ORIGINAL ARTICLE

PCR-based detection of *Aspergillus fumigatus* and absence of azole resistance due to TR_{34}/L98H in a french multicenter cohort of 137 patients with fungal rhinosinusitis

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Summary

Fungal rhinosinusitis (FRS) has a worldwide distribution, comprises distinct clinical entities but is mostly due to *Aspergillus* among which *Aspergillus fumigatus* plays a major role in European countries. Although, there is accumulating evidence for the emergence of environmentally acquired-azole resistance in *A. fumigatus* (such as TR_{34}/L98H) in various clinical settings, there is few data for patients with FRS. In this study, we aimed to investigate the prevalence of *A. fumigatus* azole resistance due to TR_{34}/L98H in a multicentre cohort of patients with FRS. One hundred and thirty-seven patients with FRS admitted between 2002 and 2016 at four French medical centres were retrospectively enrolled. Clinical and mycological findings were collected. *Aspergillus fumigatus* and the TR_{34}/L98H alteration conferring azole resistance were investigated directly from clinical samples using the commercial CE-IVD marked MycoGENIE® *A. fumigatus* real-time PCR assay. Fungal ball was the more frequent clinical form (n = 118). Despite the presence of fungal hyphae at direct microscopic examination, mycological cultures remained negative for 83 out of the 137 patients (60.6%). The PCR assay proved to be useful allowing the identification of *A. fumigatus* and etiological diagnosis in 106 patients (77.4%) compared with 44 patients (32.1%) when using culture as the reference method. Importantly, neither TR_{34} nor L98H alterations were evidenced.

KEYWORDS
Aspergillus fumigatus, azole resistance, fungal rhinosinusitis, PCR-based detection, TR_{34}/L98H

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1 | INTRODUCTION

According to the International Society of Human and Animal Mycology (ISHAM) consortium held more than 10 years ago, fungal rhinosinusitis (FRS) comprises distinct clinical entities, invasive (either acute, chronic or granulomatous), or non-invasive FRS (including fungal ball and allergic FRS), each bearing its own predisposing factors, clinical management and outcome.[1] Invasive rhinosinusitis is quite rare, mainly affects immunocompromised patients and is associated with a poor prognosis requiring systemic antifungal therapy combined with surgery.[2] By comparison, fungal ball has a worldwide distribution, is characterised by no evidence of tissue invasion and most patients are immunocompetent.[3,4] Whatever the clinical entities considered, *Aspergillus* species and especially *Aspergillus fumigatus* play a major role in the disease being responsible for most of the cases in Europe.[3,4] One additional issue, mostly observed in patients with fungal ball, is that mycological cultures remain negative in about 70% of patients.[1] Hence, the diagnosis relies mostly on histopathological or direct examination of clinical samples. In this context, non-culture based methods relying on PCR can both provide mycological identification and a more rapid diagnosis.[5] This issue can lead both to underestimate the diversity of fungal species responsible for FRS, but also prevents in vitro antifungal susceptibility testing that could be useful when dealing with invasive aspergillosis and suspected azole resistance.[6]

In recent years, azole resistance in *A. fumigatus* has been increasingly reported worldwide and emerged as an important threat to human health.[7] Briefly, two main routes of acquisition of azole-resistant isolates have been proposed. The first route, identified in the late 1990s, results from in vivo drug selection through long-term azole exposure, as illustrated in patients with chronic pulmonary aspergillosis.[8,9] The second route, relies on de novo acquisition of azole-resistant *A. fumigatus* strains (usually displaying the TR$_{34}$/L98H mutations in the *CYP51A* gene), arising in our environment as a consequence of intensive azole fungicides use in agricultural and flower fields practices.[10-12] Since then, several studies have been conducted to evaluate the burden of azole resistance among patients with invasive aspergillosis, chronic pulmonary aspergillosis or patients with cystic fibrosis.[7,13,14] However, the recent description of two patients with TR$_{34}$/L98H azole-resistant *A. fumigatus*-related FRS in France[15] and India,[16] raises the question of its prevalence in this specific population. Indeed, it could be expected that patients with FRS, a disease that commonly occurs outside hospital (and usually affecting immunocompetent patients), could be at risk to inhale azole-resistant *A. fumigatus* isolates from the environment, but data on large cohort are still lacking.

