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Correlation of Nucleic Acid Amplification Based Detection and Conventional Methods of Identification of *Aspergillus Flavus* Species in Chronic Rhinosinusitis.

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ABSTRACT

This study is an attempt to draw comparison between the traditional and molecular diagnostic tools to facilitate early diagnosis and management of cases of fungal rhinosinusitis. Fungal elements were identified in nasal lavage and polyp samples from chronic rhinosinusitis patients using KOH, culture and histopathological staining and were then subjected to PCR using appropriate primer pairs. *A. flavus* was the predominant fungal isolate. There was an increase in detection rates using PCR as a diagnostic tool in nasal lavage samples as compared to its culture. The same was not true for polyp samples. Thus PCR was definitely more sensitive in nasal lavage samples for the detection of fungal elements. *Aspergillus flavus* was the commonest fungal isolate in cases of CRS. PCR on the nasal polyp/lavage samples shows promising results as it has the ability to detect even minute amounts of DNA if present, in the sample and is rapid. PCR in the nasal lavage samples has undoubtedly shown better and rapid results.

INTRODUCTION

Fungi are ubiquitous in the environment and can coexist within the sino-nasal tract of healthy host as a saprophyte or a commensal. In 10 percent of people they can trigger the development of Chronic rhinosinusitis (CRS) though the exact cause for this is unknown. It is estimated that at any point in time 20 percent of the world population is affected by CRS^[1]. The exact burden of fungal rhinosinusitis in patients with chronic rhinosinusitis in India is not known but the incidence of these infections is increasing and no population based data is available. The fungi which are the cause of hypersensitivity reside in the mucin and provide continued stimulation. The dematiaceous group in the west while *Aspergillus* species particularly *A. flavus* is the most common etiological agent in India and Middle east^[2].

The correct and timely diagnosis of fungal rhinosinusitis is a must as the treatment and prognosis varies significantly. Conventional diagnostic methods may not be accurate and vary in different laboratories, take longer time and often may fail to identify the sparsely present fungal elements in the allergic mucin or tissue.

Polymerase chain reaction (PCR) has thus been applied in an attempt to meet these requirements as documented by many researchers^[3,4,5].

Fungal rhinosinusitis produces a significant burden of disease in India. Though exact data on the burden is not available but a rising trend has been noted. Therefore it becomes essential to utilize nucleic acid based techniques to rapidly detect them. This study is an attempt to draw a comparison between the traditional and molecular diagnostic tools to facilitate early diagnosis of cases of fungal rhinosinusitis.

MATERIALS AND METHODS

A prospective analytical cross-sectional study was undertaken at the departments of Otorhinolaryngology, Microbiology and Pathology of our tertiary care hospital from November 2010 – April 2012. The study was approved by the institutional ethical committee and informed consent was taken from patients before collection of samples. 30 patients, aged ≥ 15 years with clinical signs and symptoms of chronic rhinosinusitis and nasal polyposis for a period of more than 12 weeks were recruited from the Otorhinolaryngology department of the hospital. Patients with any history of intake of antimycotic therapy in last 15 days were excluded from the study. 30 healthy volunteers without any history of nasal/sinus surgery formed the control group. Preoperatively nasal lavage and peroperatively tissue biopsy samples from nasal polyps were taken from the patients and sent in normal saline and formalin to the laboratory of our hospital. Nasal lavage was also taken from the healthy control group.

All samples were processed under laminar flow. Biopsied tissue were cut into small pieces with sterile scissors and a part was homogenized in a mortar by gentle grinding and then inoculated on SDA (Sabouraud Dextrose Agar with antibiotics :chloramphenicol – 0.4 g/L; gentamicin – 0.04 g/L and without cyclohexamide) and incubated at 25°C for 4 weeks with weekly monitoring before being considered as negative for fungal growth. Direct microscopic examination of tissue specimen after digestion with 10 percent potassium hydroxide(KOH) was performed to screen for any fungal elements. Identification of different mycelial isolates was based on the macroscopic characteristics and lactophenol cotton blue microscopic examination of the fungal colonies. Histopathological examination of the tissues using Gomori Methenamine Silver staining was performed to identify fungal hyphae, eosinophils, charcot layden crystals, inflammatory cells and other evidence of tissue invasion^[6]

Further, the tissue and lavage samples were subjected to PCR following DNA extraction using commercially available DNA extraction kit (Invitrogen Purelink Genomic DNA Kit,USA) as per the manufacturers guidelines. The tissue samples were snap frozen in liquid nitrogen to aid lyses. PCR from the extracted DNA was done utilizing the primer pairs as shown below.

