# A new sensitive molecular marker for *Aspergillus'* Calmodulin gene detection in biological samples, used as a supplementary diagnosis for invasive Aspergillosis (IA)

# Um novo marcador molecular sensível para a detecção do gene Calmodulin em amostras biológicas do *Aspergillus*, utilizado como diagnóstico suplementar para a Aspergilose invasiva (IA)

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**ABSTRACT**
Introduction: Aspergillosis is caused by the fungi of *Aspergillus* genus. After inhalation, *Aspergillus'* conidia can invade the tracheal bronchus and lungs, resulting in airway colonization, inflammatory granuloma and invasive aspergillosis (IA), which is most likely to occur in neutropenic and immunosuppressed patients. Presently, IA has been associated with COVID-19 in patients admitted to intensive care units (ICU) for longer

periods of time. Objective: The objective of this is to design a new molecular marker for detection of *Aspergillus* spp. in biological samples, in order to develop a new technique for IA diagnosis which is faster, more efficient and safer. Methodology: The primer was designed *in silico* using conserved sequences of the Calmodulin gene. After confirming the results *in silico*, *in vitro* evaluation was performed using the PCR technique. Results: The designed primer showed high specificity for the *Aspergillus* species. Conclusion: In this study the standardized PCR reaction with the designed marker proved to be safely utilized as a diagnostic method for IA. The development of a test to aid in the diagnosis of IA is of great importance, since traditional methods are in turn time consuming and generally confused with other diseases. Early diagnosis contributes to faster treatment initiation and increasing the patient's chance of survival. We can conclude that the standardized PCR with the designed primer can be used safely in the development of a diagnostic test for IA, being of low cost, and accessible to patients treated by the public health system. A Patent application for this molecular marker has been submitted, with the number BR1020190281294.

**Keywords:** Invasive aspergillosis, Diagnosis, Polymerase Chain Reaction, Molecular marker, COVID-19.

**RESUMO**

Introdução: A Aspergilose é causada pelos fungos do gênero Aspergillus. Após a inalação, a conidia do Aspergillus pode invadir o brônquio traqueal e os pulmões, resultando em colonização das vias aéreas, granuloma inflamatório e aspergilose invasiva (AI), que é mais provável que ocorra em pacientes neutropênicos e imunossuprimidos. Atualmente, a IA tem sido associada à COVID-19 em pacientes admitidos em unidades de terapia intensiva (UTI) por períodos mais longos de tempo. Objetivo: O objetivo disto é projetar um novo marcador molecular para detecção de Aspergillus spp. em amostras biológicas, a fim de desenvolver uma nova técnica para diagnóstico de IA que seja mais rápida, mais eficiente e mais segura. Metodologia: O primer foi projetado em silico utilizando seqüências conservadas do gene Calmodulin. Após a confirmação dos resultados em silico, a avaliação in vitro foi realizada utilizando a técnica de PCR. Resultados: O primer projetado mostrou uma alta especificidade para as espécies Aspergillus. Conclusão: Neste estudo, a reação PCR padronizada com o marcador projetado provou ser utilizada com segurança como um método de diagnóstico para IA. O desenvolvimento de um teste para auxiliar no diagnóstico da IA é de grande importância, uma vez que os métodos tradicionais, por sua vez, são demorados e geralmente confundidos com outras doenças. O diagnóstico precoce contribui para o início mais rápido do tratamento e aumenta a chance de sobrevivência do paciente. Podemos concluir que a PCR padronizada com o primer projetado pode ser usada com segurança no desenvolvimento de um teste diagnóstico para IA, sendo de baixo custo e acessível aos pacientes tratados pelo sistema de saúde pública. Foi apresentado um pedido de patente para este marcador molecular, com o número BR1020190281294.

