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MICROBIOLOGY

Molecular identification of *Histoplasma capsulatum* in patients with disseminated histoplasmosis and acquired immunodeficiency syndrome

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Abstract: Histoplasmosis is caused by the fungus *Histoplasma capsulatum* and is often fatal for individuals with acquired immunodeficiency syndrome (AIDS). Delayed diagnosis is a major factor in worsening coinfection, as it can be mistaken for other diseases. Thus, rapid identification of *Histoplasma* in immunocompromised patients is essential. Molecular techniques, particularly polymerase chain reaction (PCR), were used in this study to identify *H. capsulatum* in patients coinfected with histoplasmosis and AIDS. Blood samples from 14 individuals with AIDS and disseminated histoplasmosis were collected and analyzed. The PCR method successfully amplified the fungal region in whole blood samples, while PCR-RFLP analysis confirmed a consistent profile in the samples. Genetic sequencing further confirmed the fungal species. Compared to clinical tests such as fungal culture and urinary antigen detection, molecular analysis proved faster, more sensitive, and cost-effective. These molecular markers can potentially be incorporated into routine diagnostics in the future. Further studies are needed to expand and enhance this diagnostic approach, particularly in patients with nonprogressive clinical forms of histoplasmosis.

Key words: AIDS, Coinfection, Fungal infection, HIV, Neglected disease, Polymerase Chain Reaction.

INTRODUCTION

Histoplasmosis, caused by the fungus *Histoplasma capsulatum*, is a systemic mycosis with worldwide distribution that is more prevalent in the Americas (Carreto-Binaghi et al. 2019, Messina et al. 2020). Latin America is the region with the highest number of cases, including Brazil, but the real epidemiology of the disease is still unknown and underestimated (Bezerra et al. 2021, Moreira et al. 2022, Nacher et al. 2022). It is an opportunistic infection that affects immunocompromised patients such as human immunodeficiency virus (HIV) patients (Basso et al. 2021, Kuate et al. 2021).

According to the World Health Organization (Unaids 2022, WHO 2022a) there are 38.4 million people living with HIV, and disseminated histoplasmosis is the most frequent defining infection of acquired immunodeficiency syndrome (AIDS) and the leading cause of death. Mortality is approaching 50%, especially in those with severe manifestations (Samayoa et al. 2017, Oladele et al. 2018, Nacher et al. 2021).

Due to non-specific symptoms, histoplasmosis is a neglected disease in many countries and is often misdiagnosed as tuberculosis (TB) (Adenis et al. 2018, Cáceres et al. 2018, Torres-González et al. 2018, PAHO 2020, Perez et al. 2021, Nacher et al. 2022).

The standard methods used for diagnosing the disease are based on cultivation techniques and/or detection of the presence of fungal antigens. The first ones take time, and the immunological ones can cross-react with other fungal antigens and are not available in many countries (Pérez-Lazo et al. 2017, Baker et al. 2020).

Polymerase chain reaction (PCR) and sequencing methods are sensitive and specific tools for laboratory diagnosis and have been widely used to identify fungal species (Falci et al. 2017). In filamentous fungi and yeast, the 18S rDNA, 5.8S rDNA and 28S rDNA gene regions, as well as the regions between these genes, known as ITS (Internal Trascriber Spacer), are targets that can be used for the genetic differentiation of these microorganisms (Leite et al. 2020, Santos et al. 2020). The PCR technique using the fungal universal region as a target (18S rDNA, 5.8S rDNA and 28S rDNA and ITS) followed by the restriction fragment length polymorphism (RFLP) method has been used as an analysis capable of discriminating species of fungi and yeasts in the laboratory (Fatima et al. 2017, Leite et al. 2020, Santos et al. 2020, Antunes et al. 2022).

Thus, this study aimed to identify *Histoplasma capsulatum* in blood samples from patients with coinfection of disseminated histoplasmosis and acquired immunodeficiency syndrome by molecular methods.

