Physiological, morphological, and immunochemical parameters used for the characterization of clinical and environmental isolates of *Acanthamoeba*

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SUMMARY

The factors that characterize *Acanthamoeba* strains as harmless or potentially pathogenic have not been elucidated. Analysing the *in vitro* and *in vivo* parameters of *Acanthamoeba* samples, including heat tolerance at temperatures close to that of the human body, cytopathic effects, and their ability to cause infections in animals, has been proposed to identify their pathogenic potential. Another promising criterion for differentiating strains is the analysis of their biochemical and immunochemical properties. In this study, a comparative evaluation between clinical and environmental *Acanthamoeba* isolates was performed on the basis of physiological, morphological, and immunochemical criteria. Crude antigens were used to characterize the protein profiles by electrophoresis and immunize mice to produce polyclonal and monoclonal antibodies. The antibodies were characterized using ELISA, Western blotting, and immunofluorescence techniques. The results obtained with polyclonal antibodies suggest the presence of specific proteins for each studied isolate and co-reactive immunochemical profiles among conserved components. Ten monoclonal antibody clones were obtained; mAb3 recognized 3 out of 4 samples studied. The results of this study may help standardize criteria for identifying and characterizing *Acanthamoeba* strains. Taken together, our results support the view that a set of features may help differentiate *Acanthamoeba* species and isolates.

Key words: *Acanthamoeba*, amoebic keratitis, monoclonal antibodies, polyclonal antibodies, protein profile, environmental strain.

INTRODUCTION

The amoebae of genus *Acanthamoeba* are aerobic protozoa that can be found as free-living organisms in a variety of environments (Schuster and Visvesvara, 2004*a*). Despite their free-living nature, *Acanthamoeba* may eventually be associated with severe infections in humans, acting as a parasite. Amoebae can affect the nasopharyngeal region, skin, lung, and central nervous system (Schuster and Visvesvara, 2004*b*). Infections of the central nervous system are known as granulomatous amoebic encephalitis and occur via haematogenic or pulmonary dissemination. Most cases of granulomatous amoebic encephalitis are related to immunodepression (Martinez and Visvesvara, 1997).

Acanthamoeba can also colonize the human eye, causing an invasive infection of the cornea called *Acanthamoeba* keratitis (AK). The increasing number of AK cases in recent years is related mainly to the use of contact lenses (Radford *et al*. 1998). AK is characterized by an inflammatory process that causes pain, tearing, eye redness, and a typical ring-shaped ulcer when amoebas move from the epithelium and reach the stroma (Dart *et al*. 2009). The mechanism of corneal infection involves previous events such as trophozoite adhesion via glycoprotein–lectin interactions, the secretion of proteases that cause tissue disorders, and cellular phagocytosis (Panjwani, 2010).

Several *Acanthamoeba* species have been described according to the morphological characteristics of their cysts (Pussard and Pons, 1977; Page, 1988). However, the most widely used classification at present is based on 18S rDNA sequences, which allows the identification of about 15 genotypes (Stothard *et al*. 1998; Horn *et al*. 1999; Hewett *et al*. 2003). Although genotype T4 is the most common among infection-causing *Acanthamoeba* (Booton *et al*. 2005), the factors that determine whether a species or strain is harmless or potentially pathogenic have not been fully elucidated.

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Accordingly, we evaluated the *in vitro* and *in vivo* parameters of *Acanthamoeba* samples maintained in culture, including tolerance to temperatures close to normal human body temperature, cytopathic and cytotoxic effects on cell lines, and their ability to infect experimental animals (Schuster and Visvesvara, 2004*a*).

An additional promising approach for differentiating *Acanthamoeba* samples is the evaluation of biochemical and immunological properties (Schuster and Visvesvara, 2004*a*). Possible markers of pathogenicity can be assessed by studies on protein expression as reported by Walochnik *et al*. (2001, 2004). Furthermore, identifying relevant antigens can aid the production of diagnostic tests for *Acanthamoeba* infections. Thus, the aim of this study was to comparatively evaluate clinical and environmental samples of *Acanthamoeba* according to various physiological, morphological, and immunochemical criteria and identify antigenic components that could be similar or distinct among them.

MATERIALS AND METHODS

Acanthamoeba *isolates*

Four isolates were used in this study. Sample KS (keratitis strain) was isolated from a corneal scraping in a patient with keratitis. Isolate ES (environmental strain) was obtained from the dust of the domicile of the same patient. Another 2 isolates of *Acanthamoeba polyphaga* were included for comparison in physiological, morphological, and immunochemical tests. Isolate ATCC 30461 was obtained from a corneal scraping of a keratitis patient, while isolate ATCC 30872 is a freshwater isolate. Herein, the clinical and freshwater reference isolates are designated KAP and EAP, respectively. Isolates were cultured axenically in peptone yeast extract glucose (PYG) medium at pH 6·5 and 28 °C in 25 cm^3 tissue-culture flasks without shaking, supplemented with 10% fetal bovine serum (Rowbotham, 1980). For the experiments, *Acanthamoeba* trophozoites were harvested at the exponential growth phase by centrifugation at 500 ***g*** for 10 min. Trophozoite pellets were washed twice with PBS, counted, and used for further analysis.

Growth curves and encystment assay

To compare the growth of the isolates, 2×10^5 trophozoites ml^{-1} were inoculated in 55×15 mm tubes containing 2 ml of PYG medium. Amoebae were counted using a haemocytometer after 12, 24, 36, 48, 72, and 96 h of cultivation. Generation times were calculated according to the method of Madigan and Martinko (2006).

