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Conventional vs molecular methods in diagnosis of fungal Rhinosinusitis – A comparative study

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Rhinosinusitis is characterized by inflammation of nasal and paranasal sinus mucosa. Fungal infections of the sinuses have recently been reported to cause most cases of Chronic Rhinosinusitis (CRS). Patients from all age group were included in the study conducted from March 2017 till April 2018, with a history of nasal obstruction, nasal discharge, headache, allergy to dust and fumes, etc.Nasal secretions were collected and mycological analysis was performed (direct examination, culture on Sabouraud Dextrose agar(SDA), blood agar, etc. Incubation was done at 25° and 37° C and cultures were observed up to 4 weeks before reporting it as negative for fungi. Polymerase chain reaction (PCR), was performed directly from nasal specimen. Amplification products were detected by gel electrophoresis and sequencing was done. Out of 275 samples collected, 38 fungi were isolated in culture (13.8%) most common being *Aspergillus flavus*, others were *Fonsecaea* spp., *Aspergillus glaucus*, *Rhizopus* spp., *Acremonium* spp. and *Penicillium* spp. 14 culture-negative and PCR positive samples, were confirmed by sequence analysis.

Key words: Allergy, fungi,fungal rhinosinusitis, sinusitis

INTRODUCTION

Rhinosinusitis (RS) is a common disorder affecting approximately 20% of the population. Chronic Rhinosinusitis (CRS) affects upto 31 million people (12.5% of the population) in the United States alone(Hamilos,2011) . The disease is characterized by inflammation of nasal and paranasal sinuses mucosa. Acute rhinosinusitis lasts up to 4 weeks with complete resolution of symptoms, subacute between 4-12 weeks whereas the chronic form persists beyond 12 weeks (Ann and Miriam, 2016; Rosenfeld *et al.*2015) . Fungal infections of the paranasal sinus can manifest as 2 distinct entities- Non-invasive and invasive fungal rhinosinusitis.

Non-invasive fungal rhinosinusitis includes allergic fungal sinusitis, sinus mycetoma/ball, Eosinophilic fungal sinusitis and saprophytic fungal infection (Chakrabarti *et al.* 2009). Diagnostic criteria for Allergic fungal sinusitis include: type 1 hypersensitivity by history, skin tests, or serological testing, nasal polyposis, characteristic findings on computed tomography (CT) scans, eosinophilic mucin without fungal invasion into sinus tissue, and positive fungal staining of sinus contents. Sinus mycetoma/ball is sequestration of fungal hyphal elements within the sinus without any invasive/granulomatous changes and normal nasal mucosa. Eosinophilic fungal sinusitis could be caused by abnormal cell-mediated immunity to fungal proteins with increased levels of IgE in these patients. Saprophytic fungal infection is fungal colonization of the sinonasal tract usually following a surgical procedure or traumatic event that results in inflamed and ulcerated/crusted sinonasal mucosa with the presence of surface fungal infection without tissue invasion.

Invasive fungal rhinosinusitis commonly occurs in patients with diabetes or in immunocompromised and is characterized by its invasiveness, tissue destruction, and rapid onset(Suresh *et al*.2016). Early detection and treatment are vital for these infections because of the high mortality rate. Invasive fungal sinusitis includes the acute fulminant invasive type, the non-granulomatous chronic invasive type, and granulomatous types.

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Fungal Rhinosinusitis is an increasingly recognized entity in India, the commonest form being Allergic Fungal Rhinosinusitis(AFRS). Allergic fungal sinusitis is seen to range in a wide percentage of patients with chronic rhinosinusitis from 5 to 10% in some studies to a much higher percentage in others(Kaur *et al*. 2016) . Majority of reported cases of AFRS are located in more temperate regions where relative humidity is high (Gupta *et al*.2012). The diagnosis of AFRS is a multi-disciplinary approach including the imaging, histopathology, mycology, molecular and immunological investigations.