**Palavras-chave:** Aspergilose invasive, Diagnóstico, Reação em cadeia da polymerase, Marcador molecular, COVID-19.

## 1 INTRODUCTION

Aspergillosis is an infectious disease caused by the fungi of the genus *Aspergillus,* developing into a wide spectrum of chronic or acute clinical forms [1]. Aspergillosis is transmitted through the airways, as hundreds of the *Aspergillus* species are anemophilous, distributed all over the world [2]. Consequently, it's spores may be present in the lungs as part of a transient microbiota or characterizing an infection [3]. Depending on the patient's immune status, the fungal cells may spread to other organs, causing invasive Aspergillosis (IA), usually associated both with high morbidity and mortality. IA has been recurrent in neutropenic and immunosuppressed patients admitted to intensive care units (ICU) for longer periods of time, who may present fever [1-3]. Advanced chronic obstructive pulmonary disease (COPD) and the prolonged use of corticosteroids are also associated with an increased risk of IA [4].

Invasive Aspergillosis (IA) or Invasive Pulmonary Aspergillosis (IPA) has been described as a complicating factor in respiratory syndromes caused by viruses such as H1N1 [5]. Presently, some studies have already shown that IA is highly related to respiratory syndromes caused by COVID-19. In these studies, the presence of fungal infections has been suggested as a risk factor, increasing mortality rates. Yang et. al. (2020) reported the presence of co-infections with different microorganisms, including *Aspergillus flavus*, *A. fumigatus* e *Candida albicans* [6]. Chen et. al. (2020) also drew attention to the presence of co-infection in critically ill patients. When using fungus cultures, the present authors detected the presence of *A. fumigatus*, *C. albicans* e *C. glabrata* in 4% of the samples analyzed, which were obtained from patients with COVID-19, confirmed by RT-PCR [7].

Due to the severity of the disease, in which patients become weakened through the action of respiratory syndromes, rapid diagnosis for IA is essential to ensure effective treatment, decreasing mortality rates [1]. The diagnosis of this disease is predominantly clinical, since the histopathological analysis and culture of the fungi take a long time to work and have low accuracy [8]. The serological test Enzyme-Linked Immunosorbent Assay for Detection of Galactomannan (EIA-GM®) has proven to be an important tool to monitor patients hospitalized in the ICU for long periods, as well as neutropenic patients [1,9].

To increase the sensitivity and specificity of *Aspergillus* detection in biological samples, some authors have described the PCR technique as a molecular method [10]. Some authors have demonstrated that primers for the Calmodulin gene region are

successfully used to amplify *Aspergillus* DNA from IA patients at different stages of the disease [11].

Accordingly, the present study aimed to perform the *in silico* design of a new pair of primers to amplify the Calmodulin gene of *Aspergillus* genus, which can be used for its detection in biological samples by the Polymerase Chain Reaction (PCR).

## 2 METHODOLOGY

*In silico* primer design was performed using the genome of *Aspergillus* spp., which has already been described and is available in the non-redundant GenBank database. To direct the design of the new primer, the Calmodulin gene sequences were investigated, which were also available in the non-redundant GenBank database. The files were used to locate the specific region for *Aspergillus* genus, using the program MEGA (version 7.0.26) for alignment.

The Blast*n* program was used to investigate the homology of the drawn primer, to verify if it is not complementary with the genomes of other organisms, mainly agents of other respiratory diseases, such as tuberculosis and other mycoses. Complementarity of the primer with the human genome was also verified.

After *in silico* studies, *in vitro* analyses were performed to evaluate the sensitivity of the PCR technique using the marker designed. For this, DNA was extracted from blood samples of Positive and Negative patients for the test EIA-GM®, using the Invitrogen kit (USA). In addition, DNA was extracted from cultures of International Standard fungal strains: *A. terreus* (URM 7309), *A. ochraceus* (URM 7313) and *A. fumigatus* (URM 7315), *A. flavus* (URM 7326), included in the Fungus collection denominated URM, from the Federal University of Pernambuco using the same kit for DNA extraction. After being extracted and purified, the DNA was assayed by spectrophotometry using Nanovue Plus (GE Healthcare Life Sciences, Sweden).

