Allergic fungal sinusitis: Detection of Universal Fungal, *Aspergillus* and *Bipolaris* DNA in sinus aspirate using polymerase chain reaction and evaluation of *Aspergillus* specific IgE.

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Allergic fungal sinusitis (AFS) is believed to represent a hypersensitivity reaction to fungal antigens. The pathophysiology of AFS is still not clearly understood. It is believed that it is not a true fungal infection, but an allergic response to fungal organisms that have colonized the sinus mucosa and secondarily cause a hypersensitivity reaction in the host. However, some patients with AFS do not have allergy to the fungi identified in their eosinophilic mucous but may have elevated IgE levels to other fungi. The patients are usually atopic to multiple aeroallergens. Early reports noted primarily *Aspergillus* species in allergic mucin, but more recently, the dematiaceous fungi, which include *Bipolaris*, *Curvularia*, *Alternaria* and *Helmenthosporium* species have been identified in most AFS cases. PCR is significantly more sensitive than nasal swabs cultures in detecting the presence of fungi in nasal mucosa. In our study, 68 cases were selected and sinus aspirates were withdrawn whereas a part of the mucous was used for fungal culture and the other part was used for PCR assay for universal fungal, *Aspergillus* and *Bipolaris* DNA. Measurement of *Aspergillus* specific IgE in sinus aspirate and serum total IgE were done. A control group (10 cases) was included. Among the total number of AFS (68), only 42 cases gives positive fungal growth with a percentage of 61.7% while among 10 control cases, only 3 cases gives positive growth with a percentage of 30%. Regarding AFS (42 cases), Dematiaceous family was the most common as it was isolated from 30 cases (71.4%). *Bipolaris* was the most common isolated species (18 cases) followed by *Curvularia* (11 cases) and *Alternaria* (1 case). *Aspergillus* family was isolated from 11 cases (26.1%). *Aspergillus fumigatus* was more common as it was isolated from 8 cases followed by *Aspergillus niger* (3 cases). The results of PCR assay assured the detection of fungal DNA in all cases of AFS group (68 cases) and in 4 cases of control group (40%). *Aspergillus* DNA was detected in 15 cases (22.05%) while *Bipolaris* DNA was detected in 27 cases (39.70%). Ten patients were positive for *Aspergillus fumigatus* specific IgE (14.7%) out of 68 patients and the mean value was 11.32 ±4.12 IU/ml which was significantly higher than the mean value of this specific IgE in our control group which was 0 IU/ml. Also, only 7 patients from the above 10 patients were positive to *Aspergillus fumigatus* by PCR (5 only gives positive culture) and this indicates that 3 patients were negative to *Aspergillus fumigatus* either by culture or PCR but they showed *Aspergillus fumigatus* allergen specific IgE, on the other hand, 8 cases were positive to *Aspergillus fumigatus* by PCR and 3 cases were specific by culture failed to show any *Aspergillus fumigatus* specific IgE indicating that the presence of fungus is not essentially accompanied with allergic process.

**INTRODUCTION**

Fungal sinusitis can be divided into four primary categories: [1]acute/fulminant (invasive), [2]chronic/indolent (invasive), [3]fungus ball, and [4] AFS. Each subtype has unique immunologic, pathologic, and clinical features. AFS is the most common form (1).

Milar et al.,(2) and Katzenstein et al., (3) first described AFS in the early 1980s. Ence et al.,(4) suggest that at least 7% of patients requiring surgery for chronic sinusitis have AFS.

