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Molecular Diagnosis Compared to Conventional Diagnosis for Rapid Detection of Resistant Strains of *Candida* Spp.

Diagnóstico Molecular Comparado ao Diagnóstico Convencional para Detecção Rápida de Cepas Resistentes de *Candida* Spp.

Diagnóstico Molecular Comparado con el Diagnóstico Convencional para la Detección Rápida de Cepas Resistentes de Candida Spp.

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Abstract
Yeast *Candida* spp. can cause serious infections, especially in patients of the hospital environment. Variation in the virulence profile of *Candida* clinical isolates and high mortality rates indicate the importance of rapid and accurate identification, as well as susceptibility testing for successful candidiasis treatment. A total of 20 strains were isolated from patients from Tertiary Hospital of the Northern Zone of Ceará. PCR amplification followed by agarose gel electrophoresis (PCR-AGE) and the manual method (culturing on CHROMagar-Candida) and VITEK automated method were used to test a total of 20 fungal strains from clinics sources. Five strains of *C. albicans*, 2 of *C. tropicalis* and 4 strains of *C. parapsilosis* which were cultured for the determination of MIC (Minimum Inhibitory Concentration). The best sensitivity rate was demonstrated by automatic identification and PCR-AGE, where all 20 strains isolated with a sensitivity profile of 100% (20/20) were completely identified. According to the data obtained, resistance to amphotericin B (CIM ≥ 1 µg/ml) was observed in some isolates of *C. tropicalis* and *C. parapsilosis*. However, for the other antifungals, these same species presented 100% sensitivity (MIC ≤ 0.125 µg / ml; MIC ≤ 8 µg / ml). Isolates of *C. albicans* were sensitive to all antifungal agents with 100% sensitivity, although 75% of the isolates showed a tendency for less susceptibility to amphotericin B. In this study, we confirmed the efficacy and applicability of PCR-AGE in the identification and differentiation of yeast *Candida* spp. from clinical samples.
Keywords: *Candida* Spp; Molecular identification; Antifungal activity.

Resumo
A levedura *Candida* spp. pode causar infecções graves, principalmente em pacientes no ambiente hospitalar. A variação no perfil de virulência dos isolados clínicos de Candida e as altas taxas de mortalidade indicam a importância da sua identificação rápida e precisa, bem como do teste de sensibilidade para o sucesso do tratamento da candidíase. Foi utilizado um total de 20 cepas isoladas de pacientes de um Hospital Terciário da Zona Norte do Ceará. A amplificação por PCR seguida de eletroforese em gel de agarose (PCR-AGE) e o método manual (cultivo em

CHROMagar-Candida) e o método automatizado VITEK foram usados para identificar um total de 20 cepas fúngicas oriundas de quadros clínicos diversos. Cinco cepas de *C. albicans*, duas de *C. tropicalis* e quatro cepas de *C. parapsilosis* foram utlizadas para determinação de CIM (Concentração Inibitória Mínima). Os testes que demonstraram maior sensibilidade foram a identificação automática e PCR-AGE, onde todas as 20 cepas testadas apresentaram perfil de sensibilidade de 100% (20/20) apresentando completa identificação. De acordo com os dados obtidos, resistência à anfotericina B (CIM ≥ 1 μg/ml) foi observada em alguns isolados de *C. tropicalis* e *C. parapsilosis*. Entretanto, para os demais antifúngicos, essas mesmas espécies apresentaram-se sensíveis (CIM ≤ 0,125 μg/ml; CIM ≤ 8 μg/ml). Os isolados de *C. albicans* foram sensíveis a todos os antifúngicos, embora 75% das cepas tenham tendência a menor sensibilidade à anfotericina B. Este estudo mostrou evidências sobre a eficácia e aplicabilidade da PCR-AGE na identificação e diferenciação da levedura *Candida* spp. a partir de amostras clínicas.
Palavras-chave: *Candida* Spp; Identificação molecular; Atividade antifúngica.

Resumen
La levedura *Candida* spp. pode causar infecciones graves, principalmente em pacientes no ambiente hospitalar. Una variación en el perfil de virulência de dos aislados clínicos de Candida y como altos taxones de mortalidad indica una importancia de su identificación rápida y precisa, como prueba de suscetibilidad para el tratamiento exitoso de la candidiasis. Foi utilizado en un total de 20 cepas foram aisladas de pacientes del Hospital Terciário da Zona Norte do Ceará. Una amplificación por PCR seguida de electroforesis en gel de agarosa (PCR-AGE) y un método manual (cultivo en CHROMagar-Candida) y un método automatizado VITEK para probar un total de 20 cepas fúngicas de fuentes clínicas. Cinco cepas de *C. albicans*, dos de *C. tropicalis* y cuatro cepas de *C. parapsilosis* que foram cultivadas para determinación de CIM (Concentração Inibitória Mínima). Una mejor taxa de sensibilidad foi demostrada pela identificação automática e PCR-AGE, una vez que 20 cepas aisladas con perfil de sensibilidad de 100% (20/20) foram totalmente identificadas. De acuerdo con los dados obtenidos, la resistencia a la anfotericina B (CIM ≥ 1 μg/ml) se observó en algunos aislados de *C. tropicalis* y *C. parapsilosis*. Entretanto, para os demais antifúngicos, essas mesmas espécies apresentaram 100% de sensibilidade (CIM ≤ 0,125 μg/ml; CIM ≤ 8 μg/ml). Los aislados de *C. albicans* fueron sensibles a todos los antifúngicos, aunque el 75% de las cepas tendieron a ser menos sensibles a la anfotericina B. Este estudio mostró evidencia sobre la eficacia y aplicabilidad de PCR-AGE en la identificación y diferenciación de *Candida* spp. de muestras clínicas.
Palabras clave: *Candida* Spp; Identificación molecular; Actividad antifúngica.

