Original Article

Clinico-pathological Correlation in Diagnosis of Fungal Rhinosinusitis: A One-Year Study

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Abstract

Background. Allergic fungal rhinosinusitis (AFRS), the most common form of fungal rhinosinusitis (FRS) results from an allergy to fungus in immunocompetent patients. There is no consensus on the diagnostic criteria for AFRS and confusion prevails due to difficulty in demonstrating fungal hyphae in the mucin.

Methods. We classified patients with FRS (n=30) using various clinical, histopathological and microbiological parameters. The patients underwent computed tomography of nasal and para-nasal sinuses, absolute eosinophil count and testing of serum immunoglobulin E levels. Fungal elements were identified in nasal lavage and polyp samples from 30 patients with chronic rhinosinusitis using potassium hydroxide (KOH), culture, histopathological examination, polymerase chain reaction (PCR) and were categorised into eosinophilic mucin rhinosinusitis, eosinophilic fungal rhinosinusitis, AFRS and fungus ball categories.

Results. Allergic fungal rhinosinusitis was evident in 5 (20.8%) patients (EMRS 1; EFRS 4, based on histological examination). Diagnosing the aetiological agent in suspected cases of FRS requires not only a high index of clinical suspicion, but a thorough microbiological and pathological work-up of the samples also and should always be supported by computed tomography findings and immunological work-up for atopy as these not only constitute important diagnostic criteria in cases of AFRS, but also are important pre-operative predictor for the condition.

Conclusions. Histopathological examination remains the gold standard for diagnosing chronic FRS but speciation can be possible only with culture or PCR on appropriate samples. The rapid methodology of PCR with appropriate primer pairs has shown promising results in our study and in collaboration with radiological and immunological work-up would provide the complete picture for the diagnosis of FRS.
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Key words: Chronic rhinosinusitis, Allergic fungal rhinosinusitis, Polymerase chain reaction.

Introduction

Fungal rhinosinusitis (FRS), a disease characterised by fungal colonisation of the nose and para-nasal sinus, has become an increasingly recognised entity over the past decade. It was previously thought to contribute 5% to 15% of all the cases of chronic rhinosinusitis; however later data suggest that the burden of FRS seems to be much more.[1] It is categorised as invasive or non-invasive, based on the presence/absence of fungi in sinus mucosa (submucosa, vessels or bone). The invasive disease includes: (i) acute invasive (fulminant) FRS, (ii) granulomatous invasive FRS, and (iii) chronic invasive FRS.[2] The non-invasive forms include: (i) saprophytic fungal infection, (ii) fungus ball, and (iii) eosinophil related FRS [which includes allergic fungal rhinosinusitis (AFRS), eosinophil fungal rhinosinusitis (EFRS) and eosinophilic mucin rhinosinusitis (EMRS)].[2]

Allergic fungal rhinosinusitis is the most common form of FRS.[3] It is defined as a condition in an immunocompetent patient with an allergy to fungus. The fungi reside in the mucin and provide continued stimulation causing a hypersensitivity reaction. It is extremely common in India and a rising trend has been noted, but no population-based data are available.[4] The diagnostic criteria for AFRS vary; the Bent and Khun criteria being most widely accepted.[5] However, uncertainity still prevails due to difficulty in demonstrating fungal hyphae in the mucin. The laboratory findings in possible AFRS group can be variable, making the diagnosis quiet controversial. This one-year study is an attempt to classify cases of FRS using various clinical, histopathological and microbiological parameters.