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University Varanasi comprising a total of 275 clinically suspected cases of fungal rhinosinusitis, including 105 females and 170 males of all age groups attending Otorhinolaryngology Out Patient Department (OPD) clinic at Sir Sunderlal Hospital, Institute, Banaras Hindu University during March 2017 – April 2018. Clinically suspected cases of fungal rhinosinusitis (FRS) patients were included in the study depending on their clinical presentation, nasal endoscopy, radiological evidence from OPD clinic of Department of Otorhinolaryngology, Sir Sunderlal Hospital after obtaining informed consent from the patient. Patients with at least two major or one major and two minor criteria were considered for inclusion as described earlier. Major criteria included Facial pain/fullness, nasal obstruction, postnasal discharge, hyposmia/ anosmia and fever. Minor criteria included Headache, halitosis, fatigue, dental pain, cough, ear pain/fullness. All patients taking or started on antifungal therapy were excluded.

Nasal secretions/swabs were collected from 275 subjects according to their clinical presentations and examined by direct microscopy for fungal elements/hyphae using Gram stain and Potassium Hydroxide (KOH) mount. The sample was inoculated in duplicate on Sabouraud Dextrose Agar (SDA), SDA with chloramphenicol and blood agar plates and incubated at 25 ^{o}C and 37 ^{o}C and checked daily for growth for 7 days, and then twice a week through the second week and once a week till 4 weeks before it was called negative for any growth.

Once growth was observed in culture media plates, it was examined for its colony morphological characteristics like growth rate, the presence of mycelium, color, obverse and reverse of growth or any pigment production.

Lacto-phenol cotton blue (LPCB) mount or Gram stain preparation (as indicated) was examined for further identification of the organism. Slide culture was also performed.

Susceptibility of isolates to antifungal drugs was assessed by Disc Diffusion Method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines M51- A (for molds) and M44-A (for yeast).

Total IgE testing was done by taking serum samples from the patients using serum IgE detection kit(Demeditec diagnostics). Serum IgE e”100 IU/ml in an adult patient was considered positive.

Fungal DNA was extracted from clinical samples by in-house standardized conventional lyticase - proteinase K- lysis buffer - phenol-chloroform method. The DNA content and purity of the extracted DNA in TE buffer (i.e. 260/280 ratio and 260/230 ratio) were estimated using spectrophotometer (Nanodrop®). Then, the extracted DNA was stored at -20^{o}C until further used. nested Polymerase Chain Reaction(PCR) was performed using universal panfungal primers internal transcribed spacer(ITS) 1 and ITS 4 in the primary cycle (Table 1) and ITS 1 and ITS 2 in nested cycle. (Table 2).

25.0 µl master mix for PCR was prepared using 2.5 µl Taq 10X buffer, 2 µl 10 mM dNTP mix, 0.33 µl Taq Polymerase (3U/ µl), 1 µl (10 pmol) forward and reverse primers {GeNei, Merck} along with 5 µl extracted DNA as template for the first cycle and 1 µl of first-round PCR product for second round nested PCR.

Using thermal cycler (Bio-Rad, USA), the reaction mixture was subjected to 10mins of initial denaturation at 95°C, followed by 40 cycles consisting of 45 secs of denaturation at 95°C, 45 secs of annealing at 48°C and 51°C respectively for first and second round PCR and 90 secs of extension at 72°C, followed by 10 mins of final extension at 72°C. The amplified PCR product (10

µl) was analyzed by electrophoresis in a 2% agarose gel (Hi-Media, RM 273) stained with ethidium bromide with Tris Borate EDTA (TBE) buffer (Fig. 2).

The PCR amplification of the human β-globin gene sequence was employed as an internal control to assess extraction of adequate amplifiable DNA and the absence of PCR inhibitory substances in the extracted DNA. The DNA extraction and PCR protocol were optimized using various fungal reference strains.

DNA sequence analysis was carried out at AgriGenome for the nested PCR amplicons obtained from 2 clinical samples which were both culture and PCR positive for standardization and validation of PCR protocol and all 14 clinical samples which were culture negative and PCR positive for fungal identification.

RESULTS AND DISCUSSION

The study comprised a total of 275 subjects with 170 males (61.8%) and 105 females (38.2%).