AFS accounts for approximately 6% to 8% of all chronic sinusitis requiring surgical intervention and has become a subject of increasing interest to otolaryngologists and related specialists. Although certain signs and symptoms, as well as radiographic, intraoperative, and pathologic findings, may cause the physician to suspect AFS, no standards have been defined for establishing the diagnosis. It is extremely important to recognize AFS and differentiate it from chronic bacterial sinusitis and other forms of fungal sinusitis because the treatments and prognosis for these disorders vary significantly.(5)

Up to 10% of patients suffering from chronic rhinosinusitis may carry the diagnosis of AFS, and atopy is very common in these patients. The pathophysiological process that has been proposed involves the atopic host being exposed to the fungi, resulting in an inflammatory response (IgE mediated), subsequent tissue edema, obstruction of sinus ostia, sinus stasis, further proliferation of fungus, increased antigenic exposure, etc.,
with a cycle that becomes self-perpetuating. Ultimately, the sinuses become filled with the characteristic allergic mucin that is the surgical hallmark of the disease, and the development of nasal and/or sinus polyposis also may ensue. In the atopic patient, a reaction ensues leading to the development of AFS, whereas in a nonreactive individual, the formation of a fungus ball or “sinus mycetoma” may occur. AFS remains an underdiagnosed condition due to not only a lack of awareness among physicians but also the inability to demonstrate the presence of fungi in many suspected cases. Atopy is characteristic of AFS, roughly two thirds of the patients report a history of allergic rhinitis and 90% show elevated specific IgE to one or more fungal antigens. Approximately 50% of the patients had asthma. Infections caused by Bipolaris/Exserohilum and Aspergillus show many clinical and pathologic similarities despite the lack of taxonomic relationship between these fungi. Both cause disseminated disease in immunocompromised patients that is characterized by tissue necrosis and vascular invasion. Both cause central nervous system disease, osteomyelitis, and sinusitis and are associated with allergic bronchopulmonary disease. Sinusitis, the most common form of disease caused by Bipolaris and Exserohilum, occurs in otherwise healthy patients with nasal polyposis and allergic rhinitis.

The diagnosis of fungal infections remains a significant problem. The clinical presentation is difficult to interpret, and the findings of noninvasive methods (computed tomographic scanning and X ray) are not specific. Culture results are available at the earliest in 2 to 3 days, and blood and deep-tissue sample cultures from infections with focal lesions are frequently negative. Direct microscopy and histopathological examination are rapid, but they do not always allow identification of the infecting agent to the species level. In contrast, even though the latest generation of monoclonal antibody-based enzyme-linked immunosorbent assays (ELISAs) for circulating Aspergillus and Candida antigens are specific, they lack sensitivity. Thus, rapid methods that are sensitive and specific are needed, and PCR has been applied to fulfill these requirements.

In our work, we aimed to compare the detection of fungi in sinus aspirate by culture and PCR assay for universal fungal, Aspergillus and Bipolaris DNA and try to clarify the relation between the presence of fungi in nasal sinus and type of fungal allergen causing the disease. Measurement of Aspergillus fumigatus specific IgE in sinus aspirate and serum total IgE will further clarify this relationship.

**PATIENTS AND METHODS**

A definite AFS diagnosis is done if they met five of the following six criteria: [1] history and physical not suggesting another etiology, [2] sinus computed tomography consistent with allergic fungal sinusitis, [3] typical allergic mucin, [4] fungus isolated from allergic mucin, [5] presence of fungal-specific IgE, and [6] elevated total IgE. Sixty eight cases were selected according to the previous criteria. For all cases, sinus aspirates were withdrawn and placed on a saline-moistened nonstick sheet to avoid absorbing of large part of the mucous which was later collected in 1.5 ml tubes and transferred directly to laboratory whereas part of the mucous was used for fungal culture and the other part was frozen at - 30°C till used for PCR assay and measurement of Aspergillus fumigatus specific IgE. Serum samples from all patients were frozen and used later for measurement of total IgE. All patients were examined for the presence of characteristic fungal mucin and stained smears were done using Giemsa stain. A control group (10 cases) was included, in which no history of nasal or paranasal sinus disease, with no symptoms of inhalant allergy and with normal-appearing mucosa and sinus aspirate was done due to other surgical conditions.

**Culture:**

Sinus aspirates were cultured on Sabaraud dextrose agar, incubated at 25°C for a period ranging from 5 days to 1 month and examined regularly and identified systematically.