1. Introduction

Candida is a genus of yeast considered the most common cause of fungal infections worldwide (Irinyi *et al*., 2015; Calvo, 2016). In the hospital environment, *Candida* infections account for 80% of all fungal infections, including mainly infections of the bloodstream, urinary tract and surgical site (Tamura *et al*., 2007; Guimarães *et al*., 2012). They are commonly found among the microorganisms present in the mucous membranes of the respiratory and genital tract of animals, being part of the natural flora of microorganisms in humans (Greene & Chandler, 1998; Sidrim & Rocha, 2004; Jadhav; Pal, 2006; Suchodolski *et al*., 2008).

These commensal and opportunistic yeasts may become pathogenic when the immune system is compromised and may cause localized infections in immunosuppressed patients, patients receiving long-term corticosteroids, prolonged antimicrobial therapy, cytotoxic chemotherapy, and patients with diabetes mellitus (Berberi; Noujeim; Lima, 2015; Andes, 2016; Rocha; Sobrinho 2017). The exact detection and identification of the microorganism is extremely important because the virulence of *Candida* isolates differ according to the species and, in addition the onset of candidemia and localized infections caused by species resistant to traditional antifungal agents has increased significantly (Fujita & Weinstein *et al*., 2001; Nunes & Furlaneto, 2011; Sardi *et al*., 2013; Paramythiotou *et al*., 2014).

Azole antifungals such as itraconazole and fluconazole act to inhibit the enzyme lanosterol 14α-demethylase *(Grossman et al., 2014). The drug-induced change generates a methylated toxic sterol and affects the biosynthetic pathway of ergosterol, a key component of the fungal cell membrane, which depletes the functions of the plasma membrane (Sims et al; Lazo 2006;* Morschhäuser, 2016). However, microorganisms adapt to changes in their environment by properly adjusting gene expression and cellular activities, making them resistant. In addition, some widely used azolic derivatives, such as fluconazole, are considered fungistatic drugs that inhibit growth but do not kill the fungus, thus providing the opportunity for the

development of resistance *(Azeredo et al., 2013; Paramythiotou et al., 2014;* Morschhäuser, 2016). Especially during repeated or prolonged treatment of recurrent episodes of oropharyngeal candidiasis in patients with AIDS, the evolution of fluconazole-resistant strains of *C. albicans* has been commonly observed (Paulique, 2017; Da Silva *et al*., 2020).

Fungal infections of the genus *Candida* have increased their prevalence in the last three decades (Buitrón *et al*., 2009). Although *C. albicans* remains the most common pathogen, a significant increase has been described in other species classified as *Candida* non-albicans (Gokce *et al*., 2007; Guinea, 2014; Yapar, 2014). Thus, opportunistic infections, and in particular those caused by various *Candida* species, have gained considerable significance as a cause of morbidity and often mortality (Kaplan *et al*., 2000; Sardi *et al*., 2013; Koehler; Tacke & Cornely, 2014).

Currently, mycoses are important opportunistic infections in immunosuppressed individuals, and their diagnoses and treatments are decisive, since, in some cases, these can lead to a possible death (Silveira, 2017). Several systems for yeast culture capable of identifying these pathogens have been developed and require a time between 4 to 72 hours for analysis of the results, being widely available and commercialized (Espinel-Ingroff *et al*., 1998; Silva; Candido, 2005; Oliveira *et al*., 2006). Although correct identification of clinically important yeast strains can be achieved with these systems, incomplete or incorrect identifications can occur when new and emerging yeast strains are tested (Pfaller *et al*., 1996; Morschhäuser, 2016).

Early detection of infection has a major impact on the clinical course of many infectious diseases (Back-Brito *et al*., 2009). Morphological features and biochemical tests useful to identify isolated fungi may take days or even weeks to grow in culture, and evaluation of test results requires knowledge of mycology (Oliveira *et al*., 2006; Back-Brito *et al*., 2009). In recent years, several DNA-based methods have been developed to improve the diagnosis of mycotic infections and the identification of pathogenic fungi (Luo & Mitchell, 2002; Morschhäuser, 2016). The Polymerase Chain Reaction (PCR – *Reaction Chain Polimerase*) has shown great efficiency and promising results in the identification of pathogenic fungi from several clinical sources, presenting great sensitivity, specificity and simplicity (Fujita, 2001; Massonet & Moretti *et al*., 2004; Back-Brito *et al*., 2009; Almeida, 2021).

Molecular identification of yeasts is based on regions of DNA widely sequenced in the molecular ecology of fungi, recognized as universal fungal barcodes sequences, termed ITS and IGS (Schoch, 2012). These sequences are conserved regions in the ribosomal cluster of fungi, whose size may vary between species of the genus *Candida* (Fujita, 2001; Peay *et al*., 2008; Morschhäuser, 2016). Due to its greater degree of variation than other rDNA gene regions (for small and large subunit rRNA), the variation between individual rDNA repeats can be observed in the ITS and IGS regions (Morschhäuser, 2016). Thus, a PCR technique is based on the use of fungal universal primers for the amplification of such regions, followed by restriction analysis, sequencing or size determination of the amplified fragmente (Fujita, 2001; Massonet et a., 2004; Bolckelmann *et al*., 2008; Back-Brito *et al*, 2009).