MATERIALS AND METHODS

Study Area

The study site was the Hospital Estadual de Doenças Tropicais Dr. Anuar Auad - HDT. The hospital is a reference for the treatment of infectious and dermatological diseases and is located in Goiânia-GO, the second largest metropolis in the Midwest of Brazil (Ferreira et al. 2017). The present study was part of a clinical trial registered at *ClinicalTrials.gov* with the identifier number NCT04059770 and was approved by the HDT Research Ethics Committee (Report No. 4,556,040/2021).

Previous laboratory tests

As an inclusion criterion, participants should test positive for HIV and *Histoplasma*. For this purpose, HIV tests were carried out during the clinical trial using the ELISA method (Enzyme Linked Immuno Sorbent Assay) and histoplasmosis tests using urinary antigen detection or classical mycological methods.

In addition, they should have the HIV virus in an advanced stage of AIDS (CD4 T lymphocyte count <200 cells/μL) and the disseminated form of histoplasmosis. This was defined according to the criteria/clinical manifestations of invasive fungal disease of the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC/MSGREC) (Donelly et al. 2020).

Study patients and samples

Fourteen patients were included in this study. The period established for collection was from February 2021 to December 2021. For carrying out molecular analyses, blood was collected in tubes containing anticoagulant EDTA (ethylene diamine tetracetic acid) according to the standard HDT procedure and stored in a freezer at -20°C. These were transported on dry ice to the Health Sciences Research Laboratory (Hospital Universitário Clemente Faria-HUCF,

State University of Montes Claros, Montes Claros, Minas Gerais, Brazil). In this laboratory, the samples were stored in a -80°C freezer until nucleic acid extraction was performed.

Blood samples for performing fungal culture were also collected. These were collected following the standard procedure of the Central Laboratory of Public Health (LACEN) and were sown in their own media: Sabouraud and Mycosel agars (used together). The blood was inoculated into the flasks with the media so that they covered the entire surface of the medium and were left in a vertical position so that the blood was absorbed by the medium. After collection, the blood culture tubes were transported at room temperature to LACEN.

Histoplasma antigen was detected in urine samples using the commercial monoclonal *Histoplasma* Galactomannan enzyme immunoassay (HGM EIA) kit (IMMY, Norman, OK, USA). The test procedure was performed according to the manufacturer's instructions.

Fungal culture

Fungal culture was performed at LACEN-SES-GO with Sabouraud and Mycosel agars. These are highly selective media used in the isolation of pathogenic fungi. The cultures were incubated for up to 30 days to release the results. *Histoplasma*-positive cultures initially exhibit white, cotony-like, slow-growing colonies that later become darkened (brownish). Under microscopy, they exhibited nipple-like microconidia and macroconidia (Kauffman 2007).

DNA extraction from patient samples

The patients' whole blood samples were subjected to DNA extraction. The protocol described by Sambrook & Russell (2001) was followed, with modifications as described below: 100 µl of blood was aliquoted in a 2.0 ml polypropylene tube, and 300 µl of PBS (1X) was added and homogenized in a vortex. Subsequently, 500 µl of lysis buffer (0.1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, 0.2% SDS and 1 M NaCl) was added to the tube, homogenized in a vortex and incubated at 65°C in a water bath for 1 hour. After incubation, 300 µl of chloroform and isoamyl alcohol (24:1) were added to the tubes and shaken vigorously. The tubes were centrifuged at 12,000 RPM for 10 minutes so that the aqueous phase could be transferred to a new tube. This process was repeated twice, and 300 µl of ice-cold isopropanol was added to the supernatant and incubated at -20°C for 20 minutes. After this period, the tube was centrifuged at 12,000 RPM for 10 minutes. The isopropanol was discarded, and the sediment was washed with 300 µl of ice-cold 70% ethanol in centrifugation at 12,000 RPM. The 70% ethanol was discarded, and the sediment after drying was resuspended in 100 µl of sterile water.