Male:female ratio=1.6:1.The subjects were divided into six age groups, with the maximum number of patients in the age group of 21-25 years, followed by 16-20 years(Fig1). Majority of patients suffering from rhino-sinusitis were agricultural workers (28%). Others being housewives (24%), students (21%), businessman (16%) and office workers (11%). Rhino-sinusitis was found to be most commonly reported in months of July – October.

Table 1: Primers for primary cycle PCR for fungal detection

Forward	ITS 1	TCCGTAGGTGAACCTGCGG
Reverse	ITS 4	TCCTCCGCTTATTGATATGC

Table 2 : Primers for nested cycle PCR for fungal detection

Forward	ITS 1	TCCGTAGGTGAACCTGCGG
Reverse	ITS 2	GCTGCGTTCTTCATCGATGC

Inferior turbinate hypertrophy (21.1%) and nasal polyps (17.4%) were the most important associated findings among subjects (Table 3).

Nasal discharge (95%) was the most common presenting complaints amongst patients with fungal rhinosinusitis, 82.9% presented with nasal obstruction and 78.9% presented with headache with unknown etiology (Table 4).

On Gram staining, fungal elements were seen in 18/275 (6.54%) nasal secretions/swabs, all of which were culture positive. The KOH mount was positive for fungal elements in 27/275 (9.8%) nasal secretions/swabs, out of which 23 samples were culture positive and 4 were culture negative. (Table 6).

A total of 38 out of 275 nasal secretions/swabs (13.8%) yielded fungal growth on culture. The fungal isolates were enlisted in Table 5.

Table 3: Associated findings in different subjects of Rhinosinusitis

Findings	OPD subjects (Nasal secretions/swabs) (n=275)	Fungal etiology detected (n=52)	Non -Fungal rhinisinusitis (n=223)	Statistical significance (sig/non-sig)
Nasal Polyps	48 (17.4%)	28 (53.8%)	20 (9%)	p< 0.05 sig
Diabetes mellitus	3 (1.1%)	2 (3.8%)	1 (0.4%)	P<0.05 sig
DNS	22 (8%)	10 (19.2%)	12 (5.4%)	P<0.05 sig
Inferior turbinate hypertrophy	58 (21.1%)	28 (53.8%)	30 (13.5%)	P<0.05 sig
Use of nasal decongestants	5 (1.82%)	1 (1.9%)	4 (1.8%)	P=0.95 Not sig
Family history of allergy	20 (7.27%)	11 (21..2%)	9 (4%)	P<0.05 sig
No significant findings	167 (60.7%)	0	167 (74.9%)	

Table 4: Clinical presentations in cases of Rhinosinusitis

Clinical presentation (signs and symptoms)	Total cases of rhinosinusitis (n=275)	Fungal rhinisinusitis (n=52)	Non-Fungal rhinisinusitis (n=223)	Statistical significance (sig/non-sig)
Nasal discharge	262	52	210	p< 0.05
	(95.3%)	(100%)	(94.2%)	sig
Nasal obstruction	228	52	176	P<0.05
	(82.9%)	(100%)	(78.9%)	sig
Headache	217	32	185	P<0.05
	(78.9%)	(61.5%)	(83%)	sig
Anosmia /hyposmia	166	52	114	P<0.05
	(60.4%)	(100%)	(51.1%)	sig
Allergy to dust, pollen, perfumes etc	200	50	150	P<0.05
	(72.7%)	(96%)	(67.3%)	sig
Fever	50	19	31	P<0.05
	(18.2%)	(36.5%)	(13.9%)	sig
Ear pain/fullness	88	10	78	P<0.05
	(32%)	(19.2%)	(35%)	sig
Facial pain/Swelling	14	8	6	P<0.01
	(5.1%)	(15.4%)	(2.7%)	sig
Ocular pain /proptosis	5	4	1	P<0.01
	(1.8%)	(7.7%)	(0.45%)	sig