Encystment rates were determined by transferring 2×10^5 trophozoites ml^{-1} into 2 ml of Neff encystation saline (Neff and Neff, 1969). The cysts were quantified in a haemocytometer daily for 4 days.

Scanning electron microscopy

Trophozoites (2×10^5) grown on glass cover slips were fixed for 1 h with 2·5% glutaraldehyde diluted in PBS, washed 4 times with the same buffer, and post-fixed for 15 min in the dark with 2% osmium tetroxide made up in 0·1 M cacodylate buffer. Following extensive washing with PBS, samples were dehydrated in increasing amounts of ethanol and critical-point dried with CO_2 as the transitional fluid (Samdri 780; Tousimis Research Corp.). The samples were then coated with a thin layer of gold by an ion-sputtering device (Jeol-JFC I 100) and observed under a scanning electron microscope. Digital photomicrographs were captured with appropriate software (DSM-982 Gemini; Zeiss). Acanthopodia were quantified using Image J® in 10 randomly selected trophozoites in the photomicrographs.

Antigen extract preparation

Trophozoites were sonicated with 10×1 min 40 W pulses at 30 s intervals on a tissue sonicator (Sonifer W185D; Heat Systems Inc.) with a microtip. Trophozoite disruption was confirmed by microscopic observation. The suspension was centrifuged for 10 min at 10 000 ***g***, and the protein concentration of the supernatant was determined.

Immunization protocols

Polyclonal antibodies. After pre-immune sera were collected, 16 mice were injected subcutaneously with 30 μg antigen emulsified in complete Freund's adjuvant (day 1). Booster injections were performed subcutaneously 15, 30, and 45 days later with the same dose in incomplete Freund's adjuvant. The control group received Freund's adjuvant alone under identical conditions. Immune sera were collected on day 52.

Monoclonal antibodies (mAbs). After pre-immune sera were collected, BALB/C mice received an initial injection of 30 μg KS isolate crude antigen following the same protocol described above. Animals were bled, and blood samples were collected 1 week after the last injection. An ELISA was used on the KS antigens. Mice whose serum resulted in absorbance (492 nm) higher than 1·2 (serum dilution 1:100) were given an intravenous boost of 5 μg KS in 100 μl of PBS. Three days later, the spleens from hyperimmunized mice were collected and their splenocytes were fused to Sp2/0 myeloma cells (Alvarenga *et al*. 2003). The supernatants of hybridomas were ELISA-screened using KS crude antigen. KS-positive

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hybridomas were subsequently expanded, retested, and cloned by 2 rounds of single-cell limiting dilutions. Anti-KS mAbs were tested using the ELISA described below. The mAbs were purified by single-step immunoaffinity chromatography by using an immobilized protein A CNBr–Sepharose column prepared according to the manufacturer's instructions (Pharmacia). The isotype of anti-KS mAbs was determined using the IsoStrip Mouse Monoclonal Antibody Isotyping kit (Roche Diagnostics Corporation, Alameda, CA, USA).

All procedures performed with mice were approved by the Ethics Committee on Animal Experimentation from Universidade Federal do Paraná (No. 23075.087228/2009-74) according to the guidelines of the Brazilian College of Animal Experimentation (COBEA).

ELISA

Anti-*Acanthamoeba* sera, control sera, or anti-KS hybridoma supernatant (1:100 to 1:12 800 dilution) were tested against KAP, KS, EAP, and ES crude antigens by ELISA. Falcon flexible microtitration plates (Becton Dickinson, France) were coated overnight at 4 °C with 100 μl of 10 μg/ml solution of each antigen in 0·02 M sodium bicarbonate buffer (pH 9·6). The assay was conducted in duplicate as described previously (Chávez-Olórtegui *et al*. 1991).

Crude antigen electrophoresis and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed to assess the protein profile of each *Acanthamoeba* isolate and verify the reactivity of the polyclonal antibodies. Briefly, 25 μg crude antigen of each strain and molecular weight markers were electrophoresed in 12·5% resolving gel at 100 V for 90 min at room temperature. After electrophoresis, the gels were silver stained or transferred to a 0·45 mm nitrocellulose membrane for polyclonal antibody recognition. For the immunoblot assay, after membrane blocking with PBS with 0·3% Tween 20, 3 mm-wide strips were cut and incubated (60 min at room temperature) with a range of dilutions of anti-*Acanthamoeba* sera in PBS-T (PBS with 0·05% Tween 20) according to their titre obtained by ELISA (anti-KAP, 1:200; anti-KS, 1:400; anti-EAP, 1:600; anti-ES, 1:2000). Following washing, the membrane was probed with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:4000 in PBS-T. After several washes, the reaction was developed by the addition of 3′,3′ diaminobenzidine and chloronaphthol.

Anti-KS mAb supernatants were assayed against *Acanthamoeba* crude antigen blots after resolving proteins under non-denaturing conditions. The procedure was similar to that described above, except SDS was replaced with 0·1% Nonidet P-40.

Immunofluorescence assay

Trophozoites were incubated on cover slips in PYG medium for 24 h at 28 °C, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0·2% Triton X-100 for 20 min at room temperature. After 2 careful washes with PBS and blocking solution (2% casein/PBS, 60 min at room temperature), the trophozoites were incubated with polyclonal or monoclonal antibodies for 2 h at room temperature. Following washing with PBS, reactivity was detected by incubation with FITC-labelled anti-mouse IgG-AlexaFluor. To promote nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI) was added. Negative controls without primary antibodies or using the pre-immune sera were assayed in each test.