To fill this gap, our aim was to investigate the prevalence of *A. fumigatus* and azole-resistance driven by TR$_{34}$/L98H, the most frequent environmentally acquired mutation in France, in a large multicenter cohort of patients with FRS. In addition, to overcome the limitation of using cultures that are regularly negative in patients with FRS, detection of *A. fumigatus* and the TR$_{34}$/L98H mutation conferring azole resistance was performed directly from clinical samples by a commercial CE-IVD marked PCR assay.

2 | MATERIALS AND METHODS

To this aim, 137 unselected patients with a clinical diagnosis of FRS and a positive direct examination for fungal hyphae (regardless the results of fungal cultures), admitted between March 2002 and August 2016 at four French University Hospitals, were retrospectively enrolled (n = 147 samples): Nantes (n = 59), Hôpital Européen Georges Pompidou-Paris (n = 45), Amiens (n = 22) and Poitiers (n = 11). At each laboratory, mycological cultures were performed according to the local procedure, consisting of incubation on Sabouraud agar slants at 30-35+/2°C for 2-3 weeks. Clinical samples were cryopreserved below −20°C until the study. Detection of *A. fumigatus* and of the TR$_{34}$ and L98H mutations was performed directly from clinical samples by PCR using the CE-IVD marked Mycogenie® *A. fumigatus* Real Time PCR kit (Ademtech, Pessac, France). Briefly, prior to automated DNA extraction using the AutoMag system (AdemTech), each sample was subjected to external lysis with 300 μL of Tissue Lysis Buffer (supplied in the kit) for 15 minutes at room temperature. Detection of *A. fumigatus*,

TABLE 1 Characteristics of the patients with fungal rhinosinusitis (FRS; n = 137)

	Number of patients (%, when applicable)
Sex ratio (M/F)	0.61 (52/85)
Mean age, years (range)	60 (18-82)
Type of FRS (%)	
Fungal ball	118 (86.1)
Invasive sinusitis	6 (4.4)
Allergic FRS	3 (2.2)
Mixed disease	2 (1.5)
Not available	8 (5.8)
Localisation of the disease (%)	
Maxillary sinus	88 (64.2)
Sphenoidal	17 (12.4)
Ethmoidal	4 (2.9)
Mixed location	22 (16.1)
Not available	6 (4.4)
Mycological findings (%)	
Negative culture	83 (60.6)
Positive culture	54 (39.4)
Species identified by culture[a] (%)	
Aspergillus fumigatus	44 (81.5)
Aspergillus flavus	1 (1.9)
Unidentified *Aspergillus* sp.	2 (3.7)
Mucorales	2 (3.7)
Others[b]	6 (11.1)

[a]A single patient had *A. fumigatus* in mixed culture with a mucorales species (*Rhizomucor* sp.)
[b]Others included: *Paecilomyces* sp. (n = 1), *Scedosporium* sp. (n = 1), *Candida lipolytica* (n = 1), sterile hyphae (n = 1), dematiaceous fungi (n = 2, including 1 *Curvularia* sp.).

the TR$_{34}$ and L98H mutations and a PCR inhibition control (added to the sample before the extraction step), was performed on 10 μL of DNA extract using the commercial four-plex PCR assay on a Rotor-Gene Q platform (Qiagen, Courtaboeuf, France). Positive and negative controls were included in each set of experiments (supplied in the kit). Samples negative for both *A. fumigatus*, TR$_{34}$ and L98H and also negative (or with a cycling threshold >35 cycles) for the internal control, were considered uninterpretable due to PCR inhibition. Performances of this PCR assay in terms of sensitivity/specificity (limits of detection of 6 and below 1 copy per genome for the TR$_{34}$ and L98H mutations and *A. fumigatus* respectively) had been previously confirmed both on clinical isolates and on a large collection of clinical samples.[17] The following clinical and mycological data were collected: age, sex, clinical form (type and location) and antifungal therapy for FRS, species identification when available (for culture-positive samples). The protocol was approved by our local committee under no RC16_0426.

