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PCR-REA as an important tool for the identification of *Cryptococcus neoformans* and *Cryptococcus gattii* from human and veterinary sources

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ABSTRACT

The extraordinary ability of *Cryptococcus* species to cause disease has focused the attention of scientists on finding ways to improve their identification methods. In this study, PCR-REA, manual methods (morphological and biochemical characteristics), API 20C and VITEK 2 were used to test identify a total of 30 *Cryptococcus* spp. from human and veterinary sources. PCR-REA was performed using the capsular region as amplification target followed by restriction with the enzymes *Age*I, *Bsm*FI and *Hpa*II. PCR-REA identified the strains as *C. neoformans* var. *grubii* ($n = 19$) and *C. gattii* ($n = 8$). There was no significant difference between the API 20C AUX and VITEK 2 when compared to manual methods for the identification of *Cryptococcus* spp. However, none of these non-manual methods were able to detect *C. gattii* samples. PCR-REA showed a greater level of concordance with the manual method, besides being faster and more sensitive than the other methods. Therefore, it is indicated for routine identification of *Cryptococcus* spp. strains.

1. Introduction

Cryptococcus spp. are capsulated yeasts that cause disease in a great variety of hosts, including mammals and birds (Huston and Mody, 2009). *C. neoformans* and *C. gattii* are the most common etiological agents of cryptococcosis in humans and animals (Li and Mody, 2010). *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are considered opportunistic pathogens, affecting immunocompromised hosts. On the other hand, *C. gattii* has mainly been reported among immunocompetent individuals (Duncan et al., 2006; Springer and Chaturvedi, 2010).

Early microorganism identification is of great importance for the patient, since cryptococcosis can be a life-threatening disease (Li and Mody, 2010). The laboratory investigation of *Cryptococcus* spp. in clinical specimens is based on direct microscopy, culture and subsequent biochemical identification (Huston and Mody, 2009), but results are often imprecise because of the subjective interpretation of phenotypical tests (Borman et al., 2008). Besides this, automated and semi-automated methods can also be inconclusive because they frequently require

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supplementary tests to conclude microorganism identification (Gundes et al., 2001; Massonet et al., 2004). Several molecular methods for the identification of major molecular types of the *Cryptococcus* species complex from clinical specimens and cultures, such as PCR-restriction enzyme analysis, PCR multiplex and the Luminex xMAP technique based on DNA hybridization, have been available for some time (Bovers et al., 2007; Enache-Angoulvant et al., 2007; Ito-Kuwa et al., 2007; Feng et al., 2008; Leal et al., 2008), but they are not routinely used (Sidrim et al., 2010).

The goal of this study was to use a comparative approach to evaluate manual, automated and PCR restriction enzyme analysis (PCR-REA) for the identification of strains of the *C. neoformans* species complex from human and veterinary sources.

2. Materials and methods

2.1. Fungal strains

A total of 30 strains from the culture collection of the Specialized Medical Mycology Center were included in this study. They were chosen by the following criteria: *Cryptococcus* spp. obtained from human clinical cases ($n = 12$), animals ($n = 5$) and pigeon excreta ($n = 13$), previously identified as *Cryptococcus* sp. by at least two of the three methods (manual, API32 and VITEK2). These isolates were stored at −20 °C, on 2% potato dextrose agar, supplemented with glycerol. A total of eight reference strains obtained from Evandro Chagas Clinical Research Institute, Brazil (IPEC/FIOCRUZ) were used as controls: *C. neoformans* var. *grubii* WM 148 (serotype A, VNI/AFLP1), *C. neoformans* var. *grubii* WM 626 (serotype A, VNII/AFLP1A), *C. neoformans* WM 628 (serotype AD, VNIII/AFLP2), *C. neoformans* var. *neoformans* WM 629 (serotype D, VNIV/AFLP3), *C. gattii* WM 179 (serotype B, VGI/AFLP4), *C. gattii* WM 178 (serotype B, VGII/AFLP6), *C. gattii* WM 175 (serotype B, VGIII/AFLP5), and *C. gattii* WM 779 (serotype C, VGIV/AFLP7) (Meyer et al., 2003).

2.2. Phenotypical identification

Initially the *Cryptococcus* spp. strains were phenotypically identified through manual methods, according to their morphological and biochemical characteristics. Micromorphological characteristics were analyzed on cornmeal-Tween 80 agar (Difco Laboratories, Detroit, USA), after incubation for 48 h at 30 °C, for visualization of blastoconidia, with no hyphae or pseudohyphae (De Hoog et al., 2000). The following biochemical tests were used: (1) urease test, on Christensen's urea agar (Difco Laboratories, England); (2) phenoloxidase activity on birdseed (*Guizotia abyssinica*) agar, supplemented with biphenyl (0.1%); and (3) chemotyping on CGB medium (L-canavanine, glycine, bromothymol blue), through the detection of growth of *C. gattii* (Kwon-Chung et al., 1982; De Hoog et al., 2000). After phenotypical identification, the same strains were also identified through the API 20C AUX semi-automated system (BioMérieux, France) and VITEK 2 automated system (BioMérieux Vitek, Hazelwood, France), according to the manufacturers' instructions.