DNA quantification was performed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Yield was determined by absorbance measurements at 260, 280 and 230 nm. DNA purity was determined by the absorbance ratio 260/280. DNA integrity was assessed by electrophoresis on a 1% agarose gel stained with ethidium bromide, visualized by UV light and photodocumented in an L-Pix Touch Loccus photodocumenter (Loccus, Brazil). DNA extraction and quantification by NanoDrop were performed at the Health Sciences Research Laboratory (LPCS) at HUCF.

Identification of the genomic profile by PCR--RFLP

To obtain DNA fragments from the samples of approximately 1000 base pairs (bp), oligonucleotides 5'-CTGGTCATTTAGAGGAAGTAA-3' and 5'-CCGTGTTTCAAGACGGG-3', called OligoITS1F and OligoLR3, described by Raja et al. (2017), were selected. The fragments obtained with

the oligonucleotides selected for the study comprise a sequence that represents a universal signature for fungi and yeasts, as described by Leite et al. (2020).

Fungal universal region PCR was performed in a mix containing 2X Go Taq Green Master Mix (PROMEGA, USA), $MgCl_2$ (2.5 mM), 10 µM of each primer and 50 ng of yeast/filamentous fungus DNA in a final reaction volume of 50 µl. A second PCR cycle was performed using the product of the first cycle as a template. PCRs were performed in a Veriti Applied Biosystems thermocycler. The amplification conditions followed the parameters described by Leite et al. (2020). The amplicons were visualized on a 1.8% agarose gel, stained with ethidium bromide and photodocumented on an L-Pix Touch Loccus photodocumenter (Loccus, Brazil).

For restriction analysis of the PCR products, approximately 1000 bp was digested with the restriction enzyme TaqαI (Promega Corporation, USA) according to the chemical and thermal conditions described by Leite et al. (2020). The PCR products digested with restriction enzymes were subjected to electrophoresis in a 3.0% agarose gel, stained with ethidium bromide and photodocumented in an L-Pix Touch Loccus photodocumenter (Loccus, Brazil). The analysis of the restriction profile was carried out by two observers, and tables were created with the sizes of the restriction fragments obtained.

As a positive PCR control, the standard strain *Candida albicans* ATCC 10231 was used, and as a negative control, water for injections. Universal gene PCR and PCR-RFLP/agarose gel electrophoresis techniques were performed at the Unimontes Microbiology Laboratory.

Sequencing of Universal Fungal Region PCR products

The PCR products (approximately 1000 bp amplicon corresponding to the fungal universal region) were sequenced by the Sanger method (Ludwig Biotech Ltda, RS, Brazil - ACTGene Analisis Moleculars Ltda) using the primers described by Raja et al (2017). To obtain the sequence amplified by PCR, the sequencing strands were aligned by the omega cluster (European Bioinformatics Institute -https://www.ebi.ac.uk/Tools/msa/clustalo/), and the differences were visualized in the electropherogram (Chromas v.2.6. 5 – www.technelysium.com.au) and corrected. The obtained sequences were submitted to BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the similarity with the sequences deposited in GenBank was analyzed with the objective of identifying the fungi at the species level.

RESULTS

In this study, DNA was initially extracted from 14 blood samples from patients coinfected with HIV and *Histoplasma capsulatum*. PCR amplification of the fungal universal region was performed. Of the 14 samples, 12 amplified an ~1000 bp fragment (Fig. 1a). A second round of PCR was performed using the product of the first cycle as a template (Fig. 1b). Of the 14 samples, 85.7% (12/14) were PCR positive.

Patients coded 36 and 41 did not have good DNA extraction from the whole blood sample; therefore, these samples were not amplified in the fungal universal region PCR technique.

