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RESEARCH ARTICLE

TOXIGENICITY AND MOLECULAR DIAGNOSIS OF FUNGAL INFECTIONS IN PATIENTS PRESENTING WITH OTOMYCOSIS

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ABSTRACT

Introduction: Fungi causing otomycosis are mostly saprophytic in nature. Pathogenicity of fungi is attributable to presence of various virulence factors like biofilm formation, presence of enzymes like urease, phospholipase, hemolysin and production of mycotoxins like aflatoxin. **Aim:** To compare the toxigenicity, molecular and conventional methods in diagnosis of otomycosis. **Methods:** All clinically diagnosed and untreated cases of otomycosis were included in the study. History of otalgia, aural pruritus, ear discharge, diminished hearing, ear block, and tinnitus was elicited. **Results:** Biofilm production was seen with 6 isolates of Candida albicans (29%). Hemolysin was produced in 12 isolates of Aspergillus niger (23.53%). Aspergillus niger species showed higher production of Aflatoxins B1 and G1. 2 of the rare isolates were subjected to PCR followed by Sanger sequencing for speciation and identified as Cladosporidium cladosporioides. **Conclusion:** PCR and Gene sequencing are reliable methods for identification and speciation of rare fungi. Analysis of virulence factors showed biofilm formation in Candida. Production of Urease and Hemolysin were identified as virulence factors among Aspergillus species.

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INTRODUCTION

Otomycosis is a superficial fungal infection of the external auditory canal. *Aspergillus* and *Candida* are the most common organisms causing otomycosis. Other fungi like *Penicillium, Mucor, Rhizopus, Cladosporium, Chrysosporium* and *Absidia* are also involved (Jagdish Chander). Fungi causing otomycosis are mostly saprophytic in nature. Pathogenicity of fungi is attributable to presence of various virulence factors, which allows them to survive at elevated body temperatures and helps to overcome the host defense mechanisms (Jagdish Chander). Direct fungal damage on tissue may occur due to tremendous force of hyphal extension into cells. The fungi also secrete various metabolic products such as mycotoxins and enzymes like phospholipases, elastase and hemolysins. These factors facilitate adherence and cause hydrolysis of cells of the host contributing to its virulence (Moharram, 2016 and Raksha, Gurjeet Singh, 2017 and Boonen Jente, 2012). Biofilm formation among Candida species is associated with poor response to treatment and increased risk of recurrence. Mycotoxins, are secondary metabolites produced by many species of fungi, that are toxic to humans and animals.The most important mycotoxin produced by the Aspergillus group of fungi are the aflatoxins. There are over 16 types of aflatoxins. The important ones are aflatoxins B1, B2, G1, and G2 of which aflatoxin-B1 is the most toxic. Aflatoxin has carcinogenic, mutagenic and immunosuppressive properties. Aflatoxin B1 is a category 1 human carcinogen which usually enters the body through ingestion or occasionally by inhalation. Recent studies have demonstrated property of aflatoxin B1 to enter the body by permeating through the skin (Boonen Jente, 2012). In immunocompromised patients, complications like invasive otitis externa can occur. Complications like tympanic membrane perforation, mastoditis with bone erosion are also known to occur. The pathophysiology of the tympanic membrane perforation is attributed to avascular necrosis of the tympanic membrane as a result of mycotic thrombosis in the adjacent blood vessels. In recent years there has been an increased awareness about fungal infections and a high degree of clinical suspicion among doctors in diagnosing otomycosis. More in-depth studies on the various otomycosis causing fungi, different methods of diagnosis, their virulence profile and their degree of sensitivity

to currently available antifungal drugs will help the clinician in diagnosing and refining treatment of otomycosis.

Aim: To compare the toxigenicity, molecular and conventional methods in diagnosis of otomycosis.

