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ANTIFUNGAL SUSCEPTIBILITY TESTING OF ISOLATES OF ASPERGILLS SPECIES FROM RHINOSINUSITIS IN A TERTIARY CARE CENTRE IN KERALA

Microbiology

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ABSTRACT

Fungal Rhino sinusitis (FRS) is recognised in persons of all age groups resulting in great socioeconomic effects including both direct and indirect costs to the society. It has significant impact on quality of life in comparison to chronic debilitating diseases like diabetes mellitus and congestive cardiac failure. Rapid increase in fungal infections is noticed now a days due to increased use of immunosuppressive agents, prolonged use of broad spectrum antibiotics, radiotherapy and chemotherapy. Newer antifungal drugs are being marked recently besides so many drugs are already available. Drug resistance has been reported for certain antifungal drugs also. Hence in vitro susceptibility testing of the isolate is mandatory for the selection of appropriate antifungal drug to treat the patient with FRS. If the appropriate antifungal agent is administered at the appropriate time, complications can be avoided. A study was conducted in the Dept. of Microbiology Govt. Medical College, Thiruvananthapuram, Kerala for a period of one year from August 2015 to July 2016 to identify the fungal pathogens causing FRS in patients attending the ENT dept. A total number of 186 clinically diagnosed cases of FRS from whom different types of clinical specimens were collected and processed in the 24 hours clinical Microbiology Laboratory attached the Govt. Medical College Hospital, TVM. A total number of 50 isolates were obtained by fungal culture. The culture positivity was 26.88%. *Aspergillus* species were the predominant isolates (62%). Among them, *Aspergillus fumigatus* was the most common isolate (42%) followed by *A.flavus A, niger* (19.35%) and *A terreus* (6.45%). Antifungal susceptibility testing of the Aspergillus isolates were done by microbroth dilution method according to CLSI (2008) guidelines M38-A2. The isolates showed 100% sensitivity to Itraconazole and 93.5% sensitivity to Amphotericin B. All of them were resistant to Fluconazole (100%). The mortality rate in the study significantly reduced (4%) due to early detection and initiation of appropriate antifungal therapy.

KEYWORDS

Fungal rhinosinusitis, Aspergillus fumigatus, Aspergillus flavus, antifungal susceptibility testing, microbroth dilution method.

Introduction

Aspergillus species are the predominant fungi causing rhinosinusitis. The first definite case of invasion of paranasal sinuses by *Aspergillus* species was reported by Schubert in 1886. C. Zarniko in 1891 identified the causative agent of rhinosinusitis as *Aspergillus fumigatus.* Later in 1897, oppe described *Aspergillus* species causing infection of sphenoid sinus with bony wal erosion and invasion of cerebrum. Safirstein in 1976 noted a combination of nasal polyposis, crust formation and sinus cultures yielding *Aspergillus* species. Till date, *Aspergillus* species are the most common fungi causing FRS. So many antifungal agents are available nowadays Drug resistance is a major problem we observe when the fungal infections are treated by clinicians. Hence it is mandatory to do antifungal susceptibility testing of the isolates obtained in the clinical laboratory. Even though, many methods are available, microbroth dilution method is considered to be the best method as it helps to detect MIC distributions and epidemiological cut off values (ECV) to identify the resistant strains.

Aim of Study

The aim of the study was to find out the proportion of FRS at Govt. Medical College Thiruvananthapuram and the predominant species of fungi causing FRS, the important predisposing factors and to determine the antifungal susceptibility pattern to the isolates obtained during the study.

Materials and Methods

Study design : Descriptive study
Study population : Clinically diagnosed cases of rhinosinusitis with age group between 18 to 90 yrs.
Study setting : Dept. of Microbiology and Dept. of ENT, Govt. Medical College, Thiruvananthapuram, Kerala
Study period : One year (from August 2015 to July 2016)

Methodology

Collection of samples

Samples were collected under sterile conditions. Sinus secretion and debris, sinonasal polyp, scrapings of necrotic tissue materials, excised sinus tissue, allergic mucin, fungal ball and mucocele were collected through Functional Endoscopic Sinus Surgery (FESS). The nasal cavity was packed with 4% lignocaine and adrenaline before surgery. First step was uncinectomy followed by middle meatal antrostomy where the maxillary sinus ostium was widened. The antrum was then inspected from any polyps, fungal ball, allergic mucin or debris. If pathologic lesion was suspected in ethmoid sinuses, the bulla ethmoidalis was opened and all ethmoid air cells were removed. Sphenoid sinus was opened last. All the samples collected by endoscopic sinus surgery were sent to the 24 hrs clinical microbiology laboratory immediately after collection.