The conventional PCR reaction was performed in the final volume of 10µL of a solution containing 1x PCR buffer; 3 mM $MgCl_2$; 0.2 µM dNTPs; each primer F and R 0.4 µM; 1.5 u / µL Taq DNA polymerase and $H_2O$ mili-q. For each sample, 2µL of DNA at 20 ng/ µL was used. The amplification cycles were as follows: 1 cycle of 95°C for 5 min; 40 cycles of 95°C for 1 min, 62°C for 30 sec. and 72°C for 1 min.; finalized by a cycle of 72°C for 5 min. After cycling, the PCR´s products were visualized in 1% polyacrylamide gel electrophoresis, stained by 7% silver nitrate.

To confirm the *in vitro* specificity of the primers, the PCR reactions were performed using genomes extracted from other pathogenic microorganisms: *Fonsecaea pedrosoi* (URM 3161), *Sporothrix schenkii* (URM 5111), *Trychophyon rubrum* (URM 6753), *Penicillium expansum* (URM 7317) and *Exophiala dermatitidis* (URM 7460), also included in the URM Fungus Collection of the Federal University of Pernambuco. For these PCR reactions, the reagent concentrations and cycling conditions were the same as in the previous tests.

The research was appropriately approved by the Ethics Research Committee of the Santa Casa of Belo Horizonte Hospital, under the number CAAE 55549216.2.0000.5138.

## 3 RESULTS

According to the Calmodulin sequences alignment, using the program Mega (version 7.0.26), it was possible to observe preserved regions in the *Aspergillus* genus, as the Forward primer calmodulin 1 (5’ GAG-TAC-AAG-GAG-GCC-TTC-TC 3’) described by O’Donnell et al. (2010) (12). For this reason, this Forward primer was chosen to delineate the design of the new Reverse primer, with the help of the Oligo Analyzer tool. The Reverse primer designed was named AspR-5 ' - TCC-TTR-GTG-GTR-ATC-TGG-CCT-R - 3'. According the program, it was verified that the primers had a close Melt Temperature (TM) to one another and the product size of the PCR would be between 230bp and 280bp, varying according to the different species of *Aspergillus* genus (Figure 1).

Figure 1 represents the chosen primer and its placement in the target sequence. Highlighted in yellow, the primer Cmd 1 - 5'-GAGTACAAGGAGGCCTTCTC-3' (foward), and in green the primer AspR - 5'-TCCTTRGTGGTRATCTGCCTR-3' (reverso).

*Aspergillus nidulans*

5'GTTTCCGAGTACAAGGAGGCCTTCTCCCTATTTGTAAGTGCCATTGGTTACTGTTATATCAAAATCGAATTTGTATTGAG
AGTATACTAATACATTCCGCACTAAACAGGACAAGGATGGCGATGGTTAGTGCATCTGTCCCCCCAGGCTTGATCGCATT
CGCCCAGCATGTCTGCTGTAGCTCTATATAACCGTTTCTGACAAACGGCGACAGGCCAGATTACCACTAAGGAGCTTGG
CACTGTCATGCGCTCGCTCGGTCAGAATCCTTCAGAGTCTGAGCTTCAGGACATGATCAACGAAGTTGACGCCGACAAC
AATGGCACCATTGACTTTCCAGGTACGCGAACTCCCCAATCTACTTCGCACCAGCCTAGAAATGTACTAATGCTAAACAG
AGTTCCTTACCATGATGGCCAGAAAGATGAAGGACACCGATTCCGAGGAGGAAATTCGGGAGGCGTTCAAGGTCTTCGA
CCGTGACAACAATGGTTTCATCTCCGCTGCTGAGCTGCGTCACGTCATGACCTCGATCGGTGAGAAGCTCACCGATGAC
GAAGTCGACGAGATGATCCGCGAGGCGGACCAGGATGGCGACGGCCGAATTGACTGTACGTT3'

