Fungus culture and PCR in nasal lavage samples of patients with chronic rhinosinusitis

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Chronic rhinosinusitis (CRS) affects approximately 15 % of the adult population in industrialized countries. Fungi have been recognized as important pathogens in CRS in the immunocompromised host. Recently, fungi have been detected in more than 90 % of nasal lavages (NLs) in immunocompetent patients with CRS. Employing NLs of immunocompetent patients with CRS in the present study, the detection rates for fungi by culture techniques were compared with the results of different fungus-specific PCR assays. Standard fungal cultures were performed on NLs from 77 patients with CRS. NLs were also tested for the presence of fungal DNA by a panfungal assay with and without specific probes for Candida spp. and Aspergillus spp./Penicillium spp., and an Aspergillus-specific nested PCR assay. Nineteen of the 77 samples (25 %) grew fungi. Fungus-specific DNA was detected in 34 of 77 NLs (44 %). Twelve samples were positive for both culture and panfungal PCR, whereas seven specimens grew fungi in culture, but were negative in panfungal PCR, and an additional seven samples were positive in panfungal PCR, but negative in culture. The combination of culture and all employed PCR assays detected fungi in 39 patients (50 %). This study demonstrated that PCR and conventional culture techniques could be complementary diagnostic techniques to detect fungi in nasal specimens from CRS patients.

INTRODUCTION

Chronic rhinosinusitis (CRS) in adults is an inflammatory disease of the nasal and paranasal sinus mucosa defined by persistence of symptoms for longer than 8 weeks, or by more than four episodes of rhinosinusitis occurring within a year (Bachert et al., 2003). CRS affects approximately 15 % of the adult population in Western industrialized countries (Benson & Marano, 1998). It is unclear whether nasal polyps are a distinct form of chronic sinusitis of unknown aetiology or whether they reflect an advanced stage of chronic sinusitis in general (Pawankar, 2003). Frequently, nasal polyps are associated with asthma or aspirin sensitivity (Bateman et al., 2003). Eosinophils are abundantly found in the tissue of most nasal polyps (Pawankar, 2003) and also within the mucus blanket covering the nasal and paranasal mucosa (Ponikau et al., 1999). This eosinophilic infiltration is independent from atopy in most patients (Bachert et al., 2003).

Fungi have been increasingly recognized as important pathogens in sinusitis. Fungal infection, mainly by moulds, can impose a severe acute and chronic sinusitis in the immunocompromised host (Malani & Kauffman, 2002). In contrast, fungi are regarded as frequent innocent bystanders when cultured from the respiratory tract of immunocompetent hosts (Uffredi et al., 2003). Allergic fungal rhinosinusitis (AFRS) is an IgE-mediated hypersensitivity reaction to fungal colonization of the paranasal sinus mucosa. Besides positive skin tests for fungal allergens, AFRS is defined by distinct clinical features including nasal polyps, frequently unilateral opacification of the paranasal sinus system with hyperdense spots on CT scans, peanut-butter-like 'allergic mucin' within the sinus lumen, and demonstration of fungi by culture or histology (Bent & Kuhn, 1994). The reported prevalence of AFRS is subject to considerable geographical variations and ranges between 1 and 20 % of patients with CRS (Ferguson, 2000). The characteristic clinical appearance of AFRS may also occur without detectable fungi. This condition was addressed as eosinophilic mucin rhinosinusitis (Ferguson, 2000; Lara & Gomez, 2001).

 Recently, fungi have been specified as the main aetiological agent of CRS in immunocompetent patients (Ponikau et al.,...
In this study, we compared the detection of fungi in culture in patients suspected of having cystic fibrosis or immotile cilia syndromes or chronic infectious diseases except CRS were eligible for inclusion. Patients with severe CRS with a minimum CT score of 20 and volunteers gave their written consent to participate in the study. The study was approved by the ethics committee of the University of Ulm.

Fungal cultures. To prevent fungal detection in patients suffering from CRS has not yet been evaluated, particularly in NLs pretreated with a mucolytic agent.