Panfungal gene^[7] : Forward primer – 5´ GAGGGCAAGTCTGGTGCCAGC 3´
(501bp) Reverse primer – 5´ CCGATCCCTAGTCGGCATAG 3´
A.flavus gene^[8] : Forward primer – 5´CGACGTCTACAAGCCTTCTGGAAA3´
(200bp) Reverse primer – 5´CAGCAGACCGTCATTGTCTTGTC3´

The panfungal primers targeted the highly conserved regions in members of the fungal kingdom including *Candida*, *Aspergillus*, *Fusarium*, the dimorphic fungi and the Zygomycetes group .The master mix of 25µl for each reaction mixture was prepared with 2.5µl of buffer, 0.75µl(10mM) of dNTP mix, 0.75µl (50mM) of MgCl₂, 0.5µl (5U/µl) Taq DNA polymerase , 1.0µl (100pmol) of each forward and reverse primers (SIGMA,USA) and distilled water to make up the volume. Eppendorf tubes were placed into the thermal cycler (Eppendorf mastercycler Gradient,Germany). Thermal cycling parameters were the following: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1min, annealing at 60°C (Panfungal gene and *Aspergillus flavus* gene) for 45 s, extension at 72°C for 1min and a final extension at 72°C for 7 min.

PCR products were subjected to electrophoresis in 1.5% agarose gels utilizing Tris acetate as running buffer, and visualized by ethidium- bromide staining with detection under UV light.

Statistical data was analyzed by SPSS 17. One way ANOVA test followed by Tukeys test was used for comparative analysis.

RESULTS

A total of 30 patients with clinically suspected CRS having a mean age of 29.06(± 2 S.D) years were included in the study. Majority of the patients (96.66 percent) presented with nasal obstruction. Other common symptoms included nasal discharge (73.33 percent), hyposmia (56.66percent) and headache (53.33percent) .The duration of the presenting symptoms ranged from 3–11 months in 19/30 patients (63.33percent). Rest of the patients were suffering from the stated complaints for more than 1–2 years. 6/30 (20 percent) of the patients gave a past history of sinus surgery.

Fungal profile of patients with Chronic Rhinosinusitis: On examination, 14/30 (46.66 percent) nasal polyp were found positive for hyphae by direct KOH microscopic examination and 13/30(43.33 percent) were positive for culture as well. Out of 14 polyp samples with a positive KOH examination, one alone failed to grow on culture. None of the 30 nasal lavage samples revealed any fungal elements on KOH examination, however in 5 of them fungi were isolated on culture.

Polymerase chain reaction: Fungal DNA was detected in 10/30 (33.33 percent) nasal polyp samples by PCR using panfungal primers. Among these 6 were positive on culture for fungal growth while 4 remained culture negative. 7 of these 10 samples were further detected positive for *Aspergillus flavus* using the species specific primers(Fig 1A&1B).

Figure 1A: Agrose gel electrophoresis showing amplification of representative samples from patients of chronic rhinosinusitis using *Aspergillus flavus* specific primers. Lane M represents 100bp DNA ladder, lane 1 shows amplification of a positive control of size 200bp. Lane 2,5,7,10 show a band at 200bp from nasal polyp samples. Lane 4,9 show a band at 200bp from nasal lavage respectively. Lane 11 shows the negative control.

