* strains. Similar conclusions can be made from isoenzyme analyses. Using isoenzyme analyses, we were successfully able to differentiate *A. polyphaga* ATCC 30871 and CCAP 1501/3c using ES, MDH, PEPD and IDH. Previously, several groups have used analyses of isoenzyme electrophoretic patterns to test the morphological classification scheme. These studies discovered extensive diversity among isolates of *Acanthamoeba*. For example, De Jonckheere (1983), Daggett et al. (1985) and Costas and Griffiths (1986), studied large groups of isolates (30, 71 and 37 strains, respectively). Each study divided isolates of *Acanthamoeba* into several different groups that often were

inconsistent with species and morphological group designations. In addition, isoenzyme patterns have been shown to vary when amoebae are grown under different conditions i.e., axenic vs monoxenic (Weekers and De Jonckheere 1997). In addition, the requirement of a large number axenically grown parasites is also a shortcoming. In contrast, ARDRA is a sensitive method as amplifications can be achieved from a single cell (Abbaszadegan et al. 1991). This method is increasingly being used to identify parasitic organisms including *Plasmodium* (Waters and McCutchan 1989, Mathiopoulos et al. 1993), *Leishmania* (Uliana et al. 1991), *Toxoplasma* (Cazenave et al. 1991, Guay et al. 1993, MacPherson and Gajadhar 1993, Verhofstede et al. 1993), *Entamoeba histolytica* (Garfinkel et al. 1989, Bracha et al. 1990), *Naegleria* (Sparagano 1993), *Cryptosporidium parvum* (Laxer et al. 1991, 1992) and *Giardia* (Mahbubani et al. 1991, 1992). Usually ARDRA can be performed within 2–3 hours, and samples can be directly assayed without the prior need of cultivation making it a rapid assay. The restriction banding patterns depend on the nucleotide sequence recognised by restriction enzymes. Genetic polymorphism resulting even from a single base pair change can yield different electrophoretic patterns, if this change creates or eliminates a restriction enzyme site, offering a powerful and direct probe into the parasite genotype as observed in this study. Distinct restriction patterns were observed between *A. polyphaga* ATCC 30871 and CCAP 1501/3c using a number of restriction enzymes. Comparing restriction patterns from *A. polyphaga* CCAP 1501/3c with other strains of *Acanthamoeba* revealed that *A. polyphaga* CCAP 1501/3c gave similar patterns to *A. palestinensis* (data not shown). We further confirmed these differences in our study by rDNA sequencing of *A. polyphaga* CCAP 1501/3c, GenBank Ref. No. AF239298, which supported the conclusions from restriction enzyme analyses. Overall these methods have proven valuable and cost-effective in species differentiation of *Acanthamoeba*. However, these methods do not provide information to determine an isolate's pathogenic potential. Previously, we and others have shown that temperature tolerance (De Jonckheere 1980, Khan et al. 2001), osmotolerance (Khan et al. 2001), and extracellular proteases (Khan et al. 2000) can be used as potential pathogenic traits to determine an isolate's pathogenicity. Indeed, *A. polyphaga* ATCC 30871 trophozoites were able to grow at high temperatures, were osmotolerant and produced higher extracellular proteases (indicative of pathogenic strains), which was

not observed with *A. polyphaga* CCAP 1501/3c, indicating that *A. polyphaga* CCAP 1501/3c may not possess pathogenic potential. To further confirm these findings, cytotoxicity assays were performed using corneal epithelial cells. *Acanthamoeba polyphaga* ATCC 30871 but not CCAP 1501/3c produced cytotoxicity on corneal epithelial cells in *in vitro* assays. At present, mechanisms of *Acanthamoeba*-induced cytotoxicity are incompletely understood but we have shown that pathogenic *Acanthamoeba* strains can be easily differentiated on the basis of these assays.

Over time we have observed the development of more detailed and reproducible methods for the differentiation of *Acanthamoeba* species. Here we demonstrate that morphological, biochemical, physiological and molecular methods have and will continue to serve as useful tools for species differentiation. Our data correlate well with the findings of Stothard et al. (1998), who based on rDNA sequencing showed that *A. polyphaga* ATCC and CCAP 1501/3c are distinct and reclassified the later as *A. palestinensis* (we further confirmed these differences in our study by rDNA sequencing, GenBank Ref. No. AF239297 [*A. polyphaga* ATCC 30871] and AF239298 [*A. polyphaga* CCAP 1501/3c]). In addition, *A. polyphaga* ATCC 30871 was placed among T4 pathogenic clade, while *A. polyphaga* CCAP 1501/3c was placed in T2 clade i.e., mostly weak and/or non-pathogens. When tested for their pathogenic traits (i.e., higher temperature tolerance, osmotolerance, extracellular proteases and *in vitro* cytotoxicity), we observed *A. polyphaga* ATCC 30871 to be pathogenic while *A. polyphaga* CCAP 1501/3c as non-pathogen thus correlating well with the data obtained by rDNA sequencing. In comparison with other assays, rDNA sequencing is more sensitive as more genetic characters are surveyed and thus serves as a sensitive marker of genetic change and is therefore more useful in identification of species and strains of the parasites. Our data together with these studies will aid in diagnostic and differentiation of pathogenic *Acanthamoeba* species. Ultimately, the identification and differentiation of pathogenic *Acanthamoeba* strains can be achieved by any one or combination of the methods described above promising to accelerate the pace of our understanding of this important and interesting class of pathogens.

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