METHODS

Patients. Patients referred for paranasal sinus surgery to the tertiary rhinologic referral centre between April 2001 and April 2003 were recruited. Patients with severe CRS with a minimum CT score of ≥ 20 (Lund & Kennedy, 1997), a minimum symptom score of ≥ 15 (Lund & Kennedy, 1997) and a minimum endoscopy score of ≥ 3 (Malm, 1997) were included. Only immunocompetent adults without a history of malignancies or chronic infectious diseases except CRS were included. Only immunocompetent adults without a history of malignancies or chronic infectious diseases except CRS were included. Nine healthy volunteers served as control group. All patients and volunteers gave their written consent to participate in the study. The study was approved by the ethics committee of the University of Ulm (Nr. 82/2001).

NL. After mucosal decongestion, patients were asked to recline their head about 45° and to breathe in and hold. Then, both nostrils were flushed with 5 ml of sterile isotonic saline solution using a sterile disposable syringe. After 10 s, the patients vigorously blew the solution into a sterile glass container.

Fungal cultures. All samples were further processed under laminar flow to prevent contamination with airborne fungal spores. For microbiological examinations, 2–5 ml of the lavage fluids were treated with equal volumes of sterile DTT (0·3 mg ml⁻¹) for 15 min at room temperature in order to dissolve viscous mucus (Poronkay et al., 1999). Then, 0·5 ml of the samples were inoculated on two Sabouraud/glucose (4 %) agar plates (Becton-Dickinson) each containing chloramphenicol (0.4 g l⁻¹) and gentamicin (0·04 g l⁻¹). The plates were incubated for 30 days at 37 and 30 °C, respectively.

The remainder of the lavage sample was centrifuged at 3000 g for 10 min; the supernatants were discarded, leaving approximately 1·6 ml for resuspension of the sediment by vortexing. An aliquot (0·5 ml) was cultured on Sabouraud agar and in Sabouraud bouillon for 30 days at 30 °C. The remaining 0·5 ml was utilized for PCR.

All fungal isolates were identified morphologically and biochemically by standard methods (de Hoog et al., 2000).

In addition, a direct microscopic examination of these fluids for the presence of hyphal elements, using the optical brightener Blankophor, was performed (Ruchel & Saffrinski, 1999).

Fungal PCR assays. General recommendations to prevent PCR assay contamination were followed (Kwok & Higuchi, 1989). Sample preparation, PCR set-up and PCR analysis were performed in separate rooms under laminar flow. A negative control containing 500 µl sterile saline paralleled all processing steps. Controls for each PCR run included 10 pg and 1 pg Aspergillus fumigatus DNA as positive controls and two negative controls: one fully processed DNA-free sample preparation and one DNA-free mixture of PCR reagents.

Of the stored samples, 500 µl were centrifuged at 14 000 g for 10 min. The supernatants were discarded, and 50 µl of 1 M NaOH and 50 µl of 2 % SDS were added to the pellets. The mixtures were thoroughly vortexed and alkaline lysis was performed by boiling for 5 min in a water bath. After neutralization with 50 µl of 1 M HCl and 50 µl of 1 M Tris, pH 8·0, the samples were processed by a purification and concentration procedure for DNA using the GeneClean II kit (Bio 101), according to the recommendations of the manufacturer. The DNA preparations were finally dissolved in 25 µl sterile water.

For panfungal PCR the broad-range primers S1 and CUF1 were used, amplifying a 194 bp segment of the 18S rRNA gene of a wide variety of fungal species (Kappe et al., 1998; Rimek et al., 1998). PCR amplifications were carried out in 100 µl volumes with 10 µl of prepared sample added. The mixtures contained 0·5 µM of each primer, 200 µM each of dATP, dCTP, dTTP and dGTP, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris/HCl (pH 9) and 1 unit of Thermoprime Plus DNA polymerase (ABGene). PCR runs were performed in a thermocycler (Tpersonal; Biorad) as follows: 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 1 min at 53 °C, 1 min at 72 °C; and a final extension segment for 3 min at 72 °C; the mixtures were then kept at 4 °C.