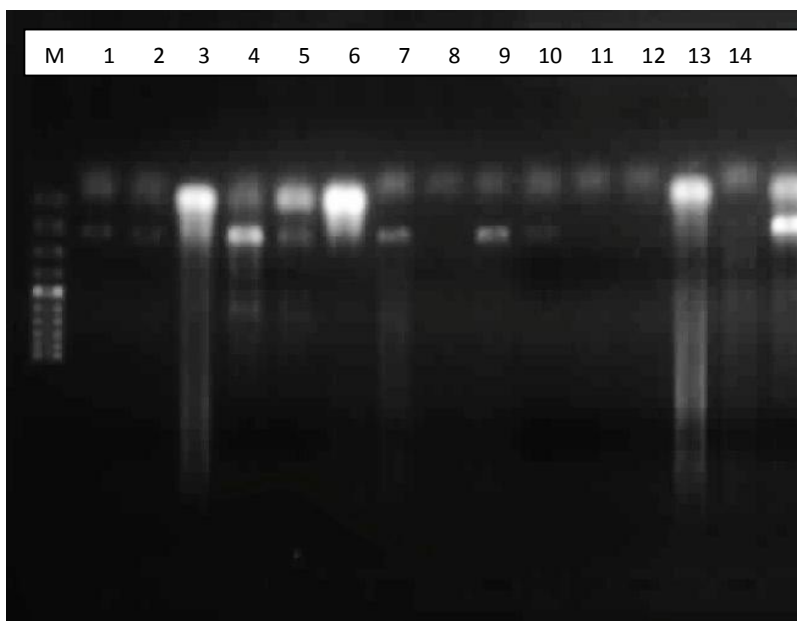
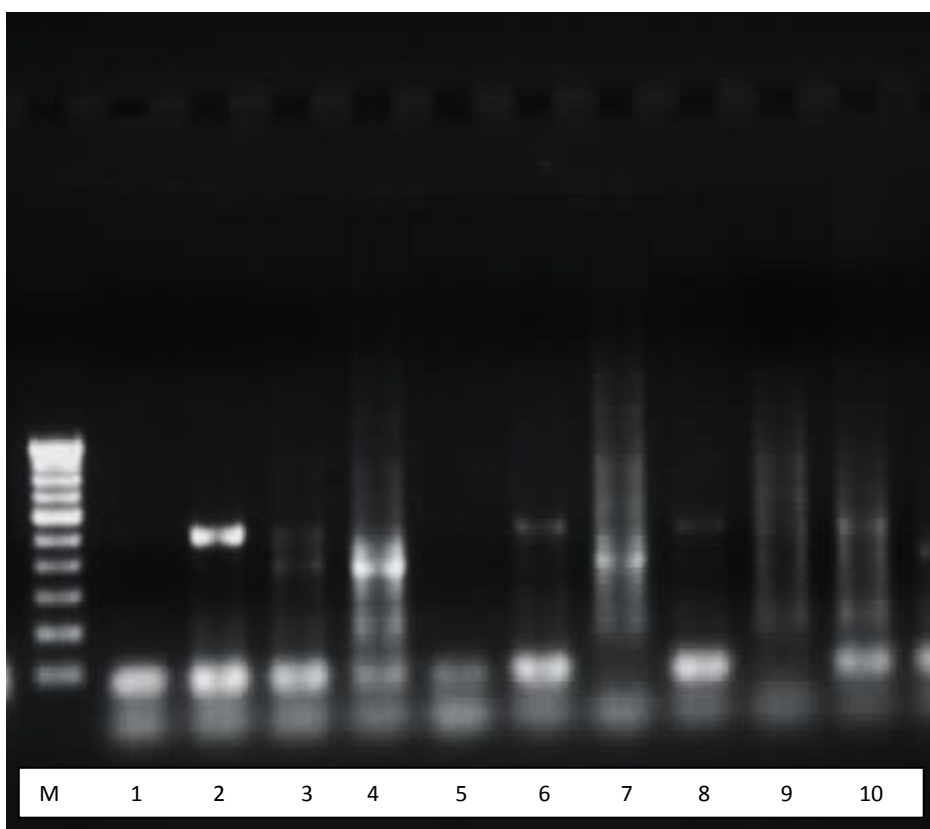


Figure 1B: Agrose gel electrophoresis showing amplification of representative samples from patients of chronic rhinosinusitis using Panfungal primers. Lane M represents 100bp DNA ladder for comparison, lane 1 is the negative control, lane 2 shows amplification of a positive control of size 501bp, lane 3,6 show a band at 501bp from nasal polyp samples and lane 8,10 show a band at 501bp from nasal lavage samples.



The sensitivity and specificity of PCR was 45.45percent and 61.53percent respectively. PCR using Panfungal primers on nasal lavage could demonstrate the presence of fungus in 12 out of 30 samples of which only 4 were positive on culture, 10/ 12 were confirmed as *A. flavus*. Nasal lavage of healthy controls did not reveal any fungal hyphae on direct KOH examination. On culture 7/30 samples of control group were positive for *A. flavus*. The PCR using panfungal primers was also positive in the above 7 control (Table 1) .