To validate the presence of amplifiable DNA, PCR of the human endogenous beta actin gene was performed in parallel (data not shown) for all samples, and a fragment of approximately 250 bp of *Homo sapiens* DNA was amplified, except for patients 36 and 41, which reinforces the understanding that the genetic material was degraded. These were excluded from the sample.

To carry out the molecular identification of the products obtained by PCR, they were

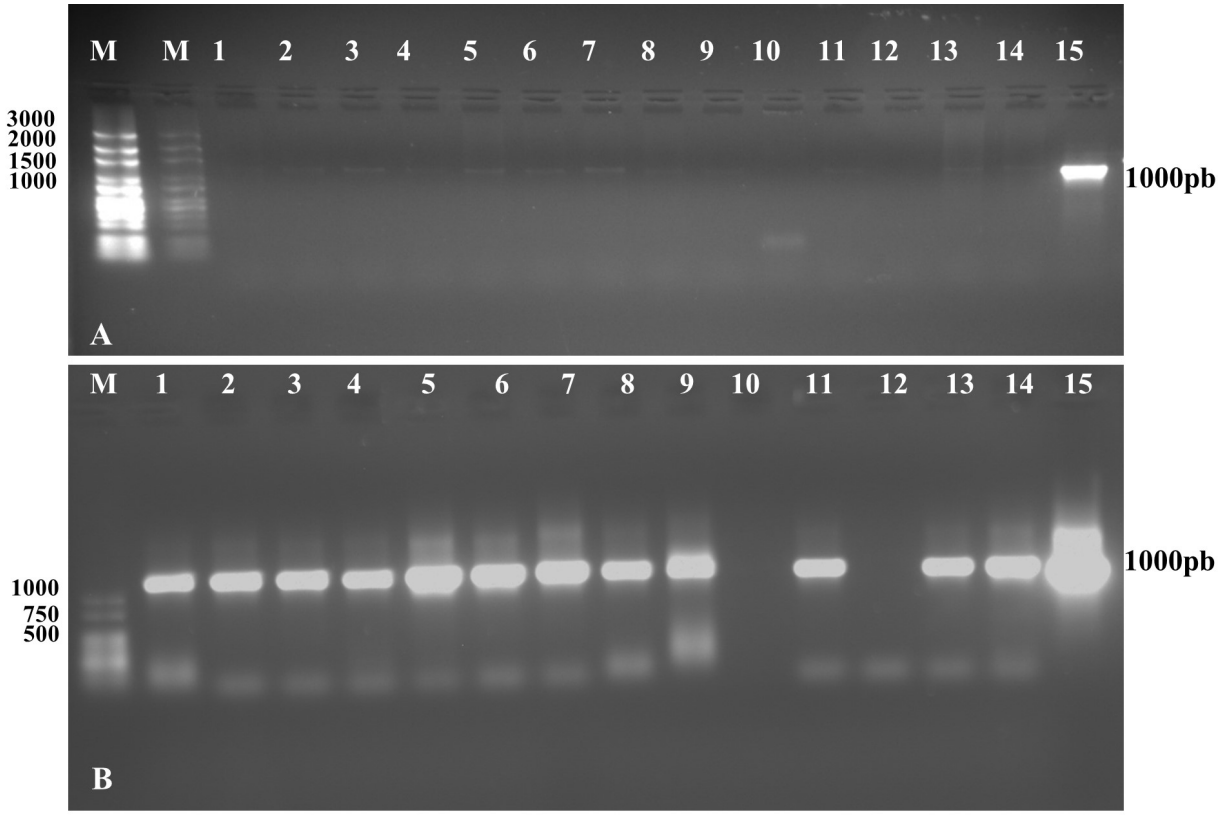

Figure 1. **Fungal universal region PCR. Panel A: PCR amplification of the approximately 1000 bp fragment of DNA samples extracted from the blood of HIV patients coinfected with *Histoplasma capsulatum*. M: Mid-range Molecular Mass Marker – 10 and 5 µl applied in two different lines (Cellco Biotecnologia). Lines 1 to 14: Results of PCR with blood DNA samples from patients coded 04,06,08,09,12,13,16,26,35,36,37,41,42,10. Line 15: Positive control (1000 bp fungal universal region amplified from *Candida albicans* ATCC 10231 DNA. Panel B: Amplification of the PCR products identified in panel A in a new PCR for fungal universal region detection. M: Mass Marker Molecular 50pb (Ludwig Biotecnologia) Lines 1 to 14: Results of the PCR with DNA samples from patients' blood coded 04,06 ,08,09,12,13,16,26,35,36,37,41,42,10. Line 15: Positive control (1000 bp universal fungal region amplified from DNA of *Candida albicans* ATCC 10231). Electrophoresis in 1.8% agarose gel.**