**Germ tube test for Bipolaris:**

A drop of distilled water is incubated with a piece of fungus with a cover slide in a covered plate to prevent dryness at room
temperature for 8-24 hours. A drop of distilled water may be added sometimes directly before microscopic examination.

**Assay of total serum IgE:**

Serum IgE were measured by ELISA kit (Elitech diagnostics - France). ELISA reader (Spectra III, Austria) was used.

**Determination of* Aspergillus-fumigatus* specific IgE:**

RIDASCREEN Spezifisches IgE (R-Biopharm AG, Darmstadt, Germany) ELISA kit was used. Fifty µl of each of the five standards, positive and negative controls and sinus aspirates were pipetted into wells containing* Aspergillus fumigatus* allergen discs (after removal of the buffer from wells by suction) and plate was incubated at 37°C for 60 minutes. Wells were washed using washing buffer and 50 µl conjugate were added to each well and incubated at 37°C for 60 minutes. Wash was repeated and 100 µl of allergy substrate were added to each well and incubated at 37°C for 60 minutes. Fifty µl stop solution were added and absorbance was measured using ELISA reader (Spectra III, Austria) at 405/620nm where standard curve was constructed and sinus aspirate* Aspergillus-fumigatus* specific IgE were calculated.

**DNA isolation:**

QiAamp DNA mini kit (QIAGEN Inc., Valencia, CA, USA) was used. Sinus aspirate (usually viscid) was diluted with equal volume of 0.9% sodium chloride and mixed well by vortexing and 200 µl of sinus aspirate was put into microfuge tube and centrifuged at 7500 rpm for 10 min. and the supernatant was discarded and 180 µl buffer ATL were added to the pellet. Twenty µl Proteinase K were added and mixed by vortex. After incubation at 56°C for 1 hour, 200 µl of buffer AL were added and mixed by pulse-vortexing for 15 seconds and incubated at 70°C for 10 min. 200 µl ethanol 100% were added to the sample and mixed by pulse-vortexing for 15 seconds and the mixture was applied to QIAamp spin column and centrifuged at 8000 rpm for 1 min. 500 µl buffer AW1 were added to the QIAamp spin column and centrifuged as before. 500 µl buffer AW2 were added to the QIAamp spin column and centrifuged at 14000 rpm for 3 min.. 200 µl buffer AE were added to the QIAamp spin column and incubated at room temperature for 5 min. and then centrifuged at 8000 rpm for 1 min. and the elute was collected in sterile tubes and stored at -20°C till PCR reaction was done.

**Primers:**

Three primers have been selected (15). Universal fungal primer (ITS) was used to detect any fungus growth in the samples as it amplify a highly conserved region of fungal DNA that is present in most fungi and is not shared with human or bacterial DNA, *Aspergillus* specific primer detects Alkaline protease of both *Aspergillus fumigatus* and *Aspergillus flavus* and *Bipolaris* specific primer which amplifies a sequence of the Brn-1 gene found in all *Bipolaris* species. These primers sequences are shown in table (1).

**Table (1): Synthetic oligonucleotides used in PCR.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5' to 3')</th>
<th>Size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal fungal</td>
<td>ITS3 GCA TCG ATG AAG AAC GCA GC TCC TCC GTC TAT TGA TAT GC</td>
<td>300</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Alp 11 AGC ACC GAC TAC ATC TAC GAG ATG GTG TG TGC TGC GC</td>
<td>747</td>
</tr>
<tr>
<td>Bipolaris</td>
<td>Bipol A73 TCA TGC TGA CAA CGC TCC AG TAC CGA TGG CCA TGG ACC T</td>
<td>518</td>
</tr>
<tr>
<td></td>
<td>Bipol B572</td>
<td></td>
</tr>
</tbody>
</table>
PCR: (15)

