UNIVERSIDADE FEDERAL DE MINAS GERAIS

Faculdade de Medicina

Programa de Pós-graduação em Medicina Molecular

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REAÇÃO EM CADEIA DE POLIMERASE COMO UMA FERRAMENTA AUXILIAR PARA O DIAGNÓSTICO DE LESÕES ORAIS E MAXILOFACIAIS DE ASPERGILOSE

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Thaís Ellen Chaves Gomes

Reação em Cadeia de Polimerase como uma ferramenta auxiliar para o diagnóstico de lesões orais e maxilofaciais de aspergilose.

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como requisito parcial à obtenção do título de Mestre em Medicina Molecular.

Orientador: Prof. Dr. Ricardo Santiago Gomez

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Às 14:00 horas do dia seis de agosto de dois mil vinte e quatro, no formato on-line, realizou-se a sessão pública para a defesa da Dissertação de **THAIS ELLEN CHAVES GOMES** , número de registro 2022688974, graduada no curso de ODONTOLOGIA, como requisito parcial para a obtenção do grau de Mestre em MEDICINA MOLECULAR. A presidência da sessão coube ao professor Ricardo Santiago Gomez- Orientador (Odontologia). Inicialmente, o presidente fez a apresentação da Comissão Examinadora assim constituída: Prof. Ricardo Santiago Gomez- Orientador (Odontologia),Profa. Roberta Rayra Martins Chaves - Coorientadora, Prof. Ricardo Alves Mesquita (UFMG), Prof. Mário Romañach (UFRJ). Em seguida, a candidata fez a apresentação do trabalho que constitui sua Dissertação de Mestrado, intitulada: **"REAÇÃO EM CADEIA DE POLIMERASE COMO UMA FERRAMENTA AUXILIAR PARA O DIAGNÓSTICO DE LESÕES ORAIS E MAXILOFACIAIS DE ASPERGILOSE".** Seguiu-se a arguição pelos examinadores e logo após, a Comissão reuniu-se, sem a presença da candidata e do público e decidiu considerar aprovada a Dissertação de Mestrado. O resultado final foi comunicado publicamente à candidata pelo presidente da Comissão. Nada mais havendo a tratar, o presidente encerrou a sessão e lavrou a presente ata que, depois de lida, se aprovada, será assinada pela Comissão Examinadora.

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Dedico este trabalho aos meus amados pais, Ana Maria e José Marques, ao meu irmão Paulo e aos meus avós Cidalina, Conceição, Nelson e Thimóteo, que são meus maiores exemplos e sempre me apoiaram e me apoiam em todos os momentos da vida.

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“Toda tarefa na Terra, no campo das profissões, é convite do Pai para que o homem penetre os templos divinos do trabalho.”

Clarêncio (Nosso Lar)

Resumo

A aspergilose é uma doença fúngica causada pela inalação de esporos fúngicos do gênero *Aspergillus* spp. Este fungo afeta principalmente os pulmões, mas pode se espalhar e infectar a região maxilofacial através da corrente sanguínea ou inoculação do fungo após a extração ou tratamento endodôntico, especialmente nos dentes posteriores superiores. A doença tem manifestações clínicas inespecíficas que dificultam seu diagnóstico precoce. Embora a técnica da Reação em Cadeia da Polimerase (PCR) seja promissora como uma ferramenta diagnóstica para aspergilose, os serviços de análise anatomopatológica não adotam rotineiramente esse método. Portanto, o presente estudo teve como objetivo avaliar a aplicabilidade da PCR e padronizar as técnicas de preparação de amostras biológicas para a detecção das três espécies: *Aspergillus niger*, *Aspergillus fumigatus* e *Aspergillus flavus*. Seis amostras de tecido fixado em formalina e incorporado em parafina (FFPE) com um diagnóstico histopatológico sugestivo de aspergilose foram investigadas usando PCR. Como controle positivo para a reação de PCR, culturas morfologicamente e geneticamente caracterizadas foram usadas, com suas sequências depositadas no NCBI sob os códigos de adesão MW837777 (*A. fumigatus*) e MW837779 *(A. niger*). A cultura de *A. flavus* é de referência RC 2053. Quatro das seis amostras avaliadas foram positivas para *Aspergillus* spp., das quais uma apresentou coinfecção com as espécies *A. fumigatus* e *A. flavus*, duas foram positivas apenas para *A. flavus* e uma amostra foi positiva apenas para *A. fumigatus*. Esses achados sugerem que a PCR pode ser usada como um método auxiliar para diagnosticar a aspergilose. No entanto, este foi um estudo piloto, e a expansão do tamanho da amostra e a avaliação da PCR em comparação com outros testes de diagnóstico para aspergilose são essenciais para determinar a precisão do método.