submitted to the RFLP technique with the Taq$^{\alpha}$1 restriction enzyme (Fig. 2), and the fungal isolates exhibited a homogeneous pattern of bands. A positive control strain of *Candida albicans* was used to standardize the technique (Fig. 2). The results of the PCR-RFLP analysis of the fungal ITS1, 5.8S rDNA and ITS2 regions are shown in Table II. Patients with disseminated histoplasmosis and acquired immunodeficiency syndrome showed bands of size 100, 160, 350 bp.

The samples included in the study were from patients previously diagnosed with disseminated histoplasmosis and AIDS by microbiological and/or immunological methods. Table I shows the culture and antigenuria results for histoplasmosis of the patients selected for the study.

All PCR amplified products were sequenced using the Sanger method. The sequences obtained were first checked by BLAST to assess the genetic similarity with other *H. capsulatum* sequences deposited in GenBank, and the results showed that the isolates showed similarity greater than 80% (Table I). Only isolate number 16 did not find similarity with *H. capsulatum*, and it is believed that failures occurred in its

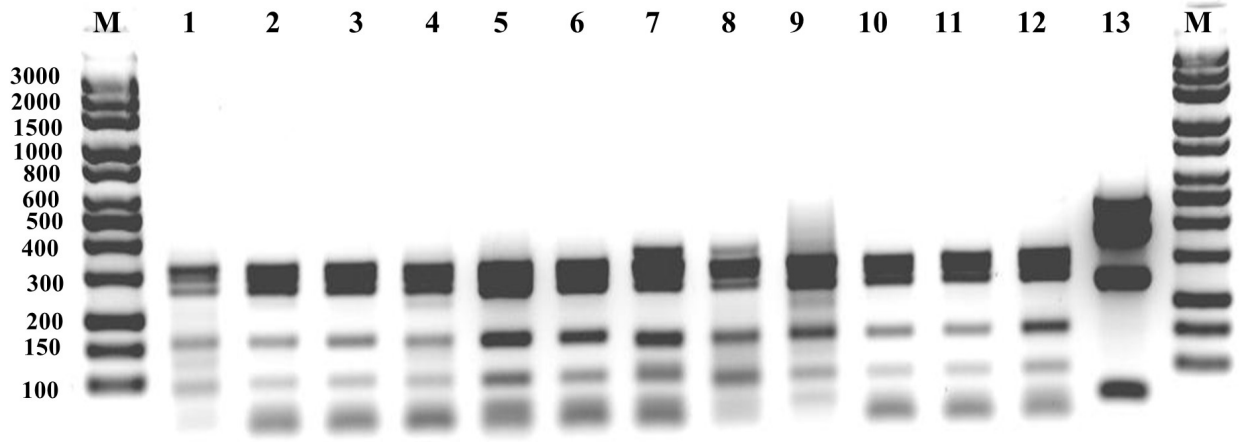

Figure 2. Restriction profile of 1000 bp PCR products amplified and digested with Taqα1 among fungal isolates from patients in the study. M - Mid Range Molecular Mass Marker (Cellco); Lines 1 to 12: Results of the PCR-RFLP reaction with samples from patients coded 04,06,08,09,12,13,16,26,35,37,42,10. Line: 13: Positive control (1000 bp universal fungal region amplified from *Candida albicans* ATCC 10231 and digested with Taqα1). M -M - Mid Range Molecular Mass Marker (Cellco). Electrophoresis in 3% agarose gel.

sequencing. The similarity with other fungal species was also verified in BLAST, and the result showed similarity with *Aspergillus sydowii*.