Two µl of DNA, 1 µl of sense primer and 1 µl of antisense primer were added to 46 µl of master mix containing 3 µl MgCl2 solution (1.5 mm), 5 µl of 10X PCR buffer, 100 mM Tris HCl (Ph 8.3), 500 mM KCl/µl, 1 µl dNTPs mixture (10 mM/µl), 0.4 µl Ampli Taq DNA polymerase (5 units/µl) all in 36.6 µl distilled water. Using (Norwall, CT, USA) thermal cycler. Initial denaturing step at 94 oC for 3 min., then up to 35 PCR cycles were performed, each consisting of three steps: denaturing step (15 seconds at 94 oC), annealing step (20 seconds at 55 oC) and primer extension step (30 seconds at 72 oC) followed by a final extension step at 72 oC for 5 minutes. Aliquots were taken from each tube and mixed with gel loading buffer and run on 1.5% agarose gel in Tris-borate buffer (54 gm Tris amino methane , 27.5 boric acid and 20 ml 0.5 M EDTA[pH 8.0] per liter, prepared as concentrated x5 stock), stained with ethedium bromide (0.5 mg/ml). Photographs were taken under ultraviolet illumination using digital camera.

RESULTS

Fungal culture:

Among the total number of AFS cases (68), only 42 cases gives positive fungal growth with a percentage of 61.7 % while among 10 control cases, only 3 cases gives positive growth with a percentage of 30%. Regarding AFS which showed positive growth (42 cases), Dematiaceous family was the most common as it was isolated from 30 cases (71.4%). Bipolaris was the most common isolated species (18 cases) followed by Curvularia (11 cases) and Alternaria (1 case). Aspergillus family was isolated from 11 cases (26.1 %). Aspergillus fumigatus was more common as it was isolated from 8 cases followed by Aspergillus niger which was isolated from 3 cases (Table 2, Fig. 1).

Table (2): Distribution of AFS and control cases with positive culture.

<table>
<thead>
<tr>
<th></th>
<th>AFS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Dematiaceous family:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bipolaris</td>
<td>30</td>
<td>71.4</td>
</tr>
<tr>
<td>Curvularia</td>
<td>18</td>
<td>42.8</td>
</tr>
<tr>
<td>Alternaria</td>
<td>11</td>
<td>26.1</td>
</tr>
<tr>
<td>Aspergillus species:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>11</td>
<td>26.1</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>3</td>
<td>7.1</td>
</tr>
<tr>
<td>Mucor</td>
<td>1</td>
<td>2.38</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>100</td>
</tr>
</tbody>
</table>

Total serum IgE:

The mean value of total IgE in AFS group was 665.2 ±219.3 IU/ml which was statistically higher than that of normal control (70.3 ±25.8 IU/ml) (Table 3).

Table (3): Assay of total serum IgE:

<table>
<thead>
<tr>
<th></th>
<th>AFS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total serum IgE</td>
<td>665.2</td>
<td>70.3</td>
</tr>
<tr>
<td>±</td>
<td>219.3</td>
<td>25.8</td>
</tr>
</tbody>
</table>

Aspergillus-fumigatus specific IgE:

Ten patients were positive for specific IgE (14.7 %) out of 68 patients with AFS and the mean value was 11.32 ±4.12 IU/ml which was significantly higher than the mean value of this specific IgE in our control group which was 0 IU/ml. Also, only 7 patients from the above 10 patients were positive to *Aspergillus fumigatus* by PCR (5 only gives positive culture) and this indicates that 3 patients were negative to *Aspergillus fumigatus* either by culture or PCR but they showed *Aspergillus fumigatus* allergen specific IgE (Table 4).
Table (4): *Aspergillus fumigatus* allergen specific IgE in allergic patients to *Aspergillus fumigatus* (10 patients) and control group.

<table>
<thead>
<tr>
<th></th>
<th>Aspergillus fumigatus allergic patients (10)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±</td>
<td>Mean ±</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> allergen-specific IgE (IU/ml)</td>
<td>11.32 4.12</td>
<td>0 0</td>
</tr>
</tbody>
</table>

**PCR assay:**
The results of PCR assay assured the detection of fungal DNA in all cases of AFS group (68 cases) and in 4 cases of control group (40%). *Aspergillus* DNA was detected in 15 cases (22.05%) while *Bipolaris* DNA was detected in 27 cases (39.70%) (Table 5, Fig. 2-4).