2.3. Genotypical analysis

2.3.1. DNA extraction

First, *Cryptococcus* spp. strains were grown on potato dextrose agar (Difco Laboratories, Detroit, USA) for 48 h, at 28 °C. Then, a fungal suspension of approximately 2×10^8 cells, which was determined through spectrophotometry (Ultrospec 1100 pro, GE Healthcare Life Sciences, Buckinghamshire, UK), was prepared for each sample in 1.0-mL microtubes containing PBS (phosphate buffered saline). The suspension was vortexed for 15 s and centrifuged twice at $1500 \times g$. Afterwards, approximately 1 mL of 2% Triton X-100 (Sigma Aldrich, Germany) was added to the fungal suspension, which was incubated in a bath at 100 °C for 10 min. After this procedure, the mixture was left at 28 °C for 5 min and centrifuged at $16{,}000 \times g$ for 1 min. The supernatant was collected, transferred to another microtube and frozen at −20 °C. Before performing PCR, each DNA sample was quantified through spec trophotometry (Ultrospec 1100 Pro).

2.3.2. Amplification of CAP59 and restriction enzyme analyses (REA)

The target region chosen for PCR-REA was the *CAP59* gene, which is specific for *C. neoformans* and *C. gattii* and encodes for capsule production (Enache-Angoulvant et al., 2007). PCR was performed in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). The estimated amplicon size was approximately 400 bp. After the amplification reaction, PCR products were submitted to enzymatic digestion with three restriction enzymes, *Age*I, *Bsm*FI and *Hpa*II (New England BioLabs, USA), according to the manufacturer's instructions. The fragments were separated in 6% polyacrylamide gel containing 100 bp molecular weight marker to estimate band sizes and stained with ethidium bromide at 0.25 μL/mL in TBE 1× solution. Electrophoresis was conducted in 1× TBE (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 50 V for 3 h. Restriction fragments were use for *Cryptococcus* identification and serotyping, as described by Enache-Angoulvant et al. (2007). The *BsmFI* enzyme generates fragments of 206 bp and 191 bp in serotype A strains; *AgeI* enzyme generates fragments of 269 bp and 100 bp in serotype B strains; *HpaII* enzyme generates fragments of 270 bp and 101 bp in serotype B strains, 256 bp and 101 bp in serotype C strains and 211 bp and 162 bp in serotype D strains.

2.4. Statistical analysis

Fisher's exact test was applied to analyze the correspondence ($P < 0.05$) among manual identification method, API 20C, VITEK 2 and PCR-REA. The manual method was considered the gold standard (Brito et al., 2009).

3. Results

Through the manual methodology, the majority of the strains were identified as *C. neoformans* ($n = 12$) or *C. gattii*

($n = 12$). A total of six strains were not recognized as belonging to the *C. neoformans* species complex, as they did not produce melanin and showed weak urease activity (Table 1). The phenotypical identification of *Cryptococcus* strains based on morphological and biochemical characteristics took five to seven days.

No significant agreement was observed between the manual identification method and either the API 20C AUX ($P = 0.2720$) or VITEK 2 ($P = 1.0000$) method. When using the API 20C AUX semi-automated method, identification took a maximum of 72 h and the isolates were identified as *C. neoformans* ($n = 20$) and *C. laurentii* ($n = 10$). With the VITEK 2 automated system, the time required for identification was 48 h and the isolates were identified as *C. neoformans* ($n = 24$), *C. albidus* ($n = 2$), *C. laurentii* ($n = 1$) and *Candida parapsilosis* ($n = 3$) (Table 1). Reference strains were identified as *C. neoformans* by API 20C AUX and VITEK 2. It is noteworthy that the API 20C AUX and VITEK methods did not identify any *C. gattii* isolates. In addition, five *C. gattii* strains were misidentified as *C. laurentii* by API 20C AUX.