DISCUSSION

H. capsulatum is a pathogen that enters the lung, and the disease can be present in only one site or in disseminated form (Carreto-Binaghi et al. 2019). In recent years, an increase in the disseminated form in patients has been reported (Damasceno et al. 2019). According to the World Health Organization (WHO 2022b), disseminated histoplasmosis has been responsible for one in every six AIDS-related deaths in the Americas.

A retrospective study by Ferreira et al. (2017) carried out in Brazil in the center west of Goias revealed that coinfection with HIV and disseminated histoplasmosis was associated with high mortality rates (53%) in the referral hospital for infectious diseases. Rapid detection of histoplasmosis in HIV patients is essential because it reduces mortality rates due to late diagnosis (Cáceres et al. 2018, Perez et al. 2021, Basso et al. 2022, Oladele et al. 2022, Pasqualotto et al. 2023).

For this, it is important to invest in diagnostic methods for identifying *H. capsulatum*, since culture, the gold standard method for diagnosis, can take up to 6 weeks to obtain the result, since the fungus that causes it is slow-growing and requires advanced biosafety to its handling (Falci et al. 2017, Torres-González et al. 2018, Hatakeyama et al. 2019, Samayoa et al. 2019). In addition, other laboratory methods, such as histopathological analysis and antibody tests, have low sensitivity in immunocompromised people (Falci et al. 2017, Cáceres et al. 2018, 2020, Baker et al. 2020).

This study showed that molecular methods can be viable alternatives for diagnosing *H. capsulatum* in whole blood samples. Molecular tools for the identification of fungi have several advantages, such as high sensitivity and specificity (Carreto-Binaghi et al. 2019). Molecular methods capable of detecting fungal DNA directly from clinical samples have the potential to improve the diagnosis, since systemic fungal infections can occur even in the presence of

Table I. Results of culture, urinary antigen and molecular analysis of samples (blood and urine) from patients with disseminated histoplasmosis and acquired immunodeficiency syndrome.

Isolate	Fungi identified with fungus culture	Urinary antigen for *H. capsulatum**1	Molecular Analysis PCR ITS1, 5.8SrDNA, and ITS2*2	Identified fungi (identified nucleotide similarity, % identity with *H. capsulatum* deposited sequence in GenBank/ID accession number)
04	*Histoplasma capsulatum* +	+	+	468/541 (87%)/ID:FJ897698.1
06	-	+	+	825/915 (90%)/ID:FJ897699.1
08	*Histoplasma capsulatum* +	+	+	575/660 (87%)/ID:FJ897699.1
09	*Histoplasma capsulatum* +	+	+	829/911 (91%)/ID:FJ897699.1
12	*Histoplasma capsulatum* +	+	+	853/939 (91%)/ID:FJ897698.1
13	-	+	+	831/911 (91%)/ ID:FJ897699.1
16	*Histoplasma capsulatum* +	+	+	No similarity
26	*Histoplasma capsulatum* +	+	+	335/420 (80%)/ ID:KT155309.1
35	NR	+	+	490/586 (84%)/ID: FJ897699.1
36	*Histoplasma capsulatum* +	+	-	NR
37	*Histoplasma capsulatum* +	+	+	714/789 (90%)/ ID:FJ897699.1
41	*Histoplasma capsulatum* +	+	-	NR
42	*Histoplasma capsulatum* +	+	+	831/911 (91%)/ID:FJ897699.1
10	*Histoplasma capsulatum* +	+	+	508/570 (89%)/ID:FJ897699.1
CP	Candida albicans ATCC 10231	NR	+	No similarity