Table (5): PCR amplification of fungal DNA, *Aspergillus* and *Bipolaris* in AFS and control cases.

<table>
<thead>
<tr>
<th>Type of PCR amplified</th>
<th>AFS</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Universal fungal (fungal DNA)</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td>15</td>
<td>22.05</td>
</tr>
<tr>
<td><em>Bipolaris</em></td>
<td>27</td>
<td>39.70</td>
</tr>
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</table>

Table (6): Distribution of cases showing *A. fumigatus* -specific IgE and its relation to presence of *A. fumigatus* by culture and PCR (18 positive cases only are shown from 68 cases).

<table>
<thead>
<tr>
<th>Cases</th>
<th><em>A. fumigatus</em> (culture)</th>
<th><em>A. fumigatus</em> (PCR)</th>
<th><em>A. fumigatus</em> -specific IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>5</td>
<td>+</td>
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<td>6</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>8</td>
<td>+</td>
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<td>9</td>
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<td>+</td>
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<td>13</td>
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<td>+</td>
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<tr>
<td>17</td>
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<td>+</td>
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<tr>
<td>18</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. (1): (Left) Macroscopic appearance of *Bipolaris* colonies which grows rapidly and become mature within 7 days. The colony is wooly olive green with raised grayish periphery and depressed center and the reverse is also darkly pigmented and olive to black in color. (Right) Microscopic appearance of *Bipolaris* germ tube test after 8 hours showing germ tubes at one or both ends along the conidial axis. The typical picture of *Bipolaris* are seen including elongated conidiophore, excess conidia which are dark cylindrical with 3-5 transverse septa.

Fig. (2): The ethedium bromide staining pattern of agarose gel (1.5%) electrophoresis of Universal fungus DNA. All Lanes (1-8) show 300 bp bands.
Fig. (3): The ethidium bromide staining pattern of agarose gel (1.5%) electrophoresis of *Aspergillus* DNA. Lanes 1,3,4,6 show 747 bp bands.

Fig. (4): The ethidium bromide staining pattern of agarose gel (1.5%) electrophoresis of *Bipolaris* DNA. Lanes 1,2,11 show 518 bp bands.

**DISCUSSION**

AFS is believed to represent a hypersensitivity reaction to fungal antigens. The pathophysiology of AFS is still not clearly understood. It is believed that AFS is not a true fungal infection, but an allergic response to fungal organisms that have colonized the sinus mucosa and secondarily cause a hypersensitivity reaction in the host. The patients are usually atopic to multiple aeroallergens.

Matsuwaki et al., prospectively studied the incidence, pathogenesis and diagnosis of AFS in Japan among 102 patients with chronic sinusitis who underwent endoscopic sinus surgery. Four cases (3.9%) were diagnosed with AFS based on the criteria of Bent and Kuhn. This incidence is slightly lower than that reported in Europe and the United States. They concluded that IgE antibody-mediated type I allergy may be involved in the pathogenesis of AFS. Allergic mucin was found in 97 (96%) of 101 consecutive surgical cases of chronic rhinosinusitis. AFS was diagnosed in 94 (93%) of chronic rhinosinusitis cases based on histological findings and culture results. This 93% incidence of AFS in chronic rhinosinusitis is considerably higher than the incidence reported in previous retrospective reviews.

In our work, we evaluated the incidence of fungal colonization of nasal sinuses by culture method and among the total number of AFS (68), only 42 cases gives positive fungal growth with a percentage of 61.7 % while among 10 control cases, only 3 cases gives positive growth with a percentage of 30%. Regarding AFS (42 cases), Dematiaceous family was the most common as it was isolated from 30 cases (71.4%). *Bipolaris* was the most common isolated species (18 cases) followed by *Curvularia* (11 cases) and *Alternaria* (1 case). *Aspergillus* family was isolated from 11 cases (26.1%).
Aspergillus fumigatus was more common as it was isolated from 8 cases followed by Aspergillus niger which was isolated from 3 cases.

