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**Contribution of molecular tools for the diagnosis and epidemiology of fungal  
chronic rhinosinusitis**

**Short title: molecular diagnosis of fungal chronic rhinosinusitis**

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## Abstract

Chronic rhinosinusitis (CRS) rank second at chronic inflammatory diseases in industrialized countries and are an important public health concern. Diagnosis relies on a set of arguments including clinical signs, imaging, histopathologic and mycological analyses of sinus specimens, collected during nasal endoscopy. The sensitivity of fungal cultures is reported to be poor, even when direct examination is positive, thus the epidemiology of fungal chronic sinusitis is ill-known.

This study evaluated the sensitivity of molecular diagnosis in 70 consecutive samples (61 patients with CRS) analysed at the University Hospital of Rennes during a three-year period. DNA detection was performed using a conventional PCR method targeting the ITS1/ITS2 sequence and the resulting amplification products were sequenced.

Fungal CRS was proven in 42 patients (69%), of which only 20 (48%) had a positive culture. 37/42 (88%) patients were diagnosed with a fungus ball, 3 with allergic fungal CRS and 2 with undetermined fungal CRS. PCR was positive in all 42 cases and direct sequencing allowed to identify fungi in all cases but one, and detected multiple infection in 3. *Aspergillus fumigatus* was present in 69% of patients; *Cladosporium cladosporoides* in 9.5%, *Scedosporium sp*, *A. nidulans* and *A. flavus* in 7% each. In 2/19 patients with negative direct examination, sequencing analysis revealed the presence of *Capnobotryella sp* and *C. cladosporoides*, in clinical settings compatible with fungal sinusitis.

In conclusion, ITS1/ITS2 PCR had a twice better sensitivity than culture, and combined sequencing provides accurate epidemiological data on fungal CRS.

## INTRODUCTION

Chronic rhinosinusitis (CRS) is a common disorder ranking second among chronic inflammatory diseases in industrialized countries, affecting the health-related quality of life <sup>1</sup> <sup>2</sup>. It is considered to affect 10 to 17% of people and to be the fifth clinical setting leading to antibiotics prescription <sup>3,4</sup>. CRS etiology may be due to multiple factors including anatomical, mechanical (inadequate muco-ciliary clearance system), immunological or infectious factors. The relative prevalence of viral, bacterial and fungal infections in CRS is difficult to appreciate in the absence of systematic biological diagnosis, as the diagnosis usually relies on imaging and clinical findings. Fungal agents can be responsible for acute invasive sinusitis, granulomatous invasive rhinosinusitis and chronic invasive rhinosinusitis, but also for non-invasive CRS clinical entities, i.e. fungus ball (FB), and allergic fungal CRS (AFRS), also described as eosinophil related fungal rhinosinusitis <sup>5</sup>. Recent studies have shown that fungi accounted for 15-20% of maxillary CRS, most of them being associated to *Aspergillus* fungus ball <sup>6,7</sup>.

A fungal etiology can be suspected in case of antibiotherapy failure or CT scan imaging showing evocative features (unilateral sinusal opacity, intrasinusal foreign bodies of dental or endogen origin, microcalcifications or osseous remodeling), but needs confirmation by microscopic examination and culture of sinusal secretions or pus collected during surgical lavage. However, fungal cultures are reported to be frequently negative despite the observation of fungal hyphae after microscopic examination, thus the epidemiology of fungal CRS remains largely ill-known <sup>8</sup>.

In this study, we evaluated the performance of molecular diagnosis for the diagnosis of fungal CRS. All samples analyzed in our lab during a 3-year period were retrospectively

analyzed by PCR and sequencing of the ITS1/ITS2 sequence, and the results were compared to the routine mycological diagnostic methods and to anatomopathologic examination.