Statistical analysis

All statistical analyses were performed using SigmaStat Windows version 3.5 (Systat Software Inc., Erkrath, Germany). Data were analysed using Student's *t*-tests for paired data or the Mann–Whitney *U*-test and One-Way ANOVA for unpaired data. The results are expressed as mean ± S.E.M. The level of significance was set at $P < 0{\cdot}05$.

RESULTS

Growth in PYG medium

The *Acanthamoeba* isolates studied here exhibited similar growth profiles with a typical exponential phase starting after 24 h and ending at 72 h (Fig. 1). The cell density at each time-point was significantly distinct among the isolates ($P < 0{\cdot}05$). The growth rate ranged from 0·031 to 0·036 generations per hour. Early on in incubation (i.e., at 24 and 36 h), clinical isolates (i.e., KAP and KS) exhibited higher growth rates than environmental ones (i.e., EAP and ES) (Fig. 1).

Encystment assays

The dynamic of encystment in Neff medium indicated that the environmental isolates, EAP and ES, switched from trophozoites to cysts more rapidly than the clinical isolates, KAP and KS. The difference in cyst density between the clinical and environmental groups was remarkable after 48 h ($P < 0{\cdot}05$, Student's *t*-test). After 72 and 96 h, all isolates reached more than 80% encystment (Fig. 2).

Amoebae morphology by scanning electron microscopy

A typical amoeboid shape with acanthopodia was observed in all studied isolates (Fig. 3). KAP, KS, and ES exhibited significantly higher numbers of projections than that of the reference environmental

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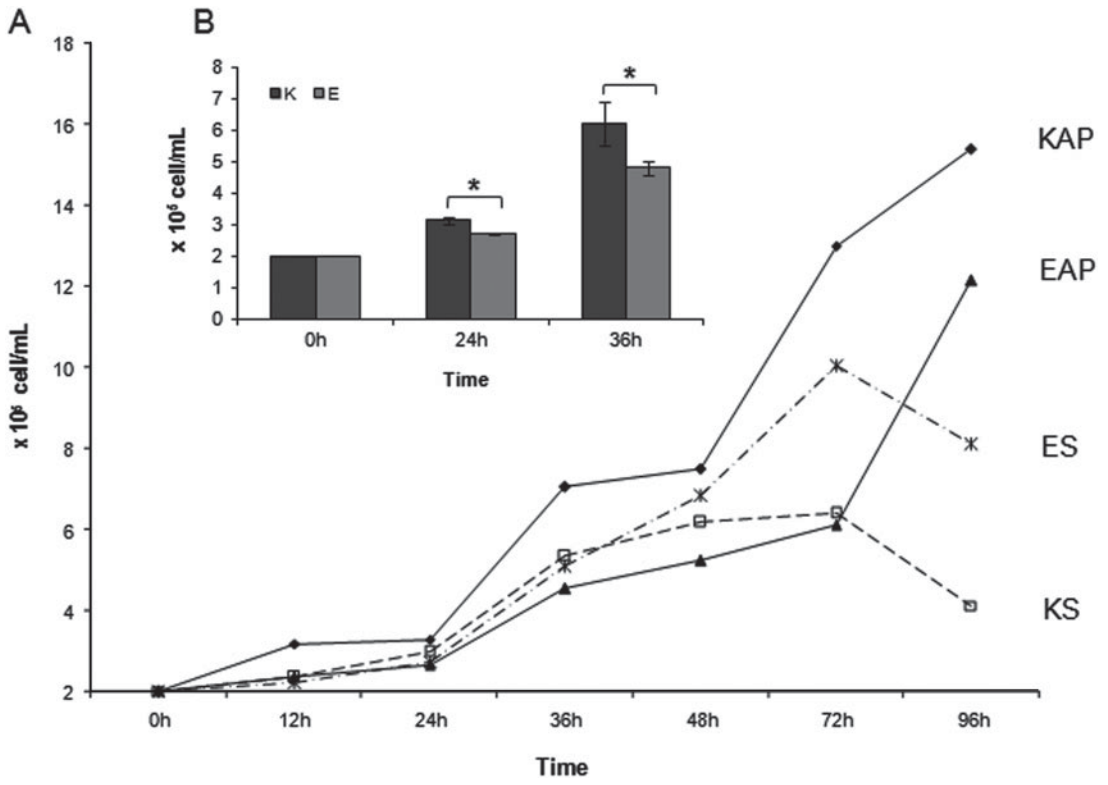

Fig. 1. (A) Growth curves of the 4 *Acanthamoeba* isolates in PYG medium with an initial inoculation of 2 × 10 (exp5) trophozoites ml^{-1}. Significant differences ($P < 0{\cdot}05$, one-way ANOVA) in cell density were observed in most of the intervals of time studied. (B) The growth of *Acanthamoeba* keratitis isolates (K), KAP and KS, was clustered and compared with the group of environmental isolates (E), EAP and ES, during incubation in PYG medium with an initial inoculation of 2 × 10 (exp5) trophozoites ml^{-1}. Differences between the clusters were significant at 24 and 36 h as represented by the asterisks ($P < 0{\cdot}05$, Student's *t*-test).