This study aimed to isolate strains of *Candida* spp from hospitalized patients diagnosed with candidiasis, to identify pathogens by manual, automated methods and PCR-AGE, performing a comparative analysis between each method, followed by strains sensitivity test to itraconazole, fluconazole and amphotericin B by the broth microdilution method.

2. Methodology

2.1 Clinical Examinations and Sample Collection

A total of 20 strains were isolated from patients from Santa Casa de Misericórdia de Sobral (Tertiary Hospital of the Northern Zone of Ceará). All research was approved by the ethics committee (N° opinion 644.365). The isolates were obtained from primary cultures, and yeast strains were presumptively identified according to the morphological characteristics and staining of colonies grown in CHROMagar-Candida medium (Paris, France), strains identified as *Candida* spp., unidentified strains were submitted to VITEK 2 automated system (BioMérieux Vitek, Hazelwood, France) and PCR-AGE analysis. Type

Culture Collection (ATCC), american strains of *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258) were also included as quality controls.

2.2 Extraction of genomic DNA

After presumptive identification in chromogen medium, the strains were isolated and grown in liquid medium for 24-48 hours at 37°C, and then extracted the genetic material. Cells were recovered by centrifugation at 4500 x g for 2 minutes and resuspended in 200 µL of buffer 1 (2% triton x 100; 1% de SDS; 10 mM Tris / HCL (pH 8); 100 mM NaCl). Then, 200 µL of phenol-chloroform-isoamyl alcohol (25: 24: 1) and 300 µg of glass bead were added. After shaking for 4 minutes, 200 µL of buffer 2 (10mM Tris / HCL (pH 8) EDTA 1mM) were added. The samples were centrifuged (13.000 x g) for 5 minutes and the DNA extracted into the supernatant was precipitated by the addition of ethanol (1mL). Then the samples were centrifuged at 13,000 x g for 5 minutes and the pellet was resuspended in 400 µL of buffer 2 (10mM Tris / HCL (pH 8), EDTA 1mM). The samples were incubated in a water bath for 30 minutes at 50°C for removal of the RNA, and after centrifugation at 13,000 x g the pellet was air-dried and resuspended in 50 µL of buffer 2 (10mM Tris / HCL (pH 8), EDTA 1mM). DNA yield was done spectrophotometrically using a BioPhotometer (Eppendorf, Hamburg, Germany) (Ausubel *et al*., 2002).

2.3 Molecular identification

The procedure for performing PCR was followed according to the method previously described by Fujita *et al*., (2001). ITS1 primers (5_-TCCGTAGGTGAACCTGCG-3_) and ITS3 (5_-GCATCGATGAAGAACGCAGC-3_), and the reverse ITS4 (5_-TCCTCCGCTTATTGATATGC-3_) were used to target regions conserved in the ribosomal DNA cluster, 18S, 5.8S and 28S, respectively, being supplied by Invitrogen (Carls -bad, CA). Samples were identified using two independent PCR reactions: one using ITS1-ITS4 primers and another, using the ITS3-ITS4 primers. The pair of primers ITS1-ITS4 was used to amplify the intermediate region 5.8S of rDNA and the adjacent regions ITS1 and ITS2, the pair of ITS3-ITS4 primers was used to amplify a larger portion of 5.8S rDNA and the region adjacent to IST2. PCR amplification was performed with a volume of 25 µL. The amount of sample added to the blend (2.5 µL of buffer, 0.5 µL of dNTPs: 2.0 µL of each primer and 0.3 µL of TAq DNA polymerase Invitrogen, Carlsbad, CA) for each reaction was established according with the yield of DNA obtained, with the remaining volume consisting of distilled water. For the two PCR reactions, the amplification parameters consisted of an initial denaturation at 94°C for 4 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55 ° C for 30 seconds, and extension at 72°C for 1 minute; and a final extent at 72°C for 6 minutes as described by Fujita *et al*. (2001). PCR was performed on an Ep Gradient Mastercycler S Thermal Cycler (Eppendorf, Hamburg, Germany). Negative control reactions without any template DNA were performed simultaneously. The lengths of the amplified fragments were determined by agarose gel electrophoresis (AGE) followed by image analysis. Agarose gel electrophoresis (2.0%) was performed with 1 x TBE buffer (0.1 M Tris, 0.09 M boronic acid, 1 mM EDTA) at 5 V / cm for 2 hours. A DNA molecular marker with 100 base pairs (Invitrogen, Carlsbad, CA) was used to aid in the size determination of the amplified fragments. The software package ImageQuant TL (Amersham Biosciences Corp) was used to calculate the molecular sizes. The size of the fragments were measured three times for each strain on different gels.

2.4 Sensitivity Test

For the microbiological assay the broth microdilution test recommended by CLSI (2008) was used. Five strains of *C. albicans* (LABMIC 0101, LABMIC 0102, LABMIC 0103, LABMIC 0104, LABMIC 0105), two of *C. tropicalis* (LABMIC 0201 and LABMIC 0202) and four strains of *C. parapsilosis* (LABMIC 0301, LABMIC 0302, LABMIC 0303, LABMIC 0304) which were cultured on Sabourand agar at 35 ° C for 48 hours. For the preparation of the inoculum, a fragment of the

Candida strains was transferred into test tubes containing 9 mL of saline. Subsequently, the suspensions were diluted 1: 2000 with RPMI 1640 medium to obtain final inoculum concentrations of approximately 5 x 104 CFU / mL. For the determination of MIC, the assay was performed in 96-well, U-shaped plates (Elisa Plate). Initially, 100 µL of RPMI 1640 medium was dispensed into all wells and 100 µL of the drug (itraconazole, fluconazole and amphotericin B) was then pipetted to a concentration of 64 mg / ml, then the serial dilution technique was performed, from the withdrawal of a 100 µL aliquot from the more concentrated cavity to the successor cavity. 100 ul aliquots of the inoculum corresponding to each strain tested were dispensed into the orifices of each column. As a negative control, the viability of the strains was verified by inoculating the fungal suspension in RPMI medium without the addition of antifungal agents. Readings were taken to determine the MIC at 24 and 48 hours after the test, where the formation or not of clusters of cells at the bottom of the wells of the plate was considered.

