Evaluation of in vitro activities of extracellular enzymes from Aspergillus species isolated from corneal ulcer/keratitis

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1. Introduction

Mycotic/fungal keratitis is a suppurative, generally ulcerative infection of the cornea. The filamentous fungi, Aspergillus spp. are the second leading cause of mycotic keratitis, particularly in India. Aspergillus spp. produce a range of extracellular enzymes that are used to break down complex molecules and used for growth and reproduction, also for survival on/in host organism. The current study was designed with an objective to screen in vitro extracellular enzyme activity of Fusarium and Aspergillus isolates from mycotic keratitis patients and to correlate the same as a putative virulence factor. Extracellular enzymes viz., deoxyribonuclease (DNase), protease, lipase, elastase, keratinase, etc., produced by Aspergillus have key role in keratomycosis and hence their (n = 85) in vitro activities were investigated. It was found that, the majority of the Aspergillus isolates produced protease (n = 75; 88% of 85) followed by lipase (n = 59; 69% of 85), DNase (n = 35; 41% of 85), elastase (n = 26; 31% of 85) and keratinase (n = 13; 15% of 85). The enzyme activity indices (EAI) for DNase, elastase, protease and lipase ranged between 1.01 and 1.98, whereas elastase EAI varied between 1.26 and 1.92. DNase, protease and lipase showed a maximum EAI of 1.98 and lowest EAI value of 1.01, respectively. Extracellular enzymes of Aspergillus spp. may have potential role in the onset and progression of keratitis.

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lular enzymes that are used to break down complex polysaccharides into simple sugars to be assimilated and used for growth and reproduction, also for survival on host organism.

Research on extracellular enzymes production as a virulence factors for Aspergillus isolated from ocular infection remains unexplored (Bouchara et al., 1995; Latgé, 1999; Tonne and Kauffman, 2000). Fungi secrete several extracellular hydrolytic enzymes such as keratinases, collagenases, gelatinases, phospholipases, lipases, and acid proteinases in culture media (Khan et al., 2010). Extracellular proteinases help in the adherence and survival of the pathogen on mucosal surfaces (Borg and Rüchel, 1988), invasion of host tissues (Odd’s, 1985; Rüchel, 1986) and digestion of immunoglobulins (Rüchel, 1986; Yuan and Cole, 1987) and corneal matrix degradation (Gopinathan et al., 2001). Park et al. (2013) (Park et al., 2013) reported that lipolytic enzymes also have been implicated in fungal virulence and has been extensively studied in Candida species. Khan et al. (2010); (Alp and Arikan, 2008; Khan et al., 2010) stated that lipase of Aspergillus species has a role in tissue damage. Elastase cleaves the peptide bonds in elastin, aiding the digestibility of this elastic protein. The keratomycosis aided by the extracellular enzymes of Aspergillus thus will add to the severity of the infection. Against this background, the present in vitro analysis was undertaken with the objective of examining the role of the extracellular enzyme activities as putative virulence factors in Aspergillus keratitis.

2. Materials and methods

2.1. Isolation and identification of Aspergillus spp.

Corneal scrapings were collected by an ophthalmologist from the patients with suspected keratomycosis at Aravind Eye Hospital and Postgraduate Institute of Ophthalmology (Coimbatore, Tamilnadu, India) during 2013-2015. The collected material was inoculated directly onto 5% sheep blood agar, Chocolate agar, brain heart infusion broth and potato dextrose agar (PDA) (HiMedia, Mumbai, India) and also spread on a glass slide for direct microscopy after 10% KOH wet mount. The Culture plates were incubated at 37 °C (for bacteria) and 27 °C (for fungi), examined daily, and discarded after 1 week if no growth were present. The fungi that were initially identified based on colony morphology on SDA were further characterized microscopically after lactophenol cotton blue staining (Harris, 2000). Suspected A. fluvis isolates were further screened on Aspergillus differentiation agar (ADA) to differentiate other similar morphological species of Aspergillus genera (Rodrigues et al., 2007). All the isolates were stored in screw capped tubes containing 0.85% saline at 4 °C.

