

Evaluation of nested PCR in diagnosis of fungal rhinosinusitis

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ABSTRACT

Background and Objective: Given the importance of rapid diagnosis for fungal rhinosinusitis, this study aimed to evaluate the use of nested PCR to identify *Aspergillus* and *Mucor* species in clinical samples from patients with suspected fungal rhinosinusitis.

Methods: Functional endoscopic sinus surgery specimens were collected from 98 patients with rhinosinusitis from 2012 to 2013. All samples were ground and cultured on sabouraud dextrose agar. The isolated fungi were identified based on their macroscopic and microscopic features. Fungal DNA was extracted from the tissue samples and nested PCR was performed with two sets of primers for *Mucor* and *Aspergillus*.

Results: Direct microscopic showed that 5.1% contained fungal components and 9.2% exhibited growth of fungi in culture. The most common agents isolated were *Aspergillus fumigatus* (n= 3), *Aspergillus flavus* (n=2), *Penicillium sp* (n=3) and *Alternaria sp.* (n=1). *Mucor sp.* was identified in the pathology smear from 1 patient. Positive results for fungal rhinosinusitis were obtained for a total of 10.2% by culture or pathology smear. Positive PCR results were obtained in 72 samples for *Aspergillus* and 31 samples for *Mucor*.

Conclusion: Our results suggest that endoscopic sinus surgery specimens are not suitable for nested PCR, probably because of the accumulation of fungi that contaminate the environmental air. This drawback is a limiting factor for diagnosis with nasal cavity specimens. Therefore, molecular methods and conventional culture techniques are helpful complementary diagnostic methods to detect fungal rhinosinusitis and determine appropriate management for these patients.

Keywords: functional endoscopic sinus surgery, rhinosinusitis, nested PCR, *Aspergillus*

INTRODUCTION

Rhinosinusitis (RS) infections are caused by various organisms such as bacteria, viruses, fungi and environmental pollutants. The incidence is

increasing worldwide, and RS is now estimated to affect approximately 31 million Americans and result in 18 to 22 million medical visits annually in the USA (1, 2). According to 2008 National Health Interview Survey data, RS infection occurred in approximately 1 of every 7 adults (3). Fungal rhinosinusitis (FRS) is unusual and is mostly observed in patients with a compromised immune system, although it can also appear in immunocompetent persons (4). The prognosis in immunocompromised patients is poor and mortality has been reported to range from 60% to 80% (5).

Aspergillus is the most frequent etiologic agent in chronic invasive and granulomatous FRS, and in

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acute fulminant infections *Rhizopus* spp., are the most commonly isolated fungus (6).

The clinical symptoms and radiological signs of RS infection are the same but treatment varies depending on whether the etiological agent is bacterial, viral or fungal. The gold standard method for the diagnosis of FRS is isolation of the etiologic agent by culture. The sensitivity of culture under normal condition varies depending on the fungal species. For example, sporulation may fail in mucoral fungi, and the results of culture from clinical specimens are often negative because of nonviable organisms in necrotic tissues.

Molecular methods to diagnose fungal infections do not necessarily require the existence of viable organisms, and unlike culture methods, the former can detect very small amounts of the agent in the sample volume. Among the molecular approaches currently available, polymerase chain reaction (PCR)-based techniques have been used to identify fungi in clinical samples. Several studies have reviewed the specificity and sensitivity of molecular methods to detect various types of fungi (7, 8).

Functional endoscopic sinus surgery (FESS) is the usual surgical treatment for RS and nasal polyps. The aim of surgery is to enlarge the drainage pathways of the sinuses, thus avoiding the accumulation of mucus and pus. Given the importance of rapid diagnosis of FRS infections, this study was designed to evaluate the use of nested PCR with specific primers to identify species of *Aspergillus* and *Mucor* in FESS samples from patients with suspected FRS.

MATERIALS AND METHODS

Samples. In this cross-sectional study the FESS specimens were collected from 98 patients with RS (diagnosed by clinical manifestations and computed tomography scans) from 2012 to 2013 and examined in the Mycology Department of Professor Alborzi Clinical Microbiology Research Center, Nemazi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran. Demographic data of the patients were collected from their medical files. Briefly, samples from the paranasal sinus were obtained by surgical procedure under general anesthesia. The FESS samples were divided into two parts, one for pathological and the other for mycological testing.

Definition. Patients with a positive fungal culture or pathology smear, or both, were considered as cases

of documented fungal infection.