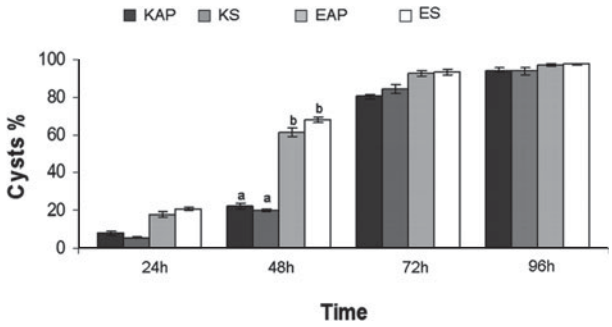

Fig. 2. Kinetics of encystment of *Acanthamoeba* isolates. Trophozoites (2 × 10 (exp5) amoebae ml^{-1}) were incubated in Neff's encystment solution for up to 96 h, and the cysts were quantified by light microscopy. The experiment was performed in triplicate. EAP and ES: environmental isolates, KAP and KS: clinical isolates. Distinct lower-case letters (a, b) represent significant differences ($P < 0{\cdot}05$, one-way ANOVA). The bars represent standard errors of the mean.

isolate, EAP (Fig. 4). ES seems to be an intermediate between the clinical isolates and the reference.

Protein profiles

The protein profiles of the *Acanthamoeba* crude antigens are shown in Fig. 5. Complex profiles with multiple proteins in each isolate were observed; molecular weights ranged from 10 to 200 kDa. Among the conserved proteins, prominent bands were visible around 40 and 130 kDa. Each sample also exhibited specific bands in its protein profile.

Characterization of polyclonal antibodies

To evaluate the cross-reactivity of sera from mice immunized with different *Acanthamoeba* antigens, we employed an ELISA and Western blotting. In these experiments, sera were tested at a dilution of 1:400 (Fig. 6) and at different dilutions: anti-KAP, 1:200; anti-KS, 1:400; anti-EAP, 1:600; anti-ES, 1:2000 (Fig. 7), respectively. The immune sera presented cross-reactivity against protein extracts from clinical and environmental samples. Immunoblot analysis was used to identify immunogenic and antigenic proteins that may be specifically present in each *Acanthamoeba* crude antigen (Fig. 7). The immunoblot profiles of *Acanthamoeba* antigens exhibited reactivity with the same proteins present in the silver-stained profiles.

The ES antigens were more immunogenic than the others considering the high concentration of anti-ES antibody (titre >1:2000); however, these antibodies had less specific antigen-binding reactivity. Anti-EAP

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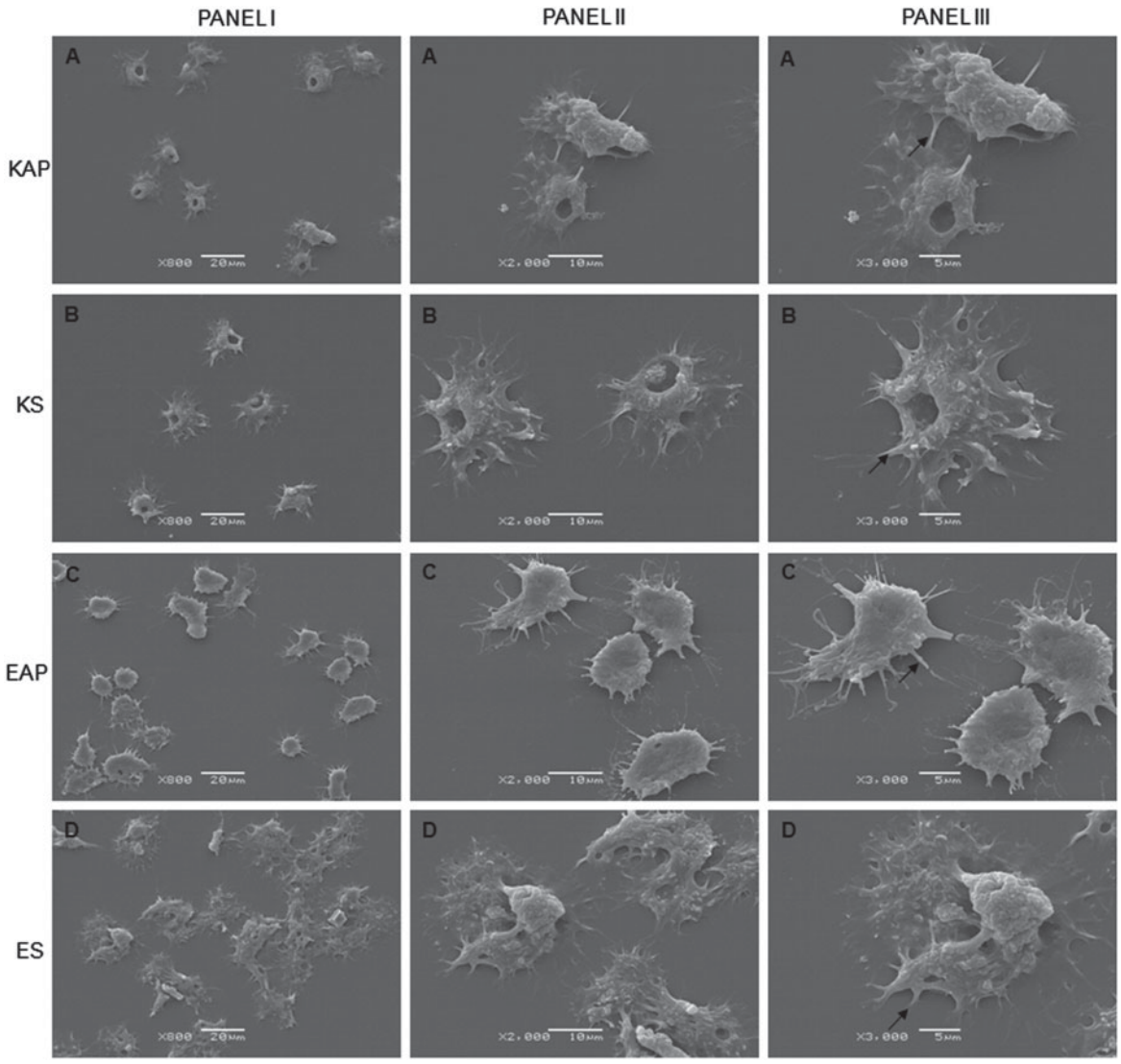