2.2. Fungal inoculum preparation

The test isolates Aspergillus were grown on potato dextrose agar slants and incubated at 28 °C for seven days. Sterile saline (0.9% NaCl, 2 mL) was added to the culture slant, and the conidia were harvested after gentle vortexing and the mycelial remnants from the conidial suspension were separated by filtration through sterile cotton-wool. The conidial suspension was used as inoculum.

2.3. Extracellular enzyme assays

DNase test agar, rose Bengal elastin agar, tributyrin agar and skim milk agar were aseptically prepared and autoclaved for assays of DNase, elastase, lipase and protease, respectively. All the chemicals were purchased from HiMedia, Mumbai. For keratinase assay, basal medium (BM) overlaid with keratin azure (Sigma-Aldrich, USA) was used (Scott and Unterreiner, 2004). The assay media were inoculated with 30 μl of spore suspension and incubated at 28 °C in darkness. After incubation, DNase assay plates were flooded with 1 N hydrochloric acid for clarity in the zone of hydrolysis (Sánchez and Colom, 2010). Elastase (Kothyary et al., 1984), lipase (Griebeler et al., 2011) and protease assay plates (J Sharma et al., 2005) were observed for the zone of clearance after fungal mats were removed with the help of a sterile cotton swab (Mythili et al., 2014). Keratinase activity was evaluated visually from the release of azure dye into the colourless lower layer of BM after third day and within three weeks of inoculation (Scott and Unterreiner, 2004).

For DNase, elastase, lipase and protease enzymes, enzyme activity index (EAI) was calculated by the following formula (Blanco et al., 2002):

\[ \text{Enzyme activity index (EAI)} = \frac{\text{Zone diameter} (ZD)}{\text{Growth diameter} (GD)} \]

The mean EAI was calculated from three observations and the calculated values were grouped under four classes as high EAI (2.0 to 1.75), medium EAI (1.75 to 1.25), low EAI (1.25 to 1.10) and negligible or no EAI (1.10 to 1.00).

3. Results

A total of 1628 ocular specimens were collected from suspected cases of keratitis, from which 85 isolates of Aspergillus species were obtained. These isolates were further analysed for microscopic and macroscopic morphology so as to identify at the species level. Microscopic characteristics such as stipes colour, surface appearance, vesicle serration, shape of vesicle and conidia surface were observed after staining with LCB and compared with the standard morphological characteristics (Diba et al., 2007) and they were identified as A. flavus (n = 53), A. fumigatus (n = 14), A. terreus (n = 9), A. tamarii (n = 6) and A. niger (n = 3).

The extracellular enzyme assay revealed that all the A. flavus isolates had one or the other enzyme activities and each isolate were found to have different enzyme activity indices (EAI) ranging from 1 to 1.97 (Table 1). Among the 47 isolates tested for DNase activity, medium EAI was observed for 16 isolates, low EAI value for 30 isolates, negligible or no EAI value for one isolate and none of the isolate showed high EAI value (Fig. 1). The highest (1.59) and lowest (1.08) was exhibited by the isolate AF29 and AF18 respectively. For elastase enzyme 26, 9, 4 and 8 isolates exhibited high, medium, low and negligible or no EAI, respectively. The elastase EAI ranged from 1.0 to 1.97, the highest value of 1.97 (AF05) and the minimum value of 1.0 (AF10, AF11, AF12, AF13, AF17 and AF18). While analyzing lipase activity indices, it was found that 12, 29 and 6 isolates had medium, low and negligible or no EAI respectively. Of 47 isolates tested for protease activity a total of 12, 30 and 5 isolates exhibited medium, low and negligible or no EAI respectively. The highest value for protease activity was found to be 1.55 and the least value of 1.00. A total of five isolates (AF04, AF08, AF14, AF21 and AF27) were positive for keratinase production.