Because fungi are found nearly everywhere in nature and form airborne spores, it is reasonable to assume that most people have some level of fungal colonization in their nasal cavity. Ponikau et al., found that all 14 (14%) volunteers with absolutely no chronic rhinosinusitis did in fact culture fungi from the nose using an irrigation technique. Their study further demonstrated no difference in the spectrum of fungi isolated between normal volunteers and patients with chronic rhinosinusitis. Using a sensitive assay. Unfortunately, because 100% of the control group also had fungi present, its presence alone is insufficient to implicate it as a pathogen in sinusitis. While the sensitivity of the irrigation assay proves convincingly the ubiquitous nature of fungi, it fails to show true pathogenesis of the fungi present. This study although found a very high percentage of fungi in control cases is in accordance with us regarding that the presence of fungi in sinus is not essentially a pathogenic process.

Our results regarding incidence and species of fungal growth concur that of Manning and Holman. In their study, 263 cases of AFS were identified, of which 168 cases yielded positive fungal cultures. Of those 168 positive cultures, 87% were dematiaceous genera including Bipolaris, Curvularia, Exserohilum, Alternaria, Drechslera, Helminthosporium and Fusarium, whereas only 18% yielded Aspergillus.

Early reports noted primarily Aspergillus species in allergic mucin, but more recently, the dematiaceous fungi, which include Bipolaris, Curvularia, Alternaria and Helminthosporium species have been identified in most AFS cases and this is in agreement with our high incidence of dematiaceous fungi. Also our work yielded only single species for every case and this is similar to Feger et al., who indicated that fungal cultures from allergic mucin are often, but not always, positive with a single species isolated in most cases.

However, Castelnuovo et al., studied 45 cases (4.3%) out of a total of 1050 patients who had undergone endoscopic surgery for sinusopathy. Following the Katzenstein classification, the cases were broken down into non-invasive chronic mycoses or fungus ball (34 cases), allergic mycoses (7), chronic indolent invasive mycoses (3) and fulminating invasive mycosis (1 case). The mycetes most often involved was Aspergillus fumigatus but this author isolated fungi from chronic sinusitis cases and not only AFS and that is why they isolated a higher incidence of Aspergillus species.

Our results concerning the isolation of a high percentage of Bipolaris is in agreement with Schubert, who made the diagnosis of AFS from surgical histopathology with or without an associated positive surgical sinus fungal culture. The histopathology shows extramucosal allergic mucin that stains positive for scattered fungal hyphae and eosinophilic-lymphocytic sinus mucosal inflammation. They indicated that Bipolaris spicifera is the most common fungus cultured.

Eight patients with culture positive Bipolaris AFS were prospectively compared with 10 control subjects with chronic rhinosinusitis. All 8 patients with AFS had positive skin test reactions to Bipolaris antigen. Eight of the ten control subjects demonstrated negative results to both skin and serological testing, thus implicating the importance to allergy to fungal antigens (Both in vivo and in vitro) in the pathophysiology of AFS.

In our study, we measured total serum IgE to assure the diagnosis of AFS. The mean value of total IgE in AFS group was statistically higher than that of normal control. The value of measurement of serum IgE was emphasized by Kuhn and Javer who stated that the total serum IgE level can be used as an important marker for a legitimate recurrence of the disease. Also the total and or fungal-specific IgE levels can help considerably in differentiating a legitimate AFS from a bacterial infection, because they do not appear to rise with bacterial infection even if the fungus is cultured.