2.5 Statistical analysis

To obtain the sensitivity of each method used, the yeast identifications were classified as: (a) complete, when the species was established beyond any doubt; (b) not identified when the method was unable to identify the strain. The data of each method were expressed as a percentage of the tests. Then, to calculate the specificity of each procedure, the automated methods (VITEK) and PCR-AGE were compared with analysis of the morphological characteristics in CHROMagar-Candida medium (Paris, France), which was used as the standard for the final identification objective. The sizes of the fragments obtained by PCR-AGE were calculated as +/- of the standard deviation of three measurements. The frequency and antifungal sensitivity profile data were expressed by inferential statistics, admitting P value <0.05 for significant statistical differences.

3. Results

3.1 Comparison of different identification methods showed more specificity for PCR-AGE molecular approach.

The viability of the manual, automatic method (VITEK 2) and methods based on PCR to identify strains of *Candida* from clinical sources were evaluated. In addition, the sensitivity of all three methods comparing Vitek and PCR-AGE with the chromogenic medium (CHROMagar-Candida) method was analyzed. Yeast species were isolated from 20 cases of candidiasis in hospitalized patients. The complete manual identification by growth analysis in chromogenic medium required 24 hours to be completed, however the automated identification by the Vitek 2 system lasted about 24-48 hours. In comparison, the PCR-AGE method lasted 1 hour and 50 minutes for DNA isolation, 1 hour and 20 minutes for amplification, 2 hours and 30 minutes for electrophoresis, and 30 minutes for fragments analysis, requiring a time required for 5 hours and 50 minutes. The best sensitivity rate was demonstrated by automatic identification and PCR-AGE, where all 20 strains isolated with a sensitivity profile of 100% (20/20) were completely identified, however, for the conventional method in chromogen medium 70% (14/20) and 6 strains were not identified, presenting atypical chromogenic profile in the cultures used, the results are shown in table 1. A total of 20 strains were identified, table 2 summarizes the results of the manual method, Vitek and yeast identifications by PCR-AGE. The clinical specimens were *C. albicans* (6), *C. tropicalis* (4), *C. parapsilosis* (6), *C. famata* (3) and *C. krusei* (1). The frequency among the identified species are shown in Table 3, and consisted of *C. albicans* (30%), *C. parapsilosis* (30%), *C. tropicalis* (20%) e *C. famata* (15%) in isolated cases. Thus, the respective agreement rates were observed: 55% (11/20) for the three methods, 100% (20/20) for PCR-AGE and VITEK, and 40% (8/20) for PCR and conventional method in CHROMAgar-Candida medium. In this work, molecular and automated methods were more effective than the manual method, data are expressed in table 4. However, there were no statistical differences regarding the specificity in relation to the methods evaluated (p = 3583). Amplification of the DNA ITS regions by targeting conserved ribosomal gene sequences by means of conventional PCR (*Polymerase Chain Reaction*) was able to completely identify the strains tested. Resolutions of 2% agarose gel electrophoresis gels demonstrated the complete amplification of the samples used, extracted from the yeasts isolated and

identified with the methods described previously (figures 1 and 2). Restriction fragment length polymorphism analysis was performed by the ImageQuant TL software package (Amersham Biosciences Corp), which revealed molecular sizes of 415-838 base pairs (bp) for the fragments generated with the ITS3 / ITS4 primers.

Table 1 - Sensitivity of the methods Manual, Vitek 2 and PCR - AGE in the identification of yeasts (%).

Method	Full identification	Identification not completed	Not identified
Manual	70% (20/20)	70% (6/20)	70% (6/20)
Vitek 2	100% (20/20)	-	-
PCR – AGE	100% (20/20)	-	-

Source: Elaborated by the authors.

Table 2 - Comparison of methods, chromaging, vitek 2 and pcr-age for identification of yeasts of the genus C*andida* spp.

Sample	CHROMagar	Vitek 2	PCR-AGE
03	C. *Tropicalis*	C. *Tropicalis*	C. *Tropicalis*
04	C. *Albicans*	C. *Albicans*	C. *Albicans*
05	C. *Albicans*	C. *Albicans*	C. *Albicans*
06	C. *Parapsilosis*	C. *Parapsilosis*	C. *Parapsilosis*
07	C. *Krusei*	C. *Krusei*	C. *Krusei*
08	C. *Parapsilosis*	C. *Parapsilosis*	C. *Parapsilosis*
09	C. *Tropicalis*	C. *Parapsilosis*	C. *Parapsilosis*
11	C. *Albicans*	C. *Albicans*	C. *Albicans*
12	Undefined	C. *Parapsilosis*	C. *Parapsilosis*
13	Undefined	C. *Parapsilosis*	C. *Parapsilosis*
14	C. *Albicans*	C. *famata*	C. *famata*
15	C. *Albicans*	C. *Albicans*	C. *Albicans*
16	C. *Tropicalis*	C. *Tropicalis*	C. *Tropicalis*
17	Undefined	C. *Tropicalis*	C. *Tropicalis*
18	C. *Albicans*	C. *famata*	C. *famata*
19	Undefined	C. *Albicans*	C. *Albicans*
20	Undefined	C. *Parapsilosis*	C. *Parapsilosis*
21	Undefined	C. *Tropicalis*	C. *Tropicalis*
22	C. *Tropicalis*	C. *famata*	C. *famata*
23	C. *Albicans*	C. *Albicans*	C. *Albicans*

Source: Elaborated by the authors.

