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# A PCR-Based Approach for Early Diagnosis of Head and Neck Aspergillosis

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**Abstract:** Aspergillosis is a fungal disease caused by the inhalation of fungal spores of the genus Aspergillus spp. This fungus mainly affects the lungs but can spread and infect the maxillofacial region through the bloodstream. The disease has nonspecific clinical manifestations, which hinders early diagnosis. Even though the Polymerase Chain Reaction (PCR) technique holds promising potential to aid in diagnosing aspergillosis, anatomopathological analysis services have not yet routinely adopted the method. Therefore, the present study aims to evaluate the applicability and standardize the techniques of preparation of biological samples and PCR for the detection of the species *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*. Six samples of formalin-fixed, paraffin-embedded tissue (FFPE), with histopathological diagnosis suggestive of aspergillosis, were investigated by PCR. Five of the six samples evaluated were positive for the fungus *Aspergillus* spp, 2/5 of which were co-infected by *A. fumigatus* and *A. flavus* species, and the others were positive for *A. flavus* species. The findings of this study suggest that PCR may be an auxiliary method in diagnosing aspergillosis. However, the expansion of the sample size and the evaluation of PCR in comparison with other diagnostic tests for aspergillosis is essential to determine the accuracy of the method.

**Keywords:** aspergillosis; *Aspergillus*; PCR; diagnosis

## 1. Introduction

First described in 1729 by the Italian priest and biologist Pier Antonio Micheli, the fungus *Aspergillus*, from the Latin *aspergere* (to spread), received its name because it resembles an aspergillum, a device used in churches to sprinkle holy water [1]. *Aspergillus* is a filamentous fungus, ubiquitous in nature, and can be found in soil, dust, plants and even in hospital ventilation systems [2]. For example, they are important microorganisms that produce food, medicines, and enzymes [3]. However, not all species are beneficial; some can cause human illness. Its spores remain suspended for long periods and can remain viable for months in a dry environment [4,5]. Inhalation of these *Aspergillus* spores can cause an opportunistic infection known as aspergillosis, which is still frequently underdiagnosed [6]. Theodor Sluyter reported the first case of aspergillosis in humans in 1847 [7].

There are different clinical presentations of aspergillosis that can range from allergic-type disease (non-invasive) to generalized forms, leading to the risk of death (invasive form) [9]. Allergic bronchopulmonary aspergillosis (ABPA) and allergic fungal rhinosinusitis are the non-invasive

forms, while chronic pulmonary aspergillosis and invasive pulmonary aspergillosis (IPA) comprise the invasive forms of the disease [8,9].

The manifestation of each clinical type depends on the fungus's virulence and the immunological host response [10]. In immunocompetent patients, inhalation of *Aspergillus* spores rarely causes illness, as they are eliminated from the body due to the coordinated action between the innate and adaptive responses of the immune system [2,11]. However, the infection can spread through vascular invasion to other organs [10,12], principally in immunocompromised patients. The dissemination of *Aspergillus* may affect the nose, paranasal sinuses, and the oral cavity, which makes this infection of particular interest to dentistry [5,13].

Fungal sinusitis is the main manifestation of *Aspergillus* infection in the maxillofacial region in immunocompromised patients [14]. Other forms of infection include oral and maxillofacial lesions with nonspecific radiographic presentation, such as radiolucent lesions in the periapex after extraction or endodontics and ulcerations on the palate or gums [5,15]. While sinus aspergillosis lesions can be radiographically like viral or bacterial rhinosinusitis or even neoplasms [16], ulcerated lesions can be confused with infections caused by other filamentous fungi, such as various *Zygomycetes* (oral mucormycosis) [17]. These overlapping clinical findings can hinder the differential diagnosis of aspergillosis cases, delaying its early identification in the oral medicine routine [14].