Majority of the Aspergillus isolates produced protease (n = 75; 88% of 85) followed by lipase (n = 59; 69% of 85), DNase (n = 35; 41% of 85), elastase (n = 26; 31% of 85) and keratinase (n = 13; 15% of 85) (Figs. 1 and 2). The EAI for the enzymes DNase, elastase, protease and lipase ranged between 1.01 and 1.98. DNase, protease and lipase showed a maximum EAI of 1.98 and lowest EAI value of 1.01, whereas elastase EAI was found to be varying between 1.26 and 1.97. The keratinase activity index (EAI) was calculated by the following formula (Blanco et al., 2002):

\[ \text{Enzyme activity index (EAI)} = \frac{\text{Zone diameter} (ZD)}{\text{Growth diameter} (GD)} \]

The mean EAI was calculated from three observations and the calculated values were grouped under four classes as high EAI (2.0 to 1.75), medium EAI (1.75 to 1.25), low EAI (1.25 to 1.10) and negligible or no EAI (1.10 to 1.00).
(n = 3) exhibited protease activity. A total of 7, 5 and one isolates of A. flavus (13.21% of 53), A. fumigatus (35.71% of 14) and A. terreus (11.11% of 9), respectively showed keratinase activity. The extra-cellular enzyme activity indices of Aspergillus isolates were calculated and tabulated as frequency table (Table 1). On DNase test agar, isolates of Aspergillus exhibited varying magnitude of enzyme activity. One of the isolates of A. flavus showed very low/negligible EAI (1.0 to 1.10). Relatively low EAI (1.11 to 1.30) was exhibited by 17 isolates and medium EAI (1.31 to 1.60) was shown by 2 isolates and none of the isolates of A. flavus showed high EAI (1.61 to 2.0). High DNase EAI (1.61 to 2.0) was exhibited by one isolate of each A. fumigatus and A. tamarii. A. terreus had low to medium EAI (1.11 to 1.40) and A. niger exhibited low EAI (1.21 to 1.30).

4. Discussion

Certain extracellular enzymes such as DNase, elastase, protease, lipase, keratinase, etc., are known to play an important role in the process of infection by pathogenic including fungi (U Gajjar, 2019).
In the present study five clinically important enzymes viz., DNase, elastase, protease, lipase and keratinase were screened for their activity in order to evaluate the overall spectrum of extracellular enzyme activities of Aspergillus (n = 85) strains isolated from fungal keratitis.

Most of the isolates (n = 25; 96.15% of 26) of Aspergillus showed medium to high elastase activity. Fifteen isolates of A. flavus showed high elastase activity indices (1.61 to 2.0) and one isolate each of A. tamarii and A. niger showed medium elastase activity indices (1.31 to 1.60). For protease, exactly, 34 (64.15% of 53) isolates of A. flavus showed relatively low EALs (1.11 to 1.30) and high EALs were exhibited by A. fumigatus (n = 2), A. tamarii (n = 2) and A. terreus (n = 1). A total of 21 and 11 isolates of A. flavus showed relatively low and medium lipase activity, respectively. Two isolates of A. tamarii and one isolate each of A. fumigatus and A. terreus had high lipase activity.

In this study, the possible role of the extracellular enzymes as a putative virulence factor in Aspergillus keratitis was explored. It was observed that the isolates of Aspergillus were able to produce an array of extracellular enzymes. Aspergillus isolates had lesser activities of DNase (n = 35) and keratinase(n = 15) when compared to protease, elastase and lipase. The DNase enzyme activity was analysed by observing the halo zone around the colony, similar to that of Sanchez and Colom (Sanchez and Colom, 2010) where 85 Cryptococcus isolates were reported to be positive for DNase activity. In the present study, 69% (n = 59) and 88% (n = 75) of Aspergillus isolates produced lipase and protease, respectively. Higher lipase activity in Fusarium isolates was observed by St. Leger et al. (St. Leger et al., 1986). Similar to the present study, Gajjar et al. (U Gajjar, 2019) and Selvam et al. (Selvam et al., 2014) reported 83% of protease activity in Aspergillus isolates. Alp and Arikian (Alp and Arikian, 2008) investigated the production of extracellular elastase, acid proteinase, and phospholipase from the clinical isolates of Aspergillus and found that 84.9, 27.4, and 65.8% of Aspergillus isolates were able to produce elastase, acid proteinase and phospholipase, respectively and also reported that none of the A. niger isolate produced elastase. Whereas, in the present study, 31% of Aspergillus isolates produced elastase and one isolate among 3 isolates of A. niger also produced elastase activity. The production of elastin lytic enzymes by Aspergillus suggests that elastase play a role in pathogenesis (Blanco et al., 2002; Kolattukudy et al., 1993; Kothary et al., 1984; Rhodes et al., 1988). Kothary et al. (Kothary et al., 1984) stated that non-elastase producing environmental Aspergillus isolates were relatively less virulent compared with high elastase producers. Bazan (Bazan, 2005) stated that lipid and lipid mediated compounds play an important role in the recovery of corneal inflammations in a complex manner. These probably answer the critical role of extracellular lipid produced by the test isolates of Aspergillus from keratomycosis patients.