Table 5: Fungal isolates in cases of fungal rhinosinusitis

Fungal isolates identified on culture	Nasal secretions/swabs (n=275)
Aspergillus flavus	15
Aspergillus glaucus	1
Aspergillus spp.	2
Rhizopus spp.	1
Fonsecaea pedrosoi	3
Alternaria spp.	1
Acremonium spp.	2
Fusarium spp	1
Candida albicans	6
Paecilomyces variotii	1
Penicillium spp.	1
Cladosporium spp.	2
Unidentified molds	2
Total	38

Table 6 : Comparision of findings of KOH mount and gram staining with fungal culture isolation

		Total samples Nasal secretions/swabs (n=275)	
		Culture positive (n= 38)	Culture negative (n= 237)
Fungal elements on KOH mount	Positive (n=27)	23	4
	Negative (n=248)	15	233
Fungal elements on Gram stain	Positive (n=18)	18	0
	Negative (n=257)	20	237

~71% of isolates were resistant to fluconazole, ~21% resistant to itraconazole, ~12% resistant to amphotericin B, and 5 % resistant to voriconazole.

The bacterial isolates (n=21) obtained on culture in 21/309 clinical samples were Coagulase

Table 7 : Sequence analysis

Fungal species identified by DNA sequencing of nested PCR amplicon product of clinical samples	Total no.
Aspergillus fumigatus	1
Aspergillus aculeatus	1
Aspergillus fischeri	2
Aspergillus oryzae	2
Bipolaris sorokiniana	2
Aspergillus campestris	2
Penicillium digitatum	1
Scedosporium apiospermum	2
Penicillium arizonense	1

Table 8 : Comparative chart of all the test performed used for assessment of statistical parameters

		Fungal etiology	Nonfungal etiology	Total
KOH	Positive	27 (51.9%)	0	27(9.82%)
	Negative	25	223	248
	Total	52	223	275
Gram stain	Positive	18 (34.6%)	0	18(6.5%)
	Negative	34	223	257
	Total	52	223	275
Culture	Positive	38(73.1%)	0	38(13.8%)
	Negative	14	223	237
	Total	52	223	275
PCR	Positive	52(100%)	0	52(18.9%)
	Negative	0	223	223
	Total	52		275
IgE	Positive	42(80.8%)	198	240(87.3%)
	Negative	10	25	35
	Total	52	223	275

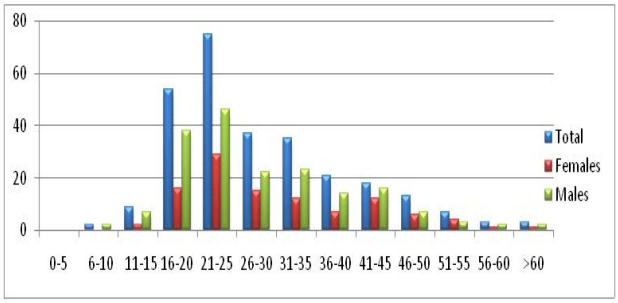

Fig. 1 : Distribution of subjects with respect to AGE AND GENDER in the study

negative *Staphylococci* (CONS) (n=5), *Staphylococcus aureus* (n=5), *Pseudomonas aeruginosa* (n=4), *Klebsiella pneumoniae* (n=3), *Proteus mirabilis* (n=2), *Klebsiella aerogenes/ Enterobacter aerogenes* (n=1) and *Citrobacter freundii* (n=1).

Table 9 : Statistical parameters of various test performed in detection of fungal rhinosinusitis

KOH mount preparation

	Fungal rhinosinusitis (n=52)	Non fungal rhinosinusitis (n=223)
KOH positive (n=27)	27	0
KOH negative (n=248)	25	223

Sensitivity=51.9
Specificity=100
Positive predictive value=100
Negative predictive value=89.9
Accuracy=90.9

Gram Staining

	Fungal rhinosinusitis (n=52)	Non fungal rhinosinusitis (n=223)
Gram staining positive (n=18)	18	0
Gram staining negative (n=257)	34	223

Sensitivity=34.6
Specificity=100
Positive predictive value=100
Negative predictive value=86.8
Accuracy=87.6

Fungal culture Association

	Fungal rhinosinusitis (n=52)	Non Fungal rhinosinusitis (n=223)
Culture positive (n=38)	38	0
Culture negative (n=237)	14	223