Table 3 - Frequency of *Candida* sp species isolated from patients of the Tertiary Hospital of the Northern Zone of Ceará (Santa Casa de Misericórdia de Sobral).

Species	Frequency	Percentage
Candida albicans	6	30%
Candida tropicalis	4	20%
Candida famata	3	15%
Candida parapsilosis	6	30%

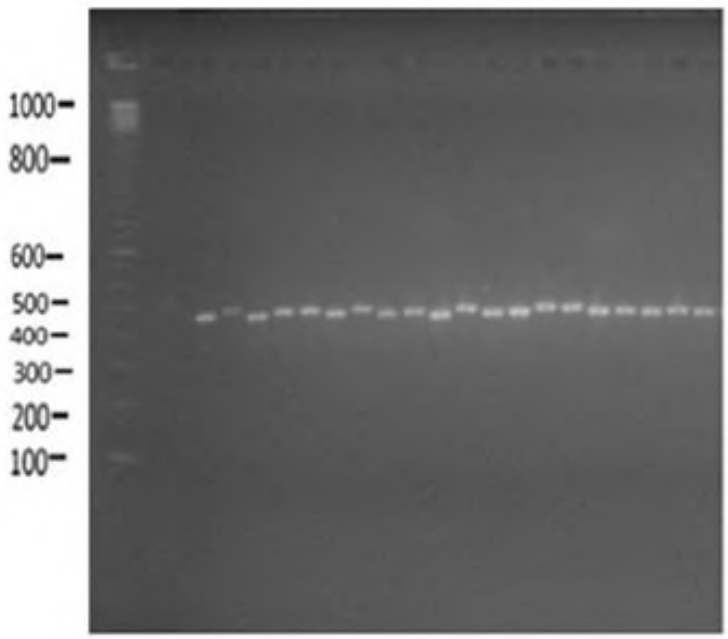
Source: Elaborated by the authors.

Table 4 - Concordance rates among the three methods used in the identification of *Candida* sp isolates: manual, automated and PCR-AGE.

Method	Agreement
Manual – Vitek 2	40%
Manual – PCR	40%
Manual – Vitek 2 – PCR	55%
Vitek – PCR	100%

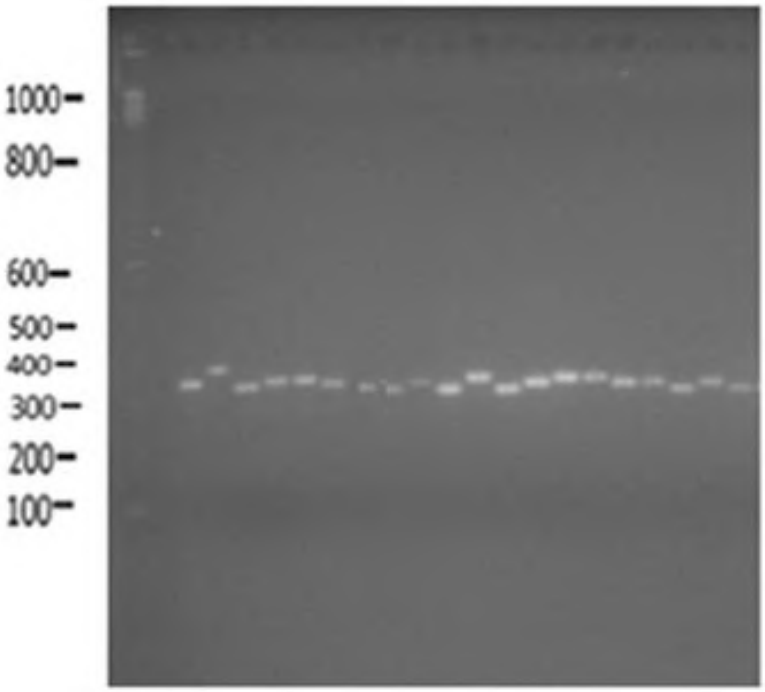
Source: Elaborated by the authors.

Figure 1 - Representative gel showing the amplification by AGE-PCR and variation in the lengths of amplicons generated with the ITS1 / ITS4 primers targeting ITS regions.



Source: Elaborated by the authors.

Figure 2 - Amplicons produced using the ITS3 / ITS4 primers. Ray 1: 100pb DNA Marker; Lane 5: *C. albicans*; Lane 6: *C. parapsilosis*; Lane 7: *C. Krusei*; Lane 9: *C. tropicalis*.



Source: Elaborated by the authors.

3.2 *Candida* spp. isolates demonstrated variable sensitivity for the tested antifungal drugs.

