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COMPARISON OF PCR OVER OTHER TECHNIQUES FOR DIAGNOSIS OF FUNGAL RHINOSINUSITIS IN CHRONIC RHINOSINUSITIS PATIENTS.

Microbiology

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ABSTRACT

Fungal rhino sinusitis (FRS) is an important infection of para nasal sinuses, which encompasses two main categories; invasive and non invasive forms according to histopathological findings. Aspergillus spp are the most common species isolated. Given the importance of rapid diagnosis for fungal rhinosinusitis, this study aimed to evaluate the use of PCR for diagnosis of fungal infection in cases suspected with FRS. Seventy six patients suspected to fungal rhino sinusitis were investigated in a cross-sectional prospective study from June 2009 to Sep 2013. All patients under went endoscopic sinus surgery following CT scan. Tissue biopsies were investigated for culture, microscopy, histopathology and PCR. In total, 76 patients were diagnosed with chronic fungal rhinosinusitis (CFR'S) over the 3 years of period of this study. Of total 43(56.58%) were male and 33 (43.42%) female. with a male to female ratio of 1.3:1. Of total 76 patients tested, 42 (55.26%) were positive by at least one of the test. All the patients/specimens were screened by all the methods and the highest positivity was found in PCR with 35.53% (n=27) positivity, followed by culture with 27.63% (n=21) positivity and KOH microscopy with 21% (n=16), while radiology and histopathology detected in 18.42% (n=14) cases. While facial pain was directly associated with FRS. The PCR assay thus provides a rapid and reliable option for laboratory diagnosis of fungal rhinosinusitis. This study demonstrated that PCR could be complementary diagnostic techniques to detect fungi in nasal specimens from CRS patients.

KEYWORDS

FRS, PCR, aspergillus.

Introduction:

One of the most common inflammation in human caused by rhinosinusitis (RS) to the aggravation of nasal and paranasal sinus mucosa due to microscopic organism (bacteria or fungi). In Western industrialized countries approximately 15% of adult population is affected by Chronic rhinosinusitis(CRS).[1] The clinical symptoms and radiological signs of RS infection are the same but treatment varies depending on whether the etiological agent is bacterial, viral or fungal. In recent years especially in North India fungi are remarkable reason for sinusitis and occurrence of such disease. Aspergillus is the most well- known pathogen in contagious rhinosinutis.

Fungal rhinosinusitis has been a known theurapeutic element for a few hundred years yet just in later circumstances the component has been further defined. FRS involve a wide range of process which differ in introduction, histological appearances and clinical importance.

This infectious element is progressively being perceived due to the understanding, better technique of specimen collection, culture of fungi and unusual staining pathological examination. The gold standard method for the diagnosis of FRS, is isolation of the etiologic agent by culture. The sensitivity of culture under normal condition varies depending on the fungal species. Early diagnosis and accurate identification of pathogenic fungal species are crucial for effective treatment and clinical decision-making.[2] Currently, diagnosis of fungal sinusitis still depends on histopathological examination and culture from nasal biopsy, but conventional culture-based phenotypic identification techniques often include significant delays and can fail to yield growth in clinical samples.[3] In a significant number of cases, fungal culture is negative and only formalin-fixed paraffin-embedded (PE) tissue specimens are available for diagnosis of fungal infection.

However, rapid diagnosis of surgical tissues is urgently needed in acute invasive infection cases. In addition, histopathological observations of fungal shape and arrangement may not be sufficient for the accurate identification of fungal species if only a limited quantity of anamorphic fungal hyphae is present. Therefore, to improve the outcome for fungal rhinosinusitis patients, the rapid and accurate detection and identification of pathogenic fungal species are needed to allow early initiation of targeted therapy.

Molecular methods to diagnose fungal infections do not necessarily require the existence of viable organisms, and unlike culture methods, the former can detect very small amounts of the agent in the sample volume. Among the molecular approaches currently available, polymerase chain reaction (PCR)- based techniques have been used to identify fungi in clinical samples. Several studies have reviewed the specificity and sensitivity of molecular methods to detect various types of fungi.