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Material and Methods

This cross-sectional study was undertaken at the departments of Otorhinolaryngology, Microbiology and Pathology of a tertiary care hospital in East Delhi, India over one-year period. The study was approved by the Institutional Ethics Committee and informed consent was taken from patients before collection of samples. Thirty patients, aged 15 years or more with clinical signs and symptoms of chronic rhinosinusitis with or without nasal polyposis, for a period of more than 12 weeks were recruited from the department of Otorhinolaryngology of the hospital. Diagnosis of chronic rhinosinusitis was based on the guidelines set up by the Rhinosinusitis Task Force in 2003 (Table 1).[6] Patients with any history of intake of antifungal treatment in the last 15 days were excluded from the study. Pre-operatively nasal lavage and per-operatively tissue biopsy samples from nasal polyps were obtained from the patients and sent in normal saline and formalin to the laboratory of the hospital. Patients baseline investigations included computed tomography (CT) of nasal and para-nasal sinuses and venous blood samples for absolute eosinophil count (AEC) and serum immunoglobulin E (IgE) levels (Calbiotech IgE ELISA kit, Spring Valley, CA, USA). Nasal lavage and biopsy tissues collected were processed under laminar flow and cultured on Sabouraud dextrose agar with antibiotics and cyclohexamide and incubated at 25 ºC for four weeks with weekly monitoring before being considered as negative for fungal growth. Direct microscopic examination of specimen after digestion with 20% potassium hydroxide (KOH) was performed to screen the 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. The tissue and lavage samples were subjected to polymerase chain reaction (PCR) following deoxyribonucleic acid (DNA) extraction using commercially available DNA extraction kit (Invitrogen Purelink Genomic DNA Kit, Carlsbad, CA, USA) as per the manufacturer's guidelines. PCR from the extracted DNA was done utilising the primer pairs for Panfungal gene[7] and *Aspergillus flavus* gene[8] (Table 2) at a standardised protocol[9] which consisted of initial denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C (for both the genes) for 45 seconds, extension at 72 °C for 1 minute and a final extension at 72 °C for 7 minutes of 25μL master mix which consisted of 2.5μL of buffer, 0.75μL (10mM) of deoxyribonucleotide triphosphate (dNTP) mix, 0.75μL (50mM) of magnesium chloride ($MgCl_2$), 0.5μL (5U/μL) thermophilus aquaticus (Taq) DNA polymerase 1.0μL (100pmol) of each forward and reverse primers (SIGMA, St. Louis, USA) and distilled water to make up the volume for each reaction mixture. Eppendorf tubes were placed into the thermal cycler (Eppendorf mastercycler Gradient, Hamburg, Germany) and the PCR products obtained were subjected to electrophoresis in 1.5% agarose gel utilising tris acetate as running buffer, and visualised by ethidium-bromide staining with detection under ultraviolet (UV) light (Figures 1 and 2).

Table 1. Factors associated with diagnosis of chronic rhinosinusitis*

Major Factors	Minor Factors
Facialpain/pressure	Headache
Nasal obstruction/blockage	Fever
Nasal discharge/purulence/ discoloured postnasal drainage	Halitosis
Hyposmia/Anosmia	Fatigue
Purulence in nasal cavity on examination	Dentalpain
	Cough
	Ear pain/pressure/fullness

*Chronic rhinosinusitis was diagnosed if ≥2 of the above major factors OR 1 major factor + 2 minor factors OR nasal purulence on examination for a duration of ≥12 weeks were present

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey test was applied for multiple comparisons. Statistical Package for Social Sciences (SPSS version 17) was used for statistical analysis.

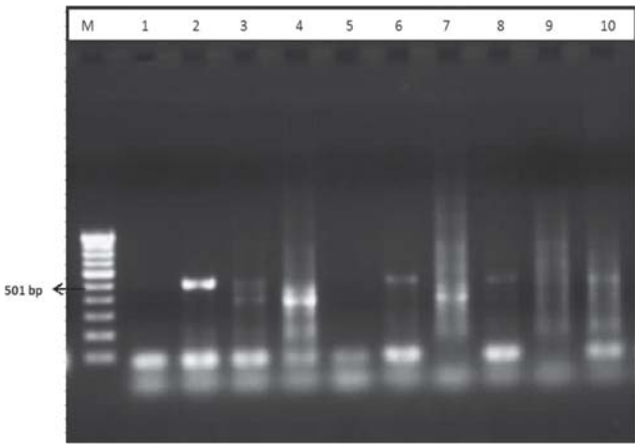

Figure 1. Presence of fungal DNA in chronic sinusitis patients using Panfungal primer pair as shown by gel electrophoresis. Lane M represents 100bp DNA ladder. Lanes 6,8,10 represent sample positive for Panfungal specific gene in DNA extracted from Nasal polyp with band showing at 501bp. Lanes 1 and 2 represent negative and positive controls, respectively.