Fig. 3. Scanning electron microscopy of *Acanthamoeba* trophozoites from clinical (i.e., keratitis) (A, B) and environmental (C, D) isolates showing typical spiny projections termed acanthopodia (black arrows). Panels I and II, low-magnification image of an amoeba isolate; panel III, high magnification of environmental isolates, EAP and ES, and clinical isolates, KAP and KS.

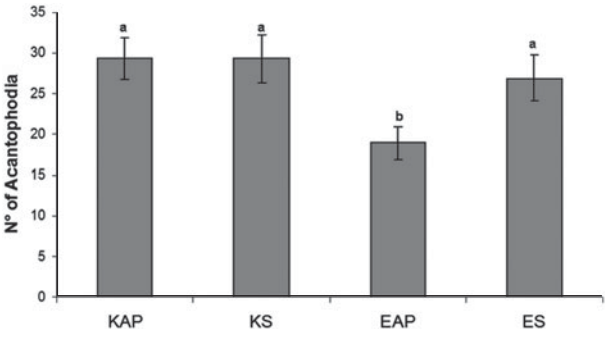

Fig. 4. Number of acanthopodia observed in clinical isolates, KAP and KS, and environmental isolates, EAP and ES. The experiment was performed in triplicate. Distinct lower-case letters (a, b) represent significant differences ($P < 0{\cdot}05$, one-way ANOVA). The bars represent standard errors of the mean.

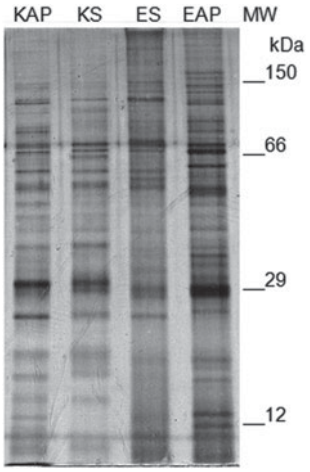

Fig. 5. Protein profiles of the *Acanthamoeba* trophozoites of clinical isolates, KAP and KS, and environmental isolates, EAP and ES, in 12·5% silver-stained SDS-polyacrylamide gels. MW, molecular weight marker.

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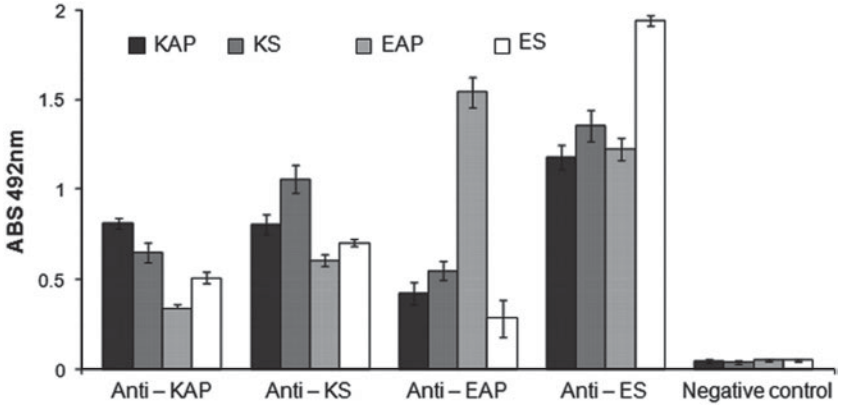

Fig. 6. Cross-reactivity of polyclonal antibodies produced against the antigens of clinical isolates, KAP and KS, and environmental isolates, EAP and ES, by ELISA. The plate was immobilized with 10 μg/ml *Acanthamoeba* trophozoite antigen. The immune sera were tested against their specific antigens at a dilution of 1:400 and against a non-specific antigen at the same dilution. The reactions were confirmed by adding anti-immunoglobulin. The bars represent standard errors of the mean.