MATERIALS AND METHODS

This Cross Sectional Descriptive study was conducted at the Government Kipauk Medical College for a period of one year from January 2016 to December 2016. Patients attending the ENT department with symptoms of Otomycosis were selected as the study population. Under strict aseptic precautions the debris in the external auditory canal was collected using 3 sterile swabs, after cleaning the pinna and adjacent areas with antiseptic solution. A repeat swab was taken on the same day after 2 hours to rule out contamination. The collected specimen was sent to the lab promptly along with a proper requisition slip and processed within 30 minutes. The fungi were identified by conventional methods like KOH mount, culture on Sabouraud's Dextrose Agar, LPCB mount. Candida species were identified by Gram stain, Germ tube test, Dalmau plating, Candida CHROMagar, sugar assimilation and fermentation tests. Tests for virulence factors were also done. Antifungal Susceptibility Testing of Filamentous Fungi was done by Microbroth Dilution method, CLSI document M38-A2 and for yeasts by Disc diffusion method according to CLSI M44 A. Aflatoxin production was detected and quantified by High performance Liquid Chromatography. Rare fungal isolates were subjected to Pan fungal PCR followed by gene sequencing for identification.

Tests for Virulence

Urease Activity (Moharram, 2013)

- Using a sterile loop, yeast colony or mould colony was inoculated onto Christensen urea medium and incubated at 25°C for 3-5 days.
- Appearance of a deep pink color indicates a positive test.

Hemolysin production (Raksha, Gurjeet Singh, 2017)

- Colonies of *Aspergillus* species were inoculated onto Blood agar plate containing 7% sheep blood mixed in Sabouraud Dextrose Agar.
- The plates were incubated at 25°C for 3-5 days.
- Appearance of hemolysis surrounding the fungal growth indicates a positive test.

Biofilm formation (Vivek Agwan, 2015)

- Using sterile loop, *Candida* colonies from a 18 -24 hours culture on SDA was taken.
- It was inoculated into 5ml of Sabouraud's Dextrose broth and the turbidity was adjusted to o.5 Mac Farland standard.
- The tubes were incubated at 37°C for 18 - 24 hours.
- The broth was poured out and washed with Phosphate Buffer Saline.
- 0.5% crystal violet solution was added and stained for 10 minutes.
- A visible and uniform violet coloured film in the tube indicates presence of biofilm.

Toxicity Testing for Aflatoxin by High Performance Liquid Chromatography (HPLC) (Lisker, 1993 and Miguel, 1986)

- Fungus to be tested was cultured on Sabouraud's Dextrose Agar slope
- 50 gms of the test portion was transferred into a 500 ml Erlenmeyer flask and 5gms of NaCl was added.
- The test portion was extracted with 300 ml methanol extraction solvent and 100 ml hexane in a blender at high speed for 3 minutes.
- The extract was filtered through a fluted filter paper.
- 10 ml of clear filtrate was added to 60 ml of Phosphate buffered saline (PBS) and applied to the affinity column containing antibodies to aflatoxins B1, B2, G1 and G2.
- 10 ml of the PBS solution with the filtrate was applied to the top of the column and allowed to pass at a speed of 2-3ml/min through the column by gravity.
- Aflatoxins were eluted from the column with 0.5ml methanol.
- Post column derivatization was done using pyridinium hydrobromide perbromide (PBPB).
- 200 mL of aflatoxin standards were eluted through the liquid chromatography. Aflatoxins elute in the order G2, G1, B2, and B1 with retention times of 6, 8, 9, and 11 min respectively.
- 200 mL of the test extract was injected into the injector and each aflatoxin peak and retention times in the chromatogram was identified and compared with the corresponding reference standard by fluorescence detection. Quantity of each aflatoxin was also derived from the standard curve.

MOLECULAR DIAGNOSIS

Polymerase Chain Reaction: Pan fungal DNA is measured at 270 bp was identified by Polymerase Chain Reaction.

Gene Sequencing (Anna, 2010): The PCR products were purified with a Qiagen PCR purification kit. Both strands were sequenced by Sanger's dideoxynucleotide sequencing method. The sequences were obtained as overlapping runs of the two flanking primers (primers ITS-1 and NL-4), as well as runs of two internal primers (primers ITS-4 and NL-1).

Sequence analysis: The sequence data were assembled and analyzed by the use of Sequence analysis software. Individual nucleotide-nucleotide searches are done with the BLASTn algorithm at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). The outputs from the BLAST searches are sorted on the basis of the maximum identity and were recorded as they appeared without modification of genus or species names that may have been synonyms or teleomorphs of other genus or species names in other Gen Bank records. Sequence-based identities with a cutoff of 97% or greater were considered significant in this study, and the best hit was defined as the sequence with the highest maximum identity to the query sequence.