*Aspergillus niger*

5'TCCGAGTACAAGGAGGCCTTCTCCCTCTTTGTGAGTGCTCCCTGAATGACCCTCCGATCATCCTGATCGATGAGCTATC
TTTACCGGAGCATAATGCTAATGTGTTTTCGGACTTAATAGGACAAGGATGGCGATGGTGGGTGGAATTCTATCCCCTTC
ACATTATACCTGTAGCGCTCGATCCGACTGCGGGATTTCGACAGCATTTTCCAGAACGATTTGGATCATAATACTAATTTA
AATCGGTGAATCAGGCCAGATCACCACCAAGGAGCTCGGCACTGTGATGCGCTCCCTTGGCCAGAACCCCTCCGAGTC
TGAGCTTCAGGACATGATCAACGAGGTTGACGCTGACAACAACGGAACGATCGACTTCCCCGGTATGTGTTAGATTTAC
GCCTGTAAGGCGGAAATGCGGGCTGGATTGTGATTGACTTTTGCCGCCAGAATTCCTTACCATGATGGCTCGTAAGATG
AAGGACACCGACTCCGAGGAGGAAATCCGCGAGGCTTTCAAGGTCTTCGACCGCGACAACAATGGTTTTATCTCCGCCG
CGGAGCTGCGCCACGTTATGACCTCTATCGGA3'

*Aspergillus fumigatus*

5'TCCGAGTACAAGGAGGCCTTCTCTCTCTTCGTAAGTGAACTGTCCAAGTCCCTGGTCGTTGTATAGGAGGGATCTCCA
GAATATTGAGGGTGTGCGCTGACACGAGATTTGACGTATAGGACAAGGATGGTGATGGTTAGTGACCCTTTTTCCACTCC
TCGAACTTCGGCTTCCATGCGATCATGTTCAAACGCCGACTCACAATATCCGGAAATGACCCGTCAGTACTGATAATATC
TATGTTTGACTATCAGGCCAGATCACCACCAAGGAATTGGGCACTGTAATGCGCTCTCTGGGCCAGAACCCTTCCGAGT
CAGAGCTGCAAGATATGATCAACGAGGTGGATGCTGACAACAACGGCACCATCGATTTCCCCGGTATGTGATACTTTCG
GTATGAACTCGGGAGGGGAGAGAACAATCATTAACTTGTAATCAGAATTCCTTACCATGATGGCTCGGAAGATGAAGGAC
ACCGACTCCGAAGAGGAAATTCGGGAAGCTTTCAAGGTCTTCGACCGCGACAACAACGGTTTCATCTCCGCTGCGGAGC
TGCGCCACGTTATGACCTCTATCGGA'3

*Aspergillus terreus*

5'CCGAGTACAAGGAGGCCTTCTCCCTCTTCGTATGTCTTGACTTACCCCTCTTCCCTGAATCGACCATCTCTCCGTGTTC
CAGGCTCGTTTTCCGAAACACAAGCTAACTGGGATTCTCCTCTGTTTTAGGACAAGGATGGTGATGGTTAGTGCAATTTC
CCGCTCCGATTGCCTCATGCGACCGATCGATTGCATCGTTCTATGTCGAATCTCGAGTCTTGACTTTATCTTCTGTTATGG
TCGATCATTTAACACCATGTAGGCCAGATCACCACCAAGGAGCTGGGAACCGTCATGCGCTCGCTGGGCCAGAACCCCT
CCGAGTCGGAGCTCCAGGACATGATCAACGAGGTTGATGCTGACAACAACGGCACCATTGACTTCCCTGGTACGTTTCG
TTCTACACGAGTCGCTGGAACCAGTTGTTGACATTTCCTTGAACAGAGTTCCTCACGATGATGGCCCGCAAGATGAAAGA
CACCGACTCCGAGGAGGAAATCCGGGAAGCTTTCAAGGTCTTTGACCGCGATAACAACGGTTTCATCTCCGCCGCCGAG
CTGCGCCACGTTATGACCTCTATCGG3'

According to the BLASTn analysis, it was demonstrated that the designed Reverse primer was 100% complementary to the Calmodulin gene of *Aspergillus* spp. However, this primer was also complementary to sequences of the species of *Ophiostoma* sp., *Byssochlamys spectabilis, Sporothrix brasiliensis, Stagnosporopsis citrulli* and *Calonectria pentaseptada*.