PCR products were subjected to ethophoresis in 2 % agarose gels utilizing 1 × Tris/borate/EDTA as running buffer, visualized by ethidium-bromide staining and detected under UV light. A band of 194 bp indicated a positive result in the panfungal PCR. The amplified fungal DNA was hybridized with the biotinylated oligonucleotide probes V2AS (10 ng ml⁻¹, specific for Aspergillus spp. and Penicillium spp.) and V2CA (50 ng ml⁻¹, specific for Candida spp.) at 48 °C. Hybridization products were detected with the GEN-ETI-K DNA enzyme immunoassay (DEIA; BykDiaSorin), according to the instructions of the manufacturer. The DEIA cut-off value was defined as 0·15 OD units above the mean value of the negative controls (Fig. 1).

For the Aspergillus-specific nested PCR two-step amplification, primers AFU75S and AFU7AS (first step) and primers AFU5S and AFU5AS (second step) were used, amplifying a 405 bp and a 236 bp segment of the 18S rRNA gene of Aspergillus spp. in the first and second step, respectively (Składny et al., 1999). PCR amplifications were carried out in 100 µl volumes with 10 µl of prepared sample added. The mixtures contained 0·5 µM of each primer, 200 µM each of dATP, dCTP, dTTP and dGTP, 2 mM MgCl₂, 50 mM KCl, 10 mM Tris/HCl (pH 9) and 1 unit of Thermoprime Plus DNA polymerase. PCR runs were performed in a Tpersonal thermocycler as follows: for the first PCR, 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 1 min at 65 °C, 1 min at 72 °C, and a final extension segment for 5 min at 72 °C; then the mixtures were kept at 4 °C; for the second PCR, 2 min at 94 °C, 35 cycles of 40 s at 94 °C, 1 min
samples two different fungal species were isolated resulting in Nineteen of the 77 NL samples (25 %) grew fungi. In eight this study. The mean age was 48 years (range 25–77 years). In total, 77 patients, 27 female and 50 male, were included in 

RESULTS

Statistics. The level of agreement between the results of fungal cultures and the corresponding PCR assay was estimated by using the Cohen kappa coefficient (Agresti, 1990). In detail, the following agreement levels were determined: panfungal PCR and growth of all fungi, hybridization with the Aspergillus spp./Penicillium spp. probe and growth of Aspergillus spp. or Penicillium spp., hybridization with the Candida probe and growth of Candida spp., and Aspergillus-specific nested PCR and growth of Aspergillus spp. The Cohen’s kappa coefficient with a 95 % confidence interval and the exact two-sided $P$ value were calculated employing StatXact 4.01 (Cytel Software).

Correlation of culture and PCR findings

NL samples were considered fungus-positive if either culture and/or any of the PCR assays yielded a positive fungal result. NLs of 38/77 patients with CRS were fungus-negative by culture and by every one of the four types of PCR assays employed, leaving 39 patients in which at least one of the investigational techniques yielded a positive result. Five of the latter were exclusively positive by culture, 20 by PCR and 14 by both culture and PCR.

In NLs from healthy controls ($n = 9$), no fungal elements were detected either by PCR or by culture ($P < 0.005$).

Comparing the culture results for all fungi and panfungal PCR, in 12 samples both the culture and the PCR were positive. In seven cases a positive PCR was found with a negative culture result, which could be due to the exclusive presence of non-viable fungal elements. The Cohen’s kappa coefficient was calculated as 0.52 ($P = 0.001$). For Aspergillus spp. and Penicillium spp. PCR and cultures yielded concordant results in 58/77 samples, Cohen’s kappa coefficient equalled 0.1 ($P = 0.4$). Concordant results for Aspergillus spp. in culture and Aspergillus-specific nested PCR were found in 61/77 lavage samples. In 13 cases the PCR detected fungal elements without a simultaneous demonstration of fungi by culture. For this situation, the Cohen’s kappa coefficient was calculated as 0.24 ($P < 0.05$).