Palavras-chave: aspergilose, *Aspergillus*, PCR, diagnóstico.

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 or inoculation of the fungus after extraction or endodontic treatment, especially in the upper posterior teeth. The disease has nonspecific clinical manifestations that hinder its early diagnosis. Although the Polymerase Chain Reaction (PCR) technique holds promise as a diagnostic tool for aspergillosis, anatomopathological analysis services do not routinely adopt this method. Therefore, the present study aimed to evaluate the applicability of PCR and standardise the techniques of preparation of biological samples for the detection of the three species: *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*. Six samples of formalin-fixed, paraffin-embedded tissue (FFPE) with a histopathological diagnosis suggestive of aspergillosis were investigated using PCR. As a positive control for the PCR reaction, morphologically and genetically characterized cultures were used, with their sequences deposited at NCBI under accession codes MW837777 (*A. fumigatus*) and MW837779 (*A. niger*). The *A. flavus* culture used is reference RC 2053. Four of the six samples evaluated were positive for *Aspergillus* spp., of which one was co-infected with *A. fumigatus* and *A. flavus* species, while two others were positive only for *A. flavus*, and one sample was positive only for *A. fumigatus*. These findings suggest that PCR can be used as an auxiliary method for diagnosing aspergillosis. However, this was a pilot study, and expansion of the sample size and the evaluation of PCR in comparison with other diagnostic tests for aspergillosis are essential to determine the accuracy of the method.

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

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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 aspergillum, which is a device used in churches to sprinkle holy water [1]. *Aspergillus* is a filamentous fungus that is ubiquitous in nature and is found in soil, dust, plants, and hospital ventilation systems [2]. They are important microorganisms that produce food, medicine, and enzymes [3]. However, not all species are beneficial, and some can cause human illness. Its spores remain suspended in air for long periods and can remain viable for months in a dry environment [4,5]. The inhalation of *Aspergillus* spores can cause an opportunistic infection known as aspergillosis, which remains frequently underdiagnosed [6]. Theodor Sluyter reported the first human case of pulmonary aspergillosis in 1847 [7].

Clinical presentations of aspergillosis can range from an allergic-type disease (non-invasive) to generalised forms, leading to the risk of death (invasive form) [8]. Allergic bronchopulmonary aspergillosis and allergic fungal rhinosinusitis are non-invasive forms, whereas chronic pulmonary aspergillosis is an invasive form [8,9].

The manifestation of each clinical type depends on the virulence of the fungus and the immunological host response [10]. In immunocompetent patients, the inhalation of *Aspergillus* spores rarely causes illness, as they are eliminated from the body owing 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 in 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 lesions that arise after endodontic treatment or tooth extraction, particularly in the upper posterior teeth. Clinically, lesions present as necrotic ulcers on the palate that may extend to the attached gingiva, showing a nonspecific radiographic appearance [5,15]. While sinus aspergillosis lesions can

be radiographically similar to 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 and delay its early identification in the oral medicine routine [14].