For the *in vitro* confirmation, the conventional PCR assay showed amplification of the 230-280 bp fragments, as expected *in silico* analysis (Figure 2). For the specificity test, the extracted DNA from blood samples that obtained positive tested EIA-GM® was amplified. Extracted DNA from blood samples of negatively testing EIA-GM® patients, used as a negative control, was not amplified (Figure 3).

The extracted DNA from International Standard cultures of *Fonsecaea Pedrosoi* (URM 3161*), Trychophyon Rubrum* (URM 6753) and *Exophiala Dermatitidis* (URM 7460) was not amplified. The extracted DNA from International Standard cultures of *A. terreus* (URM 7309*) A. ochraceus* (URM 7313), *A. fumigatus* (URM 7315) and *A. flavus* (URM 7326) were all amplified. The extracted DNA from International Standard cultures of *Sporothrix schenkii* (URM 5111), *Penicillium expansum* (URM 7317) were also amplified, but amplifying fragments of different sizes to that generated by the amplification of *Aspergillus* species. (Figure 3).

## 4 DISCUSSION

The pair of primers performed in this study demonstrated similar melting temperatures, and the PCR product size has a specific 230 to 280 bp sequence of the Calmodulin gene of the *Aspergillus's* genome. This amplicon size is ideal for the standardization of a real-time or conventional PCR technique. In order for the Reverse primer to cover the largest number of *Aspergillus* species, it was necessary that it was designed degenerate, where the letter R stands for two possibilities of nucleotide substitution, which can be Adenine or Guanine [13]. As a result, the degenerate primer may have less specificity [14]. Analyses of the primer using BLAST*n* showed that it has a high homology with all *Aspergillus* species, providing a broad spectrum of diagnosis for all kind of aspergillosis. But at the same time, the primer also presented a match with the genome *Ophiostoma* sp*, Byssochlamys spectabilis, Sporothrix brasiliensis, Stagnosporopsis citrulli* and *Calonectria pentaseptada*.

Despite its lack of specificity, it is not a problem for the IA diagnosis application. The species of *Ophiostoma, Stagnosporopsis* and *Calonectria* are known to cause diseases in vegetation, while causing no infections in humans [15,16]. *Byssochlamys spectabilis* is the teleomorphic form of *Paecilomyces variotii*, commonly found in air, soil, food, though rarely found as an etiological agent in human infections. However, it is known as one of the fungi that causes infections in immunosuppressed patients [17]. *Sporothrix brasiliensis* is the agent of sporotrichosis; its distribution is restricted to the tropical regions, causing outbreaks of the disease. The infection usually occurs through traumatic inoculation of the fungus on the skin, and rarely causes pulmonary infection [18].

According to the PCR *in vitro* analyses from patients' extracted DNA, an amplification of blood samples obtained from positive EIA-GM® which presented a

fragment of approximately 280bp, confirming what was shown in the *in-silico* analysis, was observed. Negative EIA-GM® DNA extracted from patients' blood, used as a negative control was Negative for the PCR reaction, confirming that the primers do not align with human DNA. The use of samples from healthy patients is important, since the primer cannot hybridize with human DNA. During the DNA extraction it is not possible to separate which DNA belongs to human beings and which belongs to the fungus [1,8,9,10].

The DNA extracted from cultures of International Standard *A. terreus* (URM 7309), *A. ochraceus* (URM 7313), *A. fumigatus* (URM 7315) and *A. flavus* (URM 7326) were amplified. These cultures presented different amplification patterns. The amplified samples showed the pattern of base pairs consistent with what was found in bioinformatics, from 230 bp to 280 bp, confirming the polymorphism between species of the genus *Aspergillus*.