3 | RESULTS

As shown in Table 1, most patients were female (sex ratio M/F = 0.61) and mean age was 60 years. Fungal ball was the more frequent clinical presentation of FRS (n = 118 patients) and mostly involved maxillary sinuses (n = 88). Except the few patients with acute invasive FRS (n = 6) that were given systemic antifungals, all remaining patients were cured by surgical resection alone. Despite the presence of fungal hyphae at direct microscopic examination for all patients (inclusion criteria), mycological cultures remained negative for 83 out of the 137 patients (60.6%, 90/147 samples; Figure 1). When positives, fungal cultures mostly grew *A. fumigatus* in 44 patients (47/57 samples,

82.5%). Other hyalohyphomycetes or dematiaceous fungi were only identified in a few patients (Table 1). By comparison, PCR was positive for *A. fumigatus* in 116/147 samples (78.9%, 106 patients). A near-perfect agreement was noted between PCR and culture when positive for *A. fumigatus* (46/47, 97.9%). However, the PCR assay was also able to detect *A. fumigatus* in 64 of the 90 culture-negative samples (71.1%), and in 6 of the 10 samples with a culture positive for other fungi or unidentified *Aspergillus* (Figure 1). Only 7 samples were uninterpretable due to PCR inhibition. Taken together, PCR allowed the diagnosis of FRS due to *A. fumigatus* in 106 patients compared with only 44 patients by culture. For 23 patients/samples, both mycological cultures and *A. fumigatus* PCR were negative, suggesting the involvement of other fungal species in the physiopathology of FRS. Neither TR$_{34}$ nor L98H alteration was evidenced in this cohort.

4 | DISCUSSION

In agreement with previous studies, *A. fumigatus* was identified as the main etiological agent of FRS in this cohort, although other species were occasionally involved.[1,3] This study also illustrates that a PCR-based strategy is of major interest, offering an increased sensitivity compared with mycological cultures.[5] Here, PCR-based diagnosis allowed the identification of *A. fumigatus* in 78.9% of the clinical samples of this cohort compared with only 32% when using mycological cultures as a gold standard. Compared to panfungal PCR assays combined with DNA sequencing, this commercial CE-IVD marked PCR assay can allow a more rapid identification, in <4 hours, of *A. fumigatus* and of the TR$_{34}$ and L98H mutations conferring resistance, by avoiding delays due to sequencing.[5]
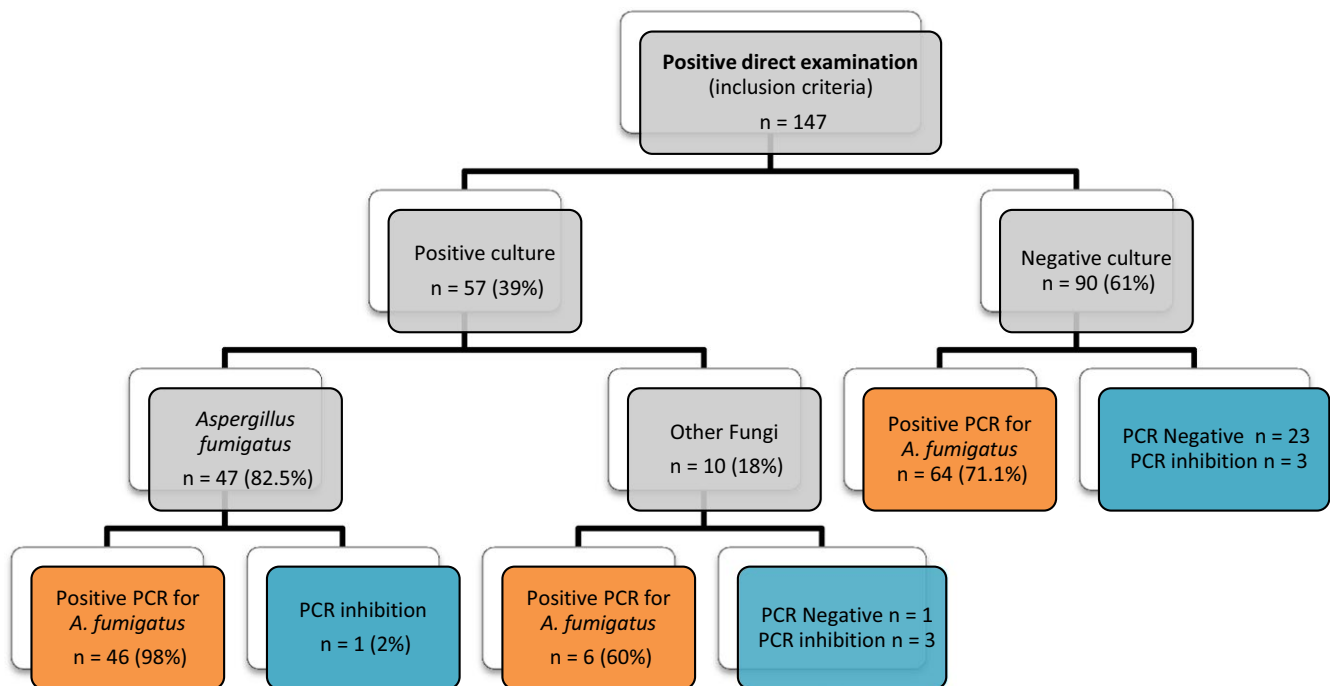

