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Profiling of cellular and secreted proteins of selected human pathogenic fungi for selection of species specific diagnostic biomarkers

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ABSTRACT

Isolates of *Aspergillus niger*, *A. flavus* and *Penicillium* sp. were recovered from blood and body fluid samples obtained from hospital. Extraction procedures were optimized for high degree of recovery of cellular and secreted proteins from the fungal species under study. Single dimension profiling of the proteins was performed by SDS-PAGE analysis to compare unique as well as differentially expressed proteins among the species. Among the differentially expressed cellular proteins, 28, 39 and 95 kDa specific to *A. niger*, 70 kDa protein of *A. flavus*, 27, 37 and 85 kDa proteins of *Penicillium* sp. were found to show prominent difference in expression. Although many proteins showed difference in the comparison of extracellular profile of the fungal species, the 25 kDa protein of *A. flavus* was unique to the species. Proteins of 37 and 46 kDa of *Penicillium* sp. expressed at a higher degree compared to other fungal species. Potential candidate biomarkers can be further characterized by analyzing the expression of the reported proteins in many isolates of these pathogens.

KEYWORDS

Human fungi;
Protein profiling;
Cellular proteins;
Secreted proteins.

INTRODUCTION

Fungal pathogens are major cause for increased morbidity and mortality in immuno-compromised patients and occupational allergy in healthy individuals. The clinical treatment suffers from lack of rapid diagnosis. The high mortality rate of invasive fungal infections is caused in part by difficulties in diagnosis at early stages of the disease and most infection is proved only at autopsy[1]. Currently available diagnosic methods including isolation and identification takes time and are more laborious process. Accuracy of serological detection techniques has cross reactivity of antibodies as the major limiting issue. Initial enthusiasm for the *Aspergillus* galactomannan test as a diagnostic tool must be tempered, as the sensitivity of the assay is lower than expected[2]. Histopathological diagnosis requires lot of tissue and can be difficult to obtain. The morphological similarities of many filamentous fungi in tissues make the specific identification difficult[3,4]. Presently, molecular assays are used as an adjunct for diagnosis along with clinical symptoms and other diagnostic markers, such

as antigen testing and radiographic findings[5]. The new challenge for nucleic acid based diagnostics is whether it can be used reliably to assess early stage primary infection. Discovery of proteome biomarkers is an emerging field of study in many biomedical areas. Application of clinical proteomics has the potential of revolutionizing disease diagnosis. With this background information, the present study has been designed to profile the cellular and extra-cellular proteins of the human pathogenic fungi with the objective to identify species specific candidate proteins for biomarker development.

EXPERIMENTAL

Blood and body fluid samples from the patients with suspected invasive fungal infections were obtained from Kovai Medical Centre and Hospital, Coimbatore, India. The samples were spot inoculated onto potato dextrose agar plates and the cultures of *Aspergillus flavus*, *A. niger* and *Penicillium* sp. were isolated and purified by single hyphal tip method. Lactophenol cotton blue stain was used to stain the cultures and the morphological characteristics *viz*., septation and branching of mycelia, conidiophores and conidia were observed. For extraction of mycelial and extra-cellular proteins, the fungal isolates were grown on mycological broth (HiMedia, India) for seven days at room temperature. The liquid cultures were filtered through Whatman No.1 filter paper under aseptic conditions. The mycelia collected on the filter paper were washed several times with sterile distilled water to remove excess media. The mycelia were carefully removed and frozen stored at -80°C until further use.