## **MATERIALS AND METHODS**

### **Patients and samples**

Patients with CRS were examined at the consultation of otorhinolaryngology of the Centre Hospitalier Universitaire de Rennes or of the Clinique Mutualiste La Sagesse (Rennes) and underwent imaging investigation (computerized tomography or magnetic resonance) to explore CRS. All patients with suspected fungal CRS benefited from mycological analyses to document the infection. In some cases, additional biological analyses were prescribed (dosage of total or specific anti-Aspergillus IgE, blood cell count). Mucosal biopsies or sinus samples were collected during endoscopic sinus surgery and sent to our lab for mycologic examination. In most instances, samples were also submitted to anatomopathological examination. All samples analyzed from May 2010 to May 2013 were included in this study. Overall, 77 anatomically distinct sinus samples from 66 patients were routinely analyzed by microscopic examination and mycologic cultures. Remaining samples were stored at -20°C for further molecular analysis. For 5 patients (7 sinus samples), the remaining frozen samples were insufficient to be included in the study. Overall, 70 samples from 61 patients were available for molecular diagnosis (Figure 1). Patients were classified as having proven fungal CRS if microscopic examination of sinus samples demonstrated the presence of fungal hyphae. Patients were categorized as having an AFRS on the basis of evocative CT-Scan imaging, presence of allergic mucin and eosinophil infiltrates by histopathologic examination and/or eosinophil infiltrates and high titers of total and specific anti-Aspergillus IgE. They

were categorized as having a fungus ball on the basis of evocative CT-Scan imaging (presence of intrasinus body with metallic densities, sinus opacity and/or microcalcifications or osseous remodeling), presence of fungal elements after mycological examination and no predominance of eosinophils nor presence of allergic mucin in histopathologic examination. In rare instances, patients were categorized as having fungal CRS of undetermined origin, if the anatomopathological examination was not done or discrepant with imaging findings.

### **Mycological diagnosis**

For direct examination, samples were crushed in a microtube containing 500 µL of sterile physiologic water; one drop was mounted with a coverslip and observed immediately under light microscopy. Several drops of the crushed material were inoculated on Sabouraud agar medium supplemented with antibiotics and cultured at 30°C and 37°C for 7 days. Identification of fungi was performed by morphologic and microscopic examination of cultures.

### **Molecular diagnosis**

200 µL of frozen samples were lyzed using Magnalyser<sup>®</sup> device (Roche Diagnostics, Meylan, France) in a microtube containing magnetic beads and 400 µL of lysis buffer (Magnalysis buffer, Roche) during 35 seconds at 7000 rph twice. Samples were left at room temperature during 10 min, then 400µL of supernatant was collected in a microtube and extracted using Magnapure compact<sup>®</sup> device (Roche). DNA was eluted in 100 µL. Ten µL of DNA diluted to 1:20 were used for ITS1/ITS2 amplification, in a final volume of 50 µL containing primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') at 0.2 µM each,

0.025U of Taq polymerase (Promega, Charbonnières les Bains, France), 3 mM MgCl<sub>2</sub> and 200 μM of each dNTP, and 1X Promega® buffer. Amplification reaction was carried on a GeneAmp® PCR system (Life Technologies, Saint Aubin, France) for 50 cycles consisting of 1 min at 95°C, 1 min at 54°C and 2 min at 72°C and 10 min at 72°C. The presence of amplification products was verified after migration in a 1.5% agarose gel, and fragments were purified using sephadex and Millipore multiscreen MAHVN 4550 filter plates (Merck Millipore, Molsheim, France). For sequence analysis, 2.5 μL of amplification product diluted to 1:10 was added to 17.5 μL of reaction mix containing Big dye Terminator® buffer (Life technologies) and 1 μL of reverse or forward primer at 10 μM. Amplification reaction was carried on a GeneAmp® PCR system for 30 cycles consisting of 0.1 min at 95°C, 0.1 min at 50°C and 4 min at 60°C. Amplification products were purified using Big dye Terminator® kit, according to the manufacturer's instructions, and sequencing was performed on an ABI 7900® device (Life technologies). Bidirectional sequences were analyzed using Seqscape® software and submitted to GenBank public database by using the BLAST Search program for comparison and species identification.