Processing of samples

Direct microscopic examination of the samples were made after wet mount preparation with 10% KOH and Lactophenol cotton blue stain. Fungal hyphae if present may be observed clearly.

Culture was done on Sabouraud's Dextrose Agar (SDA) (plain) and SDA with gentamicin SDA slants were prepared on tubes by dispensing 5 ml volume of the medium plugged with cotton and after autoclaving cooled in slanting position to make SDA slants.

SDA with gentamicin slants are prepared by adding 50 mg/L gentamicin using a sterile membrane filter after autoclaving. The medium was dispensed in 5 ml volumes in sterile test tubes plugged with cotton and allowed to cool in slanted position to make slants.

Each set of media were incubated at 37^{0}C and at room temperature. After inoculation tubes were observed for any growth after 24 hours and 48 hours. If no growth was observed it was viewed twice a week followed by once a week for 4 weeks. If no growth occurs even after 4 weeks, culture is declared as negative. Fungal growth if obtained during the period was examined for the rate of growth, colony morphology, colour of the obverse and reverse sides of the tubes for diffusible pigment production. The morphology of the fungi were identified by direct microscopy by removing a bit of fungal growth using a straight wire and after teasing with teasing needle and by making wet mount preparation with lactophenol cotton blue stain. The method is limited in usefulness because the conidia are often separated from conidiophores which makes the identification difficult in the case of filamentous fungi.

Slide culture technique was performed for the identification of filamanetous fungi which permits the microscopic observation of the undisturbed morphology of the fungi and the identification was confirmed.

For the identification of yeast like fungi, gram staining was done to demonstrate the budding yeast cells, pseudohyphae, if present. Germ tube test was performed using human serum to differentiate *Candida albicans* and non albicans. If the germ tube test was positive, preliminary identification of *Candida albicans* was made. Others who do not produce germ tubes were included in the nonalbicans group.

Antifungal susceptibility testing

Aspergillus species were the predominant fungi isolated in the study (31 isolates) (62%). Antifungal susceptibility testing of the isolates were done by microbroth dilution method according to the CLSI M38-A2 Reference method. The antifungal drugs tested were Amphotericin B, Itraconazole and Fluconazole. The medium used was RPMI 1640.

Quality control :

Aspergillus fumigatus ATCC 204305

Aspergillus flavus ATCC 204304

Antifungal drugs were obtained in the powder form from Himedia laboratories and stored at 400C in a refrigerator as recommended by the manufacturer.

Antifungal agent stock preparation

Antifungal stock solutions are prepared at concentration hundred times more than the highest concentrations tested. Amphotericin B and Itraconazole were dissolved in dimethyl sulfoxide (DMSO) and Fluconazole powder was dissolved in sterile distilled water.

The following drug concentration ranges were tested.

0.125-64 µg/ml for Fluconazole

0.0313 µg/ml for Amphotericin B and Itraconazole.

Stock solutions of 1600 µg/ml concentrations were prepared for Amphotericin B and Itraconazole and 5120 µg/ml for Fluconazole.

Stock solutions of antifungal agents do not commonly support the growth of contaminating organisms and can be considered sterile.

Weighing of powder

To determine the amount of powder needed, the following formula was used.

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{concentration } (\mu g/ml)}{\text{Assay potency } (\mu g/mg)}$$

The antifungal powders were accurately weighted for 10 ml stock solution using an analytical calibrated balance. The assay potency of Amphotericin B was 750 µg/mg and 980 µg/mg for both Fluconazole and Itraconazole. So for preparing the 10 ml stock solution, the amount required for Amphotericin B was determined as 21.33mg/ 52.2mg for Fluconazole and 16.3 mg for Itraconazole.

TEST PROCEDURE

Broth medium

The medium used was RPMI-1640 (with glutamine, without bicarbonate and phenol red as pH indicator). The buffer used was MOPS (3-[N-morpholino] propane sulfonic acid).