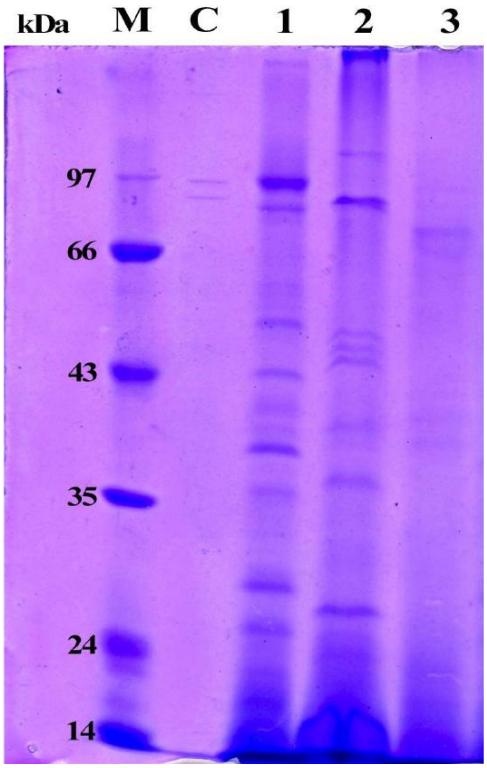
Cellular proteins were extracted by following three methods. Method 1: PIB (protease inhibitor buffer) containing 10mM tris pH 8.0, 1mM EDTA, 2% polyvinyl pyrilidone was used for extraction. The frozen mycelia were homogenized in the above buffer and centrifuged at 10,000 rpm for 20 min at 4°C. Equal volume of ice-cold acetone was added to the supernatant and centrifugation step repeated. The air dried pellet was suspended in SDS-PAGE sample buffer and heated for four min at boiling temperature before electroporesis. Method 2: Extraction buffer containing 1% SDS, 9M urea, 25mM tris pH 6.8, 1mM EDTA, 0.7M 2-mercaptoethanol was used to homogenize the frozen mycelia. The extracts were boiled for 2 min and vortexed for 1 min. The samples were then boiled again for 1 min and centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was transferred to fresh tubes and SDS-PAGE sample buffer was added and processed further as described above, for electrophoresis. Method 3: The mycelia were homogenized in the PIB buffer and proteins precipitated by adding 100% trichloro acetic acid at 1:4 ratio to the sample and centrifuging at 10,000 rpm for 10 min at 4°C. The pellet was washed with ice-cold acetone for three times and dissolved in SDS-PAGE sample buffer. To obtain secreted proteins, the culture filtrate was centrifuged at 10,000 rpm for 15 min at 4°C and the proteins was precipitated by adding 100% trichloro acetic acid at the 1:4 volume to the filtrate. After incubation at 4°C for 10 min, the filtrate was centrifuged at 10,000 rpm for 10 min at 4°C. Pellet obtained was washed three times with ice-cold acetone followed by air drying the pellet to remove traces of acetone. SDS-PAGE analysis was done in vertical slab gel as described[6]. The proteins were separated on 12% polyacrylamide gel by using equal quantities (50 µg) of protein. Electrophoresis was carried out at constant voltage of 100 V. The gels were stained with Coomassie brilliant blue stain.

RESULTS AND DISCUSSION

Among the different extraction methods of mycelial protein followed in the study, the method 3 of using PIB buffer followed by trichloro acetic acid precipitation yielded more proteins and revealing minor proteins in the gel profile. The other two methods yielded fewer amounts of proteins with less number of protein bands (data not shown). Many mycelial cellular proteins of different molecular weight were found to be expressed differently among the species of fungi tested (Figure 1).

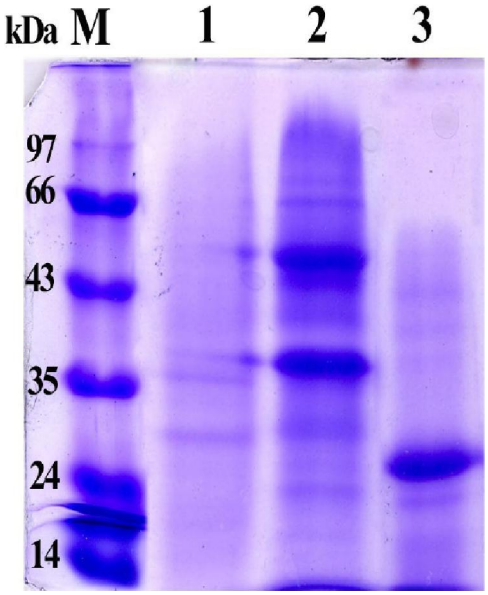
Among the proteins, *A. niger* specific proteins of 28, 39 and 95 kDa, *Penicillium* species specific proteins of 27, 37 and 85 kDa and *A. flavus* specific 70 kDa proteins were found to be prominently expressing the difference. The extra-cellular / secreted protein profile showed proteins of 30 and 36 kDa of *A. niger* as unique proteins. The unique proteins of *Penicillium* sp and *A. flavus* were 24, 31, 45, 66 kDa and 23, 25, 39, 40, 43 kDa respectively (Figure 2). The 25 kDa protein of *A. flavus* was expressed at a high level indicating the species specificity. It suggests that the clinical

isolates of different species secrete different proteins that could contribute to their pathogenesis or colonization of the host.