Material and Methods:

In a prospective cross-sectional study conducted between a period from September 2014 to August 2016. 76 patients with complaint of rhinosinusitis were enrolled from Department of ENT, KGMU, Lucknow & Department of ENT Santosh Medical University, Ghaziabad, U.P. and Department of Microbiology, KGMU and Santosh Medical University, U.P. The patients had features of acute rhinosinusitis and nasal mass/polyps in endoscopic examinations with sinus involvement in CT scan findings after obtaining informed consent were taken from each patients included in this study while patients suffering from other diseases like congenital mucocillary disorder, atrophic rhinitis, systemic disease causing problems. All the enrolled patients underwent Functional Endoscopic Sinus Surgery (FESS). Tissue biopsies were taken to evaluate histopathological, fungal culture, PCR and microscopy characteristics of these infections. Antimicrobial susceptibility testing of the fungal isolates were done by disc diffusion (M51-A) and broth micro dilution (M38-A2) methods of CLSI.

All the specimens received were processed by standard methods for fungal culture. The tissue specimens, after mincing into pieces, were subjected to initial screening by 10% potassium hydroxide (KOH) using light microscopy to look for fungal elements (septate or aseptate, hyaline or dematiaceous). Rest of the specimen was inoculated in

duplicate onto Sabouraud dextrose agar with chlormphenicol and incubated at 25°C and 37°C for 4 weeks or until culture positive whichever was earlier. This was followed by slide culture and other special techniques wherever necessary. All histological samples were stained with haemotoxylin and eosin and with periodic acid Schiff staining. If sample was negative for fungi then the gomori methamine silver staining method was done.

DNA extraction & PCR:

Fungal DNA was extracted from the 200mg of tissue samples using ZR fungal/bacterial mini prep by Zymo research, following manufacturer instruction. The optical densities were measured at 260 nm for DNA and 280 nm for proteins. The detection of Fungal DNA was carried out using 28s ribosomal DNA region. Fungal DNA was amplified by conventional PCR assay specific for ~269bp fragment for all the fungal genotype.[4] Details of primers used in amplification of are U1 (5′-GTG AAA TTG TTG AAA GGG AA-3′) and U2 (5′-GAC TCC TTG GTC CGT GTT-3′). PCR amplifications were carried out in 50-µl reaction volumes. Cycling conditions were as follows: initial denaturation at 94°C for 7 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min followed by a final extension phase at 72°C for 10 min. The aliquot of 5 l PCR product of each sample was analyzed by gel electrophoresis in 2% agarose gel after staining with ethidium bromide (0.5 mg/ml, Sigma) along with molecular weight marker (100 bp DNA Ladder; Banglore Gennie, India) and PCR products of positive and negative controls.

Results:

In total, 76 patients were diagnosed with chronic fungal rhinosinusitis (CFR'S) over the 3 years of period of this study. Overall the mean patient age was 33.32±15.16 years (age range 9-65 years). Of total 43(56.58%) were male and 33 (43.42%) female. with a male to female ratio of 1.3:1. Overall, 41 (53.95%) belonged to urban background while 35 (46.05%) belonged to ruler background.

Of the 76 patients tested, 42 (55.26%) were positive by at least one of the test. Of total positives 22 (52.38%) were male and 20 (47.62%) were females. All the patients/specimens were screened by all the methods and the highest positivity was found in PCR with 35.53% (n=27) positivity, followed by culture with 27.63% (n=21) positivity and KOH microscopy with 21% (n=16), while radiology and histopathology detected in 18.42% (n=14) cases. The positivity percentage was higher in rural population (68.9%) as compared to urban population (43.9%). While the positivity was highest in month of November (Figure 1).

Risk factors affecting fungal rhinosinusitis infection in chronic rhinosinusitis were studied. History of blood pressure, respiratory rate, hemoglobin, TLC, Blood sugar were compared between fungal rhinosinusitis and non-infected patients. Other clinical features like nasal obstruction, discharge, facial pain, fever, halitosis etc. None of the feature were clinically associated among fungal infected and non-infected except facial pain. Facial pain was found to be statistically significantly associated with fungal infection, showing high facial pain is associated with fungal rhinosinusitis (Table 1).

Aspergillus flavus was the most common etiological agent in CFRS followed by Aspergillus niger while some rare species such Syzophyllum commune were also reported (Figure 2). The specificity and sensitivity of all the tests as compared to culture showed PCR has the highest sensitivity while histopathology has the highest specificity for the detection of fungal rhinosinusitis. The details are shown in table 2.