Antifungal agent intermediate stock/dilution series preparation

Sterile plastic test tubes were used to prepare drug dilutions and sterile disposable multiwell (96-U shaped wells) to perform the tests. Intermediate concentration series ranging from 1600 µg /ml -3.13 µg/ml (for Amphotericin B and Itraconazole) and 640 µg/ml -0.3125 µg/ml) are prepared from stock solutions in DMSO. Working antifungal solutions were prepared at 10 times the highest concentration for Fluconazole and 100 times the highest concentration for Amphotericin B, Itraconazole. Ten sterile test tubes were placed in a row. Each of the intermediate concentrations are diluted (1:50) in RPMI-1640 to make the working solution. This was done to achieve the final two-fold concentration of drug needed for microdilution. Add 100 µl of the two fold antifungal concentrations into columns 1-10 of sterile disposable 96 well microdilution plates. Column 1 will contain the highest concentration and column 10 the lowest drug concentration. Add 100 µl of diluent to column 11 & 12. Column 11 serves as the growth control well and column 12 as sterility control.

INOCULUM PREPRATION

Aspergillus isolates were grown on Potato Dextrose Agar slants (PDA) incubated at 37^{0}C for 7 days until sufficient conidia were formed. Sporulating colonies were convered with 1 ml of sterile 0.85% saline. A drop of tween was added to the suspension to facilitate the preparation of inocula. Conidia were harvested by agitation with a sterile Pasteur pipette. Resulting suspension was transferred to a sterile test tube and kept for 5 minutes to allow the heavy particles to sterile down. Without disturbing gently, the upper homogenous suspension was transferred to a sterile tube, the cap was tightened and vortexed in a vortex mixer. The optical densities of the conidial suspension was adjusted at 530 nm that ranges from 0.09-0.13 in spectrophotometer. This will obtain a conidial suspension in a range of 0.4x104 to 5x105 CFU/ml to provide the most reproducible MIC data. 100 µl of conidial suspension was added to 4.9ml of RPMI-1640 (1:50 dilution), and mixed. 100 µl of this inoculum was added to each well excluding the sterility control well (column 12) The growth control well contains 100 µl of inoculum suspension and 100 µl of drug diluent. The sterility control well contains only diluent without any inoculum. The microdilution tray was incubated at 350C for 48 hours.

READING RESULTS

Minimum inhibitory concentration (MIC) is the lowest concentration of antifungal agent that inhibits the growth of organism as detected visually. The growth in each MIC well was compared with that of growth control with the aid of a reading mirror.

I. **Amphotericin B**
   End points are well defined for Amphotericin B
   MIC is defined as the lowest drug concentration without any visible growth (100%) inhibition)

2. **Fluconazole :** End points are typically less well defined.
   MIC is defined as the lowest drug concentrations at which the turbidity is reduced to at least 50% when compared to the growth control well.

3. Itraconazole

End points are typically easily defined.

MIC is defined as the lowest drug concentration without any visible growth (100% inhibition)

INTERPRETATION OF RESULTS

MIC above 2µg/ml is associated with treatment failure and below 2 µg/ml with clinical cure for Amphotericin B.

For Itraconazole MIC above 8µg/ml has been associated with treatment failure and below 8µg/ml with clinical cure.

Results

A total number of 186 samples were collected from clinically diagnosed cases of rhinosinusitis who underwent FESS during the study period under sterile conditions.

Table 1. Proportion of fungal culture positive samples

Total number of samples	Culture positive samples	Percentage	Culture negative samples	Percentage
186	50	26.88%	136	73.11%

Table 2 : Culture positivity based on gender

Total number		Culture positivity	
Male	82	44%	Culture positivity
Female	104	56%	24 (48%)
Total	186	100%	50(100%)

Table 3 : Fungal isolates from FRS cases

Sl. No.	Name of Fungi	No. & Percentage
1.	*Aspergillus fumigatus*	13 (26%)
2.	*Aspergillus flavus*	10 (20%)
3.	*Aspergillus niger*	6 912%)
4.	*Aspergillus terreus*	2 (4%)
5.	*Rhizopus species*	6(12%)
6.	*Candida species*	4 (8%)

7.	*Mucor species*	2 (4%)
8.	*Penicillium species*	2(4%)
9.	*Pseudoallescheria boydii*	2(4%)
10.	*Trichosporon species*	1 92%)
11.	*Fusarium species*	1 (2%)
12.	*Curvularia species*	1 (2%)
	Total	50 (100%

Table 4. Antifungal sensitivity testing of the Aspergillus species by microbroth dilution method