Although *Aspergillus* spores are constantly inhaled, disease is rarely observed in immunocompetent hosts. On the other hand, immunosuppressed patients (corticosteroid therapy) or critically ill patients are strongly associated with high mortality and morbidity rates due to aspergillosis [18] and, therefore, early diagnosis is important to provide a better prognosis through an assertive therapeutic intervention [14]. Histopathological analysis and culture of microorganisms are currently the gold standard for diagnosis [9]. However, culture is a time-consuming examination, and histopathological analysis is only suggestive due to the microscopic similarity between the different fungal organisms. In addition, hyphae are not always visible on histopathological examination, and other fungal infections, such as fusariosis and mucormycosis, may have histopathological features superimposed on aspergillosis [9,19].

Therefore, it is necessary to develop a tool that helps in the rapid and reliable diagnosis of aspergillosis. The Polymerase Chain Reaction (PCR) may be a potential tool for early and assertive diagnosis since its benefits are already scientifically proven [12]. However, due to the lack of a standardized technique for *Aspergillus*, PCR is not yet widely used as a diagnostic method for aspergillosis [10,20]. In the present study, we aimed to standardize PCR assays for the detection of *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus niger*, the main species responsible for human infections [9,10] and evaluate the reliability of this technique for the rapid diagnosis of aspergillosis.

## 2. Materials and Methods

This study was approved by the Research Ethics Committee of the Universidade Federal de Minas Gerais (CAAE: 74144623.4.0000.5149) in accordance with the principles of the 1964 Helsinki Declaration and its later amendments.

Digital records from the Oral and Maxillofacial Pathology Laboratory of the School of Dentistry of the Universidade Federal de Minas Gerais (UFMG) and Universidade Federal do Rio de Janeiro (UFRJ) were retrospectively accessed for all suspected cases of aspergillosis between 2000 and 2023. Six samples were analysed in the present study, all from male patients aged between 52 and 79 years old. For each case, clinical data and the corresponding FFPE samples were retrieved. The case 5 and 6 were obtained from patients with a history of frontal bone and zygomatic bone fractures, respectively. The microscopic aspects of all cases were reviewed by two Oral and Maxillofacial pathologists (RSG and MJRGS (Figure 1). Fungal cultures of each species were used as positive controls. The Mycology Laboratory of the Institute of Biological Sciences at UFMG provided us with fungal cultures of *A. fumigatus* and *A. niger*, while the species *A. flavus* was made available by the Faculty of Veterinary Medicine at UFMG, which were cultivated in medium own for seven days. Inflammatory fibrous hyperplasia sample was used as the negative control.
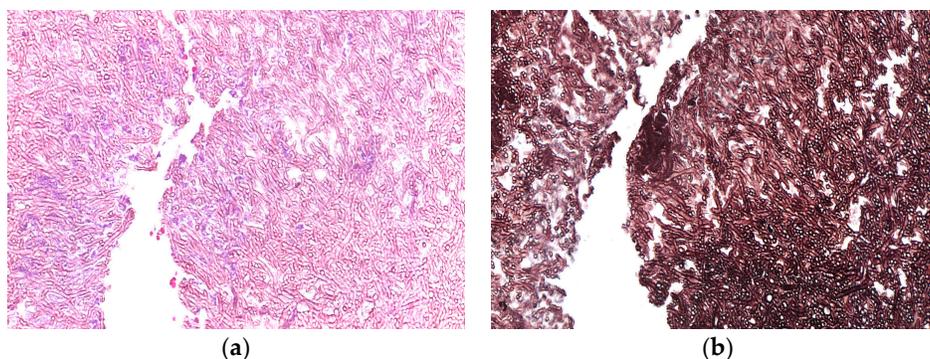
**Figure 1.** Aspergillosis histopathology. Septate hyphae, with dichotomous branching and, sometimes, forming angles of 45°, stained with HE (a) and Grocott (b). (original magnification, H&E staining, x40).