RESULTS

200 cases of clinically suspected otomycosis were analyzed out of which 105 samples showed growth of fungi, accounting for 52.5%. The fungi were identified and speciated by conventional methods. Out of the 105 fungi isolated, 81 were filamentous fungi (77%), and 24 were Candida species (23%).

The p-value was significant with <0.001. Among the fungal species identified, *Aspergillus niger* was the most common isolate in 51 cultures (49%), followed by *Aspergillus flavus* in 14 cultures (13%). *Aspergillus terreus* was isolated in 4 cultures. There were 2 isolates each of *Aspergillus fumigatus* and *Rhizopus*. The rare causative fungi isolated were *Cladosporium* (2), *Aspergillus nidulans* (1), *Mucor* (1), *Aureobasidium pullulans*(1), *Fonsecaea pedrosoi* (1), *Curvularia lunata* (1) and *Rhizomucor* (1). Of the 24 Candida species isolated, 20 were *Candida albicans*, 2 were *Candida parapsilosis*, 1 was *Candida glabrata*, and 1 isolate was *Candida krusei*. The results were statistically significant with a p-value of <0.001

Table 1. Different Species Isolated in the study (n=105)

Fungus isolated	Number	Percentage
Filamentous Fungi (n=81)		
Aspergillus niger	51	49%
Aspergillus flavus	14	13%
Aspergillus terreus	4	4%
Aspergillus fumigatus	2	2%
Rhizopus oryzae	2	2%
Cladosporium cladosporoides	2	2%
Aspergillus nidulans	1	1%
Aureobasidium pullulans	1	1%
Fonsaceae pedrosoi	1	1%
Rhizomucor pusillus	1	1%
Mucor circinelloides	1	1%
Curvularia lunata	1	1%
Non Filamentous Fungi (n=24)		
Candida albicans	20	19%
Candida parapsilosis	2	2%
Candida glabrata	1	1%
Candida krusei	1	1%

Table 2. Biofilm Formation (n=24)

CANDIDA SPECIES	BIOFILM
Candida albicans (n=20)	6 (29%)
Candida parapsilosis (n=2)	1 (50%)
Candida glabrata (n=1)	0
Candida krusei (n=1)	0

Table 3. Urease Test

Aspergillus species	Number tested	Urease positive	Percentage
Aspergillus niger	51	0	0
Aspergillus flavus	14	11	78.57%
Aspergillus terreus	4	4	100%
Aspergillus fumigatus	2	0	0
Aspergillus nidulans	1	0	0

Table 4. Hemolysin Production

Aspergillus species	Number tested	Hemolysin positive	Percentage
Aspergillus niger	51	12	23.53%
Aspergillus flavus	14	4	28.57%
Aspergillus terreus	4	3	75%%
Aspergillus fumigatus	2	2	100
Aspergillus nidulans	1	0	0

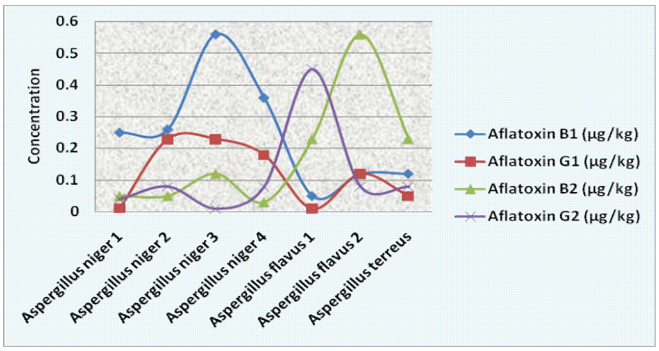

Figure 1. Aflatoxin production in *aspergillus* species

Hemolysin as a virulence factor was produced in 4 isolates of *Aspergillus niger* (7.8%), 2 isolates of *Aspergillus flavus* (1.4%), and 1 isolate of *Aspergillus terreus* (25%). *Aspergillus* isolates producing hemolysin were tested for aflatoxin production by High Performance Liquid Chromatography (HPLC). All the isolates produced Aflatoxins. Higher production of Aflatoxins B1 and G1 was seen with *Aspergillus niger* while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2.

