Serine-like proteolytic enzymes correlated with differential pathogenicity in patients with acute *Acanthamoeba* keratitis

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Abstract

Acute ocular infection due to free-living amoebae of the genus *Acanthamoeba* is characterized by severe pain, loss of corneal transparency and, eventually, blindness. Proteolytic enzymes secreted by trophozoites of virulent *Acanthamoeba* strains have an essential role in the mechanisms of pathogenesis, including adhesion, invasion and destruction of the corneal stroma. In this study, we analysed the relationship between the extracellular proteases secreted by clinical isolates of *Acanthamoeba* and the clinical manifestations and severity of disease that they caused. Clinical isolates were obtained from patients who showed typical symptoms of *Acanthamoeba* keratitis. Trophozoites were cultivated axenically, and extracellular proteins were collected from cell culture supernatants. Secreted enzymes were partially characterized by gelatin and collagen zymography. *Acanthamoeba* trophozoites secreted proteases with different molecular masses, proteolysis rates and substrate specificities, mostly serine-like proteases. Different enzymatic patterns of collagenases were observed, varying between single and multiple collagenolytic activities. Low molecular weight serine proteases were secreted by trophozoites associated with worse clinical manifestations. Consequently, proteolytic enzymes of some *Acanthamoeba* trophozoites could be related to the degree of their virulence and clinical manifestations of disease in the human cornea.

Keywords: *Acanthamoeba*, collagen, cornea, proteolytic enzymes, zymography
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Introduction

The frequency of corneal infections caused by free-living amoebae of the genus *Acanthamoeba* has increased among the Brazilian population [1]. Previous studies have shown a closest relationship between contact lens wearers and *Acanthamoeba* keratitis (AK) cases, and risk factors for disease transmission involved corneal trauma followed by improper or inadequate lens care [1–3]. In the earlier stages of this devastating infection, adherence of trophozoites to the epithelial layer is followed by invasion and destruction of the anterior cornea; the later stages are clinically characterized by ulceration of the corneal epithelium, oedema and necrosis of the stroma [4]. As the parasitic interaction between amoebae and epithelial cells involves the cytopathic effects of pathogenicity factors of *Acanthamoeba* trophozoites, proteolytic enzymes secreted in the corneal surface should play a key role in this process [5,6]. The action of extracellular proteases includes damage to the collagen shield and degradation of glycoproteins, such as fibronectin and laminin, and other proteins, such as plasminogen, fibrinogen, fibrin and haemoglobin [7].

We describe the partial biochemical characterization of extracellular proteolytic enzymes secreted by *Acanthamoeba* trophozoites isolated from infectious sites, and correlate the occurrence of proteases with clinical manifestations. Furthermore, we report the presence of different proteinase activities related to pathogenicity mechanisms of protozoa in the corneal stroma.

Materials and Methods

Patients

The research was approved by the local ethics committee and was conducted in accordance with the tenets of the

Declaration of Helsinki. Informed consent was obtained before sample collection. *Acanthamoeba* strains were isolated from ten different patients diagnosed with infectious keratitis. All patients were contact lens wearers, and their ages varied between 20 and 52 years. Clinical symptoms were contact lens wear intolerance, pain, photophobia and tear production. The severity of the degree of corneal infection by different clinical isolates was categorized into two classes: severe and moderate (previously classified as potentially and rarely sight-threatening infections, respectively) [8].

Culture

Laboratory diagnosis was based on culture of corneal samples on agar examined with an inverted phase contrast microscope at ×200 magnification. Identification of free-living amoebae of the genus *Acanthamoeba* was based on trophozoite and cyst morphology according to the Page [9] criteria. The reference strain *Acanthamoeba castellanii* from the American Type Culture Collection (ATCC 30011) was used as a non-pathogenic control [10]. In order to achieve axenic cultivation of clinical isolates, a piece of agar culture containing amoebae, cysts and trophozoites from each corneal sample was picked up aseptically and transferred to a tissue culture flask containing 5 mL of Neff's [11] broth medium.