Table 1 : Positivity of nasal samples with different diagnostic methods

Sample N=30	KOH	Culture	PCR for panfungal gene	PCR for <i>A.flavus</i> gene
Nasal polyp	14(46.66)*	13(43.33)	10(33.33)	7(70)
Nasal lavage	0	5(16.66)	12(40)	10(83.33)
Nasal lavage from Healthy Control	0	7(23.33)	7(23.33)	-

*Parentheses indicate percentage

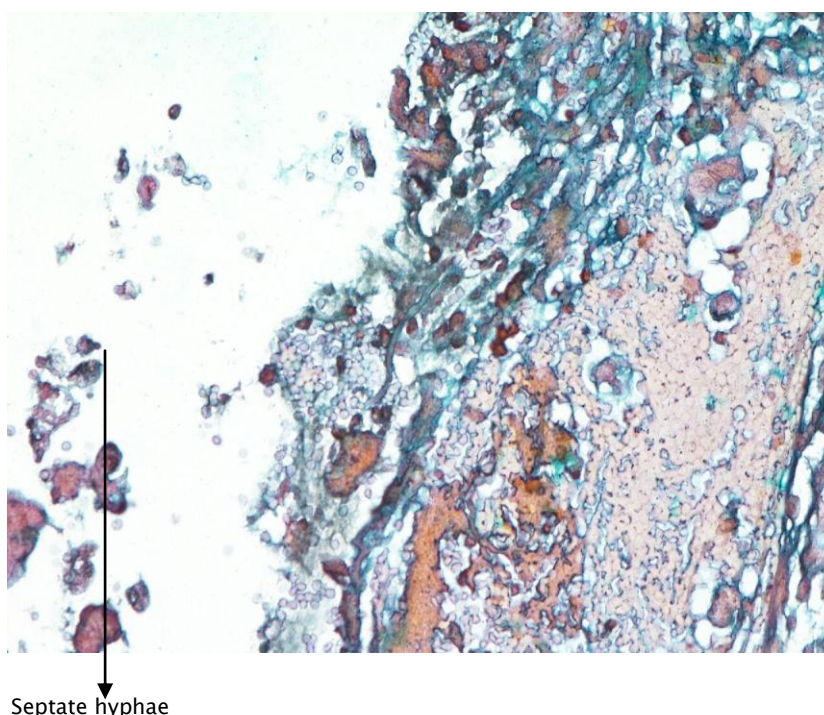
Histopathological profile of patients with CRS is shown in (Table 2: Fig 2)

Table 2: Histopathological profile of nasal polyp tissue in patients of CRS

Histopathological features	N=30
Allergic mucin and fungal hyphae and mixture of sloughed epithelial cells, eosinophils and charcot leyden crystals	6(20%)*
Only Allergic mucin	4(13.33%)
Inflammatory nasal polyps consisting of a mixtures of polymorphonuclear leukocytes, eosinophils, lymphocytes and plasma cells	14(46.66%)
Allergic polyps mixture of mild to moderate eosinophils and few polymorphonuclear leukocytes.	4(13.33%)
Fungus ball aggregates of tightly packed fungal hyphae and occasional inflammatory exudates	1(3.33%)
Cholesterol granules with mild to moderate eosinophils .	1(3.33%)

*Parenthesis indicate percentage

Figure 2: Gorcott–Gomori Methenamine Silver Staining of nasal polyp tissue showing septate hyphae



DISCUSSION

The potential role of fungi in the nose and sinus cavity is unclear. In some it simply exists as a part of normal flora and in others it acts as a pathogen causing Fungal Rhinosinusitis^[9]. Fungal Rhinosinusitis, a disease characterized by fungal colonization of the nose and paranasal sinus, has become an increasingly recognized entity over the past decade. It can be distinguished into noninvasive and invasive forms based on the absence/presence of fungi in sinus mucosa. As the treatment and prognosis of fungal rhinosinusitis is different from its other counter parts it becomes immensely important to correctly diagnose and differentiate fungal from bacterial and other causes of CRS^[3]. This study was thus conducted to compare PCR and fungal culture for identification of fungi in patients with CRS and also to study the frequency of occurrence of *A.flavus* and other fungal agents in CRS.