Discussion:

Fungal rhinosinusitis is underreported and neglected disorder.[5] Its incidence is now being increasingly reported worldwide.[6] In last 10 years, more than 200 cases had been reported in various studies. Fungal rhinosinusitis is being increasingly recognized in persons of all age groups, resulting in great socio-economic effects, including both direct and indirect costs to the society.[7] All the tissue samples collected from 76 patients suspected for CFR's enrolled during study period. Of the 76 patients tested, 42 (55.26%) were positive by at least one of the test. In the present study the incidence of FRS was 55.26%. Incidence of FRS is in accordance to others studies reporting incidence ranging from 21% to 46.7%.[8-10]

The male to female ratio was 1.3:1 among 42 fungal rhino sinusitis cases. CFR was majorly affecting young adults with mean age 35.29 yrs in our study. Urban and rural preponderance was not significant in our study. Many studies have observed CFR in adult males.[8,11-12] There is previous study from Lucknow, Uttar Pradesh by Prateek et al., 2013 reported CFR incidence in similar age and with slight male preponderance. Majority of fungal rhinosinusitis were referred and positive in winter seasons (November; 42.86%) in our study as obvious fungus grows better at low temperature.

Fungal rhinosinusitis is a great imposter. While on one hand, certain radiological appearances are characteristic for certain forms of the disease, yet there is a significant percentage of cases, wherein the best imaging techniques fail to clinch the diagnosis and warrant the use of nonradiological diagnostic modalities. Of total 76 patients, 42 were positive for fungus either of the technique. PCR has highest positivity 35.53% (n=27) positivity, followed by culture with 27.63% (n=21) positivity and direct KOH microscopy with 21% (n=16), while radiology and histopathology detected in 18.42% (n=14) cases. The specificity and sensitivity of all the tests as compared to culture showed PCR has the highest sensitivity while histopathology has the highest specificity for the detection of fungal rhinosinusitis. Whereas histopathology was found to be least sensitive and culture was least specific technique to diagnose CFR. This detection rate lies within the range of several other investigations.[13-15] PCR molecular assay has been reported to be rapid and high sensitivity method for fungus detection.[14,16-19] Methods like culture and histopathology may take several days to weeks before final results; a molecular diagnostic method (PCR) can expedite the diagnosis of fungus in CFR cases. Thus, PCR increased the rate of fungus detection in nasal polyp considerably, but was not able to replace culture techniques as later is gold standard method. However, non-viable fungus material is generally not detected by culture techniques, but could serve as an appropriate template for PCR provided the material contains fungal DNA. In this respect, PCR adds relevant information to current fungal culture techniques. Molecular methods to diagnose fungal infections do not necessarily require the existence of viable organisms, and unlike culture methods, the former can detect very small amounts of the agent in the sample volume.

Aspergillius flavus (76%) were the most common etiology recovered by any of the PCR or culture followed by Aspergilus niger (9.5%) and Aspergillus fumigates (4.76%). Other fungi like Alternaria spp and Schizophyllum communae was detected in 4.76% of CFR cases. CFR is primarily caused by *Aspergillus* spp. and is mainly found in Africa and Southeast Asia.[8,20-21] In this study majority of patient had non-invasive disease. This is similar to previous studies but different in sub type disorder frequency. In contrast with our study, Challa et al. in South India reported a low frequency of non-invasive fungal sinusitis (25%) vs. invasive form (75%).[22] FB was the most common form that was similar to previous studies. Incidence AFRS was lower than other studies but Panda et al. and Chakrabarti, et al. reported much lower incidence of this type of sinusitis.[23] Aspergillus species are the most common fungal agents of the paranasal sinuses while according to geographical conditions there is a difference between Aspergillus species. A. fumigatus has the highest frequency in some reports but in our study A. flavus had the highest frequency similar to other studies.