Supernatant analysis

Culture supernatants were taken from axenic trophozoites after 72 h of incubation at 25°C. In order to remove cell debris, supernatants were filtered through a sterile 0.22-μm cellulose membrane filter. Samples were extensively dialysed against physiological saline solution (sodium chloride 0.85%) for 72 h at 4°C. Protein contents were concentrated tenfold with Amicon Ultra-15 centrifugal filter devices with a molecular mass cut-off of 5 kDa (Millipore, Bedford, MA, USA). Protein quantification was performed by the method of Bradford [12], and the concentration was adjusted to 200 mg/L. Fifty microlitres of each concentrated protein solution was subjected to precipitation with methanol/chloroform prior to 10% SDS-PAGE under non-reducing conditions [13]. Proteins were stained with silver nitrate as described previously [14]. The apparent molecular masses of the proteins were estimated by comparison with protein molecular mass markers (Low and High Molecular Weight Markers; GE Healthcare Life Science, Piscataway, NJ, USA).

Gelatinolytic activity assays

Zymography assays were performed with 10 μL of each crude protein extract (200 mg/L). Samples were analysed in 7.5% acrylamide gels containing 0.1% gelatin as substrate (SDS-PAGE-gelatin) [15]. In order to ensure the correct size of proteins, prestained molecular mass marker was used as standard (Spectra Multicolor Broad Range Protein Ladder; Fermentas Inc., Hanover, MD, USA). After electrophoresis, the enzymes were renatured by rinsing the gels in 2.5% (v/v) Triton X-100 solution for 1 h in order to remove SDS, and this was followed by incubation at 37°C overnight in Tris-buffered saline (20 mM Tris-HCl, pH 7.5; 150 mM NaCl). Gels were stained with 0.25% Coomassie Brilliant Blue solution in 50% methanol and 10% acetic acid. In this assay, the gelatin proteolysis was detected as colourless bands on the otherwise blue gel.

Protease inhibition assays

In order to study the class of proteolytic enzymes, protein extracts from culture supernatants were mixed with protease inhibitors and incubated for 30 min at 37°C before zymography electrophoresis. The inhibitors used were phenylmethylsulphonyl fluoride (PMSF) (final concentration of 1 mM), an irreversible serine protease inhibitor, and EDTA (final concentration of 10 mM), a chelating metalloprotease inhibitor. Both inhibitors were purchased from Sigma Chemical Co. (St Louis, MO, USA) and were prepared according to the manufacturer's instructions. Zymography analysis in SDS-PAGE-gelatin was performed as described above, and the gels were visually compared with zymogram gels where the samples were not treated with protease inhibitors.

Collagen degradation assays

Type I purified collagen (bovine achilles tendon; Sigma Chemical Co.), at a concentration of 0.1%, was copolymerized with 7.5% SDS-PAGE gels. Zymography assays were performed as described above. Collagenase activity was observed as clear bands on a blue background.

Results

Acanthamoeba cysts and trophozoites were isolated from all corneal tissue samples analysed (Fig. 1). All AK patients were treated with 0.02% topical polyhexamethylene biguanide, a cationic antiseptic agent, combined with 0.1% propamidine, instilled hourly. Different protein patterns were observed with different clinical isolates (Fig. 2). The results shown in Fig. 2 indicate a single prominent protein band with a molecular mass of approximately 30 kDa, secreted by trophozoites of the ATCC 30011 strain (lane 2) as well as by those from patients 01 and 03 (lanes 3 and 5, respectively). Trophozoites from patients 04, 05 and 06 (lanes 6, 7 and 8, respectively) showed similar protein patterns, with molecular mass bands

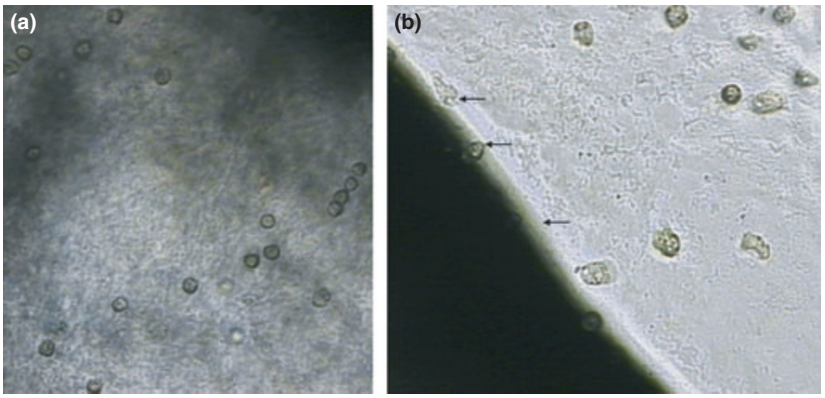

FIG. 1. Photomicrographs of the corneal tissue showing *Acanthamoeba* cysts (a) and trophozoites (b) isolated in this study. Arrows show the migration of trophozoites from corneal tissue to the agar surface seeded with inactivated *Escherichia coli*. Magnification: ×40.