Although the present study detected keratinase activity only among 15% Aspergillus, Friedrich et al. (Friedrich et al., 1999) and Sharma et al. (Sharma et al., 2011) reported keratinase activity in many of these species. Oyeleke et al. (Oyeleke and Auta, 2010) stated that Aspergillus yielded high amount of protease enzymes. In the present study, protease activity was profoundly seen among Aspergillus isolates (88% of 85). Protease is proven to be associated with cornea that showed progressive ulcer along with the presence of dense inflammatory cells (Gopinathan et al., 2001). Burda and Fisher (Burda and Fisher, 1960) and Dudley and Chick (DUDLEY and CHICK, 1964) have demonstrated extracellular protease activity in corneal matrix degradation in mycotic keratitis using rabbit model. The pathogenic role of extracellular protease in keratitis has been reported by various authors (Barletta et al., 1996; Mahmoud et al., 2007; Zhu et al., n.d.).

5. Conclusion

The present study clearly indicates the potential role of extracellular enzymes of Aspergillus spp. in the onset and progression of keratitis. However, the number of isolates Aspergillus keratitis cases is low and the problem remains to be further clarified by concordant examination of other potential virulence factors. Further studies on the multifactorial impact on virulence of the isolates causing fungal keratitis are required.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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References

Alp, S., Arikian, S., 2008. Investigation of extracellular elastase, acid proteinase and phospholipase activities as putative virulence factors in clinical isolates of Aspergillus species. J. Basic Microbiol. https://doi.org/10.1002/jobm.200700349.
Barletta, J.P., Angelia, G., Balch, K.C., Dimova, H.G., Stern, G.A., Moser, M.T., Van Setten, G.B., Schulz, G.S., 1996. Inhibition of pseudomonal ulceration in rabbit corneas by a synthetic matrix metalloproteinase inhibitor. Invest. Ophthalmol. Vis. Sci.
Bazan, H.E.P., 2005. Cellular and molecular events in corneal wound healing: Significance of lipid signalling. Exp. Eye Res. https://doi.org/10.1016/j.exer.2004.12.023.
Blanco, J.L., Montecillas, R., Bouza, E., Blanco, I., Pelaez, T., Muñoz, P., Perez Molina, J., Garcia, M.E., 2002. Correlation between the elastase activity index and invasiveness of clinical isolates of Aspergillus fumigatus. J. Clin. Microbiol. https://doi.org/10.1128/JCM.40.5.1811-1813.2002.
Borg, M., Rüchel, R., 1988. Expression of extracellular acid proteinase by proteolytic Candida spp. during experimental infection of oral mucosa. Infect. Immun. 56, 626–631.
Bouchara, J.-P., Tröchon, G., Larcher, G., Chabasse, D., 1995. The search for virulence determinants in Aspergillus fumigatus. Trends Microbiol. 3, 327–330. https://doi.org/10.1016/0962-8924(95)80895-9.
Burd, C.D., Fisher, E., 1980. Corneal destruction by extracts of cephalosporium mycetien. Am. J. Ophthalmol. 50, 926–937. https://doi.org/10.1016/0002-9394(90)90345-7.
Diba, K., Kordbacheh, P., Mirhendi, S.H., Rezaie, S., Mahmoudi, M., 2007. Identification of Aspergillus species using morphological characteristics. Pak J. Med. Sci. 23, 867–872.
Dudley, M.A., Chick, E.W., 1964. Corneal lesions produced in rabbits by an extract of fusarium moniliforme. Arch. Ophthalmol. 72, 346–350. https://doi.org/10.1001/archopht.1964.00700203436012.
Friedrich, J., Gradišar, H., Mandin, D., Chaumont, J.P., 1999. Screening fungi for synthesis of keratinolitic enzymes. Lett. Appl. Microbiol. 28, 127–130. https://doi.org/10.1046/j.1365-2672.1999.00483.x.
Gopinathan, U., Ramakrishna, T., Wilcock, M., Rao, C.M., Balasubramanian, D., Kulkarni, A., Vemuganti, G.K., Rao, G.N., 2001. Enzymatic, clinical and histologic evaluation of corneal tissues in experimental fungal keratitis in rabbits. Exp. Eye Res. 72, 433–442. https://doi.org/10.1016/j.exer.2000.0971.
Griebeler, N., Polloni, A.E., Remonatto, D., Arbter, F., Vardanega, R., Cechet, J.L., Di Marco, M., Di Michel, D., Caniato, R.L., Rigo, E., Ninow, J.L., 2011. Isolation and screening of lipase-producing fungi with hydrolytic activity. Food Bioprocess Technol. 4, 578–586. https://doi.org/10.1007/s11947-008-0176-5.
Harris, J.L., 2000. Letter to the editor: Safe, low-distortion tape touch method for fungal slide mounts. J. Clin. Microbiol.
Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., Denning, D.W., 2007. Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology 153, 1677–1692. https://doi.org/10.1099/mic.0.07641-0.
Sharma, J., Singh, A., Kumar, R., Mittal, A., 2005. Partial purification of an alkaline protease from a new strain of aspergillus oryzae AWT 20 and its enhanced stabilization in entrapped Ca-alginate beads. Internet J. Microbiol. 2.