Sensitivity=73.1
Specificity=100
Positive predictive value=100
Negativepredictive value=94.1
Accuracy=94.9

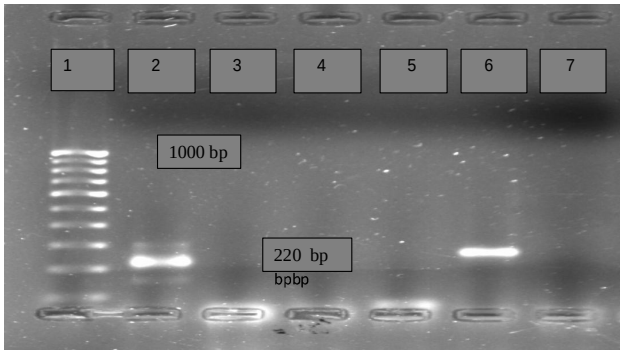

Fig. 2 : Gel electrophoresis of PCR products obtained in nested cycle using universal primers ITS 1 and ITS 2.
LANE1- 100 bp DNA Ladder
LANE 2 –220 bp amplicon
LANE 3,4,5 –Negative samples
LANE 6- Positive control
LANE 7- Negative control

A total of 52 out of 275 (18.9%) of all samples were detected to have fungal etiology by PCR. All 38 samples which were positive on fungal culture were also positive on pan-fungal PCR. But, 14 out of 237 samples which were negative on fungal culture were positive for fungal etiology on pan-fungal PCR.

In this study, 14 samples were obtained in which fungus failed to grow on culture but detected by PCR using pan fungal ITS primers. Four of those 14 samples were positive for the fungal element by KOH.

DNA sequencing analysis of Nested PCR amplicon of these 14 samples yielded identification of fungal pathogens (Table 7).

Serum IgE testing was carried out in nasal secretions. IgE was found to be raised in 42 out of 52 ((80.8%) cases in which fungal etiology was also detected.

In this study, a study subject is considered to be a case of fungal rhinosinusitis if it is either culture or PCR or both positive for fungus. So, in this study, the total number of fungal rhinosinusitis is 52 and, that of nonfungal rhinosinusitis is 223.

The comparative study of all diagnostic modalities used in the study is mentioned in Table 8 and 9.

In a study performed by Chakrabarti *et al.*(2015) FRS was diagnosed in 27.2% of Chronic rhinosinusitis (CRS) cases and 0.11% of the rural population of north India. This implies that 1.1 people per 1000 population suffer from FRS indicating a very high burden of FRS in north India, especially when 0.83 billion people live in rural India. In this study, FRS was diagnosed in 18.9% of cases of rhinosinusitis .The most common fungus isolated was *Aspergillus* (*A. flavus* being the most common species) though *Rhizopus* spp., *Fonsecaea pedrosoi, Acremonium* spp, *Cladosporium* spp. were also isolated in cases of fungal rhinosinusitis. In studies performed in India as well as Saudi Arabia also, *A. flavus* was found to be the most common fungal organism cultured in FRS and our study supported this finding(Kathleen *et al*. 2012).In Delhi-NCR, prevalence of FRS was found to be 26.6% and most frequently isolated fungus was *A.flavus (*Garg *et al*.2013).

In a study done by Pauline *et al*.(2016) DNA detection was performed in 70 consecutive mucosal biopsies/sinus samples using a conventional PCR method targeting the ITS1/ITS2 sequence and the resulting amplification products were sequenced. Fungal CRS was proven in 42 patients (69%), of which only 20 (48%) had a positive culture while PCR was positive in all 42 cases. In our study populations also, PCR was found to more sensitive as compared to culture in our effort towards improvising mycological investigations.

CRS not responding to standard therapy should be investigated for FRS. The present study was conducted to improve the laboratory diagnosis of fungal rhinosinusitis by identification of causative fungi and its correlation with the allergic component of concerned disease and to develop a molecular tool in the form of a nested PCR protocol for detection of fungus in clinical samples from patients presenting with signs and symptoms suspected of rhinosinusitis.

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