Significant agreement was only observed when comparing the manual identification method to PCR-REA ($P = 0.0112$). PCR-REA required 35 min for DNA extraction, around 90 min for amplification reaction and 4 h for enzymatic digestion and electrophoresis, for a total of 6.05 h. It was the quickest method. Restriction analysis revealed only two restriction patterns among the 30 tested strains, based on applied enzymes and the resulting band sizes. The enzyme *Bsm*FI cleaved the PCR product of 19 strains into two fragments of approximately 200 bp and 190 bp; no cutting sites were detected after reaction with both *Hpa*II and *Age*I. Taken together this restriction profile was compatible with *C. neoformans* var. *grubii*. Molecular identification of eight strains of *C. gattii* was achieved after digestion of *CAP59* gene by *Age*I and *Hpa*II enzymes into

Table 1
Comparison of the performance of conventional laboratory diagnostic procedures and PCR-REA for identifying *Cryptococcus neoformans* and *C. gattii* from human and veterinary sources.

Strains	Origin	Manual	API 20C AUX	VITEK 2	PCR-REA
Human					
CEMM 05-01-084	CSF	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-072	CSF	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-073	CSF	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
CEMM 03-02-068	CSF	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-077	Sputum	*C. gattii*	*C. laurentii*	*C. neoformans*	*C. gattii*
CEMM 03-02-070	CSF	*C. gattii*	*C. laurentii*	*C. neoformans*	*C. gattii*
CEMM 03-02-062	CSF	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
CEMM 03-02-057	Blood	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-069	Skin	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
CEMM 03-02-071	CSF	*C. gattii*	*C. laurentii*	*C. neoformans*	*C. gattii*
CEMM 03-02-059	Skin	*C. gattii*	*C. laurentii*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 05-01-072	CSF	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
Veterinary					
CEMM 03-02-061	Snot biopsy (cat)	*Cryptococcus* sp.	*C. neoformans*	*C. parapsilosis*	*C. neoformans* var. *grubii*
CEMM 05-01-090	Lymphonode (dog)	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 05-01-076	Crop (cockatiel)	*Cryptococcus* sp.	*C. laurentii*	*C. albidus*	ND
CEMM 05-01-082	Cloaca (cockatiel)	*Cryptococcus* sp.	*C. laurentii*	*C. laurentii*	ND
CEMM 05-01-087	Crop (cockatiel)	*Cryptococcus* sp.	*C. laurentii*	*C. albidus*	ND
Environmental					
CEMM 03-02-064	Pigeon feces	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-063	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-078	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-067	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-060	Pigeon feces	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-076	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-058	Pigeon feces	*Cryptococcus* sp.	*C. laurentii*	*C. parapsilosis*	*C. neoformans* var. *grubii*
CEMM 03-02-066	Pigeon feces	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-079	Pigeon feces	*C. gattii*	*C. laurentii*	*C. neoformans*	*C. gattii*
CEMM 03-02-074	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. gattii*
CEMM 03-02-065	Pigeon feces	*Cryptococcus* sp.	*C. laurentii*	*C. parapsilosis*	*C. neoformans* var. *grubii*
CEMM 03-02-080	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
CEMM 03-02-075	Pigeon feces	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
Controls					
WM 148 (VNI/AFLP1)	[a]	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
WM 626 (VNII/AFLP1A)	[a]	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *grubii*
WM 628 (VNIII/AFLP2)	[a]	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* (hybrid)
WM 629 (VNIV/AFLP3)	[a]	*C. neoformans*	*C. neoformans*	*C. neoformans*	*C. neoformans* var. *neoformans*
WM 179 (VGI/AFLP4)	[a]	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
WM 175 (VGIII/AFLP5)	[a]	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
WM 178 (VGII/AFLP6)	[a]	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*
WM 779 (VGIV/AFLP7)	[a]	*C. gattii*	*C. neoformans*	*C. neoformans*	*C. gattii*

CSF: Cerebral Spinal Fluid.
ND: Not detected by primers designed to the *C. neoformans* species complex.
[a] Reference strains obtained from the Cryptococcal Culture Collection (Laboratório de Micologia, Instituto de Pesquisa Clínica Evandro Chagas, Brazil).

two fragments of approximately 270 bp and 100 bp; amplicon remained unchanged after treatment with *Bsm*FI. Besides the manual identification method, only PCR-REA was able to identify *C. gattii* strains. Strains that did not produce melanin (CEMM 05-01-076, CEMM 05-01-082, CEMM 05-01-087) were not amplified by primers designed for the *C. neoformans* species complex, as shown in Table 1. Strains that exhibited weak urease activity (CEMM 03-02-061, CEMM 03-02-058, CEMM 03-02-065) were identified as *C. neoformans* var. *grubii* by PCR-REA.

The reference strains used as controls showed *CAP59* gene fragments within the expected sizes, after restriction with *BsmFI*, *HpaII*, and *AgeI*. The negative control was not amplified during the tests.