DNA extracted from cultures of *Fonsecaea pedrosoi* (URM 3161), *Trychophyon rubrum* (URM 6753) and *Exophiala dermatitidis* (URM 7460), used for the specificity test, was not amplified, while the DNA of *Sporothrix schenkii* (URM 5111), and *Penicillium expansum* (URM 7317) was amplified, as shown in the *in-silico* analysis. In fact, the Calmodulin gene has a high degree of preservation between species [19]. According to Halling et al. (2016), the yeast Calmodulin gene identifies at 60% with the vertebrate Calmodulin gene [19]. Therefore, a cross between those species is expected without jeopardizing the diagnosis of AI, since PCR has the function of helping in the diagnosis of diseases and its results are considered in association with other clinical, serological and mycological parameters. The benefit of using this tool is that it is faster than other tests and can be used to detect the presence of the fungus in pre-asymptomatic patient blood, enabling the treatment to be given more efficiently, and thus saving lives in a matter of days.

The PCR technique is a simple method that aims to detect the DNA of circulating fungus in biological samples. A positive result can indicate the presence of the fungus in the blood sample, providing for prompt treatment [1-9]. In general, the PCR technique is not a high cost tool, and is able to be implemented in hospitals and clinics that assist less affluent patients, where there are a greater number of infectious diseases.

## 5 CONCLUSION

The *in-silico* analyzes showed a high specificity of the designed marker to the *Aspergillus* species, discarding complementarity with human DNA. Because the primer had to align with several *Aspergillus* species, this created a possibility of alignment in with other species of fungus, albeit species that are not common in causing respiratory infections.

The PCR *in vitro* analyses confirmed a high specificity of the pair of primers to the *Aspergillus* species and the non-complementarity with the human genome. Despite the confirmation of the complementarity with the species *Sporothrix schenckii* and *Penicillium expansum*, the results can be considered satisfactory because they indicate a method to standardize the technique, which will assist in providing a faster and more sensitive diagnosis of AI.

The patent of the designed primer sequence was filed with the National Institute of Industrial Property (INPI), and registered under case number BR 10 2019 028129 4 entitled: `INITIATORS, KIT AND METHOD FOR MOLECULAR DIAGNOSTIC FOR ASPERGILLOSIS, AND USE`.

**Conflict of Interests**

The authors declare that they have no conflict of interests.

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**Ethical approval**

The authors confirm that the Ethics Policies of the journal, as noted in the author's guideline page, have been adhered to. Our study was approved by the Ethics Committee of the Santa Casa Hospital of Belo Horizonte, Brazil (CAAE 55549216.2.0000.5138).

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## FIGURE CAPTIONS

Figure 1: Sequences of the Calmodulin gene of the four *Aspergillus* species, obtained from the non-redundant GenBank database, indicating the chosen initiator and its placement in the target sequence. Highlighted in yellow, the primer Cmd 1 - 5'-GAGTACAAGGAGGCCTTCTC-3' (foward), and in green the primer AspR - 5'-TCCTTRGTGGTRATCTGCCTR-3' (reverse).

Figure 2: Polyacrylamide gel 7% stained by 2% silver nitrate. Channels: PM: Molecular Weight. 1 - blood of a patient with Positive GM EIA test; 2 - blood of a patient with Negative GM EIA test; 3 - Negative reaction Control; 4 - *A. terréus*, 5 - *A. ochraceus*, 6 - *A. fumigatus*; 7 - *A. flavus*.

Figure 3: Polyacrylamide gel 7% stained by 2% silver nitrate. Channels: PM: Molecular Weight; 1 -*Fonsecaea pedrosoi,* 2 - *Sporothrix schenkii* 3 – *Trychophyon rubrum* 4 - *A. terréus*, 5 - *A. ochraceus*, 6 – *A. fumigatus*, 7 – *Penicillium expansum*, 8 - *A. flavus*, 9 - *Exophiala dermatitidis*, 10 – Negative reaction Control.