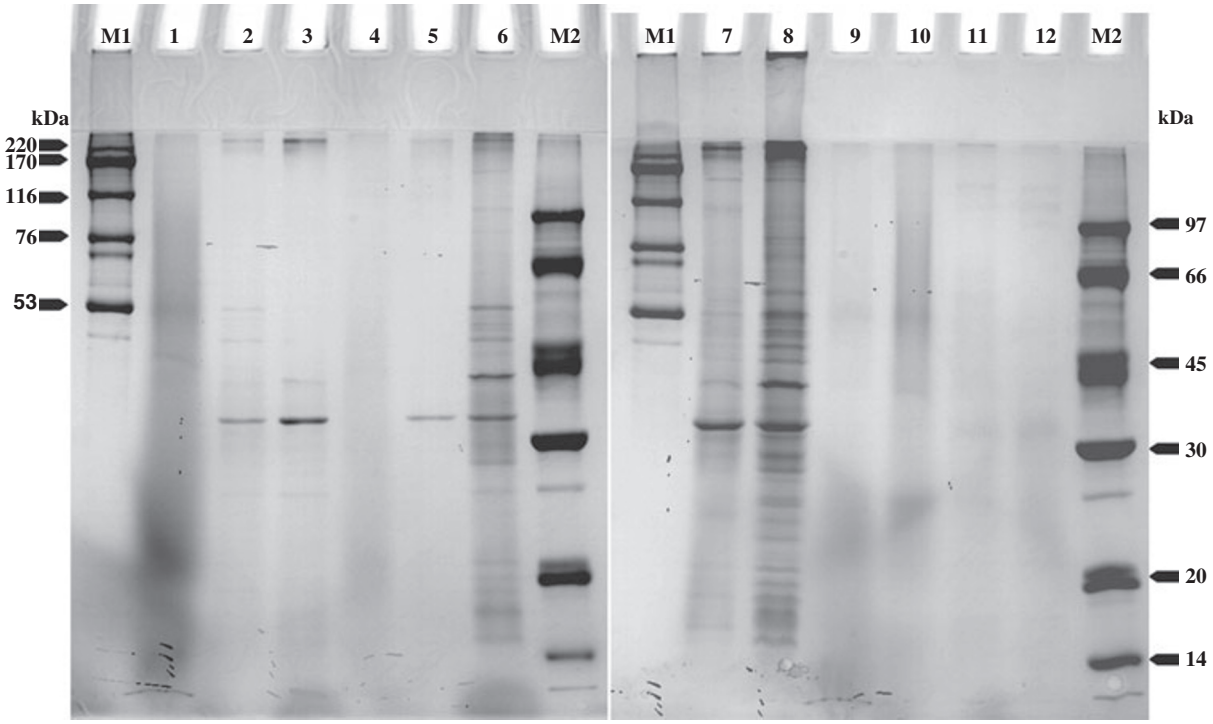

FIG. 2. SDS-PAGE analysis of crude protein extracts secreted by *Acanthamoeba* trophozoites isolated from different patients. Lane M1: high molecular mass marker (53–220 kDa). Lane M2: low molecular mass marker (14–97 kDa). Lane 1: negative control (sterile Neff's culture medium). Lane 2: *Acanthamoeba castellanii* ATCC 30011. Lanes 3–12 : *Acanthamoeba* keratitis patients (lane 3, patient 01; lane 4, patient 02; lane 5, patient 03; lane 6, patient 04; lane 7, patient 05; lane 8, patient 06; lane 9, patient 07; lane 10, patient 08; lane 11, patient 09; lane 12, patient 10).

ranging from approximately 14 to 170 kDa (Fig. 2). In addition, three main bands were observed in the crude protein extracts with molecular masses of approximately 53, 45 and 30 kDa (Fig. 2). Extracellular proteins secreted by trophozoites from patients 02, 07, 08, 09 and 10 showed as a smear pattern in polyacrylamide gels, and specific eletrophoretic profiles could not be determined. Interestingly, a single band of high molecular mass (>220 kDa) was observed in all samples analysed, including negative controls (sterile culture medium). This may have come from Neff's broth medium, which contains a high amount of protein-rich compounds such as proteose peptone and yeast extract compounds.