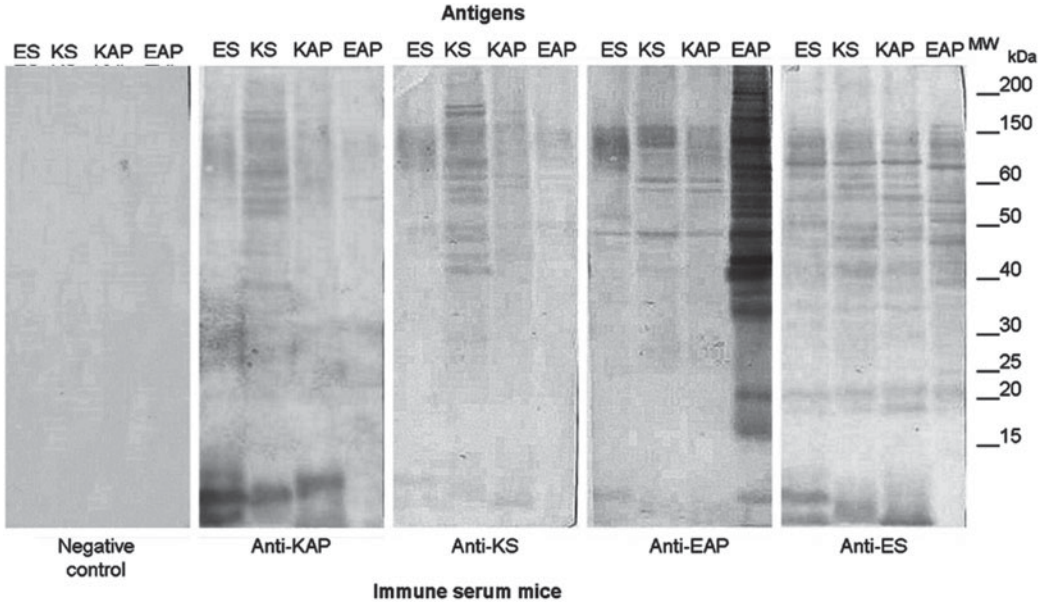

Fig. 7. Western blot analysis to identify antigenic proteins in *Acanthamoeba* extracts. Blots were probed with positive sera (i.e., anti-KAP, anti-KS, anti-EAP, and anti-ES), immune mouse serum, and negative sera (i.e., pre-immune serum). Clinical (KAP and KS) and environmental (EAP and ES) isolates were used. The proteins of isolates were separated by electrophoresis on a 12·5% polyacrylamide gel and transferred to nitrocellulose membranes. The immunoreactivity of the polyclonal antibodies was evaluated against both specific and non-specific antigens. MW, molecular mass marker.

antibodies were the most specific; their reactivity remained the same only against their own antigens when higher dilutions were assayed (data not shown). EAP pre-immune serum did not react with any proteins in the extracts. Even though anti-KAP antibodies had failed to detect the most of KAP antigens after denaturation (Fig. 7), their reactivity were confirmed by immunofluorescence (Fig. 8).

Polyclonal antibodies were subjected to immunofluorescence assays to verify their capacity for recognizing the *Acanthamoeba* trophozoites from each isolate (Fig. 8). The amoebae were first labelled with DAPI—a reagent with affinity for nuclear structures—to ensure the presence of trophozoites attached to the blade. The reactivity of the polyclonal antibodies against *Acanthamoeba* trophozoites was detected by adding an anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC). All antibodies were able to recognize the trophozoites.

Characterization of monoclonal antibodies

Twenty hybridomas secreting monoclonal antibodies reactive to *Acanthamoeba* antigens were generated after 15 days from the fusion of Sp2/0 tumor cells with spleen cells from BALB/C mice immunized

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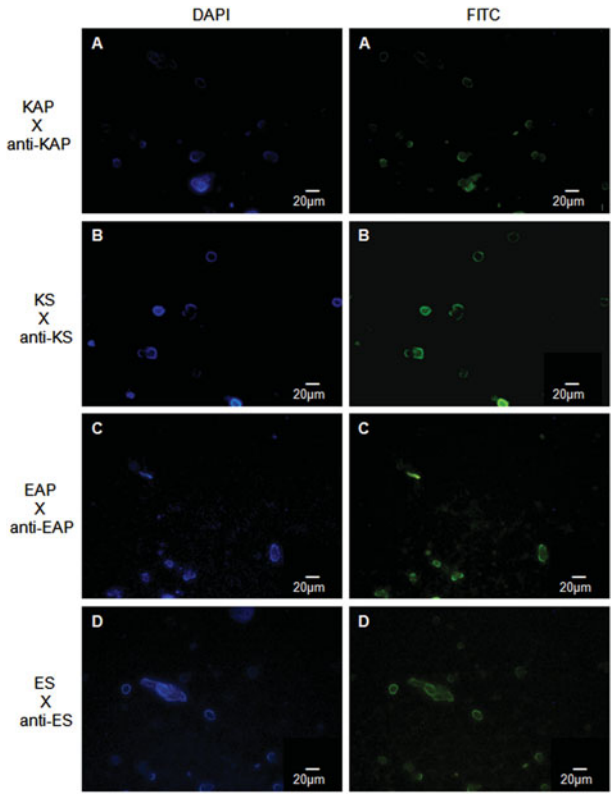

Fig. 8. Indirect immunofluorescence with polyclonal antibodies. Trophozoites KAP, KS, EAP and ES were incubated with mouse sera i.e., anti-KAP, anti-KS, anti-EAP, and anti-ES, respectively. After intensive washing, bound antigens were detected with specific antibodies. All antigens were tested in the presence of murine pre-immune serum as well as the conjugate only (i.e., anti-mouse FITC); neither showed reactivity (data not shown).

with the clinical antigen of KS. Ten subclones were also tested by an ELISA against antigens from KAP, EAP, and ES (Table 1). Monoclonal antibodies exhibited less reactivity than polyclonal antibodies but were able to recognize different antigens from the samples. Hybridoma 3A1A3 (mAb3) was chosen to be purified and characterized because it was the only one able to recognize 3 of the 4 isolates with similar intensity by ELISA. The antibody mAb3, isotyped as IgG1λ, was tested against proteins from the clinical and environmental isolates; it was able to recognize high-molecular-weight proteins from clinical isolates and from the environmental strain, ES, but did not recognize any protein components from the environmental reference sample, EAP (Fig. 9). mAb3 also was tested by immunofluorescence, and the same recognition patterns of the 3 samples (i.e., KAP, KS, and ES) were observed (Fig. 10).