FIGURE 1 Mycological findings for the samples obtained from patients with fungal rhinosinusitis by culture and PCR (n = 147 samples)

Till now, azole resistance in *A. fumigatus* has been investigated in various respiratory samples with screening being mostly performed by determining in vitro antifungal susceptibility to azoles of *A. fumigatus* isolates grown in culture.[8,9,14,15] However, such strategy can lead to interpretation bias because (i) not all colonies are usually tested when working on cultures; (ii) cultures might be negative despite azole resistance (due to non-culturable isolates), as nicely illustrated in patients with chronic aspergillosis.[13] Taken together, there is an obvious interest for the detection of azole resistance directly from clinical samples. Although azoles are usually not required in most case of FRS (except invasive diseases), in light of the recent isolation of TR_{34}/L98H isolates in patients with FRS,[15,16] we thought timely to determine the extent of environmentally acquired azole resistance in *A. fumigatus* in this specific population, through a large multicenter study. In addition, this study would also provide some data about the potential exposition of azole-naïve patients (such as patients with FRS) to environmental azole-resistance mediated by TR_{34}/L98H, in the real-life setting, outside hospitals.

Interestingly, even though we used a PCR strategy directly on clinical samples to increase the sensitivity (allowing the analysis of non-culturable isolates), no TR_{34}/L98H isolate was evidenced in this cohort of patients with FRS. Although the *CYP51A* gene exists as a single copy in the *A. fumigatus* genome, the large amounts of fungal DNA material contained in fungal balls, makes rather unlikely the hypothesis that some TR_{34}/L98H isolates could have been missed. Moreover, there is also no experimental data supporting that TR_{34}/L98H could be associated with a fitness cost, to explain this low prevalence of *A. fumigatus* in the context of FRS.[22] Therefore, a reliable hypothesis to explain our findings is that azole resistance due to TR_{34}/L98H could be low in patients with FRS (here below 1%). In other patient groups, especially in immunocompromised or in CF patients, receiving azole therapy sometimes for long periods (a situation that is clearly not observed in patients with FRS), selective drug pressure exerted by azoles could both facilitate the colonisation of the respiratory tract by environmentally acquired azole-resistant isolates and potentiate in vivo acquisition of azole resistance, enhancing azole resistance rates, as observed among CF patients.[14,23] The findings of van der Linden et al, also supports the hypothesis that prevalence of azole resistance can vary greatly according to countries and underlying diseases.[24,25] Nevertheless, our study has some limitations: (i) although the PCR-based strategy used here to detect azole resistance is an obvious strength of this study, providing the opportunity to investigate both culturable and non-culturable isolates, the retrospective design did not allow in vitro antifungal susceptibility testing on the few isolates grown in culture; (ii) we cannot strictly rule out that a few patients could have been infected with other environmentally acquired mutations such as TR_{46}/Y121F/T289A that cannot be detected by this PCR assay (although TR_{34}/L98H is yet the most frequent environmental mutation conferring azole resistance among clinical samples in France);[23,26,27] (iii) despite the large number of patients and multicenter design, this cohort is not strictly representative of all clinical entities of FRS, but at least of the most common that is fungal ball.

To conclude, although we did not identified the *A. fumigatus* TR_{34}/L98H mutations conferring resistance, suggesting a low prevalence in patients with FRS in France, our study clearly demonstrates that a PCR-based strategy is much more reliable than standard culture for the identification of *A. fumigatus* in FRS samples. Whether our data are applicable to other countries and other environmentally acquired mutations now remains to be determined.

DISCLOSURE OF CONFLICT OF INTEREST

Nothing to declare.

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