Five different patterns were recognized among the 20 fungal strains tested based on both amplicons' length. The standard deviations (SDs) of the calculated molecular sizes ranged from 1.20-2.96 with the primers ITS1-ITS4 and 2.33-3.31 with the ITS3-ITS4 primers and are shown in Table 5. The broth microdilution test was performed according to CLSI document M27-A3, where the following antifungals were tested: itraconazole, fluconazole and amphotericin B. Among the 20 strains of *Candida* spp., 19 isolates were submitted to the sensitivity test by the microdilution method, performed on sterile Elisa plaques for the determination CIM (Minimum Inhibitory Concentration) being *C. albicans* (42.1%), *C. tropicalis* (21.05%) and *C. parapsilosis* (87.5%). Table 6 summarizes in vitro susceptibility to the three antifungal agents tested for the species identified with the methods previously described. According to the data obtained, resistance to amphotericin B (CIM ≥ 1 µg/ml) was observed in some isolates of *C. tropicalis* and *C. parapsilosis*. However, for the other antifungals, these same species presented 100% sensitivity (CIM ≤ 0.125 µg / ml; CIM ≤ 8 µg / ml). Isolates of *C. albicans* were sensitive to all antifungal agents with 100% sensitivity, although 75% of the isolates showed a tendency for less susceptibility to amphotericin B.

Table 5 - Length of the amplicons of Candida species determined by PCR-AGE.

Organism (Nº. of strains)	**Mean of fragment sizes (± SD), in bp, found by PCR-AGE with the following primers.**	
	ITS1 – ITS4	**ITS3 –ITS4**
Candida albicans	535 ± 2.73	337 ± 2.77
Candida tropicalis	521 ± 2.33	327 ± 1.02
Candida famata	533 ± 3.31	370 ± 2.85
Candida parapsilosis	519 ± 3.50	314 ± 2.79
Candida krusei	496 ± 3.01	335 ± 3.02

Source: Elaborated by the authors.

Table 6 - Minimum inhibitory concentration (MIC) to fluconazole, itraconazole and amphotericin B.

***Candida* spp. N**	**MIC (µg/mL)**		
	Itraconazole	**Fluconazole**	**Amphotericin B**
C. tropicalis 4	= 0,5 (1) = 0,006 (3)	= 4 (1) = 2 (2) = 0,5 (1)	=2 (3) =1 (1)
C. albicans 8	= 0,25 (1) = 0,12 (3) = 0,06 (4)	= 4 (3) = 2 (1) = 0,5 (1) = 0,25 (3)	=2 (2) = 1 (6)
C. parapsilosis 7	= 0,12 (1) = 0,06 (2)	= 4(6) = 2(1) = 0,03 (4)	= 4 (2) = 2 (5)

Source: Elaborated by the authors.

4. Discussion

For the identification of yeasts, there are methods available, including the use of conventional methods such as chromogenic substrates, agar micromorphology and biochemical characterization. For the latter, several products are commercially available, such as Auxacolor (Bio-Rad), Vitek and API ID32C (bioMérieux) (Ods *et al*., 1994; Pires *et al*., 2015; Stefaniuk *et al*., 2016). The genus *Candida* includes 50 more species of yeasts, some of which are associated with infections in humans and animals and *C. albicans*, is the most frequently isolated species worldwide (Nadăş *et al*., 2014; Ali *et al*., 2015; Castro, 2017). However, the last decades have shown an increase in the number of non-albicans species, such as *C. krusei*, *C. parapsilosis*, *C. auris*, and these species are less susceptible to azole derivatives (Swinne *et al*., 2005; Ali *et al*., 2015; Doe *et al*., 2016; Du Han *et al*., 2020; Černáková *et al*., 2021). The rapid and accurate identification of yeasts has become increasingly essential, due to the development of new antifungal agents with different activities against several species, which differ in their characteristics in their virulence (Hazen; Wingard, 1995; Fujita, 2001; Swinne *et al*., 2005 Asadzadeh *et al*., 2015). In addition, the emergence of new emerging strains has made treatment difficult, especially in hospitalized patients, who have compromised immunity, and where some strains of *Candida* spp present intrinsic or potential resistance to traditional antifungals widely used (Gokce *et al*., 2007; Kiraz *et al*., 2010; Vieira *et al*., 2017; Du Han *et al*., 2020; Černáková *et al*., 2021).

The identification of yeasts using conventional methods is time-consuming, expensive and laborious, so that several commercial products and systems have been developed in order to circumvent the difficulties faced by clinical microbiology laboratories in the diagnosis of *Candida* spp infections, such as agar containing chromogens, kits or panels semi or fully automated for the presumed or definitive identification of the most prevalent species (Pfaller *et al*., 1996; Graf *et al*., 2000; Meletiadis *et al*., 2011; Kumar *et al*., 2013). However, the time to obtain the identification results is extremely important, since the rapid and precise clinical diagnosis directs the treatment with the appropriate antifungal (Shokohi *et al*., 2010).

In this study, the molecular identification was more rapid when compared to the methods of chromogenic analysis (24 hours) and Vitek (24-48 hours), corroborating with the data of Back-Brito *et al*. (2009) who reported the same time to obtain of the results, in relation to the molecular identification by PCR-AGE. In addition to a shorter time for the analysis of the data, the molecular methods offer excellent possibilities of application: taxonomical definitions, epidemiological and diagnostic (Barry *et al*., 2017). In human medicine, the identification of *Candida* DNA by a PCR method with subsequent analysis of the fragments presents high sensitivity when compared to the manual and automatic methods (Morace *et al*., 1999; Massonet, 2004; Back-Brito, 2009; Castro, 2017).