DISCUSSION

Members of the genus *Acanthamoeba* are some of the most common protozoans found in the environment.

Table 1. Reactivity of monoclonal antibody supernatants against clinical and environmental isolates of *Acanthamoeba* by ELISA

	Reactivity*			
mAb	KAP	KS	EAP	ES
1F10G3	0·051	0·159	0·046	0·049
2C3A11	0·219	0·250	0·045	0·118
3A1B4	0·112	0·160	0·047	0·132
3A1A3	0·259	0·248	0·044	0·215
3A1F4	0·141	0·163	0·049	0·065
4E5G5	0·050	0·178	0·044	0·059
4F6E10	0·055	0·144	0·045	0·067
4F6E11	0·058	0·199	0·042	0·072
4F6G11	0·059	0·241	0·038	0·078
4H3B8	0·061	0·221	0·049	0·043

* Absorbance 490 nm.

This genus comprises both harmless species as well as ones that cause infections in humans. Even among phylogenetically related species, some strains exhibit significantly different pathogenic potential (De Jonckheere, 1980; Walochnik *et al*. 2000; Khan and Tareen, 2003).

To elucidate the factors and mechanisms that cause an amoeba to be a pathogen, the morphological, physiological, and immunochemical properties of 2 clinical and 2 environmental *Acanthamoeba* isolates were investigated in this study. Comparing the growth curves of the isolates revealed distinct patterns with significant differences in cell density at the most intervals of time studied. Individual characteristics and different abilities to adapt to *in vitro* culture conditions could explain this variation. Analysis of the early parts of the curves revealed that clinical isolates tended to grow faster than environmental isolates, which is corroborated by previous reports (Walochnik *et al*. 2000). According to Walochnik *et al*. (2000), the higher growth rates of clinical *Acanthamoeba* isolates could be related to an adaptation to live as parasites because faster multiplication would enable such amoebae to establish an infection. Regarding the encystment dynamics of the *Acanthamoeba* studied here, environmental isolates formed cysts faster than clinical isolates. Our results contradict those of Da Rocha-Azevedo *et al*. (2007), who reported that the encystment of *Acanthamoeba* isolated from ocular keratitis occurs faster than that of the same species of environmental origin. Rapid encystment is considered an important strategy for adaptation to new and challenging environments as well as survival in hostile conditions (Walochnik *et al*. 2004). Both the external environment and host tissue may create adverse situations that require rapid encystment. Considering this perspective and taking into account the difference between our results and those of the previous work, it is possible that the

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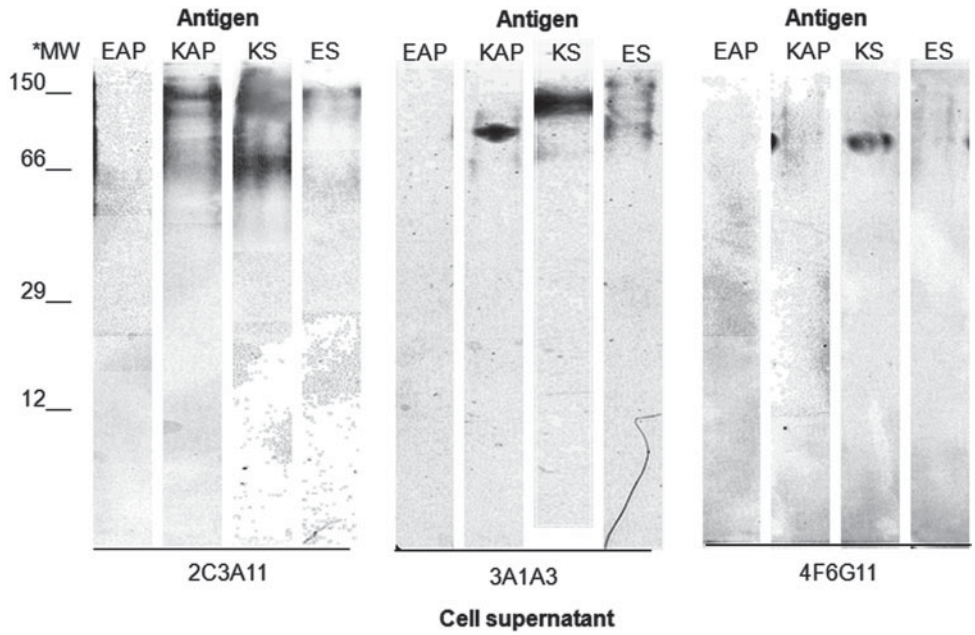

Fig. 9. Western blotting of mAb2, mAb3, and mAb4. The proteins of clinical (KAP and KS) and environmental (EAP and ES) isolates were separated by non- denaturating electrophoresis. *MW, molecular mass marker corresponds to the molecular mass profile of the non-denaturating gel.