4. Discussion

Identifying species of the *C. neoformans* complex has been routinely performed through morphological, biochemical and physiological characterization of the microorganism. These manual procedures are considered the gold standard for the diagnosis of these species (Feng et al., 2008). However, result variation and subjective interpretation often cause dubious identification (Huston and Mody, 2009). These issues have been overcome by new technologies, such as those based on DNA and RNA tests, as well as the matrix assisted laser induced desorption ionisation (MALDI) connected to the time of flight (TOF) channel (Marklein et al., 2009; McTaggart et al., 2011a,b). Together, both strategies have brought a revolution in identification of microbial pathogens (Marklein et al., 2009). Although MALDI-TOF is a very time-saving diagnostic tool with a strong discriminating power (Marklein et al., 2009; McTaggart et al., 2011a,b), initial acquisition of its apparatus is expensive and so, many laboratories worldwide are still focused on molecular approaches.

Some authors have discussed the role of phenotypical characteristics in identifying the main pathogenic species of the *Cryptococcus* genus (Mandal et al., 2005). Concerning melanin production, it has been shown that most *C. neoformans* and *C. gattii* strains produce brown pigment when grown on culture media enriched with phenolic substrates, something that is rarely observed among non-pathogenic species (Petter et al., 2001). Some studies have demonstrated the possibility of using this resource as a tool to differentiate species of the *C. neoformans* complex from other species of the genus (Cafarchia et al., 2006), while some other studies have shown that *C. albidus*, *C. laurentii* and *C. uniguttulatus* can produce melanin, though at lower levels than those of *C. neoformans* and *C. gattii* (Ikeda et al., 2002). In the present study, three strains did not exhibit pigmentation on birdseed agar, raising the suspicion of other *Cryptococcus* species. Some phenotypical tests, such as melanin production, can help microorganism identification, even though they have low specificity and take additional time.

Phenotypical methods are still the most frequently used to identify *Cryptococcus* spp., especially with CGB medium to differentiate *C. gattii* from *C. neoformans* (Leal et al., 2008). However, there are reports of the existence of *C. neoformans* strains able to resist high concentrations of L-canavanine (Khan et al., 2003). Normally, result interpretation is easy when the strain is *C. gattii*, because the color of the medium intensely changes to cobalt blue. However, when investigating *Cryptococcus* species other than *C. gattii*, CGB test interpretation can be unreliable, requiring greater attention from the laboratory technician. In this study, we failed to manually identify six *Cryptococcus* sp. strains. These isolates presented ambiguous results or did not present any of the pivotal characteristics of the *C. neoformans* species complex, i.e., melanin production, urease activity and absence of filamentation.

Based on microorganisms' ability to assimilate biochemical and enzymatic substrates, API 20C AUX and VITEK 2 have been used for yeast identification (Klein et al., 2009; Meletiadis et al., 2011). However, these techniques have limitations because they frequently depend on supplementary tests for complete identification, such as assimilation of new sugar sources (Gundes et al., 2001). Besides this, interpretation subjectivity may occur for API 20C AUX semi-automated system, even though it is an easily performed technique (Sheppard et al., 1998). Another important issue regarding API 20C AUX and VITEK 2 is that they cannot properly detect *C. gattii* strains, which in this study were misidentified as *C. neoformans*. Such ambiguous identification may have some practical outcome, as these species have clearly different epidemiological characteristics (Kidd et al., 2004), as well as distinct antifungal susceptibility profiles, since *C. gattii* is less susceptible to antifungal drugs (Soares et al., 2008). In spite of these disadvantages, both commercial systems are still frequently used because they can be stored easily, are easy to use and generate results within 48–72 h (Cafarchia et al., 2006; McTaggart et al., 2011a,b; Meletiadis et al., 2011).

We observed that PCR-REA presented the highest agreement with the manual method. Additionally, through this technique it was possible simultaneously to determine varieties and serotypes of *C. neoformans* and *C. gattii* strains. Among the various techniques of DNA manipulation and analysis, PCR has provided the greatest advances in the diagnosis of cryptococcosis and precise identification of the agent (Wengenack and Binnicker, 2009), as it has satisfactory sensitivity and specificity to detect a minimum load of the fungus (Soares et al., 2008). Protocols with restriction enzymes are frequently employed to identify these species (Frasés et al., 2009). During past years, several studies based on molecular approaches have enabled the identification of serotypes and molecular types of the *Cryptococcus* species complex (Bovers et al., 2007; Enache-Angoulvant et al., 2007; Ito-Kuwa et al., 2007; Feng et al., 2008; Leal et al., 2008). Although it is not possible to assume each would be the best method, we decided to test the protocol described by Enache-Angoulvant et al. (2007) because it has an easy-to-use approach and seems suitable for routine laboratory identification.

In conclusion, PCR-REA showed a greater level of concordance with the manual method, besides being faster and more sensitive than the other methods, therefore being indicated for routine identification of species of the *C. neoformans* complex.

Conflict of interest statement

The authors declare that they have no conflicting interests in relation to this work.

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