### **Anatomopathological diagnosis**

Fresh mucosal biopsies and sinus samples were formalin-fixed. Paraffin sections were stained with hematoxylin and eosin-safran (HES) and Periodic Acid Schiff (PAS) for light microscopy. The slides were reviewed by a dedicated pathologist. On HES staining, eosinophilic infiltrate and edema were in favor of allergic etiology. Fungal agents were identified by PAS staining.

## RESULTS

Direct examination was positive for 42 out of 61 patients (47 of 70 samples). A positive culture was obtained for 20 out of the 42 patients with positive direct examination (48%). The histological analysis showed the presence of fungal hyphae in 15 out of 20 patients with positive culture and 20 out of 22 patients with negative culture (Figure 1). PCR amplification was positive in all patients with positive direct examination. Thus, the relative sensitivity of culture, anatomopathological examination and PCR was 48%, 83%, and 100%, respectively. In three additional patients with positive culture, the histological examination showed the presence of allergic mucin and eosinophil infiltrates, supporting the diagnosis of AFRS, but no fungal hyphae.

The clinical characteristics and imaging findings of the 61 patients investigated for fungal CRS are summarized in Table 1. Patients with proven fungal CRS (positive direct examination) had an older age ( $p < 0.05$ ), presented more frequently with unilateral maxillary sinus impairment ( $p < 0.05$ ). Conversely, nasal polyps or isolated non-specific mucosal inflammation were more frequently observed in patients with CRS of other etiology ( $p < 0.05$  and  $p < 0.001$ , respectively).

Sequencing of ITS1/ITS2 region allowed fungus identification in 46/47 samples (41/42 patients). In one sample, the sequence did not allow fungal identification when submitted to GenBank (“uncultured fungus clone”); in that patient the culture was negative (Table 2). The genus *Aspergillus* was the most frequently observed (37/47 samples, 79%), and *A. fumigatus* accounted for 66% of all samples (31 out of 47). *A. nidulans*, *Cladosporium cladosporoides* and *Scedosporium* sp were detected with similar frequencies (8.5% of samples). Two different species were identified in 3 patients (Table 2). ITS1/ITS2 sequencing allowed



species identification in 3 patients for whom fungi obtained in culture were not identifiable (sterile hyphae). The concordance of species identification by microscopic examination of cultures and by sequence analysis was good, with a percent agreement of 83% (19/23 samples). The discrepancy consisted of i) a misidentification of the *Aspergillus* species in 3 cases, underlining the difficulty of *Aspergillus* species identification by morphotyping<sup>9</sup>, and ii) of molecular identification of *Scedosporium* sp in a sample which cultured positive with *A. niger* in one case. This latter discrepancy can be easily explained by i) the difficulty to grow *Scedosporium* sp, and ii) the rapidity of growth of *A. niger* which had probably overwhelmed the other species. In three patients, 2 fungal species were identified by sequencing, whereas only one of the two species was grown in culture (1 patient) or no fungus was grown at all (2 patients), despite positive direct examination. Finally, sequence analysis allowed to identify *Terfezia olbiensis* (GenBank accession number KU886548) and *Sporobolomyces roseus* (GenBank accession number KU886546) which could not be obtained in culture (Table 2).

Among the 42 cases with proven fungal CRS, the clinical signs, imaging finding and histological examination allowed to classify the patients as having a fungus ball, AFRS, and undetermined fungal CRS in 37 (88%), 3 (7%), and 2 (5%) cases, respectively (Table 3). *Aspergillus* sp. was found in 29/37 (79%) of FB samples. The role of endodontic treatment in fungus ball development was confirmed by CT-scan imaging showing the presence of intrasinus body with metallic densities in 57% of patients (21/37). Maxillary sinus involvement was mainly associated with the diagnosis of FB ( $p < 0.001$ , Table 3). Non-FB cases were mostly associated with multiple sinus involvement ( $p < 0.001$ ). There was no particular fungus species, nor specific clinical signs associated to a type of fungal sinusitis (Table 3).