Standard mycological methods. In the mycology laboratory, the fluids and fresh tissue materials were ground. One part of samples were observed under a light microscope using 10% potassium hydroxide (KOH), another were cultured on Sabouraud dextrose agar (Merk, Darmstadt, Germany) for 14 days at 30 °C. The isolated fungi were identified based on their macroscopic and microscopic features by lactophenol cotton blue smear.

Molecular investigation. Fungal DNA was extracted from the tissue samples by adding 100 µL distilled water, 100 µL lysis buffer, 20 µL proteinase K and 20 µL carrier RNA, then incubated overnight at 56 °C and washed with normal saline. The cell walls of fungi were lysed by sonicating the samples for 90 s at 150 Hz. Extraction was completed with a DNA extraction kit (Invetech, Berlin, Germany) according to the manufacturer's recommendations. The optical densities were measured at 260 nm for DNA and 280 nm for proteins.

Nested PCR was performed with two sets of primers for *Mucor* and *Aspergillus*. The outer primers of *Mucor* used in this study to detect a 175-bp DNA fragment were ZM₁ (5'-ATT ACC ATG AGC AAA TCA GA-3') and ZM₂ (5'-TCC GTC AAT TCC TTT AAG TTT C-3'), and the inner primers were ZM₁ and ZM₃ (5'-CAA TCC AAG AAT TTC ACC TCT AG-3') (9). The outer primers of *Aspergillus* for the first step were AFU5S (5'-AGG GCC AGC GAG TAC ATC ACC TTG) and AFU5AS (5'-GG G(A)GT CGT TGC CAA C(CT)C (CT)CC TGA-3'), and the inner primers for the second step were AFU7S (5'-CGG CCC TTA AAT AGC CCG-3') and AFU7AS (5'-GA CCG GGT TTG ACC AAC TTT-3'), which amplified a of 236-bp fragment (10). PCR was performed with a thermocycler (5530 Mastercycler, Eppendorf, Hamburg, Germany) under the following thermal conditions: a denaturation step for 5 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C for *Mucor* and 63 °C for *Aspergillus*, extension for 1 min at 72 °C, a final extension step for 8 min at 72 °C.

Sensitivity of PCR assays. To determine the sensitivity of the nested PCR assay, serial dilution of the extracted DNA from *A. fumigatus* and *Mucor* were amplified and measured.

Table 1. Correlation between PCR results and positive or negative results of fungal culture in 10 patients with documented fungal rhinosinusitis

NO	Age/Sex	Pathophysiological findings	KOH 10%	Culture on MEA	Aspergillus PCR result	Mucor PCR result
1	37/F	Nasal polyposis	Negative	<i>A. fumigatus</i>	+	Negative
2	18/F	Chronic fungal sinusitis	Negative	<i>Alternaria</i> sp.	+	Negative
3	28/M	Allergic fungal sinusitis	Negative	<i>A. fumigatus</i>	+	Negative
4	25/F	Allergic fungal rhinosinusitis	Positive	<i>A. flavus</i>	+	+
5	42/M	Nasal polyposis	Positive	<i>A. fumigatus</i>	+	Negative
6	43/M	Negative	Negative	<i>Penicillium</i>	+	Negative
7	51/F	Negative	Positive	<i>Penicillium</i>	+	Negative
8	28/F	Allergic fungal rhinosinusitis	Positive	<i>Penicillium</i>	Negative	Negative
9	35/M	Nasal polyposis	Negative	<i>A. flavus</i>	+	Negative
10	64/F	Purulent rhinorrhea	Positive	<i>Mucor</i> sp.	Negative	+

All statistical analyses were done with SPSS software for Windows (Statistical Product and Service Solutions, version 15.0, SPSS Inc, Chicago, IL, USA).

Ethical considerations. The ethics committee of the Clinical Microbiology Research Center at Shiraz University of Medical Sciences approved this study, which was carried out in accordance with the Declaration of Helsinki. All patients provided their informed consent in writing to participate in the research.

RESULTS

A total of 98 patients [56 (57%) males and 42 (43%) females] who underwent FESS for suspected RS according to clinical or radiological findings were enrolled in the study. Their mean age was 36.7 years and they ranged from 13 to 74 years. Most of them had radiologic evidence of infection (90/98, 92%). The patients had a history of asthma, nasal polyp, hypothyroidism, hyperthyroidism, lupus and chronic rhinosinusitis. A history of allergy was noted in 6 (5.9%), and 14 (13.7%) of the patients were smokers.