Table 5. Aflatoxin Production by HPLC

	Aflatoxin B1 (µg/kg)	Aflatoxin G1 (µg/kg)	Aflatoxin B2 (µg/kg)	Aflatoxin G2 (µg/kg)
Aspergillus niger 1	0.25	0.012	0.05	0.04
Aspergillus niger 2	0.26	0.23	0.05	0.08
Aspergillus niger 3	0.56	0.23	0.12	0.01
Aspergillus niger 4	0.36	0.18	0.03	0.08
Aspergillus flavus 1	0.05	0.01	0.23	0.45
Aspergillus flavus 2	0.12	0.12	0.56	0.08
Aspergillus terreus	0.12	0.05	0.23	0.08

Aspergillus isolates producing hemolysin were tested for aflatoxin production by High Performance Liquid Chromatography (HPLC). All the isolates produced Aflatoxins. Higher production of Aflatoxins B1 and G1 was seen with *Aspergillus niger* while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2.

Table 6. Speciation of Rare Filamentous Fungi: MOLECULAR METHOD

Number Isolated	Pan Fungal PCR	Gene Sequencing	Species Identified
2	POSITIVE	AAATCTAAAACTAAGGAA GGCCCTATATCGGGGGCA TGCTGTTCGAGCGT CATTTCACCACTCAAGCC TCGCTTGGTATTGGGCAA CGCGGTCCGCCGCG TGCCTCAAATCGACCGGC TGGGTCTTCTGTCCCCTAA GCGTTGTGGAAAC TATTCGCTAAAGGGTGCT CGGGGAGGCTACGCCGTA AAACAAACCCATTT CTAAGGTTGACCTCGGAT CAGGTAGGGATACCCGCT GAACTTAAGCATAT CAATAAAGCGGGAGAAA	*Cladosporiumcladosporioides*

2 of the rare isolates were subjected to PCR followed by Sanger sequencing. 270 bp Pan-fungal genome was identified and sequenced by Sanger's dideoxynucleotide sequencing method. Individual nucleotide-nucleotide searches were done with the BLASTn algorithm at the NCBI website. Sequence-based identities with a cutoff of 97% was obtained for both isolates and the fungi were identified as *Cladosporidium cladosporioides.*

DISCUSSION

Of the 200 clinically suspected cases of otomycosis analyzed, 105 samples showed fungal growth on culture accounting for 52.5%. Among the fungi isolated *Aspergillus niger* was the most common isolate in 51 cultures (49%), followed by *Aspergillus flavus* in 14 cultures (13%). *Aspergillus terreus* was isolated in 4 cultures. There were 2 isolates each of

Aspergillus fumigatus and *Rhizopus*. The rare causative fungi isolated were *Cladosporium*(2), *Aspergillus nidulans* (1),*Aureobasidium pullulans*(1), *Fonsaceae pedrosoi* (1), *Curvularia lunata* (1), *Mucor* (1), and *Rhizomucor* (1). Of the 24 *Candida* species isolated, 20 were *Candida albicans*, 2 were *Candida parapsilosis*, 1 was *Candida glabrata* and 1 isolate was *Candida krusei*. Virulence factors in fungi, determine the severity of the disease and its response to antifungal treatment. So testing for fungal virulence factors is an integral part of the laboratory diagnosis of fungi. 6 isolates of *Candida albicans* (29%) and 1 isolate of *Candida parapsilosis* were biofilm producers. This is similar to studies by Analy S Meloet al [9] where biofilm formation was more among *Candida albicans* than *Candida parapsilosis*. Urease production was seen in 78.57% of the *Aspergillus flavus* isolates and all 4 *Aspergillus terreus* isolates (100%). Urease production was not seen with *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus nidulans* which matches findings by A.M.Moharram et al[2].