Similarly, no particular clinical characteristics, nor fungal species was found to be associated to the immune background of the patients (data not shown).

Direct examination and culture were negative in 19 patients for whom the diagnosis of fungal CRS was not retained; in two of them, PCR was positive and sequencing identified *Capnobotryella* sp (GenBank accession number KU886547) and *Cladosporium cladosporoides*, respectively. Anatomopathological examination was in favor of allergic CRS in the former and dental material was visualized on CT-scan in the latter.

## **DISCUSSION**

The diagnosis of fungal CRS usually relies on the mycological and histological examination of sinus samples obtained during endonasal sinus surgery. This 3-year retrospective study underlines the importance to combine them because false negative results of histological examination may occur, particularly in case of AFRS where fungal hyphae may be sparse <sup>5</sup>.

The aim of this work was to evaluate the sensitivity of ITS1/ITS2 PCR on sinus samples, and its direct use for further sequencing. All samples with positive direct examination were successfully amplified by PCR. This PCR sensitivity of 100% confirms its superiority, compared to mycologic cultures, as only 48% of samples with positive direct examination were grown in culture. Furthermore, fungi in culture lacked fruiting bodies or macroconidia in 3 cases, making microscopic identification impossible. Such a poor sensitivity is in line with other studies which reported a culture sensitivity ranging from 25% to 51% <sup>6-8 10 11</sup>, and could be attributed to the poor viability of fungal elements in FB.

ITS1/5.8S/ITS2 sequencing is a widely used target for identifying filamentous fungi <sup>12 13</sup> and allowed us to achieve fungus identification in all cases but one. As already reported, A.

*fumigatus* accounted for the majority of FB cases (24/37, 65%)(Table 3) <sup>7 14</sup>. Sequencing also allowed to identify *Terfezia olbiensis* and *Sporobolomyces roseus*. The former is an Ascomycete belonging to Macromycetes. Other Macromycetes has been described as agents of fungal CRS in previous reports <sup>15-17</sup>. The responsibility of the latter species, *Sporobolomyces roseus*, in CRS is not known, but Patovirta et al. <sup>18</sup> described an association between sinusitis and elevated mould-specific IgG-levels, including anti-*Sporobolomyces salmonicolor* IgG, in teachers during a follow-up after an extensive mould remediation process in school buildings.

FB was the most predominant cause of fungal CRS (88% of cases) in this study, which is consistent with previous European studies <sup>19</sup>. Not surprisingly, FB was mainly associated with endodontic treatment, as already observed <sup>20 21</sup>, and mostly involved unilateral maxillary sinus (73%), followed by sphenoidal sinus. The average age of 62 years was consistent with previous studies <sup>6</sup>. Our study found no gender predominance, in accordance with the study of Klossek et al. <sup>22</sup>, but in disagreement with other French studies describing a female-to-male ratio of around 2 <sup>19 23</sup>.

As typically observed in other studies, patients suffered from non-specific symptoms of sinusitis such as rhinorrhea and nasal obstruction <sup>10</sup>. Dufour et al. <sup>6</sup> reported that maxillary sinus FB were asymptomatic in 10% of cases. In our study we found that about 30% of FB cases were asymptomatic, mostly in immunocompromised patients who benefited from systematic screening. Chakrabarti et al. <sup>5</sup> defined asymptomatic carriage as saprophytic fungal infestation, which can lead to the formation of FB <sup>24</sup>, as observed here.