Direct microscopic examination showed that 5 out of 98 samples (5.1%) contained fungal elements,

and 9 (9.2%) samples exhibited growth of fungi in culture. The agents isolated most frequently were *Aspergillus fumigatus* (3 patients, 3.1%) followed by *Aspergillus flavus* (2, 2%), *Penicillium* spp. (3, 3.1%) and *Alternaria* sp. (1, 1%). *Mucor* sp. was reported in the pathology smear in 1 patient. Ten patients had documented FRS. Of these 8 had positive results for both culture and nested PCR (80% of the positive cases). The characteristics of the patients with documented FRS are presented in Table 1.

The sensitivity (minimum concentration) of nested PCR was 3 ng/ μ L for *Aspergillus* and 1 ng/ μ L for *Mucor*. Of 392 nested-PCR reactions, 103 were positive for *Aspergillus* (72 cases) or *Mucor* (31 cases). The sensitivity, specificity, positive and negative predictive values for *Aspergillus* were 100%, 28.2%, 8% and 100%, and the corresponding values for *Mucor* were 100%, 31%, 3.1% and 100%, respectively.

DISCUSSION

Sinusitis significantly affects the patient's perceived general health, vitality and social function. In most cases the diagnosis is established by examination of sinus tissue and mucus obtained during sinus surgery.

In this study, the incidence of FRS was 10.2% among patients with RS, based on direct smear and culture results (9 cases with a positive culture and 1 with a positive pathologic smear). Earlier studies reported rates of FRS from 7.3% to 25 % in nasal lavage samples (11,12). Our patient sample was similar to that reported by Kordbacheh *et al.*, in which 9 of 100 (9%) polyp samples examined microscopically and by culture yielded positive findings for fungal agents (13).

The diagnosis of FRS requires pre-operative information from physical examination, along with radiologic findings and signs of infection, which are frequently identified in computed tomography (CT) scans. The sensitivity of CT scanning to detect cases was 45.5% in an earlier study (11). In this study, 90% of all patients and 100% of those with FRS had radiologic signs.

Rhinosinusitis is a clinical entity produced by several kinds of organisms, and the management of each type requires identification of the etiologic agent through direct smear and culture, which are valuable in the diagnosis. Direct smear with KOH or pathology smear are rapid methods for diagnosing the infection. The sensitivity of direct smear compared to culture in our study was 55.6%, whereas in a study by Satish *et al.* the sensitivity in tissue samples was reported as 90.9% (11). This difference may be due to the type of sample collection used in the study. The sensitivity of culture as the gold standard for the diagnosis of fungal infections varies. In the present study, 9 of 10 infected patients had a positive fungal culture and only 1 patient had a negative culture result and a positive pathology smear; therefore the sensitivity of culture was 90%. The range of sensitivities in different studies varies widely between 30%-50% and 64%-100% (3, 14). Sensitivity rates are related to the type of etiologic agent, and infection with *Mucors* sp. can decrease the sensitivity of culture. In this study, *Aspergillus* sp. was the etiologic agent isolated most frequently, a result consistent with findings in other regions (15).

Fungal spores are ubiquitous in the environment. "Some genera of airborne fungal spores such as *Alternaria*, *Aspergillus* and *Cladosporium* are found throughout the world. The airborne spores of these fungi are generally considered as important causes of both allergic rhinitis and allergic asthma (16). Therefore, a positive fungal culture is insufficient to confirm the diagnosis of infection. For this reason, the sample should be cultured in three plates and

if the same agent is isolated from all plates, a final diagnosis can be made. To improve diagnostic accuracy, a combination of culture and direct smear is recommended. In recent years PCR methods have become established as sensitive and rapid detection assays for infections in different populations. The specificity of the molecular PCR method tested in the current study was low, probably because of fungal colonization of the paranasal sinus mucosa. The sensitivity and specificity of nested PCR have been reported 100% and 28.2% for *Aspergillus* and 100% and 31% for *Mucor*, respectively. The sensitivity of a panfungal PCR method used by Polzehl *et al.* was 44.2% (34 of 77 samples), whereas the sensitivity of culture was 24.7% (19 of 77)(12). The molecular detection of mucormycetes is complicated by several factors; therefore no standard and favorable protocol has been established so far. However, few methods for the detection of mucormycetes have been published (17), and only some methods have been evaluated in clinical samples (18, 19).

CONCLUSION

Several protocols are available for the detection of bacteria and fungi in a variety of clinical samples. In the present study we analyzed FESS tissues with a PCR method. The results suggest that FESS specimens are not suitable for nested PCR because of the accumulation of fungi that contaminate the environmental air. This drawback is a limiting factor for diagnosis in specimens from the nasal cavity. Because this factor cannot be excluded, nested PCR is of limited use for the detection of FRS in the upper airways. Therefore, molecular methods and conventional culture methods should be used as complementary diagnostic techniques to detect FRS and determine appropriate management for these patients.

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