Although *Aspergillus* spores are constantly inhaled, disease is rarely observed in immunocompetent hosts. In contrast, immunosuppressed patients (corticosteroid therapy) or critically ill patients are strongly associated with high mortality and morbidity rates caused by aspergillosis [18]. Therefore, an early diagnosis is important to provide a better prognosis through assertive therapeutic intervention [14]. Histopathological analysis and culture of microorganisms are currently the gold standards for diagnosis [8]. However, culture is a time-consuming examination, and analysis of morphological characteristics of *Aspergillus* culture, such as colour and type of spores, is not reliable, as these characteristics are susceptible to the influence of environmental factors, especially the culture medium and incubation temperature [19]. Histopathological analysis is only suggestive of the diagnosis because of the microscopic similarities between 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 [8,20].

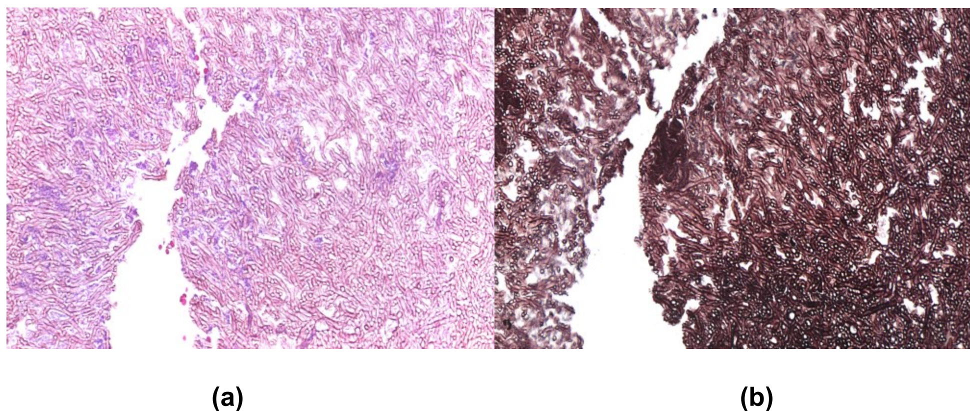
Therefore, it is necessary to develop a tool that can aid in the rapid and reliable diagnosis of aspergillosis. Polymerase Chain Reaction (PCR) is a potential tool for early and accurate diagnosis because its benefits have already been scientifically proven [12]. However, due to the lack of a standardised technique for *Aspergillus*, PCR is not yet widely used as a diagnostic method for aspergillosis [10,21]. In the present study, we aimed to standardise PCR assays for the detection of *A. fumigatus*, *A. flavus*, and *A. niger*, which are the main species responsible for human infections [8,10] 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 at the Universidade Federal de Minas Gerais (UFMG) and Universidade Federal do Rio de Janeiro (UFRJ) were retrospectively assessed for all suspected aspergillosis cases between 2000 and 2023. Six samples were analysed in the present study, all from male patients between the ages of 52 and 79. For each case, clinical data and corresponding FFPE samples were retrieved. Cases 5 and 6 involved patients with histories of frontal 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 for the PCR. The Mycology Laboratory of the Institute of Biological Sciences at UFMG provided us with fungal cultures of *A. fumigatus* and *A. niger*, while *A. flavus* was made available by the Faculty of Veterinary Medicine at UFMG and cultivated in its own medium for seven days. An Inflammatory fibrous hyperplasia sample was used as negative control.



(a) **(b)**

Figure 1. Aspergillosis histopathology. Septate hyphae, with dichotomous branching and, sometimes, forming angles of 45°, stained with H&E (a) and Grocott (b). (original magnification, H&E staining, x40)

Fungal genomic DNA (gDNA) was extracted from FFPE samples using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's protocol with some modifications. The first two 5 µm thick sections were discarded, and subsequently, ten tissue sections were collected in 1.5 mL tubes and deparaffinised with 320 µL of deparaffinization solution (QIAGEN, Hilden, Germany). After adding 360 µL of ATL