Risk factors affecting fungal rhinosinusitis infection in chronic rhinosinusitis were studied. History of blood pressure, respiratory rate, hemoglobin, TLC, Blood sugar were compared between fungal rhinosinusitis and non-infected patients. Other clinical features like nasal obstruction, discharge, facial pain, fever, halitosis etc. None of the feature were clinically associated among fungal infected and non-infected except facial pain. Facial pain was found to be statistically significantly associated with fungal infection, showing high facial pain is associated with fungal rhinosinusitis. Satish et al reported most common presenting complaints of the patients at presentation were nasal obstruction, nasal discharge and chronic headache.[24] In this study, nasal obstruction and nasal discharge were the most common symptoms presenting in 100% cases of FRS followed by headache (71.43%) and facial pain/swelling (57.14%). Lanza and Kennedy (1997)had also stated that nasal obstruction/blockage, nasal discharge, facial congestion and pain as the most prominent presentations.[25] Joshi *et al.,* in their study in Nepal reported most common clinical presentation of the patients with FRS was nasal obstruction of corresponding side with or without headache and nasal discharge.[26] After doing study on 24 patients, Rupa *et al.,* revealed nasal obstruction in 23 cases (96%), nasal secretion (88.7%), postnasal

dripping (82.2%), and cough (69.35%), common to all patients with CRS, thus, it is not important in the suspicion or presence of fungi.[27] In this study also nasal obstruction and discharge was reported as the most common presenting symptoms in all the patients (100%) followed by headache (71.43%) and facial pain/swelling (57.14).

Conclusion

Fungal rhinosinusitis patients present, most commonly, with clinical features of nasal obstruction, nasal discharge and headache, which is indistinguishable from bacterial chronic rhinosinusitis. FRS, where early diagnosis is the key to successful management. Several protocols are available for the detection of fungi in a variety of clinical samples. In the present study we analyzed all the techniques and compared with a PCR method. Diagnosis of FRS should not be based on the single method as every method has its own advantage. FRS should be evaluated by combined molecular, radiological and histopathological modalities in order to avoid both under and over diagnosis. Both false negative and false positive diagnosis should be avoided as treatment of FRS has significant implications for the patient in terms of duration, side effects and cost. Early diagnosis and management can achieve better control over the disease.

Ethical considerations

Ethical clearance was obtained .

Conflict of Interest:

The authors declare no conflict of interests.

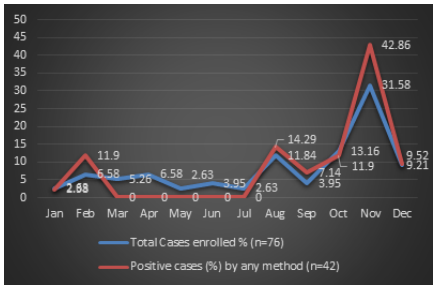

Figure 1: Month wise distribution of cases enrolled & confirmed cases

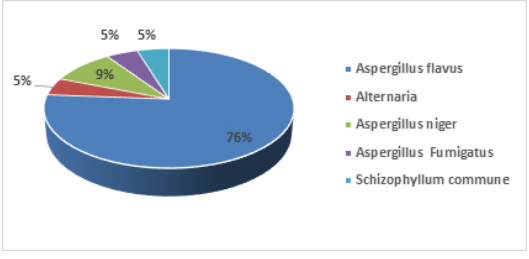

Figure 2: Distribution of culture positive isolates

Table 1.: Association of risk factors with fungal positivity

	Fungus positive	Fungus negative	p -value
Nasal obstruction /blockage	40	31	0.651
No Nasal obstruction /blockage	2	3	
Nasal discharge /purulence/discoloured post nasal discharge	33	26	1
No Nasal discharge /purulence/discoloured post nasal discharge	9	8	
Cachosmia/hyposmia/anosmia	7	6	1
No Cachosmia/hyposmia/anosmia	35	28	
Facial pain/pressure	31	7	0.0001
No Facial pain/pressure	11	27	
Purulence in the nasal cavity on examination	22	11	0.104
No Purulence in the nasal cavity on examination	20	23	
Headache	35	29	1
No Headache	7	5	

Table 2: Specificity and Sensitivity of all the test as compared to culture

	Culture Positive	Culture Negative	Sensitivity	Specificity	PPV	NPV	Accuracy
PCR Positive	15	12	71.43 %	78.18%	55.56 %	87.76%	76.32%
PCR Negative	6	43					
Radiology Positive	7	7	33.33 %	87.27%	50%	77.42%	72.37%
Radiology Negative	14	48					
Histopathology Positive	11	3	52.38 %	94.55%	78.57 %	83.87%	82.89%
Histopathology Negative	10	52					
KOH Microscopy Positive	12	4	57.14 %	92.73%	75%	85%	82.89%
KOH Microscopy Negative	9	51					

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