Definitions of abbreviation: DNA=Deoxyribonucleic acid

Table 2. Primer pairs used for polymerase chain reaction

	Forward Primers	Reverse Primers
Panfungal primers (501 bp)	5′GAGGGCAAGTCTGGTGCCAGC 3′	5′CCGATCCCTAGTCGGCATAG 3′
A. flavus primers (200 bp)	5′CGACGTCTACAAGCCTTCTGGAAA 3′	5′CAGCAGACCGTCATTGTTCTTGTC 3′

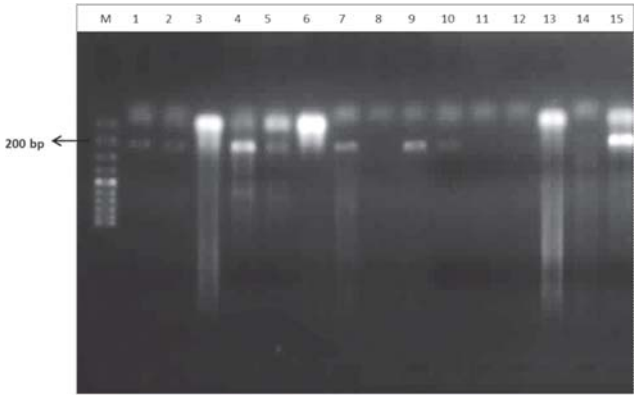

Figure 2. Presence of fungal DNA in chronic sinusitis patients using A.flavus species specific primer pair as shown by gel electrophoresis. Lane M represents 100bp DNA ladder. Lanes 1,2,4,5,7,9 represent sample positive for *A. flavus* specific gene in DNA extracted from Nasal polyp with band showing at 200 bp. Lane 14 and 15 represent negative and positive controls, respectively.

Definitions of abbreviations: DNA=Deoxyribonucleic acid; *A. flavus=Aspergillus flavus*

Results

Out of 30 clinical subjects, 24 had chronic rhinosinusitis symptoms with nasal polyps (80%) while the remaining six were without nasal polyps (20%). Majority of the patients (n=29) presented with nasal obstruction. Other common symptoms included nasal discharge (n=22, hyposmia (n=17) and headache (n=16). The duration of the presenting symptoms ranged from 3 to 11 months in 19/30 patients while the rest of the patients were suffering from the above-stated complaints for more than one to two years. Six of the 30 patients gave a past history of sinus surgery. History suggestive of asthma was associated in 2/30 patients and they also had a strong family history linked to nasal polyposis. None of the subjects had any history of aspirin intolerance in our study. Eleven of the 24 patients of chronic rhinosinusitis with nasal polyposis had FRS. Fungal profile and histological features of the patients are shown in tables 3 and 4, respectively.

Table 3. Histopathological profile of nasal polyp tissue in 24 patients with CRS

Histopathological Features	No.
Allergic mucin and fungal hyphae	6
Only allergic mucin	4
Inflammatory nasal polyps	8
Allergic polyps	4
Fungus ball	1
Cholesterol granules	1

Definition of abbreviation: CRS=Chronic rhinosinusitis

On histopathological examination of the samples of patients with AFRS, 4 of 6 patients showed the presence of allergic mucin and fungal hyphae on special staining of the tissue sections which is characteristic of the condition (Figure 3), one had a granulomatous reaction which may indicate a progression to chronic granulomatous type of fungal rhinosinusitis and 1 fulfilled all diagnostic criteria for AFRS except presence of fungal hyphae. This characteristic clinical appearance of AFRS without detectable fungi has been categorised as EMRS (Figure 4).

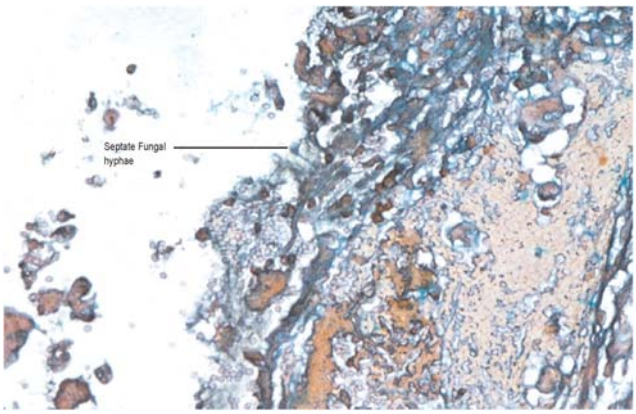

Figure 3. Photomicrograph of nasal polyp tissue showing septatehyphae within allergic mucin in a patient with AFRS (Gorcott-Gomori Methamine Silver×100).