Hemolysin as a virulence factor was produced in 12 isolates of *Aspergillus niger* (23.53%), 4 isolates of *Aspergillus flavus* (28.57%), and 3 isolates of *Aspergillus terreus* (75%). In the study by Raksha et al [10] , hemolysin was produced by 83% of *Aspergillus niger*, 57% of *Aspergillus flavus*, 77% of *Aspergillus fumigatus* and 100% of *Aspergillus terreus*. *Aspergillus niger* species showed higher production of Aflatoxins B1 and G1 while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2. Aflatoxins induce teratogenicity and mutagenicity usually on ingestion or inhalation. Studies by Boonen et al[4] and Rastogi et al[11] have documented permeation of aflatoxin through the skin. In-depth studies are needed to identify the mutagenic and teratogenic potential of fungi involved in otomycosis. 2 of the rare isolates were subjected to PCR followed by Sanger sequencing for speciation. 270bp pan fungal genome was identified and sequenced by Sanger's dideoxynucleotide sequencing method. Individual nucleotide-nucleotide searches were done with the BLASTn algorithm at the NCBI website. Sequence-based identities with a cut-off of 97% were obtained for both isolates, and the fungi were identified as *Cladosporidium cladosporioides*. MIC of Clotrimazole to *Aspergillusniger* was in the range 0.03 – 1µg/ml. MIC50 was 0.25µg/ml and MIC90 was 1 µg/ml. For *Aspergillusflavus* the MIC range was 0.03 – 1 µg/ml. MIC 50 was 0.125 µg/ml and MIC90 was 0.5 µg/ml. MIC of Amphotericin B to *Aspergillusniger* was in the range 0.03 – 1µg/ml. The MIC 50 was 0.5µg/ml and MIC90 was 1 µg/ml. For *Aspergillusflavus* the MIC range was 0.0625 – 1 µg/ml. The MIC 50 was 0.5 µg/ml and MIC90 was 1 µg/ml. MIC of Voriconazole to *Aspergillus niger* was in the range 0.03 –1 µg/ml. MIC 50 was 0.25µg/ml and MIC90 was 0.5 µg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 µg/ml. MIC 50 was 0.25 µg/ml and MIC90 was 1µg/ml. MIC of Itraconazole to *Aspergillus niger* in the range 0.03 – 1 µg/ml. The MIC 50 was 0.25µg/ml and MIC90 was 1 µg/ml. For *Aspergillus flavus* the MIC range was 0.03 – 1 µg/ml. MIC 50 was 0.125 µg/ml and MIC90 was 1 µg/ml. Among the 20 isolates of *Candida albicans* tested, all isolates were susceptible to Ampotericin B, Voriconazole and Itraconazole. 1 isolate was resistant to Itraconazole and 2 isolates were resistant to Fluconazole. Among the 2 isolates of *Candida parapsilosis*, both were susceptible to Amphotericin B, Itraconazole, Voriconazole and Clotrimazole while 1 isolate was resistant to Fluconazole. The isolate of *Candida glabrata* was susceptible to Voriconazole, Itraconazole and Clotrimoxazole. The isolate of *Candida krusei* was susceptible to Voriconazole, Itraconazole and Clotrimazole. All the patients were treated with clotrimazole and showed good response to treatment on follow up. Conventional methods provide good choice for diagnosis of fungal infections. However the time taken for diagnosis is long ranging from 7 to 21 days. Antifungal susceptibility testing by conventional microbroth dilution method is time consuming and cumbersome. With increasing emergence of resistance to antifungal agents, routine testing for antifungal susceptibility will help the clinician in choosing the most effective drug for treatment thereby reducing the morbidity and mortality in patients. Molecular methods provide a reliable and quicker alternative for both identification of fungi along with their antifungal susceptibility pattern.

Conclusion

Analysis of virulence factors showed biofilm formation in *Candida*. Production of Urease and Hemolysin were identified as virulence factors among *Aspergillus* species. On testing production of Aflatoxins among the *Aspergillus* species, *Aspergillus niger* species showed higher production of Aflatoxins B1 and G1 while *Aspergillus flavus* and *Aspergillus terreus* produced more amounts of Aflatoxins B2 and G2. More in-depth studies are needed to know the mutagenic and teratogenic potential of fungi causing otomycosis and their systemic implications. PCR and Gene sequencing are reliable methods for identification and speciation of rare fungi.

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