buffer, the samples were left at -20 ºC for a minimum of 10 min. Three stainless-steel beads were added to the tubes and vortexed for 10 min to macerate the tissue and disrupt the cell walls of the filamentous fungi. After removing the beads, 40 µL of Proteinase K (QIAamp DNA Blood Mini Kit) was added, and the sample was incubated overnight at 56°C. Then, 400 µL of AL buffer was added and vortexed for another 10 min, which was followed by incubation for 1 h at 70 ºC. The entire clear phase was transferred to a new 1.5 mL tube, and 400 µL of 100% alcohol was added. The subsequent steps were performed according to the manufacturer's protocol. gDNA extracted from purified fungal culture samples was used as a positive control following a similar protocol. Briefly, culture plates were scraped with a plastic rod and washed with sterile phosphate-buffered solution (PBS). Mycelia were collected in 15mL tubes and centrifuged to obtain pellets. The pellet was redistributed into 1.5 mL tubes and conditioned at -20 ºC until DNA extraction. Quantification and evaluation of the purity of the extracted DNA was measured on the NanoDrop® 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The A260/280 index of the deparaffinised samples varied from 1.6 to 1.82.

PCR reactions were performed following standard procedures using MyTaq HS Red Mix, 2x (Bioline Reagents, London, UK). The optimal annealing temperature was determined by gradient PCR and was 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 min at 72 ºC for final extension. Then, 300 ng of fungal gDNA was used to test the specificity of each primer set in a 25 µL PCR reaction and also served as positive control in all the experiments. For the analysis of FFPE samples, 100–200ng of gDNA was used. The gDNA extracted from fibrous hyperplasia samples was used as negative controls in the PCR. The primer sets used in this study were designed based on a search for unique sequences of the internal transcribed spacer (ITS) regions of each species available in the NCBI database. The obtained sequences were aligned, and the most conserved regions that differentiated one genus from another were selected to guide the primer design (Table 1). Then, 1µL of each primer (F and R, respectively, of each species) at 10µM concentration was added to the PCR tubes. To minimise the risk of cross-contamination, reactions for each species were performed separately. PCR products were loaded onto a 1.5% agarose gel for electrophoresis, at 200 v and 200 mA for 30 min, with 5 µL of SYBR® Saf DNA gel

stain (Thermo Fisher Scientific, CA, USA) and subsequently inspected under UV light. PCR products were then purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Foster City, CA, USA). Bidirectional DNA sequencing was performed using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and run on an ABI3130 DNA Analyzer (Applied Biosystems). ng Kit (Applied Biosystems) and run on an ABI3130 DNA Analyzer (Applied Biosystems). The chromatograms were manually inspected in the SnapGene Viewer software (v. 5.3.2, from GSL Biotech, San Diego, CA, USA; available at https://snapgene.com accessed on 19 September 2024). Sequences were then loaded on BLAST: Basic Local Alignment Search Tool (https:// blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 19 September 2024) and checked for their specificity. The GenBank accession numbers for the sequences used in the alignment were GenBank: OR939708.1 for *A. fumigatus* and GenBank: MT279323.1 for *A. flavus*.

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 sample 1. Samples 3 and 6 were positive only for *A. flavus.* Sample 4 was positive only for *A. fumigatus*, whereas samples 2 and 5 were negative for all tested species (Figures 2 and 3).

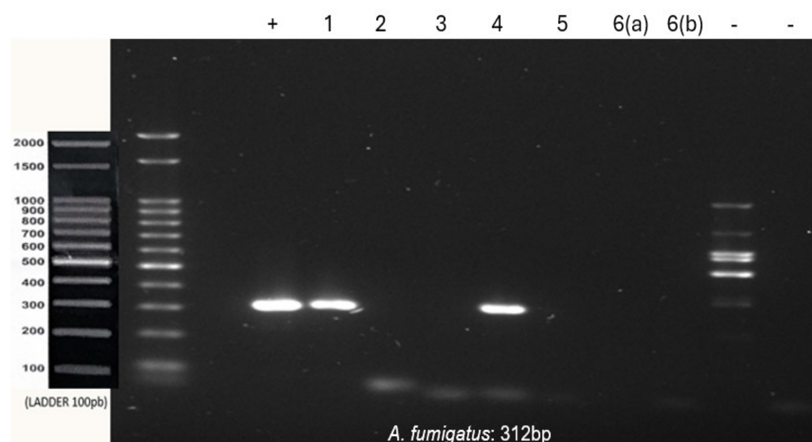


Figure 2. PCR using *A. fumigatus* primer with 100 ng of DNA from each sample. (+) DNA extracted from *A. fumigatus* culture (positive control). (-) Negative control of the reaction with and without human DNA. 1–6: Paraffin-embedded samples of lesions suggestive of aspergillosis. 6(a) and 6(b): Lesions removed from the same patient and embedded in two separate blocks.