Javer et al., concluded that identifying the fungi by culture and by speciating can be of assistance, since a fungal specific IgE level, if available, can be used to follow the patient more accurately.
In our study, we have measured Aspergillus fumigatus specific IgE and found that 10 patients were positive for specific IgE (14.7%) out of 68 patients with AFS and the mean value was 11.32 +4.12 IU/ml which was significantly higher than the mean value of this specific IgE in our control group which was 0 IU/ml. Also, we have found that only 7 patients from the above 10 patients were positive to Aspergillus fumigatus by PCR (5 only gives positive culture) and this indicates that 3 patients were negative to Aspergillus fumigatus either by culture or PCR but they showed Aspergillus fumigatus allergen specific IgE, on the other hand, 8 cases were positive to Aspergillus fumigatus by PCR and 3 cases were positive by culture failed to show any Aspergillus fumigatus specific IgE indicating that the presence of fungus alone is not essentially accompanied with allergic process.

Our results concurred that of Kuhn and Javer (25) who have often cultured fungi from the sinuses of non chronic rhinosinusitis (control) patients and they indicated that the presence of fungus alone may not be important and may even be a weak criterion for the diagnosis of AFS.

Establishing a causal relationship between the fungal culture results and the clinical presentation of AFS can be difficult, since many of the fungi isolated are ones that are more commonly considered as contaminants. Sometimes, more than one fungus may be grown. Clinical correlation is often necessary for interpretation of direct smears and fungal cultures from such patients (27). The results of Tang et al., (27) also emphasizes our results.

Some patients with AFS do not have allergy to the fungi identified in their eosinophilic mucous but may have elevated IgE levels to other fungi (28). Pant et al., (29) indicated that only 42% of AFS patients were allergic to the same fungi species identified in their eosinophilic mucous. Ponikau et al., (18) concluded that the mere presence of fungi in the nose and sinuses does not confer pathogenicity as fungi were present in the nasal cavity of 100% of healthy controls.

McCann et al.,(30) studied allergic mucin from 17 definitive AFS histologically for fungal elements. Also sera from 18 definitive AFS patients, 6 chronic sinusitis patients were tested for specific IgE to A. fumigatus and five recombinant A. fumigatus allergens (rAsps). Ten of the 17 definitive cases had hyphae morphologically resembling Aspergillus or Fusarium spp. Of definitive AFS patients, 94% showed Aspergillus fumigatus-specific IgE, and 67% were positive to one or more rAsp. The definitive group had greater mean Aspergillus fumigatus IgE versus the chronic sinusitis groups.

Our results are in accordance with that of Pant et al.,(29) who studied specific IgE levels to Aspergillus fumigatus and indicated that AFS group and AFS-like group had higher fungal-specific IgE compared with healthy control.

Corradini et al., (31) studied 24 selected patients with chronic rhinosinusitis, with a positive fungal examination of nasal secretion. Prick tests were positive for seasonal and perennial allergens in 5 patients (21%), while prick tests with fungi were positive in only 4 patients (16.6%). Total IgE levels were higher than in normal in 6 patients (25%) : In another 18 patients, total IgE were normal. Specific IgE levels for the tested fungi and eosinophilic cationic protein levels were within normal range in all patients. Nasal provocation test was negative in all patients. They concluded that the presence of fungi in nasal secretions of patients with AFS does not appear to be correlated with an allergic status to the isolated fungus. A role for IgE in either the etiology or the pathophysiology of AFS in unlikely, and probably the diagnostic criteria for allergic fungal sinusitis should not include type I hypersensitivity, since no confirmed evidence exists that IgE-mediated type I hypersensitivity is involved in the pathophysiology of AFS.

Ponikau et al.,(18) found elevated total IgE levels in fewer than 33% of their patients diagnosed as having AFS. Only 42% of the patients had a detectable type I hypersensitivity by skin test, and only 30% have an elevated fungal specific IgE by RAST. The possibility exists that local IgE production in the nasal mucosa could explain the fact that 58% of patients showed no evidence of elevated blood IgE levels to fungi (32).

There was no allergen test available for Bipolaris spicifera (33). Courley et al., (34) skin-tested 680 consecutive general allergy patients and found only a 6% incidence of
immediate skin reactivity to *Bipolaris* antigen.