Isolates	Amphotericin B		Itraconazole		Fluconazole	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
A. fumigatus	13	-	13	-	-	13
A. flavus	10	-	10	-	-	10
A. niger	6	-	6	-	-	6
A. terreus	-	2	2	-	-	2

Table 5 : Clinical samples versus culture positivity

Nature of samples	Number of samples	Percentage	Positive fungal cultures	Percentage	Negative fungal cultures	Percentage
Sinus secretion & debris	67	36.02%	18	26.86 %	49	73.13%
Sinonasal polyp	63	33.4%	6	9.52%	57	90.47%
Necrotic scraping	26	13.9%	7	26.92 %	19	73.07%
Excised sinus tissue	20	10.75%	10	50%	10	50%
Allergic mucin	6	3.22%	5	83.35	1	16.66%
Fungal ball	3	1.61%	3	100%	0	0%
Mucocoele	1	0.5%	1	100%	0	0%
Total	186	100%	50	-	136	-

Table 6 : Seasonal distribution of FRS

Season	Total no. and percentage	Culture positives with FRS No. & Percentage
Summer March – June	66(35.4%)	16 (32%)
Monsoon July – October	72 (38.7%)	22 (44%)
Winter November – Feb	48 (25.8%)	12 (24%)
Total	186 (100%)	50 (100%)

Table 7 : Predisposing factors in FRS

Sl. No.	Predisposing conditions	No. of & Percentage of patient with FRS
1	Diabetes mellitus	11 (22%)
2	Allergic rhinitis	9(18%)
3	Asthma	7 (14%)
4	Hypertension	6(12%)
5	Polypeotomy	3 (6%)
6	Dental Extraction	1 (2%)
7	None	13 (26%)
	Total	50 (100%)

Table 8 : CT scan PNS findings in FRS

CT findings	Total number	Percentage
Inflammatory mucosal thickening	16	32%
Heterogenous opacity	13	26%
Homogenous opacities	9	18%
Hyperattenuating allergic mucin	7	14%
Hyperdense lesions	3	6%
Intraorbital & intracranial invasion	2	4%

Table 9 : Outcome after treatment for FRS

Sl. No.	Total number	Percentage
1	Patients survived without complications	45 (90%)
2	Patients survived with complications	3(6%)
3	Patients died	2 (4%)
	Total no. of culture positive	50 (100%)

Table 10 : Sinuses involved in patients with FRS

Sl. No.	Name of sinus	No. of patients and percentage
1	Maxillary sinus	16(32%)
2	Ethmoid sinus	6 912%)
3	Sphenoid sinus	3 (6%)
4	Frontal sinus	2 (4%)
5	Multiple sinuses	22(46%)
	Total	50 (100%)

DISCUSSION

The value of the minimum inhibitory concentration (MIC) of the antifungal drug, Itraconazole showed good susceptibility to Aspe rgillus species.

CLSI reference method M38-A2 (CLSI-2008) is laboratories and difficult to apply in most of the clinical laboratories. Antifungal resistance is becoming an emerging problem. The azole resistance of Aspergillus species isolates has been rigorously investigated with last years. The use of azoles in agriculture has been described as a cause of the emergence of triazole resistant Aspergillus fumigatus isolates, particularly in Europe and Asia. In our study, all the Aspergillus isolates were resistant to Fluconazole.

Eventhough the main goal of antifungal susceptibility testing is to select the best treatment for a given isolate, these methods are also very important to detect resistant strains. Regardless of their advantages, the standardized broth microdilution methods of AFST are time consuming and cumbersome for clinical laboratories. Fungal spores are ubiquitous and exposure to fungus is frequent, yet disease due to tissue invasion is not common among healthy individuals. Increased incidence of invasive fungal infection observed among immunoc ompromised patients. The incidence of FRS varies widely with geographical areas and environment conditions. In our study, majority of the patients (38.7%) were admitted with clinical features of FRS during the monsoon season (July-October) Fungi thrive in warm and humid climates. Geographical and environmental conditions in different parts of the country vary. Those areas located in the tropics and receiving a heavy annual monsoon makes climatic conditions favourable for fungal proliferation. In contrast to this, study by Sunil Garg et al (2013) in Delhi observed maximum number of FRS cases during the summer season. They attributed it to the hot and dry winds blowing over the north and north western part of India during March to June which may contain large number of fungal spores. The most common predisposing factor observed in our study was uncontrolled Diabetes mellitus (30%) Mohapatra et al (2010) reported 44.8% in other study. In the present study, antifungal susceptibility testing was performed by microbroth dilution method. All Aspergillus species were sensitive to Itraconaole (100%). All Aspergillus species except Aspergillus terreus were sensitive to Amphotericin B (93.5%). This is consistent with a study conducted by Kavitha et al at Tamil Nadu and Jain et al from North India. Pfaller et al reported 100% sensitivity of Aspergillus terreus tested to Itraconazole and 25% sensitivity to Amphotericin B. Another study from Texas in which 101 isolates of Aspergillus terreus were tested, 98% were resistant to Amphotericin B. All the Aspergillus isolates were resistant to Fluconazole (100%). The increase of resistant strains associated with treatment failure highlights the need for antifungal resistance surveillance, which should ideally be made available in reference laboratories using reference procedures.