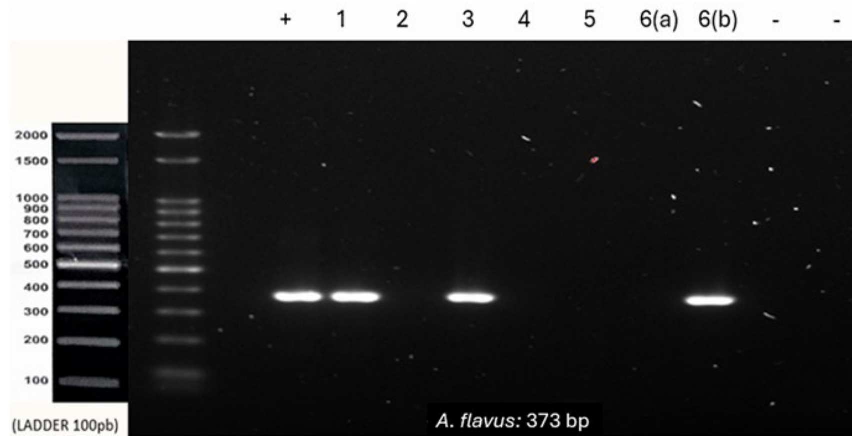


Figure 3. PCR using *A. flavus* primer with 100 ng of DNA from each sample. (+) DNA extracted from *A. flavus* culture (positive control). (-) Negative control of the reaction with and without human DNA. 1–6: Paraffin-embedded samples of lesions suggestive of aspergillosis. 6(a) and 6(b): Lesions removed from the same patient and embedded in two separate blocks.

All the samples tested negative for *A. niger* (Figure 4). The inflammatory fibrous hyperplasia samples were negative for all three species tested, reinforcing the specificity of the primers and lack of contamination during the reaction setup. The results are summarised in Table 2.

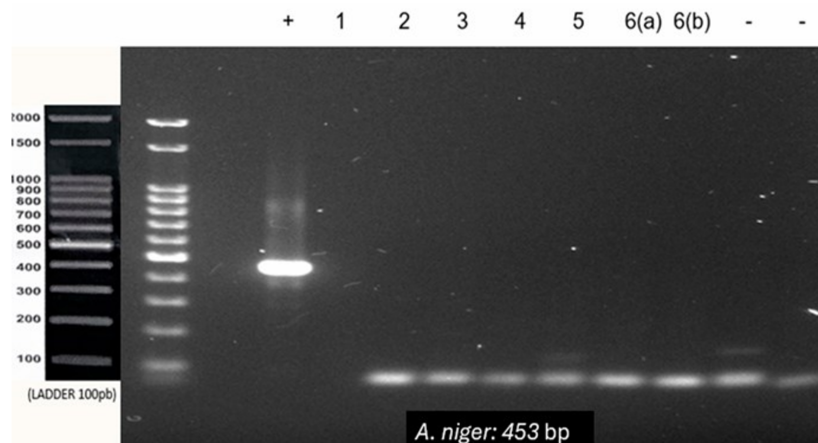


Figure 4. PCR using *A. niger* primer with 100 ng of DNA from each sample. (+) DNA extracted from *A. niger* culture (positive control). (-) Negative control of the

reaction with and without human DNA. 1–6: Paraffin-embedded samples of lesions suggestive of aspergillosis. 6(a) and 6(b): Lesions removed from the same patient and embedded in two separate blocks.

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.