*Aspergillus* was identified in 3 patients, in whom the diagnosis of AFRS was made on the basis of high titers of anti-*Aspergillus* specific IgE, a pansinusitis involvement on CT scan and

the presence of allergic mucin and fungal elements after mycological examination, in accordance with diagnostic criteria of Schubert <sup>25</sup>. Hence, these cases highlight the good complementarity between mycological and histological findings. AFRS are essentially described in American series, showing a prevalence of 5 to 10% among patients with CRS and a predominance of dematiaceous fungi <sup>26 27</sup>. However, this entity seems to be controversial and its prevalence may vary according to the criteria used for diagnosis. In a study performed in Houston <sup>28</sup>, about three quarters of the patients (34/47) were classified as having AFRS. These patients presented with eosinophilic mucin in histopathologic examination, but the presence of fungi was demonstrated in only 47% by direct microscopy or culture. Similarly, Montone et al. <sup>29</sup> reviewed 400 cases of fungal CRS and classified 45% of them as AFRS. For other authors, the sole criteria of the presence of eosinophilic mucin in histopathologic examination is not considered to be sufficient to retain the diagnosis of AFRS, <sup>1 5</sup>, as it may be only the result of inflammation dysregulation, as discussed in a recent review by Lam et al. <sup>30</sup>.

In our study, PCR was positive in two patients with negative mycological examination (direct examination and cultures). One of them was diagnosed with eosinophilic mucin and polyposis by the anatomopathologist, but the diagnosis of AFRS was not retained. Interestingly, ITS1/ITS2 sequencing demonstrated the presence of *Capnobotryella* sp, a fungus species belonging to dematiaceous fungi. This case illustrates that we may have underestimated the proportion of AFRS, as the dosage of total or specific IgE was not done systematically. In the second case, another dematiaceous (*Cladosporium cladosporoides*) was identified but was not retained as a real pathogen, though dental material was observed on CT scan in the right maxillary sinus, which could be consistent with a FB.

Overall, this study nicely shows that molecular methods are powerful tools for the diagnosis of chronic rhinosinusitis and help characterizing the accurate epidemiology of fungal CRS.

## **ACKNOWLEDGMENTS**

### **Disclosure of Conflict of Interest:**

The authors have no conflicts of interest to declare

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## FIGURE LEGEND

**Figure 1:** Flowchart of the patients and samples included in the study

ND, not done; +, positive; -, negative

**Table 1:** Clinical and biological characteristics of patients according to CRS type (n = 61)

Characteristics	Patients with proven	Patients with non-	p-value
	fungal CRS N = 42	fungal CRS N = 19	
Sex ratio	1.1	1.37	
Mean age $\pm$ SEM (yr $\pm$ SEM)	62 $\pm$ 2	55 $\pm$ 3	p <0.05
Immunosuppression, n (%)	11 (26)	8 (42)	ns (0.243)
Immunosuppressive drugs	5 (46)	1	
Hematological malignancy	1 (9)	3	
Cancer	3 (27)	3	
Transplantation	1 (9)	1	
Diabetes melitus	1 (9)	0	
Type of impaired sinus, n (%)			
Maxillary sinus (unilateral)	28 (67)	7 (37)	p <0.05
Maxillary sinus (bilateral)	2(5)	1 (5)	ns
Sphenoid sinus (unilateral)	2 (5)	1 (5)	ns
Sphenoid sinus (bilateral)	1 (2)	0	ns
Frontal sinus	1 (2)	1 (5)	ns
Maxillary sinus + sphenoid sinus	1(2)	0	ns
Maxillary sinus + ethmoid sinus	0	2 (11)	ns
Sphenoid sinus + ethmoid sinus	2 (5)	1 (5)	ns
Frontal sinus + ethmoid sinus	0	1 (5)	ns
$\geq$ 3 sinus	5 (12)	3 (16)	ns
Anatomopathology result, n (%)			
Presence of fungal hyphae	35 (85)	1 (5)	p <0.001
Eosinophils infiltrate and/or mucin	3 (7)	2 <sup>a</sup> (11)	ns
Mucosal inflammation only	2 (4)	9 (53)	p <0.001
Nasal polyps	0	3 (18)	p <0.05
Other	1 <sup>b</sup> (2)	4 <sup>b</sup> (24)	
Not done	1 (2)	2 (11)	na