We found a sensitivity of 100% (20/20) for the PCR-AGE method (20/20), where all strains were identified, presenting a concordance rate of 100% (20/20) with the Vitek method and 40 % for the method of analysis in CHROMagar medium, where 6 strains were not identified. Massonet *et al*. (2004), using the same system, found a sensitivity rate of only 67%, obtaining a lower identification performance, in relation to the molecular method of analysis of IST2 fragment polymorphism. Back-Brito *et al*. (2009) reported a sensitivity of 90.9% for the conventional method, 84.84% for vitek 2 and 99.96% for AGE-PCR, in the identification of yeasts from veterinary sources, which was similar to our results in relation to the molecular and automated method used in this study. However, there was disagreement regarding the agreement of the three methods, presenting a rate of 86.7% versus only 55% agreement in this study, in addition, the manual method presented lower sensitivity in this study. The agreement rates obtained in this study between Vitek and the manual method were closer to the data of Oliveira *et al*. (2006), who found 53.2% agreement when comparing these two methods in the identification of yeasts from animal sources and blood cultures.

Although these results showed a concordance rate of only 55% for the three methods, these results suggest that conventional methods are not ideal for the quick and accurate identification of samples from clinical sources, since they

consume time and their results are subject to errors, and additional identification tests or repetitions are required (Aubertine *et al*., 2006; Higashi *et al*., 2015). Graf *et al*. (2000) evaluated *Candida* and other yeasts using the Vitek 2-YST system in a total of 241 isolates versus automated prior version identification, myoscopy, and agglutination test for *C. krusei*. Overall, 222 samples (92.1%) were unequivocally identified, including 11 samples (4.6%) which, identified with low discrimination, were resolved by simple complementary tests. Ten samples (4.1%), for which the results were given with low discrimination, could not be unequivocally identified with additional tests, 4 strains (1.7%) were mistakenly identified and 5 (2.1%) could not be identified in any combination of methods. In this way, the authors found similar results to our data, with adequate results for automation alone of 87.5% versus our 100% of the samples.

In order to obtain a reliable and definitive diagnosis, several molecular approaches were used for the detection of fungi from clinical samples, the targets for the detection of fungi in the genus or level of species included rDNA18S (Makimura *et al*., 1994), mitochondrial DNA (Gardes *et al*., 1991), intergenic spacer regions (Radford *et al*., 1998) and ITS regions (Jackson et., 1999; Fujita *et al*., 2001; Massonet; Moretti, 2004; Back-Brito *et al*., 2009; Ali *et al*., 2015). In the last decade several techniques have been developed, since they have a high degree of reliability for epidemiological research of several strains of pathogenic yeasts (Riederer *et al*., 2002; Mirhendi *et al*., 2003). Molecular techniques are a good substitute for the identification and diagnosis of fungi like *Candida* spp., because the diagnosis of high potency is quick and easy (Chen, 2000; Mirhendi, 2003; Fatahi *et al*., 2007). This highly discriminating procedure is used in species diagnosis especially in epidemiological studies to assess the feasibility of transmission as well as to choose appropriate antifungal medicines (Cirak *et al*., 2003; Neppelenbroek *et al*., 2006; Boyanton, 2008). Several molecular methods for *Candida* spp., are currently used however, due to lack of a gold standard the ideal method has not been determined, although previous studies have demonstrated that no single approach is adequate (Shokohi *et al*., 2010).

Back-Brito *et al*. (2009) using the same set of primers and the same identification tools found similar molecular sizes with the ITS1 / ITS4 primers for *C. albicans* (536 bp), *C. parapsilosis* (519 bp) and *C. tropicalis* (522 bp), similar to our results, where *C. albicans* (535 bp), *C. parapsilosis* (519 bp) and *C. tropicalis* (521 bp) amplicons that showed similar sizes. In addition, in our studies, they showed species-specific differences in the size of the fragments generated with ITS3 / ITS4 starters, where *C. albicans* (337 bp), *C. parapsilosis* (314 bp), *C. tropicalis* (327 bp) and *C. famata* (370 bp) presented amplicons of varying sizes between the strains, being possible the correct identification. In previous studies, Fujita *et al*. (2001) performed yeast identifications by PCR-based methods, and using the same primer pairs found molecular sizes that were identical for some species: *C. parapsilosis* and *C. tropicalis*, about 520 bp; *C. utilis* and *C. neoformans*, about 554 bp; and *C. saitoana* and *Pichia anomala*, about 614 bp. The same isolates showed differences in the sizes of the fragments generated with the ITS3 / ITS4 primers, making differentiation possible. In this study, we find results that corroborate data from Back-Brito *et al*. (2009) for *C. tropicalis* and *C. albicans* that presented molecular sizes close to, and identical in *C. parapsilosis*, for the amplicons generated by the first pair of primers. However, the molecular sizes of the fragments of *C. albicans*, *C. krusei*, *C. tropicalis* and *C. famata* generated with the ITS3 / ITS4 primers corroborate with the data of Fujita *et al*. (2001).

In recent decades, the increasing incidence of fungal infections has been evident worldwide, and *Candida* infections have been frequently associated with patients such as congenital or acquired immunodeficiency adquirida (Ortega *et al*., 2010; Junqueira *et al*., 2012; Li *et al*., 2013). In addition, the severity of certain diseases such as AIDS and cancer associated with debilitating conditions of patients, causes prolonged stays and higher hospital costs, generating a great problem of public health (Back-Brito *et al*., 2009). The variability of virulence and behavior of the different *Candida* species, in addition to the growing number of isolates resistant to antifungal therapies, highlight the need for antifungal susceptibility tests to monitor the resistance of these microorganisms to widely used drugs, which may favor the choice of clinical treatment appropriate (Belazi *et al*., 2005; Li *et al*., 2013; Idelevich *et al*., 2014).