Regarding the Sanger sequencing, generated sequences displayed 100% percent identity with the respective species that were found in the investigated samples. Representative chromatograms and sequence alignments are shown in the Figure 5.

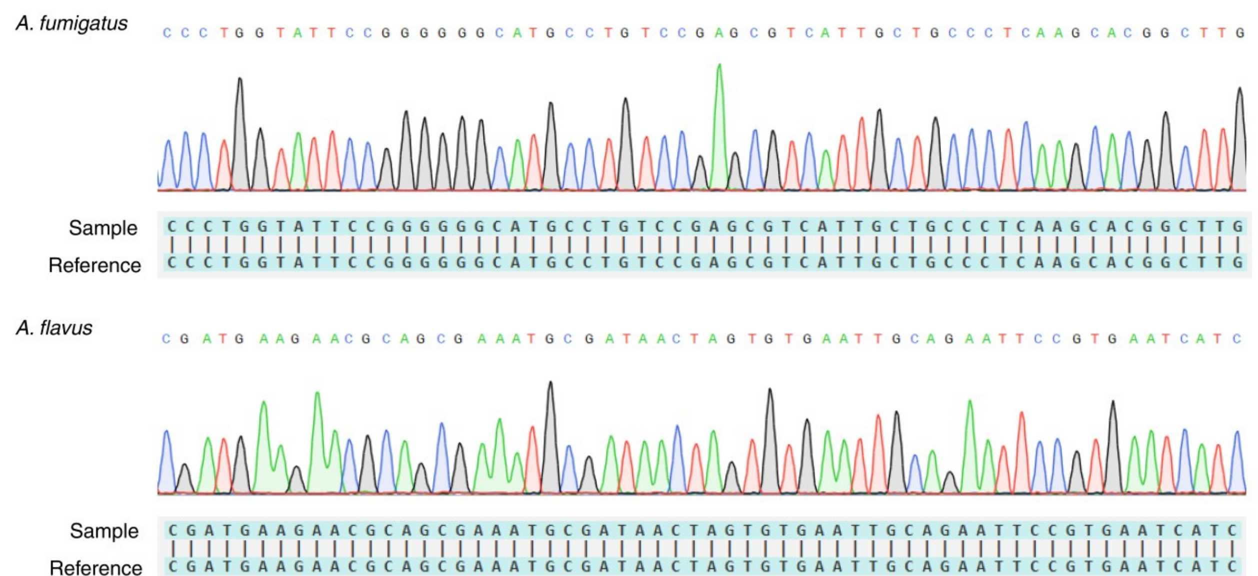


Figure 5. Sanger sequencing results and sequence alignment to reference genomes.

4 Discussion

PCR has become increasingly popular as a potential diagnostic tool for several infectious diseases, since it is faster than traditional methods and provides good sensitivity and specificity [22]. However, the early diagnosis of aspergillosis remains highly challenging. Its signs and symptoms are nonspecific, and the gold

standard test, which is culture, can take a long time to provide definitive and assertive results. The second most commonly used diagnostic tool in these cases is histopathological analysis, which has significant limitations in terms of specificity and sensitivity, because the arrangement of hyphae may resemble other fungal infections, thus requiring deep knowledge from pathologists to establish a differential diagnosis [19, 23].

With the advancement of SARS-CoV-2 and the possibility of co-infection with aspergillosis, the mortality and morbidity rates associated with fungal infections have increased since 2019 [8, 20]. This scenario warrants the need for obtaining a rapid and accurate diagnosis capable of optimising and adequately directing the therapeutic management of these patients to minimise the risk of infection.

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 the upper posterior teeth are strongly associated with maxillary sinusitis [15].

Despite recent advances in molecular studies as a diagnostic tool for many diseases, its application in aspergillosis is still incipient and restricted [22]. Its major limitation is the lack of standardization to ensure reproducibility, specificity, and sensitivity [10, 21].