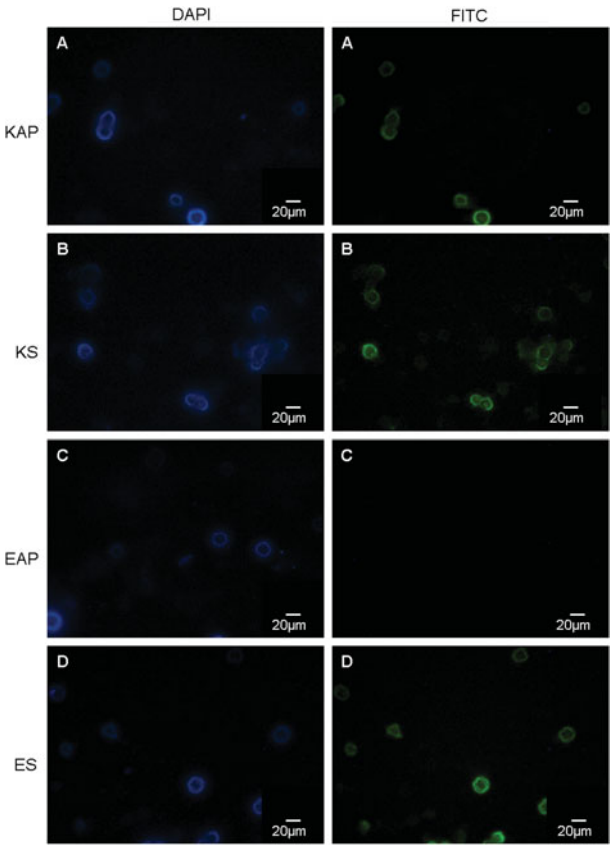

Fig. 10. Indirect immunofluorescence. Reactivity of mAb3 with trophozoites of KAP, KS, EAP, and ES. All antigens were tested in the presence of murine pre-immune serum as well as the conjugate only (i.e., anti-mouse FITC); neither showed reactivity (data not shown).

speed of encystment is an intrinsic characteristic of a sample rather than a marker of pathogenicity.

The number of acanthopodia is another feature that has been correlated with pathogenicity because these projections are associated with the binding of trophozoites to host tissues (Marciano-Cabral and Cabral, 2003). The clinical isolates (KAP and KS) and ES exhibited greater numbers of acanthopodia than that of the reference environmental isolate, EAP, suggesting that the ES isolate has a possible propensity toward pathogenicity.

In addition to the morphological and physiological criteria, the presence and/or expression levels of some components have been used as indicators of pathogenicity in *Acanthamoeba* (Niederkorn *et al*. 1999; Khan, 2001). Analysis of the electrophoretic patterns of soluble antigens in this study revealed proteins ranging from 10 to 200 kDa. A particular component in the profiles with an apparent molecular mass close to 40 kDa could correspond to actin cytoskeleton—a highly conserved component in *Acanthamoeba* trophozoites (Walochnik *et al*. 2004). As reported by Pussard and Pons (1977), the presence of a prominent band at 44 kDa is an indication that the organism belongs to *Acanthamoeba* sp. morphological group II. Therefore, the protein profile could also be used to establish classification criteria for different groups.

Polyclonal and monoclonal antibodies may be useful tools for identifying and characterizing both specific and conserved components in different *Acanthamoeba* strains and indicate the presence of molecules associated or not with pathogenicity (Imbert-Bouyer *et al*. 2004; Garate *et al*. 2005).

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Although there were some differences with respect to antigenicity and immunogenicity, all *Acanthamoeba* crude antigens used in this study induced the production of polyclonal antibodies whose cross-reactivity was confirmed by ELISA, Western blotting, and immunofluorescence. This reactivity may be justified by the conserved components present in all isolates, which may be associated with adhesion, mobility, and cell cycling (Khan *et al*. 2000).

The most antigenic and immunogenic samples were KS and ES, respectively. However, antibodies produced in mice immunized with the EAP strain were the most specific; therefore, the immunogenic profile of the EAP isolate can also be used to distinguish it from other strains by Western blotting. By using this approach, anti-KAP antibodies recognized low-molecular-weight proteins also present in the KS and ES isolates but absent in the EAP isolate, suggesting the existence of common components between the ES isolate and clinical samples.

Considering the KS sample was from a clinical case of keratitis and that this strain has not been immunochemically characterized yet, 10 hybridomas secreting monoclonal antibodies against KS crude antigens were obtained. Among them, mAb3 was chosen for further characterization due its cross-reactivity with other strains. mAb3 was able to identify a repeating epitope found in more than one component of the pathogenic isolates (KAP and KS) and the environmental strain (ES) but unable to recognize the same component in the non-pathogenic reference strain (EAP). The same mAb3 recognition pattern for the 3 samples was observed in Western blotting and immunofluorescence assays. These results reinforce the idea that these 3 samples share antigenic domains. Another piece of evidence that corroborates this phenomenon is the lack of mAb3 reactivity to some components in the environmental sample (EAP).

Previous studies indicate that *Acanthamoeba* mannose-binding protein may affect pathogenicity potential (Garate *et al*. 2005). Under both reducing and non-reducing conditions, a compound with an apparent molecular weight similar to that of mannose-binding protein ($\sim$ 130 kDa) was identified by mAb3. In addition, this monoclonal antibody recognized more than 1 band, indicating that the epitope may be shared by more than one antigenic component present in the KAP, KS, and ES crude antigens. However, further studies are required to identify and confirm the role of mAb3 in disease development.

Immunological, biochemical, and physiological criteria have been applied to identify different *Acanthamoeba* species. Information obtained through these patterns can help researchers understand the pathogenicity mechanisms of *Acanthamoeba*, including the identification of key components involved in the process (Niederkorn *et al*. 1999; Khan, 2001; Turner *et al*. 2005). Despite substantial effort to distinguish different *Acanthamoeba* isolates and consequently to identify possible indicators of pathogenicity, no reliable markers have been described until now.

The pathogenicity of *Acanthamoeba* species is represented by the sum of multiple factors that must be considered for the successful identification of different isolates (Marciano-Cabral and Cabral, 2003; Siddiqui and Khan, 2012). Taken together, our results support the idea that certain features may help differentiate *Acanthamoeba* species and isolates.

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