Management of FRS cases

Clinically diagnosed cases of FRS were treated with antifungal therapy and surgical intervention with Endoscopic sinus surgery. Aggressive surgical debridement was done in cases of invasive fungal sinusitis. Surgical excision and removal of nasal polyps and fungal ball were performed immediately after detection.

For antifungal therapy, intravenous Amphotericin B was given to patients with fungal rhinosinusitis (31%) on discharge, patients were advised to continue oral intraconazole. Patients with invasive aspergillosis were successfully treated with voriconazole (4%).

Treatment outcome

Majority of the patients (92%) responded well to the antifungal therapy along with surgical intervention. Two patients admitted with FRS were having complications like orbital cellulitis and lateral rectal palsy (4%). In spite of initiation of antifungal therapy 2 patients with invasive FRS expired during treatment. Mortality rate in our study was 4%, which was significantly reduced when compared to many other studies. A study by Sandeep Suresh et al and another study from North India have reported 10% and 8% mortality rate respectively. Antifungal susceptibility testing plays a major role in the selection of the appropriate drug for the treatment of fungal rhinosinusitis.

CONCLUSION

The study was conducted in the department of Microbiology, Government Medical college, Thiruvananthapuram for a period of one year from August 2015 -July 2016 and the samples were collected from patients with clinical and radiological features of rhinosinusitis attending ENT department during that period. A total of 186 patients with age groups ranging from 18 to 80 years of both gender irrespective of the duration of symptoms were included in the study. Most common age group was between 40-49 (34%) and males are more affected (52%) than females (48%). Majority of the cases were having chronic fungal rhinosinusitis (94%) and only 6% cases were having acute invasive fungal rhinosinusitis. Uncontrolled Diabetes mellitus was a major risk factor (30%) FRS cases. Fungal culture were positive in 26.88% of samples and negative in 73.12% of samples.

Fungal hyphae were demonstrated by direct microscopy by KOH mount in 21.5% of cases. Among the KOH positive samples, 77.5% were fungal culture positive and 22.5% were negative. Majority of the samples collected through endoscopic sinus surgery were sinus secretion and debris (36.02%) but culture positivity was 26.86%. Culture positivity was 100% in fungal ball and mucocele samples. Aspergillus species were the predominant fungi isolated in this study (62%). Among the species of Aspergillus, Aspergillus fumigatus was the most common isolate (42%) followed by Aspergillus flavus (32.25%), Aspergillus niger (19.35%) and Aspergillus terreus (6.45%) Rhizopus species and Mucor species together accounted for 16% of FRS cases followed by Candida species (8%), Pseudallescheria boydii (4%), Penicillium species (4%), Fusarium species (2%), Trichosporon species (2%) and Curvularia species (2%).

Antifungal susceptibility testing of the Aspergillus species isolates method according to the CLSI guidelines showed. 100% sensitivity to Itraconazole and 93.5% to Amphotericin B and 100% resistance to Fluconazole. Multiple sinuses were involved in 44% of with cases Fungal rhinosinusitis. The most commonly involved sinuses were maxillary sinus and ethmoid sinus (40.9%). Mortality rate was 4% in our study. The rate has been significantly reduced when compared to other studies. This is because of early detection, proper collection of samples at the proper time and isolation of the fungal pathogen in culture. Selection of appropriate antifungal agent was made possible by the in vitro antifungal susceptibility testing. Effective management of the cases could be done by appropriate antifungal therapy along with surgical intervention.

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