The fungal genomic DNA (gDNA) extraction from FFPE samples was performed using the commercial kits QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol with few adaptations. The first two 5µm-thick sections were discarded, and subsequently, ten tissue sections were collected in 1.5ml tubes and deparaffinized with 320µl of Deparaffinization solution (QIAGEN, Hilden, Germany). After adding 360µl of ATL buffer, the samples were left at -20 ºC for a minimum of 10 minutes. Three stainless steel beads were added to the tubes and vortexed for 10 minutes to macerate the tissue, disrupting the cell wall of the filamentous fungi. After removing the beads, 40µl of Proteinase K (QIAamp DNA Blood Mini Kit) were added and incubated overnight at 56°C. Then, 400µl of AL buffer were added and vortexed for another 10 minutes, followed by incubation for 1 hour at 70 ºC. The entire clear phase was transferred to a new 1.5ml tube where 400 µl of 100% alcohol were added. Subsequent steps followed the manufacturer's protocol. gDNA extraction from purified fungal culture samples, used as a positive control, followed a similar protocol. Briefly, culture plates were scraped with a plastic rod and washed with sterile phosphate buffered solution (PBS). The mycelia were collected in 15ml tubes and centrifuged to obtain a pellet. The pellet was redistributed into 1.5ml tubes and conditioned at -20 ºC until DNA extraction.

PCR reactions were performed following standard procedures using the MyTaq HS Red Mix, 2x (Bioline Reagents, London, UK) reagent. Optimal annealing temperatures were determined by gradient PCR and were settled at 57.8°C for all the primer sets. Cycling conditions were optimized as follows: 3 min - 95 ºC for initial denaturation, followed by 60 cycles (95ºC – 15s, 57.8°C – 15 s, 72°C – 30s), and 10 minutes at 72 ºC for final extension. 300ng of fungal gDNA was used to test the specificity of each primer set in a 25µl-PCR reaction and also served as positive control in all the experiments. For the analysis of the FFPE samples, an input of 100-200ng of gDNA was used in the reactions. gDNA extacted from Fibrous hyperplasia samples were used as negative controls. The primer sets used in the present study were kindly designed by Professor Dr. Leonel Mendoza from the University of Michigan, finding unique internal transcribed spacer (ITS) regions of these three fungi (Table 1). 1µl of each primer (F and R respective of each species) at 10µM concentration were added to the PCR tubes. In order to minimize the risk of cross-contamination, the reactions for each species were performed separately. PCR products were loaded into a 1.5% agarose gel electrophoresis and inspected under UV light.

**Table 1.** Sequence of primer pairs for each species studied.

| Species | Primer Sequence (5'-3') | Amplicon Size |
|---|---|---|
| *A. fumigatus* | **F**- GTCTGAGTTGATTATCGT | 312bp |
| | **R**- GGCCTACAGAGCAGGTGAC | |
| *A. flavus* | **F**- CACCACGAACTCTGTCTGATC | 373bp |
| | **R**- GATTGATTTGCGTTCGGC | |
| *A. niger* | **F**- GCCCAACCTCCCATCCGTG | 453bp |
| | **R**- CAATCCTACAGAGCATGTG | |

F: forward primer R: reverse primer.

## 3. Results

Co-infection with *A. fumigatus* and *A. flavus* was observed in samples 1 and 4. Samples 2, 3 and 6 were positive for *A. flavus,* and sample 5 was negative for all tested species (Figures 2 and 3).
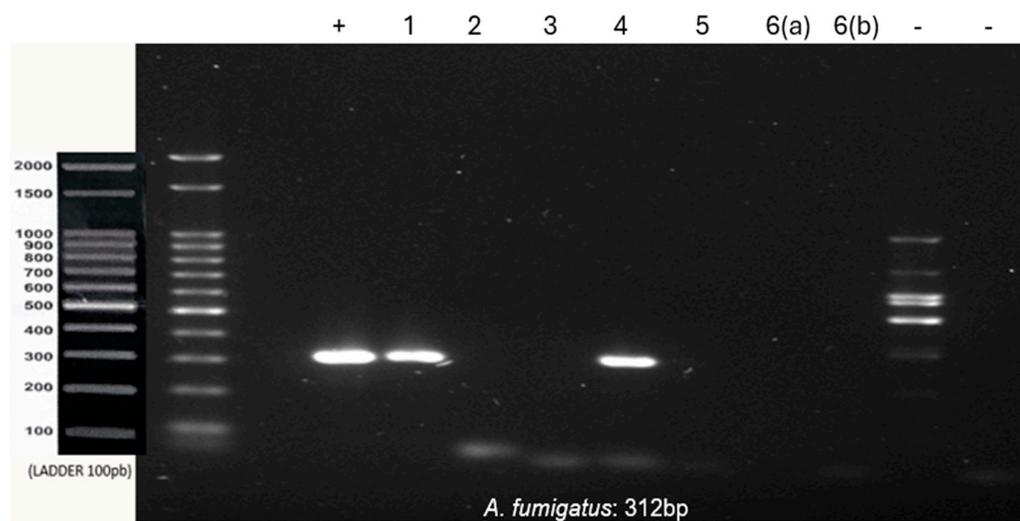