Definitions of abbreviations: AFRS=Allergic fungal rhinosinusitiss

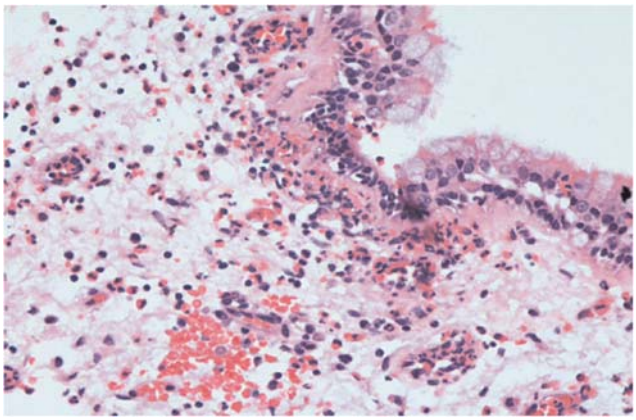

Figure 4. Photomicrograph of nasal polyp tissue showing eosinophilic (allergic) reaction with intact basement membrane as seen in EMRS (Haematoxylin and Eosin×100).

Definitions of abbreviations: EMRS=Eosinophilic mucin rhinosinusitis

Table 4. Correlation between clinical and laboratory parameters

S. No.	KOH	Culture	PCR	KOH	Culture	PCR	S. IgE# (IU/mL)	Nasal Polyposis	Allergic Mucin	Fungal Stain	Clinical Categories*	Histopathological Diagnosis**
1.	+	+	+	–	–	–	120	+	–	–	A	Saprophytic colonisation
2.	+	+	+	–	–	–	440	+	+	+	B	AFRS
3.	+	+	+	–	–	–	80	+	+	+	A	EFRS
4.	–	–	–	–	–	+	660	+	–	–	B	Saprophytic colonisation
5.	+	+	+	–	–	–	350	+	+	+	B	AFRS
6.	–	–	+	–	–	–	90	+	–	–	A	Saprophytic colonisation
7.	–	–	–	–	–	–	100	+	–	–	C	Saprophytic colonisation
8.	–	–	–	–	–	–	60	+	–	–	C	Saprophytic colonisation
9.	+	+	+	–	–	–	90	+	–	–	A	Saprophytic colonisation
10.	+	+	+	–	–	–	400	+	–	–	B	Saprophytic colonisation
11.	–	–	+	–	–	+	10	+	–	–	A	Saprophytic colonisation
12.	ND	ND	ND	–	+	+	80	–	ND	ND	A′	
13.	ND	ND	ND	–	+	+	300	–	ND	ND	B′	
14.	ND	ND	ND	–	–	+	60	–	ND	ND	A′	CRS without nasal polyp
15.	ND	ND	ND	–	–	+	75	–	ND	ND	A′	
16	ND	ND	ND	–	–	–	120	–	ND	ND	A′	
17.	+	+	+	–	–	+	400	+	+	+	B	AFRS
18.	+	+	+	–	–	+	420	+	+	+	B	AFRS
19.	+	+	+	–	+	+	425	+	+	–	B	EMRS
20.	–	–	–	–	–	+	320	+	–	–	B	Saprophytic colonisation
21.	–	–	+	–	–	–	20	+	–	–	A	Saprophytic colonisation
22	+	+	+	–	–	–	450	+	+	+	B	**AFRS**
23.	–	–	–	–	–	–	130	+	+	+	C	**EFRS**
24.	–	–	–	–	–	+	55	+	–	–	A	Saprophytic colonisation
25.	+	+	+	–	–	–	95	+	+	+	A	**EFRS**
26.	+	+	+	–	–	–	475	+	–	+	B	**FUNGAL BALL**
27.	+	+	+	–	–	–	55	+	–	–	A	Saprophytic colonisation
28.	+	–	+	–	+	+	95	+	–	–	A	Saprophytic colonisation
29.	ND	ND	ND	–	–	+	425	–	ND	ND	B′	CRS without nasal polyp
30.	+	+	+	–	–	–	25	+	+	+	A	**EFRS**

*A=CRScNP without atopy with fungus on culture and/or PCR (N=11); *B=CRScNP with atopy and fungus on culture and/or PCR (N=10); *C=CRScNP without atopy and fungus (N=3); *A′=CRSsNP without atopy (N=4) and *B′=CRSsNP with atopy (N=2). #Serum IgE cut-off values: Male=≤ 250 IU/mL; Female=≤ 175IU/mL

Definition of abbreviations: +=Positive; –=Negative; ND=Not done; PCR=Polymerase chain reaction; **AFRS=Allergic fungal rhinosinusitis; **EFRS=Eosinophilic fungal rhinosinusitis; **EMRS=Eosinophilic mucin rhinosinusitis; CRScNP=Chronic rhinosinusitis with nasal polyp; CRSsNP=Chronic rhinosinusitis without nasal polyp