Partial characterization of extracellular proteases

The proteolytic activity of each protein sample was evaluated with SDS-PAGE-gelatin. The *A. castellanii* type strain was able to secrete high molecular mass proteases. Although isolates from two patients displayed minor proteolytic activity, crude protein extracts secreted by trophozoites from the majority of patients had high hydrolytic activity against gelatin as substrate. As shown in Fig. 3a, the molecular masses of the extracellular proteases varied between 47 and 159 kDa. Trophozoites from patients 01, 02, 09 and 10 (lanes 3, 4, 11 and 12, respectively) secreted a broad range of proteases. As shown in Fig. 3a,b, all enzymes secreted by isolates from patients 01–06 (lanes 3–8, respectively) were characterized as serine proteases. The proteolytic activity of a band of approximately 72 kDa from trophozoites from patient 02 was only partially inhibited by the serine protease inhibitor PMSF (Fig. 3b). Interestingly, the proteolytic activity of enzymes secreted by trophozoites from patient 06 was inhibited both by PMSF and EDTA (Fig. 3c, lane 8). A similar proteolytic profile was observed for isolates from patients 09 and 10 (Fig. 3a, lanes 11 and 12, respectively). However, isolates from patient 09 showed proteolytic patterns with molecular masses of 157, 73 and 55 kDa whose activities were not inhibited by PMSF or EDTA and were neither serines nor metalloproteases (Fig. 3b,c). Similarly, the 157-kDa protein from trophozoites isolated from patient 10 was neither a serine nor a metalloprotease, whereas other enzymes secreted by those protozoa were characterized as serine proteases (Fig. 3a,b). Finally, the patterns of proteolytic activity of enzymes secreted by the *Acanthamoeba* strains investigated in this study were correlated with the degree of severity of corneal infection, as shown in Table 1. Surprisingly, the intensity of proteolytic activity was not correlated with the virulence of the clinical isolates. However, despite this lack of correlation, proteolytic enzymes of low molecular mass were observed in all samples from patients with severe infections.

Cleavage of native type I collagen

Protein extracts of *Acanthamoeba* trophozoites were assayed for their specific activity by digestion of type I collagen.