**Figure 2.** PCR using *Aspergillus fumigatus* primer with 100ng of DNA from each sample.
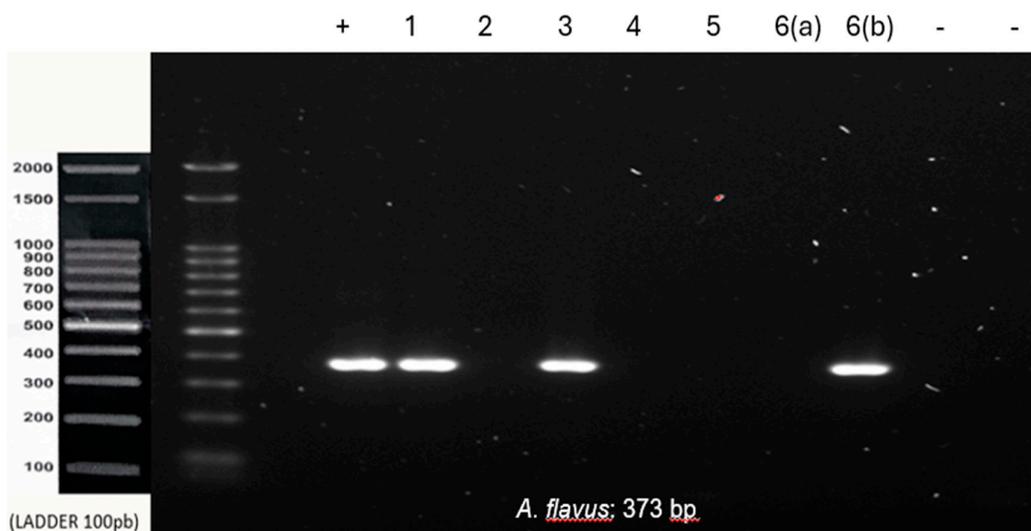


**Figure 3.** PCR using *Aspergillus flavus* primer with 100ng of DNA from each sample.

All samples tested negative for A. niger. The inflammatory fibrous hyperplasia samples were negative for all three species tested, thus reinforcing the specificity of the primers and the lack of contamination during the reaction's setup. All results are summarized in Table 2.

**Table 2.** PCR results of each sample by species.

| Samples | Location | Sex | Age | PCR test results | | |
|---|---|---|---|---|---|---|
| | | | | A. niger | A. fumigatus | A. flavus |
| 1 | Maxillary sinus | M | 79 | - | + | + |
| 2 | Maxillary sinus | M | 55 | - | - | + |
| 3 | Maxillary sinus | M | 49 | - | - | + |
| 4 | Upper gum | M | 56 | - | + | + |
| 5 | Frontal sinus | M | 57 | - | - | - |
| 6 | Maxillary sinus | M | 52 | - | - | + |

M = Male Age = Years old.