We made the following main changes made in the sample processing protocol and in the traditional sequence of gDNA extraction kits commonly used in molecular biology: freezing and agitation with 3 mm diameter beads to promote disruption of the fungal cell wall composed mainly of chitin; increasing the concentrations of the reagents used in the protocol, for example, 40 µL of proteinase K instead of 20 µL as suggested by the manufacturer. Therefore, we sought to combine chemical and mechanical methods 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 [24]. We searched for specific ITS regions in each species, resulting in three primers that allowed us to identify *Aspergillus* in the selected samples. Testing these primers in positive and negative control samples ensured their specificity for the sequences of interest.

This study also allowed us to observe the possibility of co-infection with different species in the same lesion, which may have a significant clinical impact,

given that distinct species may exhibit variable susceptibility to antifungal drugs. For example, *A. flavus* is more resistant to antifungal drugs than other known species [24]. Although our study showed that PCR can be an auxiliary tool for diagnosing aspergillosis, the low number of samples and the fact that none of the cases had the diagnosis confirmed by culture are limiting factors that should be considered. Finally, information on patient management and treatment was not available because of the retrospective nature of this study. Therefore, further prospective investigations using a larger significant number of samples are needed to validate the results presented here and to clarify the role of PCR in the clinical management of the disease. In addition, a comparative analysis of PCR with other diagnostic tests for aspergillosis is essential to evaluate the accuracy of the standardised protocol.

5 Conclusions

In conclusion, we showed in the present study that the 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 showed that co-infection with different *Aspergillus* species can occur during the oral manifestation of the disease. Further studies with a larger sample size, in which cell culture could be employed as a comparative diagnostic method, are vital for analysing the accuracy of PCR in diagnosing aspergillosis.

References

1. Baker, S.E.; Bennett, J.W. An overview of the genus aspergillus. *In The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods*; CRC Press: Boca Raton, FL, USA, 2007; pp. 3–13.

2. Paulussen C, Hallsworth JE, Álvarez-Pérez S, Nierman WC, Hamill PG, Blain D, et al. Ecology of aspergillosis: insights into the pathogenic potency of Aspergillus fumigatus and some other Aspergillus species. Microbial Biotechnology [Internet]. 2016 Jun 7;10(2):296–322. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5328810/ (accessed on 5 December 2023).

3. Batista PP. Caracterização de linhagens do grupo *Aspergillus flavus* baseada em marcadores de DNA. repositorio.ufpe.br. 2007. Available online: https://repositorio.ufpe.br/handle/123456789/6454 (accessed on 5 December 2023).

4. Egger M, Jenks JD, Hoenigl M, Prattes J. Blood Aspergillus PCR: The Good, the Bad, and the Ugly. Journal of Fungi [Internet]. 2020 Jan 27 [cited 2021

Feb 17];6(1). Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7151127/

5. Telles DR, Karki N, Marshall MW. Oral Fungal Infections: Diagnosis and Management. Dental Clinics of North America. 2017 Apr 1;61(2):319–49. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0011853216301380

6. Latgé JP, Chamilos G. *Aspergillus fumigatus* and Aspergillosis in 2019. Clinical Microbiology Reviews. 2019 Nov 13;33(1). Available online: https://cmr.asm.org/content/cmr/12/2/310.full.pdf

7. Knoke M, Bernhardt H, Schwesinger G. [Early description of a pulmonary aspergillosis 1847 from Greifswald]. Mycoses. 2003;46 Suppl 1:37–41. Available online: https://pubmed.ncbi.nlm.nih.gov/12955852/

8. Cadena J, Thompson GR, Patterson TF. Aspergillosis. Infectious Disease Clinics of North America. 2021 Jun;35(2):415–34.

9. Bongomin F, Harris C, Foden P, Kosmidis C, Denning DW. Innate and Adaptive Immune Defects in Chronic Pulmonary Aspergillosis. Journal of Fungi. 2017 May 29;3(2):26.

10. Sasani, Farzad Pakdel, Sadegh Khodavaisy, Salehi M, Salami A, Sohrabi M, et al. Mixed Aspergillosis and Mucormycosis Infections in Patients with COVID-19: Case Series and Literature Review. Mycopathologia. 2024 Jan 17;189(1).