Lanes M - protein molecular weight marker; C - control (sterile mycological broth); 1 - *Aspergillus niger*; 2 - *Penicillium* sp; 3 - *Aspergillus flavus*.

Figure 1 : Cellular protein profile of fungal isolates.



Lanes M - protein molecular weight marker; C - control (sterile mycological broth); 1 - *Aspergillus niger*; 2 - *Penicillium* sp; 3 - *Aspergillus flavus*.

Figure 2 : Extra-cellular protein profile of fungal isolates.

Proteomics is one method of choice to carry out global studies and initial efforts for the characterization of the fungal proteome. Two dimensional map of the mycelial proteome was established for human pathogenic *A. fumigates*[7]. The availability of set of proteins specific to species will contribute to deepen the understanding of the role of the central metabolism for pathogenicity of fungi[8,9]. Many fungal species have been reported to secrete proteases[10-12] and particularly the endoproteoases secreted by pathogenic fungi are aspartic proteases of the pepsin family, serine proteases of the subtilisin subfamily and metalloproteases of two different families. This also includes *A. niger* aspergillopepsin B[13,14]. The proteins that are observed in our study require further validation by screening several isolates of the fungal species. The proteins could be purified, characterized and identified using two-dimensional and mass spectrometry analysis. The preliminary proteome analytical approaches like profiling of cellular and extra-cellular proteins we report, offer a valid strategy to probe into the cellular processes in human pathogenic fungi for identifying biomarkers. These potential candidate proteins could be exploited by purification, characterization and antibodies developed against unique proteins of each species could be used for direct and simple immunological reaction based diagnosis in the clinical setup.

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REFERENCES

[1] P.Badiee, P.Kordbacheh, A.Alborzi, M.Ramzi, E.Shakiba; Infection, **36**, 580-584 **(2008)**.
[2] B.R.Herbrecht, V.Letscher-Bru, C.Oprea, B.Lioure, J.Waller, F.Campos, O.Villard, K.L.Liu, S.Natarajan-Ame, P.Lutz, P.Dufour, J.P.Bergerat, E.Candolfi; J.Clin.Oncol., **20**, 1898-1906 **(2002)**.
[3] D.W.Denning, C.C.Kibbler, R.A.Barnes; Lancet Infect.Dis., **3**, 230-240 **(2003)**.
[4] L.Kooufman, P.G.Standard, M.Jalbert, D.E.Kraft; J.Clin.Microbiol., **35**, 2206-2209 **(1997)**.
[5] D.S.Perlin, Y.Zhao; Med.Mycol., **46**, 1-10 **(2008)**.

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[6] U.K.Laemmli; Nature, **227**, 680-685 **(1970)**.
[7] M.Vodisch, D.Albrecht, F.Lebing, A.D.Schmidt, R.Winkler, R.Guthke, A.A.Brakhage, O.Kniemeyer; Proteomics, **9**, 1407-1415 **(2009)**.
[8] T.Schoberle, G.S.May; Adv.Genet., **57**, 263-283 **(2007)**.
[9] M.Brock; 'The Mycota - Human and Animal Relationships', Springer, Berlin, **(2008)**.
[10] B.Mignon, M.Swinnen, J.Bouchara, M.Hofinger, A.Nikkels, G.Pierard, C.Gerday, B.Losson; Med. Mycol., **36**, 395-404 **(1998)**.
[11] B.Hube, M.Monod, D.Schofield, A.J.P.Brown, N.A.Gow; Mol.Microbiol., **14**, 87-99 **(1994)**.
[12] F.Brouta, F.Descamps, T.Fett, B.Losson, C.Gerday, B.Mignon; Med.Mycol., **39**, 69-75 **(2001)**.
[13] H.Inoue, T.Kimura, O.Makabe, K.Takahashi; J.Biol. Chem., **266**, 19484-19489 **(1991)**.
[14] I.E.Mattern, J.M.van Noort, P.van den Berg, P.Archer, I.N.Roberts; Mol.Gen., **234**, 332-336 **(1992)**.