Twelve patients had raised total serum IgE levels with the mean serum IgE value of 241.2±183.5; p<0.001) showing a significant association of atopy with chronic rhinosinusitis; 21/30 patients had an elevated blood eosinophil count. The mean value of absolute eosinophil count (426.5±183.5) did not show any significant association (p=0.319) with CRS. In 13 cases, all the sinuses were involved, while six had involvement of only maxillary sinus and two had ethmoid sinus. Unilateral involvement was seen in 16/30 and bilateral involvement in 14/30 of the patients. The CT showed characteristic hyperattenuating

signal with sinus opacification in 9/30 of the cases (Table 5). No intra-orbital or intra-cranial extension was seen in any of the patients. Based on the above investigations, we divided our patients into five categories who were further categorised into EMRS, EFRS, AFRS and fungus ball on the basis of histopathological findings (Table 4).[1,10]

Table 5. CT findings and score in CRS patients

Patient No.	Sinuses Involved	UL/BL	CT-scan Findings	CT Score
1.	All	BL	Bilateral nasal polyposis	14
2.	All	BL	Hyperattenuated signals	14
3.	All	UL (Rt)	Hyperattenuated signals; erosion of wall	12
4.	Maxillary	UL (Rt)	Unilateral Nasal polyosis	14
5.	All	UL (Rt)	Hyperattenuated signals	7
6.	Ethmoid/Maxillary/Frontal	BL	Bilateral nasal polyosis	6
7.	All	UL (Rt)	Unilateral nasal polyps	7
8.	All	UL (Rt)	Unilateral nasal poyposis	7
9.	All	BL	Bilateral nasal polyps	14
10.	All	BL	Bilateral nasal polyps	14
11.	Maxillary	UL (Rt)	Unilateral nasal polyp extending into nasal cavity	4
12.	All	BL	Hyperattenuated signals; sclerosis	16
13.	Maxillary/Frontal	BL	Hyperattenuated signals	8
14.	Maxillary/Frontal/Sphenoidal	BL	Mucosal thickning	16
15.	All	UL (Lt)	Mucosal thickening	14
16.	Maxillary/Ethmoidal	BL	Mucosal thickening	8
17.	All	UL (Rt)	Hyperattenuated signals	9
18.	Maxillary/Frontal/Ethmoidal	BL	Bilateral nasal polyps	12
19.	Maxillary	UL (Rt)	Hyperattenuated signals; erosion of medial wall	4
20.	Ethoidal/Maxillary/Frontal	UL (Rt)	Unilateral nasal polys	12
21.	Maxillary/Sphenoid/Ethmoid	UL (Lt)	Unilateral nasal polyps with sinusitis	6
22.	Ethmoid	UL (Rt)	Right ethmoidal polyps	2
23.	Ethmoid	UL (Rt)	Hyperattenuated signals	2
24.	Ethmoid/Maxillary/Frontal	BL	Bilateral nasal polyps	10
25.	Maxillary	UL (Lt)	Left-sided nasal polyps	2
26.	All	BL	Hyperattenuated signals; sclerosis of ethmoid sinus	16
27.	Maxillary	UL (Lt)	Maxillary sinusitis present	2
28.	All	BL	Bilateral sinonasal polyps	14
29.	Maxillay	UL (Rt)	Mucosal thickning	2
30.	Frontal/Maxillary	BL	Bilateral sinonasal polyps	8

Definitions of abbreviations: CT=Computed tomography; CRS=Chronic rhinosinusitis; *UL=Unilateral *BL=Bilateral; Rt=Right; Lt=Left

Discussion

Fungal rhinosinusitis though a rare cause of chronic rhinosinusitis is showing a rising trend over the last two decades.[2,11] AFRS constituted 5% to 10% of the total cases of chronic rhinosinusitis with an increase in number of cases in North India.[3,11,12] Diagnosing AFRS can many a times be difficult, as it needs demonstration of fungal hyphae in the mucin which can further be utilised to distinguish EFRS and EMRS. Ferguson[10] suggested that eosinophilic mucin may be present, causing sinusitis without the presence of fungi. Therefore, the laboratory findings in possible AFRS group can be quiet capricious making the diagnosis contentious.