11. Carvalho-Pereira J, Fernandes F, Araújo R, Springer J, Loeffler J, Buitrago MJ, et al. Multiplex PCR Based Strategy for Detection of Fungal Pathogen DNA in Patients with Suspected Invasive Fungal Infections. Journal of Fungi. 2020 Nov 23;6(4):308.

12. Lortholary O, Gangneux JP ., Sitbon K, Lebeau B, de Monbrison F, Le Strat Y, et al. Epidemiological trends in invasive aspergillosis in France: the SAIF network (2005–2007). Clinical Microbiology and Infection. 2011 Dec;17(12):1882–9. Available online: http://onlinelibrary.wiley.com/doi/10.1111/j.1469-0691.2011.03548.x/full

13. Migott GB, Santos FM dos, Pagnussat LR, Barbosa B, Barbosa GDL, Hahn SR. Perfil Clínico e Epidemiológico de Pacientes com Suspeita de Aspergilose Pulmonar em Hospital do Estado Rio Grande do Sul, Brasil. Revista de Epidemiologia e Controle de Infecção. 2017 Jan 16;7(1).

14. Rai D, Shukla D, Bhola ND. Aspergillosis of Maxillary Sinus's Diagnosis, Management, and Association With COVID-19: A Case Report. Cureus. 2022 Oct 11; 14(10).

15. Gomes CC, Pinto LCC, Victor FL, da Silva EAB, Ribeiro A de A, Sarquis MI de M, et al. *Aspergillus* in endodontic infection near the maxillary sinus. Brazilian Journal of Otorhinolaryngology. 2015 Sep;81(5):527–32.

16. Sousa C, Romulo Antonio Pasini, Alessandro Pasqualotto, Marchiori E, Altmayer S, Irion K, et al. Imaging Findings in Aspergillosis: From Head to Toe. Mycopathologia. 2023 Jun 28;188(5):623–41.

17. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America. 2008 Feb 1;46(3):327–60. Available online: https://pubmed.ncbi.nlm.nih.gov/18177225/

18. Caggiano G, Apollonio F, Consiglio M, Gasparre V, Trerotoli P, Diella G, et al. Tendency in Pulmonary Aspergillosis Investigation during the COVID-19 Era: What Is Changing? International Journal of Environmental Research and Public Health [Internet]. 2022 Jun 9 [cited 2024 Jun 12];19(12):7079. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9222563/

19. Balajee SA, Marr KA. Phenotypic and genotypic identification of human pathogenic aspergilli. Future Microbiology. 2006 Dec;1(4):435–45.

20. Steinbach WJ. Are We There Yet? Recent Progress in the Molecular Diagnosis and Novel Antifungal Targeting of Aspergillus fumigatus and Invasive Aspergillosis. Goldman WE, editor. PLoS Pathogens. 2013 Oct 24;9(10):e1003642.

21. O’Gorman CM, Fuller HT, Dyer PS. Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. Nature. 2009 Jan;457(7228):471–4.

22. Yang S, Rothman RE. PCR-based Diagnostics for Infectious diseases: uses, limitations, and Future Applications in acute-care Settings. The Lancet Infectious Diseases. 2004 Jun;4(6):337–48.

23. Abate, M.S.; Battle, L.R.; Emerson, A.N.; Gardner, J.M.; Shalin, S.C. Dermatologic Urgencies and Emergencies: What Every Pathologist Should Know. Arch. Pathol. Lab. Med. 2019, 143, 919–942.

24. Ozhak-Baysan, Alastruey-Izquierdo A, Saba R, Dilara Ogunc, Gozde Ongut, Aysen Timuragaoglu, et al. *Aspergillus alliaceus*and*Aspergillus flavus*co-infection in an acute myeloid leukemia patient. Medical Mycology [Internet]. 2010 Nov 1 [cited 2024 Mar 14];48(7):995–9. Available online: https://academic.oup.com/mmy/article/48/7/995/1056456