Khan, M.S.A., Ahmad, I., Apil, F., Owais, M., Shahid, M., Musarrat, J., 2010. Virulence and pathogenicity of fungal pathogens with special reference to candida albicans. In: Combating Fungal Infections. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 21–43. https://doi.org/10.1007/978-3-642-12173-9_2.

Kolattukudy, P.E., Lee, J.D., Rogers, L.M., Zimmerman, P., Ceselski, S., Fox, B., Stein, B., Copelan, E.A., 1993. Evidence for possible involvement of an elastolytic serine protease in aspergillosis. Infect. Immun. 61, 2357–2368.

Kothary, M.H., Chase, T., MacMillan, J.D., 1984. Correlation of elastase production by some strains of Aspergillus fumigatus with ability to cause pulmonary invasive aspergillosis in mice. Infect. Immun.

Latgé, J.P., 1999. Aspergillus fumigatus and aspergillosis. Clin. Microbiol. Rev. 12, 310–350.

Mahmoud, Y.A.-G., Abu El-Souod, S.M., El-Shourbagy, S.M., El-Badry, A.S.M., 2007. Characterisation and inhibition effect of cetrimide on collagenase produced by Aspergillus flavus, isolated from mycotic ulcers. Ann. Microbiol. 57, 109–113. https://doi.org/10.1007/BF03175058.

Manikandan, P., Varga, J., Kocsübé, S., Anita, R., Revathi, R., Németh, T.M., Narendran, V., Vágvölgyi, C., Panneer Selvam, K., Shobana, C.S., Babu Singh, Y.R., Kredics, L., 2013. Epidemiology of Aspergillus keratitis at a tertiary care eye hospital in South India and antifungal susceptibilities of the causative agents. Mycoses 56, 26–33. https://doi.org/10.1111/j.1439-0507.2012.02194.x.

Mravič, V., Dekaris, I., Gabrč, N., Romac, I., Glavota, V., Mlinaric-Missoni, E., 2012. An overview of fungal keratitis and case report on trichophyton keratitis. African J. Biochem. Res. 5, 1–6.