Across the world, there has been an increasing increase in infections by antifungal resistant *Candida* species commonly used in clinical practice (Canuto *et al*., 2002; Vandeputte *et al*., 2005; Passos *et al*., 2007). However, in Brazil and around the world, the resistance of *Candida* non-albicans to azole antifungals has been little prevalente (ST-Germain *et al*., 2001; Pfaller *et al*., 2002; Nunes *et al*., 2012). *C. tropicalis*, is the most frequent species of *Candida* non-albicans in Brazil, being described in Ceará as being highly sensitive to the usual antifungals (Menezes *et al*., 2009; Nunes *et al*., 2012).

In the present study, isolates of *C. tropicalis* showed 100% sensitivity to azole derivatives and 75% were resistant to amphotericin B. In a study conducted by Menezes *et al*. (2009) with clinical samples from patients hospitalized at the General Hospital of Fortaleza, Ceará, 100% sensitivity of the strains to amphotericin B, itraconazole and fluconazole were found. In addition, in a study conducted by Menezes *et al*. (2016) with isolates from patients attended at the Clinical Analysis Laboratory of the Russas City Hall, Ceará, 100% sensitivity was reported for *C. tropicalis* to amphotericin B, 50% dose-dependent sensitivity on fluconazole and 50% were sensitive to itraconazole. This observation reinforces the importance of recognizing the profiles of antifungal susceptibility among these species for the adequate treatment of infections caused by these pathogens, since this complex presents varied virulence profiles (Guo *et al*.,2016). Resistant isolates have been rare in the world, however a multicenter study in Taiwan, China, revealed that 46.5% of *C. tropicalis* isolates were resistant to fluconazole at the participating center of this country (Yan *et al*., 2008). However, Nunes *et al*. (2012) warned that empirical use may lead to failure, especially if used against *C. tropicalis*, with probability of failure if the drug chosen is itraconazole, in contrast, in this study, the isolates of this species presented 100% of sensitivity.

C. albicans is the most isolated yeast species, especially in nosocomial infections, being considered the main agent of fungemia in the world (Almirante *et al*., 2005; Playford, 2010; Badiee, 2011). Isolated isolates show a high sensitivity profile to commonly used antifungals (Pfaller, 2002; ST-Germain *et al*., 2001; Diekema *et al*., 2002; Córdoba *et al*., 2011; Nunes *et al*., 2012). In this study, *C. albicans* isolates showed 100% sensitivity to all antifungal agents tested, although 75% of strains showed a tendency for lower susceptibility to amphotericin B. Although most *Candida* species show sensitivity to fluconazole, the occurrence of amphotericin B resistant isolates has increased significantly in recent years and it is not advisable to use other drugs because it is nephrotoxic (Spampinato & Leonardi, 2013; Maubon & Paramythiotou *et al*., 2014). Menezes *et al*. (2016) found an elongated resistance to fluconazole (37.5%) and itraconazole (50%) among strains of *C. albicans* isolated from urine samples, and 37.5% of isolates identified with *C. albicans* showed dose-dependent sensitivity, presenting resistance to azole antifungals higher than that found in the literature (Girmenia *et al*., 2000; Colombo *et al*., 2003). Although the sensitivity of *C. albicans* to widely used antifungals is considered common in Brazil and worldwide, variation of susceptibility profiles may occur, since microbiological resistance involves molecular mechanisms and may be either intrinsic or acquired (Wahlquist *et al*., 1991; Menezes *et al*., 2016).

Resistance to fluconazole and amphotericin B have already been observed in some *C. parapsilosis* isolates (Swinne *et al*., 2005). In this study, the isolates of this species showed 100% sensitivity to the azole derivatives and 85.7% of the strains were resistant to amphotericin B. In a study carried out by Favalessa *et al*. (2010), with isolated samples of seropositive HIV patients, *C. parapsilosis* isolates showed resistance of 25% and 12.5% to itraconazole and amphotericin B, respectively, although 60% of the isolates had susceptible to all antifungal agents tested. Many of the most commonly isolated non-albicans species are less susceptible to azole derivatives, making it difficult to treat infections (Rex *et al*., 2000; Crocco *et al*., 2004; Nunes *et al*., 2012). Although *C. albicans* is the most frequent species, in recent years there has been an increase in nosocomial infections by other species of *Candida*, *C. parapsilosis* being the second most frequent species in several countries, mainly related to blood infections (Singaravelu *et al*., 2014). Regarding the susceptibility profile, several antifungal agents, including azole, polyene, flucytosine and echinocardins, have activity against strains of *C. parapsilosis* isolated from infections (Kale-Pradhan *et al*., 2010; Menezes *et al*., 2012). Although susceptibility of *Candida* yeasts to available antifungal agents is variable

and predictable, a single isolated sample does not always follow the general pattern (Guo *et al*., 2016). Therefore, this is one of the crucial reasons that makes the susceptibility test essential for the various antifungal agents available. Furthermore, the choice of antifungal for a correct treatment of infections should consider the risks of comorbidity, the species of fungus in force, the pharmacological interaction and the patient's clinical history (Vieira *et al*., 2017).

5. Conclusion

In this study, the effectiveness of PCR-AGE in the identification and differentiation of yeast *Candida* spp from clinical samples showed better efficacy. Regarding the *in vitro* susceptibility test, most of the strains tested were sensitive to azole derivatives, however cases of resistance to amphoterician B for *Candida* non albicans were found, as well as a lower susceptibility to this antifungal for the isolates of *C. albicans*. These data reinforce the knowledge about the *in vitro* susceptibility profile of clinical isolates from patients admitted to a tertiary hospital in the northern area of Ceará.

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