***1 Source: Urine sample from patients. *2 Source: patients' blood sample (nucleic acids). CP= Standard Strain. NT= Not tested. + = positive. - = negative. **Culture of fungi in Sabouraud dextrose agar and Mycosel agar – LACEN-SES-GO.**

negative microbiological tests (blood cultures), as is the case with isolates 6 and 13 (Table I). This fact occurs due to intrinsic factors of the host, antifungal therapy and the dissemination of a variable amount of microorganisms at the time of collection, which may explain the low sensitivity of the culture and, therefore, the variation in the detection rate of the methods (Siqueira & Almeida 2018).

Urine *Histoplasma* antigen (HUAg) has been the preferred method for diagnosing disseminated histoplasmosis in patients with HIV and is the method recommended by the Pan American Health Organization (PAHO 2020). The assay uses urine, a noninvasive clinical sample, and the methodology used is the enzyme-linked immunosorbent assay (ELISA), which can be performed in less than 3 hours and is a robust technique that is highly reproducible and significantly reduces the time for diagnosis. Recently, the first point of care test that detects *Histoplasma* antigen in the serum of HIV+ patients was also developed and validated (Martínez-Gamboa et al. 2021). However, these tests are not widely available in most countries and are expensive (Pérez-Lazo et al. 2017, Falci et al. 2019, Samayoa et al. 2019, Cáceres et al. 2020), making it important to have other valid diagnostic options (Cáceres et al. 2018).

Table II. Result of the PCR-RFLP analysis of the fungal ITS1, 5.8SrDNA and ITS2 region (1000 bp digested with Taq$^{\alpha}$1 from samples from patients with disseminated histoplasmosis and acquired immunodeficiency syndrome.

Isolate	Identified fungi	*Taq*$^{\alpha}$1 restriction enzyme profile on pairs of base (pb)
04	*Histoplasma capsulatum*	100, 160,350
06	*Histoplasma capsulatum*	30,100,160,350
08	*Histoplasma capsulatum*	30,100,160,350
09	*Histoplasma capsulatum*	30,100,160,350
12	*Histoplasma capsulatum*	30,100,160,350
13	Histoplasma capsulatum	30,100,160,350
16	*Histoplasma capsulatum*	30,100,160,350
26	*Histoplasma capsulatum*	100,160,350
35	*Histoplasma capsulatum*	100,160,350
36	NT	NT
37	*Histoplasma capsulatum*	30,100,160,350
41	NT	NT
42	*Histoplasma capsulatum*	30,100,160,350
10	*Histoplasma capsulatum*	30,100,160,350
CP	*Candida albicans ATCC 10231*	50, 230, 400, 500

NT= Not tested.

Molecular tests for diagnosing *H. capsulatum* have been developed (Babady et al. 2011, Scheel et al. 2014, Alanio et al. 2021); however, none of these are commercially available and still need extensive validation to be widely used (Cáceres et al. 2018). This study demonstrated that it is possible to develop a low-cost molecular method for identifying *H. capsulatum* with an easily collected sample that is whole blood in EDTA. However, some limitations may occur with the "in house" DNA extraction method, including degradation and low yield (Pereira et al. 2019). Molecular methods, such as PCR, reveal higher positivity rates, since minimal fractions of circulating fungal DNA at the time of blood collection can be amplified and, therefore, detected with greater sensitivity and can guide therapy and contribute to a better prognosis for patients (Siqueira & Almeida 2018).

In this study, it was possible to identify *H. capsulatum* by the PCR technique followed by the sequencing of 12 patients, confirmed both in gel through amplification of the expected size fragment (~1000 bp) and through amplicon sequencing. Greater similarities with the *H. capsulatum* sequences deposited in GenBank could have been found if specific primers for *H. capsulatum* were designed in this study, as occurred in the study by Alanio et al. (2021).