Fig. 1. Ethidium bromide-stained agarose gel of PCR products obtained from NL samples from different patients with CRS, using a universal fungal PCR assay. Lanes: L, 100 bp ladder; 1–7, samples from seven different patients; 8, 10 pg A. fumigatus DNA as positive control; 9, negative control. The amplification products of patients 1 and 6 hybridized with the Aspergillus spp./Penicillium spp. probe, the product of patient 3 hybridized with the Candida spp. probe.

a total of 27 isolates (Table 1). Predominant species were Penicillium spp. and Aspergillus spp., with 11 and 7 isolates, respectively. Quantitative cultural analysis showed that the number of viable fungal elements per millilitre of NL was less than 10 c.f.u. ml$^{-1}$ in most cases. In detail, culture plates showed 1–10 c.f.u. ml$^{-1}$ in 15 cases, 11–100 c.f.u. ml$^{-1}$ in three cases and more than 100 c.f.u. ml$^{-1}$ in one case.

Direct microscopic examination detected hyphal elements in one of 77 patients.

PCR techniques

The panfungal PCR assay detected $1 \text{pg} A. \text{fumigatus}$ and $1 \text{pg}$ C. albicans DNA or $40 \text{A. fumigatus}$ conidiospores and 40 C. albicans blastospores per reaction after hybridization with the corresponding probes. The sensitivity level of the Aspergillus-specific nested PCR assay was $100 \text{pg} A. \text{fumigatus}$ DNA or $10 \text{A. fumigatus}$ conidiospores per reaction. With all PCR techniques employed, fungus-specific DNA was detected in 34 of 77 lavage specimens. Nineteen samples were positive in the panfungal PCR assay, and 12 and four samples were positive after hybridization with the Aspergillus and Candida probe, respectively. The Aspergillus-specific nested PCR detected Aspergillus DNA in 16 samples. Positive and negative results of the different PCR techniques are cross-tabulated in Table 2.

at 65 °C, 1 min at 72 °C, and a final extension segment for 5 min at 72 °C, then the mixtures were kept at 4 °C. For the second PCR, 5 μl of the first-round PCR product was used as template. The PCR products were separated by using 2 % agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.
Table 1. Fungus characterization and PCR results in NLs from 19 patients with positive fungal cultures among a total number of 77 patients with CRS

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Result of fungal culture</th>
<th>Result of fungal PCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>M</td>
<td>Mycelia sterilia</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>M</td>
<td>Candida albicans, Geotrichium candidum</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>M</td>
<td>Candida guilliermondii</td>
<td>−</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>F</td>
<td>Candida albicans, Cladosporium sp.</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>Penicillium sp.</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>M</td>
<td>Stachybotrys chartarum</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>F</td>
<td>Penicillium chrysogenum</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>49</td>
<td>F</td>
<td>Candida albicans</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>43</td>
<td>F</td>
<td>Penicillium sp.</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>67</td>
<td>F</td>
<td>Aspergillus fumigatus, Penicillium chrysogenum</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>53</td>
<td>M</td>
<td>Candida albicans, Penicillium sp.</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>32</td>
<td>F</td>
<td>Aspergillus niger, Penicillium sp.</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>45</td>
<td>F</td>
<td>Aspergillus fumigatus, Penicillium chrysogenum</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>62</td>
<td>M</td>
<td>Aspergillus fumigatus</td>
<td>−</td>
</tr>
<tr>
<td>15</td>
<td>48</td>
<td>M</td>
<td>Aspergillus fumigatus, Penicillium sp.</td>
<td>−</td>
</tr>
<tr>
<td>16</td>
<td>44</td>
<td>M</td>
<td>Penicillium chrysogenum</td>
<td>−</td>
</tr>
<tr>
<td>17</td>
<td>53</td>
<td>M</td>
<td>Aspergillus fumigatus, Penicillium sp.</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>55</td>
<td>M</td>
<td>Aspergillus fumigatus</td>
<td>−</td>
</tr>
<tr>
<td>19</td>
<td>60</td>
<td>M</td>
<td>Penicillium chrysogenum, Cephalosporium sp.</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 2. PCR results in correlation with positive and negative results of fungal culture