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Mycological diagnosis			
Fungal hyphae	42/42 (100)	0/19 (0)	nd
Positive culture	20/42 (48)	0/19 (0)	nd
Positive ITS1/ITS2 PCR	42/42 (100)	2/19 (10)	nd

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<sup>a</sup> associated with polyposis in two cases

<sup>b</sup> exogenous material of dental origin in 1 case

na; not applicable, nd; not done

**Table 2:** Results of sequencing analysis in patients with positive direct examination (n = 42)

Sequence identification	No of patients/total (%)	No of samples/total (%)	No with positive culture (%)
<i>Aspergillus fumigatus</i>	29/42 <sup>a,b</sup> (69)	31/47 <sup>a,b</sup> (66)	15 <sup>c</sup> (50)
<i>Aspergillus nidulans</i>	3/42 (7)	4/47 (8.5)	3 <sup>d</sup> (75)
<i>Scedosporium</i> sp <sup>e</sup>	3/42 (7)	4/47 (8.5)	2 (50)
<i>Cladosporium cladosporoides</i>	4/42 <sup>a</sup> (9.5)	4/47 <sup>a</sup> (8.5)	0 (0)
<i>Aspergillus flavus</i>	3/42 <sup>b</sup> (7)	3/47 <sup>b</sup> (6)	1 (33)
<i>Sporobolomyces roseus</i>	1/42 (2) <sup>f</sup>	1/47 (2)	0 (0)
<i>Terfezia olbiensis</i>	1/42 (2)	1/47 (2)	0 (0)
Uncultured fungus clone	1/42 (2)	1/47 (2)	0 (0)

<sup>a</sup> in one case, *A. fumigatus* and *C. cladosporoides* were identified in the same sample,

<sup>b</sup> in one case, *A. fumigatus* and *A. flavus* were identified in the same sample

<sup>c</sup> positive culture identified as *A. flavus* in one patient

<sup>d</sup> positives cultures identified as *A. versicolor* and *A. fumigatus*, respectively, in two patients

<sup>e</sup> consisting of 1 *S. apiospermum*, 1 *S. aurantiacum* and 1 *Pseudallescheria boydii*.

<sup>f</sup> *Sporobolomyces roseus* and *C. cladosporoides* were identified in 2 sinus samples from the same patient

**Table 3:** Clinical signs and molecular fungus identification according to the type of fungal CRS

	Fungus ball (n=37)	other fungal CRS (n = 5)	p-value
<b>Sinus type impaired, n (%)</b>			
Maxillary sinus (unilateral)	28 (76)	0	<0.001
Maxillary sinus (bilateral)	2 (5)	0	
Sphenoid sinus (unilateral)	2 (5)	0	
Frontal sinus	1 (3)	0	
≥ 2 sinus types	4 (11)	3 (60)	<0.001
<b>Clinical signs, n (%)</b>			
			ns
Fortuitous diagnosis	12 (32)	1 (33)	
Dental pain	6 (16)	0	
Nasal obstruction	10 (27)	3 (60)	
Rhinorrhea	12 (32)	3 (60)	
Headache +/- facial pain	12 (32)	3 (60)	
Other	2 (5)	4 (80)	
<b>Fungal species, n (%)</b>			
			ns
<i>Aspergillus</i> sp	29 (78)	5 (100)	
<i>A. fumigatus</i>	24 (65)	4 (80)	
<i>Scedosporium</i> sp	3 (8)		
<i>Cladosporium</i> sp	4 (11)		
Other	3 (8)		

ns; not significant

Figure 1