Current methods used to study nasal fungi have several limitations. First, fungal cultures require 4 to 6 weeks of evaluation. In a clinical setting, this is often too long to be useful. Secondly, the sensitivity of fungal cultures using swabs is low. Unfortunately, other collection techniques require irrigation and cumbersome transport techniques,\(^{(18)}\) that makes them difficult to perform particularly in an outpatient clinical setting. In addition, irrigation techniques can not differentiate between different areas of the nasal cavity\(^{(15)}\).

Use of nasal swabs, particularly with absorbent tips, results in a low yield of organisms. Cultures are often negative when fungal elements are clearly seen on histopathologic specimens\(^{(19,1)}\).

Since the viability of fungal elements in fungus balls is poor, fungi frequently fail to grow from hyphae-rich material obtained during surgery. Furthermore, other limitations, such as slow growth of many relevant fungi, delayed production, lack of characteristic fruiting bodies or macroconidia, special nutritional requirements of certain fungi, and similarities in macromorphology or micromorphology or both at the genus level, may prevent their detection and identification. Molecular techniques such as dot blot hybridization and sequence analysis may be useful tools in order to determine the distribution of fungi causing fungus balls in the maxillary sinus\(^{(35)}\).

PCR is significantly more sensitive than nasal swabs cultures in detecting the presence of fungi in nasal mucosa. The presence of fungi alone is insufficient to implicate it as the pathogen in chronic sinusitis\(^{(15)}\).

The results of our PCR assay assured the detection of fungal DNA in all cases of AFS group (68 cases) although culture methods only detected fungi in 42 cases (61.7%). Also the PCR results showed fungal DNA in 4 cases of control group (40%) while it was only 3 cases (30%) by culture methods. In our work by PCR, we detected *Aspergillus* DNA in 15 cases (22.05%) while *Bipolaris* DNA was detected in 27 cases (39.70%). The results of culture showed only 11 cases of *Aspergillus* and 18 cases of *Bipolaris*. These results indicated that the detection of fungal DNA by PCR in nasal aspirate was superior to culture method and also the use of specific primers for *Aspergillus* and *Bipolaris* was more sensitive by PCR compared to culture method for these species.

PCR analysis detected fungal DNA in 42% and 40% of control subjects and patients with chronic sinusitis while standard cultures were positive in 7% and 0% respectively. There was no statistically significant difference in the prevalence of fungi in normal control and patients with chronic sinusitis. Of the 18 normal volunteers positive for fungi using ITS primers, 2 (4%) were also positive for *Bipolaris* specific primers and 1 (2%) was positive for *Aspergillus*. There was no evidence of *Aspergillus* or *Bipolaris* DNA in any of the patients with chronic rhinosinusitis using *Aspergillus*- or *Bipolaris*-specific primers\(^{(15)}\). Our results concur that of Catten et al.,\(^{(15)}\) although they used chronic sinusitis cases and not AFS and that is why they detected lower rate of fungal DNA in their work.

A procedure based on panfungal PCR and multiplex liquid hybridization was developed for the detection of fungi in tissue specimens. The PCR amplified the fungal internal transcribed spacer (ITS) region (ITS1-5.8S rRNA-ITS2). The performance of the procedure was examined with 12 deep-tissue specimens and 8 polypous tissue biopsies from the paranasal sinuses. A detection level of 0.1 to 1 pg of purified DNA (2 to 20 CFU) was achieved. Of the 20 specimens, PCR was positive for 19 (95%), of which 10 (53%) were hybridization positive. In comparison, 12 (60%) of the specimens were positive by direct microscopy, but only 7 (35%) of the specimens showed fungal growth\(^{(36)}\).

Also, our work regarding fungal DNA are in agreement with Willinger et al.,\(^{(35)}\) who indicated that fresh surgical specimens of the maxillary sinus were extracted from patients with fungus balls of the maxillary sinus diagnosed by histopathology. PCR amplified fungal DNA in all samples. Sixteen samples (51.6%) were culture positive, 25 samples (80.7%) showed a positive result on hybridization, and 28 samples (90.3%) were positive by sequencing.
REFERENCES


Aspergillus fumigatus and Bipolaris

DNA

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