<table>
<thead>
<tr>
<th>PCR result</th>
<th>Panfungal-positive (n = 19)</th>
<th>Aspergillus spp./Penicillium spp.-positive (n = 12)</th>
<th>Candida spp.-positive (n = 4)</th>
<th>Aspergillus spp. nested-positive (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panfungal-negative (n = 59)</td>
<td>–</td>
<td>4</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Aspergillus spp./Penicillium spp.-negative (n = 65)</td>
<td>11</td>
<td>–</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Candida spp.-negative (n = 73)</td>
<td>15</td>
<td>12</td>
<td>–</td>
<td>17</td>
</tr>
<tr>
<td>Aspergillus spp. nested-negative (n = 60)</td>
<td>16</td>
<td>7</td>
<td>4</td>
<td>–</td>
</tr>
</tbody>
</table>

DISCUSSION

Fungal sinusitis is a serious disease in the immunocompromised host. Recently, it has been suggested that in immunocompetent individuals, fungi are also the main aetiological agents of CRS, a widespread pathologic condition (Ponikau et al., 1999; Braun et al., 2003). The incidence of fungal elements in nasal specimens of affected persons differed between various studies. It thus appeared necessary to further evaluate current techniques to contribute to the epidemiological analysis and to elucidate which technique might be superior for the detection of fungal elements in the upper airways.

Employing NLs pretreated with the mucolytic agent DTT to release entrapped fungal elements from the mucus film, fungi were identified in approximately 90% of immunocompetent patients with CRS (Ponikau et al., 1999; Braun et al., 2003). NL is a widely used technique to atraumatically obtain specimens of nasal secretions (Riechelmann et al., 2003). The lavage fluid reaches most areas of nasal mucosa exposed to the respiratory airway, as well as the main nasal mucus transport pathways originating in the paranasal sinus system. It is thus assumed that NL fluid has contact with the relevant mucosal areas possibly contaminated with fungal elements. The probable dilution effect inherent to NL techniques will be compensated by sedimentation of fungal elements using subsequent centrifugation steps. Thus, the higher fungus yield reported in recent studies involving the use of NLs treated subsequently with a mucolytic agent could explain the different sensitivity when compared with results of previous studies employing nasal swabs, which only reach limited areas of the nasal mucosa.

Major objectives of the present study were to determine (a) the incidence of fungal elements in DTT-treated NLs from immunocompetent patients with CRS, (b) the influence of current fungus detection techniques on positive test results, and (c) the concordance rates between current detection techniques. In 77 patients with CRS confirmed by clinical history, nasal endoscopy and CT scans, a standard NL was performed and subsequently treated with DTT. Standard culture techniques (de Hoog et al., 2000) and PCR techniques for fungal DNA identification were then employed. PCR techniques included two different PCR assays: one panfungal assay with and without specific probes for Candida spp. and Aspergillus spp./Penicillium spp., and an Aspergillus-specific nested PCR assay (Kappe et al., 1998; Rimek et al., 1998; Skladny et al., 1999).

Positive fungal cultures were obtained from 25% of patients with CRS. However, in most cases less than 10 c.f.u. ml−1 indicated low quantities of viable fungal elements within the lavage samples. This detection rate lies within the range of several other investigations (Lebowitz et al., 2002; Willinger et al., 2003; Vennewald et al., 1999). However, cultural fungus detection rates in patients with CRS in this study were far less than those described in two recent investigations employing similar sampling and culture techniques on patients from the USA and Austria (Ponikau et al., 1999; Braun et al., 2003). This difference may be explained with the established geographical variability of fungal sinusitis (Ferguson, 2000).