Mythili, A., Babu Singh, Y.R., Priya, R., Shafeeq Hassan, A., Manikandan, P., Panneerselvam, K., Narendran, V., Shobana, C.S., 2014. In vitro and comparative study on the extracellular enzyme activity of molds isolated from keratomycosis and soil. Int. J. Ophthalmol. 7, 778–784. https://doi.org/10.3980/j.issn.2222-3959.2014.05.07.

Odds, F.C., 1985. Candida albicans proteinase as a virulence factor in the pathogenesis of Candida infections. Zentralbl. Bakteriol. Mikrobiol. Hyg. A. 260, 539–542.

Oyeleke, S.B., Auta, H., 2010. Screening of Aspergillus flavus and Aspergillus fumigatus strains for extracellular protease enzyme production. J. Microbiol. Antimicrob. 2, 83–87.

Park, M., Do, E., Jung, W.H., 2013. Lipolytic enzymes involved in the virulence of human pathogenic fungi. Mycobiology 41, 67–72. https://doi.org/10.5941/MYC.2013.41.2.67.

Rhodes, J.C., Bode, R.B., McCuan-Kirsch, C.M., 1988. Elastase production in clinical isolates of Aspergillus. Diagn. Microbiol. Infect. Dis. 10, 165–170. https://doi.org/10.1016/0732-8893(88)90056-5.

Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R.R.M., Lima, N., Venâncio, A., 2007. Identification and characterization of Aspergillus flavus and aflatoxins. Commun. Curr. Res. Educ. Top. Trends Appl. Microbiol., 527–534

Rüchel, R., 1986. Cleavage of immunoglobulins by pathogenic yeasts of the genus Candida. Microbiol. Sci. 3, 316–319.

Sánchez, M., Colom, F., 2010. Extracellular DNase activity of Cryptococcus neoformans and Cryptococcus gattii. Rev. Iberoam. Micol. 27, 10–13. https://doi.org/10.1016/j.jsm.2009.11.004.

Scott, J.A., Unterreiner, W.A., 2004. Determination of keratin degradation by fungi using keratin azure. Med. Mycol. 42, 239–246. https://doi.org/10.1080/136937803100164680.

Selvam, K.P., Singh, Y.R.B., Shobana, C.S., Karunakaran, N.K., Vágvölgyi, C., Kredics, L., Al-Baradie, R.S., Manikandan, P., 2014. Extracellular enzymes and mycotoxins as virulence factors in Fusarium and Aspergillus keratitis. BioSci. Biotechnol. Res. Asia 11. https://doi.org/10.13005/bbra/1298.

Sharma, Mukesh, Sharma, Meenakshi, Rao, Vijay Mohan, 2011. In vitro biodegradation of keratin by dermatophytes and some soil keratinophiles. African J. Biochem. Res. 5, 1–6.

Srinivasan, M., 2004. Fungal keratitis. Curr. Opin. Ophthalmol. 15, 321–327.

St. Leger, R.J., Charnley, A.K., Cooper, R.M., 1986. Cuticle-degrading enzymes of entomopathogenic fungi: Synthesis in culture on cuticle. J. Invertebr. Pathol. 48, 85–95. https://doi.org/10.1016/0022-2011(86)90146-1.

Thomas, P.A., 2003. Current perspectives on ophthalmic mycoses. Clin. Microbiol. Rev. https://doi.org/10.1128/CMBR.16.4.730-797.2003.

Tomee, J.F., Kaufman, H.F., 2000. Putative virulence factors of Aspergillus fumigatus. Clin. Exp. Allergy 30, 476–484. https://doi.org/10.1046/j.1365-2222.2000.00796.x.

U Gajjar, D., 2019. Extracellular proteases from keratitis causing fusarium, aspergillus and demataceous Species. Trends Ophthalmol. Open Access J. 2. https://doi.org/10.32474/TOOAJ.2019.02.000132.

Yuan, L., Cole, G.T., 1987. Isolation and characterization of an extracellular protease of Coccidioides inimittis. Infect. Immun. 55, 1970–1978.

Zhao, W.-S., Wojdyla, K., Donlon, K., Thomas, P.A., Eberle, H.I., 1990. Extracellular proteinase of Coccidioides immitis. Infect. Immun. 55, 1970–1978.