## 4. Discussion

PCR has been increasingly consolidated as a potential diagnostic tool for several infectious diseases, being faster than traditional methods and providing good sensitivity and specificity [23]. Early diagnosis of aspergillosis is still highly challenging, though. Its signs and symptoms are nonspecific, and the gold standard test, which is culture, can take a long time to provide a definitive and assertive result. The second most commonly used diagnostic tool in these cases is histopathological analysis, which has significant limitations in terms of specificity and sensitivity, since the arrangement of hyphae may resemble other fungal infections, thus requiring deep knowledge from the pathologist to establish the differential diagnosis [19,24].

With the advance of the SARS-CoV-2 virus and the possibility of co-infection with aspergillosis, mortality and morbidity rates, associated with fungal infections, have increased from 2019 [9,19]. This scenario justifies the importance of obtaining a rapid and accurate diagnosis, capable of optimizing and adequately directing the therapeutic management of these patients to minimize the risks that this infection offers.

Aspergillosis is also of dental interest since filamentous fungi, such as Aspergillus, have been isolated in root canals of teeth with pulp necrosis and apical periodontitis. Such endodontic conditions in upper posterior teeth are strongly associated with maxillary sinusitis [15].

Despite advances in molecular studies in recent times as a diagnostic tool for many diseases, its application to aspergillosis is still incipient and restricted [23]. Its major limitation is the lack of standardization to ensure reproducibility, specificity, and sensitivity [10,20].

The main changes made in the sample processing protocol and in the traditional sequence of gDNA extraction kits commonly used in molecular biology were: freezing and agitation with 3 mm diameter beads, to promote disruption of the fungal cell wall composed mainly of chitin; increase in the concentrations of the reagents used in the protocol, for example, 40μl of proteinase K instead of 20μl as suggested by the protocol of the manufacturer of the kit used. Thus, we sought to associate a chemical and mechanical method to obtain the highest possible concentration of gDNA from the samples.

Of the more than 200 existing species of *Aspergillus*, we selected the three most important in the human pathological context [22]. We searched for specific ITS regions of each species, which resulted in three primers that allowed us to identify Aspergillus in the selected samples. Testing these primers in positive and negative control samples ensured the specificity of the primers for the sequences of interest.

The study also allowed us to observe the possibility of co-infection of different species in the same lesion, which may have a significant clinical impact, given that distinct species may exhibit

variable susceptibilities to antifungal drugs. For example, *A. flavus* is more resistant to antifungal drugs than other previously known species [22]. Although our study shows that PCR can be an auxiliary tool in diagnosing aspergillosis, the low number of samples and the fact that none of the cases had the diagnosis confirmed by culture are some limiting factors that should be considered. Finally, information regarding patient management and treatment was not available because of the retrospective nature of our study. Therefore, further prospective investigations using a more significant number of samples are needed to validate the results presented here and better clarify the role of PCR in the clinical management of the disease. In addition, the comparative analysis of PCR to other aspergillosis diagnostic tests is essential to evaluate the accuracy of the standardized protocol.

## 5. Conclusions

In conclusion, we show in the present study that clinical/pathological diagnosis of aspergillosis can be enriched by PCR, especially in challenging cases or cases where the patient is resistant to first-choice treatment. We also show that co-infection of different Aspergillus species can occur during the oral manifestation of the disease. Further studies with a higher sample size in which cell culture could be employed as a comparative diagnostic method are vital to calculating PCR's accuracy in diagnosing aspergillosis.

**Author Contributions:** Thaís Ellen Chaves Gomes: Conceptualization; investigation; methodology; writing – original draft; writing – review and editing; data curation. Victor Coutinho Bastos: Investigation; data curation; methodology; writing – review. Douglas Boniek: Data curation; methodology and writing – review. Fernanda Faria Rocha: Writing – review and methodology. Roberta Rayra Martins Chaves: Conceptualization; investigation; writing – review and editing. Ricardo Santiago Gomez: Conceptualization; investigations; methodology; formal analysis; project administration; writing – review and editing.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Universidade Federal de Minas Gerais (protocol code 74144623.4.0000.5149 and date of approval 10/30/2023).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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