As fungal cultures could last for considerable incubation periods until a positive result is obtained, a molecular diagnostic method (PCR) is supposed to accelerate the detection of fungi in NLs. Moreover, superior sensitivity of PCR for fungus detection in NLs has been reported (Willinger et al., 2003; Pham et al., 2003; Rimek et al., 1998). In this study, culture techniques alone demonstrated fungal elements in 25% of PCR techniques alone indicated the presence of fungal DNA in 44% and the combination of culture and PCR assays detected fungi in 50% of NLs. Thus, PCR increased the rate of fungus detection in NLs considerably, but was not able to replace culture techniques.

PCR assays detect small quantities of DNA. A crucial step in sample processing for PCR is the digestion of the fungal cell wall, which is subject to variations due to diverse sample preparation protocols. This and dilution procedures may cause DNA loss (Haugland et al., 1999). As a consequence, it is critical to assess the sensitivity of each PCR technique used for fungus detection. The panfungal PCR assay employed in this study detected 1 pg A. fumigatus and 1 pg C. albicans DNA or 40 A. fumigatus conidiospores and 40 C. albicans blastospores per reaction after hybridization with the corresponding probes. The sensitivity level of the Aspergillus-specific nested PCR assay was found to be 100 fg A. fumigatus DNA or 10 A. fumigatus conidiospores per reaction.

In fungal culture, the presence of at least one viable spore per specimen is theoretically sufficient for a positive result. This
extreme sensitivity was not achieved with the PCR techniques employed. However, reaching this sensitivity level still also has to be demonstrated by quantitative studies for commonly used culture techniques, though this is very likely not achievable. 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. Standard culture techniques and a panfungal PCR employing common primers yielded concordant positive results from material of 12/77 patients. However, each technique identified seven additional positive specimens, resulting in a combined detection rate of 26/77 patients. Panfungal PCR thus appeared not sufficiently reliable to serve as the only diagnostic tool for fungus detection in NLs. As a consequence, standard culture techniques and PCR are considered complementary methods for the detection of fungal elements in NLs.

NLs of a small group of nine normal controls were initially included as a laboratory reference group. Since fungal elements could not be detected in any material from this group, the clinical implication of this specific result deserves further consideration. The subjects in the control group were younger than the patients of the study group. It is not surprising to fail to detect fungal spores in NLs of normal subjects. In south Germany, approximately 200 spores per cubic metre of indoor or outdoor air have been found (Jovanovic & Piechotowski, 2000). An adult human respires 30 m$^3$ of indoor or outdoor air per day, equating to roughly 2000 spores. Due to their low aerodynamic diameter, somewhere in the order of 10% of spores are deposited on the nasal mucosa and cleared by the mucociliary transport system within a few minutes. Thus, less than one spore can be expected in the nose, when a NL is performed. In patients with CRS, the mucociliary transport system is severely impaired and thus the chance to detect inhaled spores is increased (Bertrand et al., 2000; Hafner et al., 1997). As a consequence, inhaled spores may stay longer within the nasal airways in patients with CRS than in the airways of healthy controls, increasing the chance of them being detected by sensitive assays. This may explain the striking difference between the high fungus detection rates in patients with CRS and the complete absence of fungi in the specimens of the study controls.

The ubiquitous nature of fungal spores makes it difficult to determine their aetiologic role for fungal infection in patients with CRS. The mere presence of fungi in the nose and sinuses, determined by culture or PCR assay techniques, cannot be considered as sufficient to prove their role in the pathogenesis of CRS.

In conclusion, this study detected fungal elements in DTT-pretreated NLs in 50% of patients with CRS. The rate of fungus detection is influenced by the detection techniques used. It is suggested that fungal cultures and PCR from NLs of immunocompetent patients with CRS are complementary techniques when establishing an association between the disease and the presence of a potential aetiological agent.

REFERENCES