In contrast to other studies conducted in India by Chakravarti et al which states that fungal sinusitis is more common in young adult men^[10], our study did not show any gender predilection. This is possibly due to more exposure to the external hot dry environment and fungal agents associated with sustained mucosal injury allowing them to infect the paranasal sinus and cause

chronic inflammation. As in other studies done in Iran and Egypt, our cases also presented with nasal obstruction, nasal discharge and hyposmia as the major presenting symptoms^[11,12].

In the present study 46.66 percent and 43.33 percent of the cases were positive on KOH and culture examination of nasal polyp respectively. A slightly higher isolation rate has been observed in other studies^[13,14]. This difference may often be due to released proteins by eosinophils in mucin which are toxic for fungi and can interfere with their growth on culture medium^[11].

10/30(33.33 percent) of the total nasal polyp cases were positive for fungus by PCR using panfungal primer of which 6 could be isolated on culture, and the rest 4 could not be grown. Thus PCR was able to detect additional 4 cases(13percent) that were missed on culture although clinical correlation of the fungi detected as a colonizer or a causative agent of CRS in these patients need to be ascertained. However, the sensitivity (45.45 percent) and specificity(61.53 percent) by PCR was less compared to the culture (81.81 percent; and 76.93 percent respectively). The probable reasons may be attributable to difficult fungal DNA extraction from tissues as compared to other samples and inhibitors of PCR present in tissue samples which can interfere and thus impair detection of the fungi by PCR. These findings are in concordance with study conducted by Kostamo et al where PCR is stated to accelerate the detection of fungi in nasal/sinus samples but may not always increase the accuracy of fungal rhinosinusitis diagnosis^[15,16]. The histopathological presentation of allergic mucin with fungal hyphae was comparable to positivity on fungal culture and PCR.

Nasal lavage samples collected in our study showed 16.66 percent positivity for fungal culture however none were detected with hyphae on KOH. Moreover 4(80percent) of these were also positive on PCR, except one of the sample which was positive on PCR but remained negative on culture. The culture positivity rates of nasal lavage samples were comparable with workers from South Korea, Vienna and NewYork^[5,13,17]. The inability to detect any hyphae in the KOH examination can be due to the minimal quantity of fungal elements present in the nasal lavage samples which further get diluted due to the presence of 20ml of normal saline used for the lavage samples. Despite centrifugation this would make detection of hyphae uncertain.

PCR on the nasal lavage samples had a positivity of 40 percent which was more than that obtained from culture of these samples. This is in concordance with other studies which have also reported an increase in detection rates, using PCR as a diagnostic tool in nasal lavage samples^[3,4,5]. Thus PCR was definitely more sensitive in nasal lavage samples for the detection of fungal elements.

In our study *Aspergillus* was the predominant fungus grown on culture. *A. flavus* constituted majority of the growth from the polyp samples, as also observed with PCR. Absence of dematiaceous fungi or mixed fungal growth was a conspicuous observation in our study, although *A. fumigatus* was grown in one of the samples. The remaining 30percent polyp samples were positive only for panfungal PCR. The finding in our study is not only similar to other studies conducted in India^[18-20] but to studies conducted in Iran and Saudi Arabia, where *A. flavus* has been reported to be the commonest fungal agent causing rhinosinusitis^[11,21]. 7 of the healthy controls also showed the growth of *A. flavus* in their nasal lavage samples indicating that fungi can exist as commensal in the healthy nasal flora. The reason why *A. flavus* predominates in this part of the country as compared to the West where the dematiaceous group predominates could be attributed to hot and humid climatic conditions prevalent in India favouring the growth of the fungus. Studies have shown that *Aspergillus* spores are the commonest airborne fungi in indoor and outdoor environment so exposure to these fungi will be facilitated. Another contributory factor may be related to the type of housing in the warm regions which are often open to the environment and have an open-plan style that can lead to prolonged exposure to fungi^[18].

CONCLUSION

Diagnosing the etiological agent in suspected case of Fungal rhinosinusitis, requires not only a high index of clinical suspicion but a thorough microbiological and pathological work up of the samples. PCR on the nasal polyp/lavage samples shows promising results as it has the ability to detect even minute amounts of DNA, if present in the sample with a short turnaround time. Hence it gives us an attractive and promising alternative to culture. However our results indicate that due to difficulty in fungal DNA extraction from polyps, replacing culture by PCR may not be prudent. But this may not be true for the lavage samples where PCR has undeniably shown better results. This has a definite advantage in patients with CRS where we can use nasal lavage for the early detection of fungal presence.

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