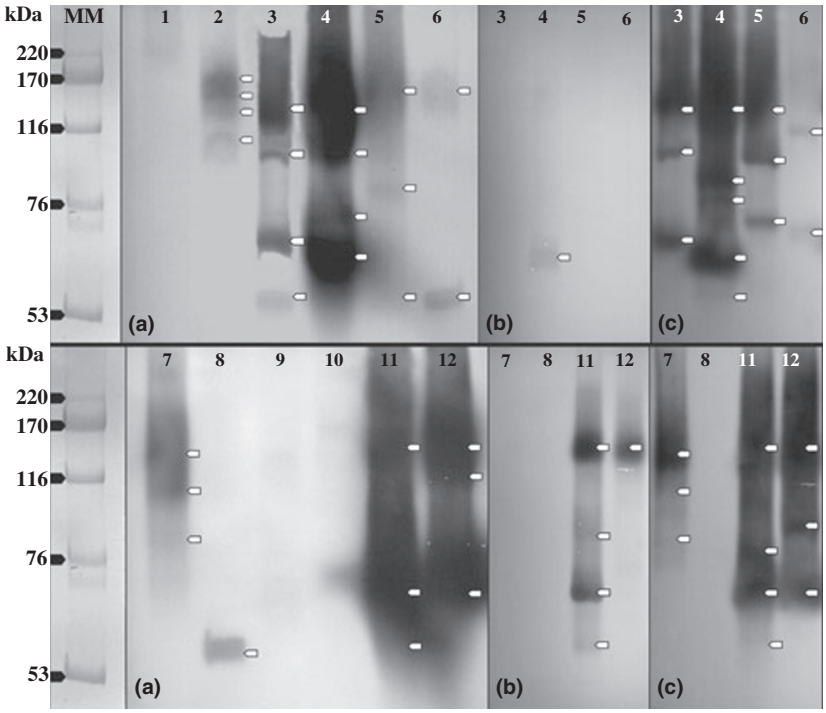

FIG. 3. Negative image of gelatin substrate gel electrophoresis of secretory products from *Acanthamoeba* trophozoites. Electrophoresis was conducted at 4°C and a constant 34 mA, for approximately 2 h, in 0.05 M glycine, 0.1% SDS, 0.025 M Tris-HCl (pH 8.3). Black bands represent proteases that have degradated the substrate in the gel: (a) gelatinolytic activity assay; (b) serine (phenylmethylsulphonyl fluoride) inhibitor assay; and (c) metalloprotease (EDTA) inhibitor assay. Lane MM: molecular mass marker. Lane 1: negative control (sterile Neff's culture medium). Lane 2: *Acanthamoeba castellanii* ATCC 30011. Lanes 3–12: *Acanthamoeba* keratitis patients (lane 3, patient 01; lane 4, patient 02; lane 5, patient 03; lane 6, patient 04; lane 7, patient 05; lane 8, patient 06; lane 9, patient 07; lane 10, patient 08; lane 11, patient 09; lane 12, patient 10).

TABLE 1. Characterization of protease secreted by clinical isolates of *Acanthamoeba* spp. and correlation with clinical manifestations observed among infected patients

Acanthamoeba isolate	Proteolytic activity characteristics	Severity of infection[a]
Patient 01	High diversity	Severe
Patient 02	Strong activity	Moderate
Patient 03	Weak activity	Moderate
Patient 04	Low molecular mass band	Severe
Patient 05	Weak activity	Moderate
Patient 06	High hydrolytic enzyme activity	Severe
Patient 09	Strong activity	Moderate
Patient 10	Strong activity	Moderate

[a]Severe: potentially sight-threatening infection. Moderate: rarely sight-threatening infection.

Collagenases were observed in amoebic extracts from six patients. As shown in Fig. 4, the same pattern of collagenolytic activity was observed with isolates from patients 01, 04 and 06 (lanes 3, 6 and 8, respectively). These isolates produced a single band with an estimated molecular mass of 36 kDa. All of these patients had severe infections. In contrast, multiple bands were observed in three other patients, and the molecular masses of those collagenases varied between 140 and 36 kDa (Fig. 4). For example, in the crude extract from *Acanthamoeba* isolated from patient 02 (Fig. 4, lane 4), three bands of 114, 95 and 50 kDa had strong proteolytic activity, whereas three other collagenases of 88, 75 and 59 kDa showed weaker activity. Similarly, collagenases secreted by trophozoites from patients 09 and 10 (Fig. 4, lanes 11 and 12, respectively) had high and low molecular mass bands.

Discussion

When AK has become associated with corneal trauma, and there is inappropriate care of contact lens equipment, binding of amoebae to the eye surface may allow invasion and destruction of the corneal stroma. For these reasons, ocular infection with *Acanthamoeba* protozoa has been characterized as a painful and vision-threatening disease [16]. Eventually, the pathophysiology of AK can result in corneal transplantation, blindness and eye enucleation [4,17]. The role of the corneal epithelium as an essential physical barrier in the prevention of amoebic infection has been described previously [18]. Most of the cytopathic factors involved in these infectious processes have been described as resulting from the activity of proteolytic enzymes secreted by trophozoites in the surface of host corneal tissue [4,5,7,19,20].

In this study, the pathogenic mechanisms of each *Acanthamoeba* isolate *in vitro* were determined by a primary culture procedure. Although the reference *A. castellanii* ATCC 30011 strain has been described as a non-pathogenic amoeba [21], we found that it secreted high molecular mass proteases. This corroborates a previous report by Khan *et al.* [20], which showed the capacity of both pathogenic and non-pathogenic *Acanthamoeba* strains to secrete proteases.