The sequencing results also showed similarity with *Aspergillus sydowii*. Similarities in the molecular structure of different organisms can occur. The greater the similarities between the sequences of the nitrogenous bases of the nucleic acids, the greater the evolutionary

proximity between the species. Taxonomically, *Histoplasma capsulatum* and *Aspergillus sydowii* belong to the same phylum and class. Mapengo et al. (2022) reported that a greater phylogenetic relationship of different fungi means that pathogen-specific assays are more difficult to develop.

In AIDS patients, histoplasmosis is much more common than aspergillosis (Falci et al. 2017), and *Aspergillus sydowii* is a saprotrophic fungus found in soil that causes aspergillosis in coral reefs but rarely affects humans (Brandt et al. 2020, Vera-Cabrera et al. 2021). Thus, knowing the previous clinical diagnosis of disseminated *histoplasmosis* and having been confirmed by other laboratory tests, the amplification of fungal genetic material demonstrates that it is *Histoplasma capsulatum*.

From PCR, an increase in the amount of DNA of a specific sequence of interest can be obtained in vitro (Oliveira et al. 2021, Karunanathie et al. 2022), which in this study was the ITS1, 5.8SrDNA and ITS2 region. The 5.8S region is a coding sequence of rDNA that evolves slowly and is highly conserved, while the internal spacer regions (ITS) evolve rapidly, presenting high polymorphism, and therefore are of great interest in phylogenetic studies of genera, species and populations (Leite et al. 2020, Santos et al. 2020). This characteristic allows its use in obtaining restriction fragment length polymorphism (RFLP) in rDNA loci through the use of restriction enzymes and allows the evaluation of genetic variability among fungi and yeasts (Oliveira et al. 2021).

PCR-RFLP analyses are based on the size of fragments generated by DNA samples after cleavage with selected restriction enzymes and are compared between the number and size of fragments that appear after DNA digestion (Oliveira et al. 2021). In the PCR-RFLP technique of the ITS region, a cleavage profile was observed between the isolates, presenting bands of 100, 160, and 350 bp after restriction with Taq$^{\alpha}$1, differing from the pattern presented by the ATCC 10231 strain of *Candida albicans* (Fig. 2). Thus, PCR-RFLP proved to be a good method for diagnosing different species, as mentioned by Torchia et al. (2021), but it was unable to assess whether there are differences in genetic profiles among samples with the Taq$^{\alpha}$1 enzyme.

According to Damasceno et al. (2019) genetic variations may exist in *Histoplasma capsulatum* and determine greater infectivity in some individuals. In addition, patients with HIV may be coinfected by two or more genotypes that may have a significant impact on the progression of the disease. In studies by Leite et al. (2020) and Santos et al. (2020), PCR-RFLP was also unable to discriminate intraspecific differences between fungi.

This study has several limitations. Ideally, a larger sample would be desirable. However, the 14 patients included in this study were all diagnosed with the coinfection of disseminated histoplasmosis and AIDS through laboratory tests and clinical records. Furthermore, the study only included patients with disseminated histoplasmosis; thus, further investigations are needed to evaluate the ability of this PCR assay to diagnose nonprogressive clinical forms of histoplasmosis. Another limitation refers to the fact that the PCR RFLP technique did not demonstrate intraspecific differences between the *H. capsulatum* isolates, and the PCR primers that amplified the fungal universal region were not specific for *H. capsulatum*. Further studies are suggested with primer design that flank a specific region of *H. capsulatum* and amplicon sequencing to reduce uncertainties.

In conclusion, the molecular markers developed in this research represent an alternative molecular approach for the identification of *H. capsulatum* in blood

samples from patients with a clinical diagnosis of disseminated histoplasmosis. These markers have the potential to be incorporated into the diagnostic routine in the future. It is crucial to conduct additional studies in patients with nonprogressive clinical forms of histoplasmosis to expand and improve this diagnostic modality.

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