It is extremely important to correctly diagnose and differentiate FRS from chronic bacterial sinusitis and other forms of sinusitis since the treatment and prognosis of these conditions vary significantly.[13] In an attempt to resolve this problem we divided our study group into five categories on the basis of presence or absence of nasal polyp, serum IgE levels and presence or absence of fungus on culture and/or

PCR. Five of the 30 patients with chronic rhinosinusitis were categorised as AFRS in our study, close to a recent report from India in which out of a total of 665 cases of rhinosinusitis, 171 (25.7%) were of non-invasive FRS, which included 160 cases of AFRS and 11 of sinus fungal ball.[12] The characteristic clinical appearance of AFRS without detectable fungi has been categorised as EMRS, but can always be disputed as a technical error in observation and may lead to mis-diagnosis[2], as seen in one of the patients in our study. The total serum IgE levels were found to be raised in all the patients of AFRS, the value of which has been emphasised by Kuhn and Javer,[14] who stated that the total serum IgE levels could be used as a marker to detect disease recurrence[14] and in differentiating AFRS from bacterial infection. CT findings in all patients of AFRS in our study included hyper-attenuating signal caused by inspissiated mucin and fungal hyphae with majority having unilateral involvement of the sinuses (n=16). These observations are similar to the study reported by Mukherji *et al*[15] who also showed predominantly unilateral involvement. CT forms an important predictor in pre-operative cases of AFRS. Dhiwakar *et al*[16] suggested that the combination of nasal polyposis, CT scan, and specific IgE titre has high pre-operative AFRS diagnostic value.

In our study, 36.4% patients with EFRS had normal serum IgE levels but the characteristic allergic mucin had predominance of eosinophils and fungal hyphae. Such a presentation could be explained as fungus being the source of antigenic stimulus responsible for eliciting eosinophilic inflammation suggestive of a type I hypersensitivity reaction. This concept is supported by *in vitro* studies in which peripheral blood mononuclear cells from patients with chronic rhinosinusitis were found to produce large quantities of interleukin-5 and interleukin-13 after exposure to certain fungal antigens. In contrast, peripheral blood mononuclear cells obtained from healthy controls failed to produce the same response. Thus, patients with chronic rhinosinusitis show evidence of sensitisation and immune activation in response to colonising fungi in the sino-nasal tract and this process may be responsible for the production of cytokines that recruit and activate eosinophils in chronic rhinosinusitis. Histopathological examination of polyps showed predominance of inflammatory nasal polyp with polymorphonuclear lymphocytes, plasma cells and mild to moderate eosinophils (33.3%), emphasising the observation of Hao *et al*[17] who stated that the incidence of such fibrotic polyp (mainly accumulation of lymphocytes and neutrophils) was relatively higher in Asian patients with nasal polyposis as compared to the west where there is an eosinophilic predominance. Microbiological work-up of the samples showed nine positive cases with culture or PCR, two positive with both and two were negative for fungus. Thus, in such cases the histological examination of tissue specimen may miss out the scant fungal elements present, however, presence of fungus on culture/PCR in these cannot be ignored. Therefore, multiple sections should be observed for the evidence of fungus histo-pathologically and correlated with culture findings and clinical presentation.

Nasal polyposis is a result of chronic inflammation of the nasal cavity and sinuses regulated by T-lymphocytes and atopy play an important role in its development. It has been recently hypothesised that atopy increases the imbalance of the T helper-17 (Th17) / T regulatory (Treg) and aggravates nasal polyposis through the role of Th17 in atopic inflammation and defective suppression of Treg on Th1 and Th2 also. Interleukin-17, a key cytokine of Th17, plays an important role in nasal polyposis by promoting eosinophilic infiltration and tissue remodeling. However, further immunological studies are required for understanding the contribution of Th1 and Th2 response and their association with Th17/ Treg in the underlying inflammatory process in AFRS.

Conclusions

Diagnosing the aetiological agent in suspected cases of fungal rhinosinusitis requires not only a high index of clinical suspicion, but a thorough microbiological and pathological work-up of the samples. Histopathological examination of nasal polyp and mucin samples is undoubtedly the gold standard for the diagnosis of AFRS but it does not speciate the fungus for which culture of the fungi on appropriate media or a judicious use of PCR with appropriate primer pairs, proves beneficial. PCR on the nasal polyp/lavage samples shows promising results and is an attractive and promising alternative to culture. However, it must be remembered that because of the ubiquitous nature of fungi, demonstration of growth on culture or a positive PCR is not always necessary to implicate the fungus as the cause of chronic rhinosinusitis. The diagnosis of FRS should always be supported by proper CT-scan findings and immunological work-up for atopy as these not only constitute important diagnostic criteria in cases of AFRS, which is one of the most common presentations of FRS, but also are important pre-operative predictor for the condition.

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