This study tested the hypothesis that different clinical manifestations of amoebic infection result from the capacity of *Acanthamoeba* trophozoites to secrete different extracellular proteases. We found that the trophozoites produced proteases with the potential to degrade gelatin

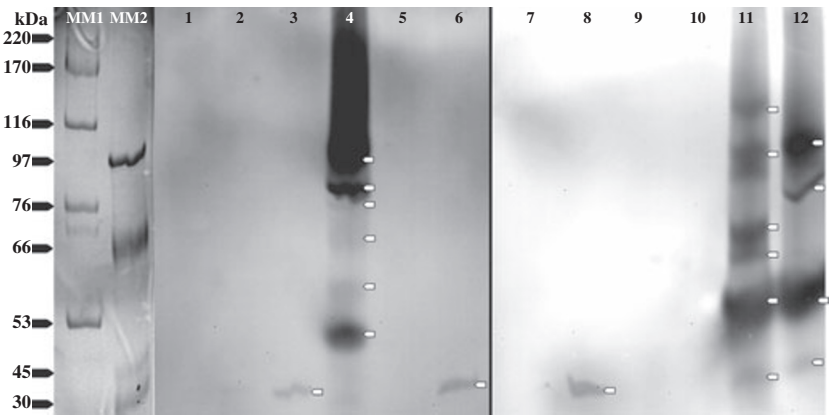

FIG. 4. Degradation of collagen type I substrate by crude extract enzymes obtained from different *Acanthamoeba* trophozoites. Overexpression of collagenolytic activity could be observed in a range of bands of approximately 50–114-kDa of patients 02, 09 and 10 (lanes 4, 11 and 12, respectively), whereas a single protein band with a molecular mass of 36 kDa was observed in three strains of patients 01, 04 and 06 (lanes 3, 6 and 8, respectively). Lanes MM1: high molecular mass marker. Lane MM2: low molecular mass marker. Lane 1: control (sterile culture medium). Lane 2: *Acanthamoeba castellanii* ATCC 30011. Lanes 3–12: *Acanthamoeba* keratitis patients (lane 3, patient 01; lane 4, patient 02; lane 5, patient 03; lane 6, patient 04; lane 7, patient 05; lane 8, patient 06; lane 9, patient 07; lane 10, patient 08; lane 11, patient 09; lane 12, patient 10).

and collagen substrates at low concentrations. The relatively low content of proteases among the extracellular proteins secreted by trophozoites can be observed when comparing the protein profile of samples on SDS-PAGE and proteolytic profile on zymography gels. Despite the fact that a distinguishable protein profile of samples from patients 02, 09 and 10 could not be observed on SDS-PAGE, those samples showed distinct proteolytic patterns and high proteolysis rates.

Enzymes produced by *Acanthamoeba* strains belong to a variety of protease classes, predominantly serine proteases and metalloproteases [22,7]. We found that a specific group of *Acanthamoeba* trophozoites were able to secrete enzymes that were blocked by both serine and metalloprotease inhibitors, suggesting the occurrence of an additional class of enzymes. Previous studies have shown that the proteolytic activities of enzymes secreted by different *Acanthamoeba* species with similar molecular masses to those observed in this study (157, 73 and 55 kDa) can be blocked by inhibitors of cysteine proteases [6,23]. A low molecular mass enzyme of the *Acanthamoeba* isolate from patient 06 was inhibited by PMSF and EDTA, suggesting the occurrence of a specific class of calcium-dependent serine proteases. Dudley *et al.* [24] have shown that the expression and activation mechanisms of proteases secreted by *Acanthamoeba* trophozoites can be calcium-dependent.

Type I collagen is the main constituent of the corneal stromal matrix. The occurrence of multiple collagenases among *Acanthamoeba* trophozoites isolated from six patients suggests that differential virulence factors and low molecular mass collagenases were correlated with the severity of clinical manifestation in patients. In this study, collagenolytic enzymes with type I collagen degradation activity seemed to have mostly serine protease activity.

Ophthalmologists have observed the occurrence of differential patterns of clinical manifestations in cases of *Acanthamoeba* infection. Our study demonstrates that strains with varying virulence isolated from patients with acute AK secrete diverse proteolytic enzymes, with a predominance of serine-like proteases. Once adhesion has been established, gelatinases and collagenases could be involved directly in the activation of pathogenic cascades in the corneal epithelium, and consequent degradation of the collagenous corneal stroma. Thus, the severity of AK could be related to the specific infecting *Acanthamoeba* strain and its ability to produce different extracellular enzymes. Further studies are underway to identify the low molecular mass serine proteases that could related to severe amoebic keratitis.

Transparency Declaration

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