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Note

Single-tube classical PCR for *Candida auris* and *Candida haemulonii* identification

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ABSTRACT

Background: *Candida auris* and *Candida haemulonii* are emerging and multiresistant pathogens. *C. auris* has produced hospital outbreaks and is misidentified by phenotypic-based methods. The only reliable identification methods are DNA sequencing and MALDI-TOF.
Aims: To develop a classical-PCR method capable of rapidly and accurately identify *C. auris* and *C. haemulonii*.
Methods: A multiplex PCR was carried out in one tube that included an internal control and oligonucleotides that specifically hybridize to the ITS2 region of *C. auris* and *C. haemulonii*. The usefulness of the new method was verified by testing a collection of 50 strains of 20 different species (previously identified by ITS sequencing). The selection of species was made in order to emulate the *C. auris* panel used by the CDC to validate diagnostic tools. In addition, other yeast species not included in the aforementioned panel were incorporated based on reported identification errors.
Results: The results obtained with the proposed protocol were in total agreement with those obtained by ITS sequencing.
Conclusions: We present a PCR method able to unequivocally identify *C. auris* and differentiate it from *C. haemulonii*. It is inexpensive, fast and it could be a useful tool to reduce the chances of a *C. auris* outbreak.

PCR múltiple para la identificación de *Candida auris* y *Candida haemulonii*

Palabras clave:
Candida auris
Identificación molecular
Candida haemulonii

RESUMEN

Antecedentes: *Candida auris* y *Candida haemulonii* son patógenos emergentes y multirresistentes. *C. auris* ha sido responsable de brotes hospitalarios y no se puede identificar por métodos fenotípicos. Los únicos métodos de identificación confiables incluyen la secuenciación y el MALDI-TOF.
Objetivos: Desarrollar un método de PCR clásica capaz de identificar rápidamente *C. auris* y *C. haemulonii*.

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Métodos: Se llevó a cabo una PCR múltiple en un tubo que incluyó un control interno y oligonucleótidos que hibridan específicamente con la región ITS2 de *C. auris* y *C. haemulonii*. Para comprobar la utilidad del método se utilizó una colección de 50 aislamientos de 20 especies diferentes (identificadas por secuenciación del ITS). La selección de especies se hizo con el fin de emular el panel de especies que ofrece el CDC para la correcta identificación de *C. auris*. Además, se incluyeron especies que son confundidas con *C. auris* y no están incluidas en el citado panel.
Resultados: Los resultados obtenidos con el protocolo propuesto estuvieron en total acuerdo con los obtenidos por la secuenciación del ITS.
Conclusiones: El método que presentamos es capaz de identificar inequívocamente *C. auris* y diferenciarla de *C. haemulonii*. Es barato, rápido y podría ser una herramienta útil para reducir la posibilidad de brotes por *C. auris*.

Candida auris and *Candida haemulonii* are closely related multiresistant yeast pathogens that are increasingly reported worldwide.[1,6–8,10,11,13] *C. auris* has become an important cause of nosocomial outbreaks. The CDC recommends all infected or colonized patients are isolated from the rest of the hospital population.[6,7,9,11,14] In order to comply with these recommendations, the correct identification of this species in clinical laboratories is essential. Phenotypic-based commercial identification systems misidentify *C. auris*. The two unique recommended methods for *C. auris* accurate identification are rDNA sequencing and MALDI-TOF.[5,9] Nevertheless, these two methods have limitations regarding speed, availability, database update and cost. The objective of this work is to propose an inexpensive single-tube classical PCR method able to quickly identify *C. auris* and differentiate it from *C. haemulonii*.

A multiplex PCR tube was assembled with four primers. It contained the ITS universal primers (ITS1 [5′-TCCGTAGGTG AACCTGCGG-3′] and ITS4 [5′-TCCTCCGCTTATTGATATGC-3′]) that were used as internal controls.[15] The remaining primers were designed to specifically hybridize the ITS2 region of *C. auris* and *C. haemulonii*. These specific primers were named as: *C. auris* (5′-CCACCGCGAAGATTGGTG-3′; orientation: antisense) and *C. haemulonii* (5′-CCGTTGGTGGATTTGTTTCT-3′; orientation: sense) (Fig. 1). The primer design was based on the following GeneBank accession numbers: HE797772.1 (*C. auris* ITS), JX459664.1 (*C. haemulonii*), JX459687.1 (*C. haemulonii* var. *vulnera*), EU881972.1 (*Candida pseudohaemulonii*) and JX459667.1 (*Candida duobushaemulonii*). ITS sequences of the species of the *C. haemulonii* complex were included in the PCR design to ensure the specificity of the primers. Oligonucleotides were purchased from GBT primers (Genbiotech-Argentina). PCR reactions were set in a 25 μl final volume following the Pegasus DNA polymerase manufacturer's instructions (PBL-Argentina). Each PCR tube contained 1 unit of Pegasus *Taq* polymerase, 50 ng of yeast DNA, 1 × PCR buffer, 250 μM dNTPs, 2 mM $MgCl_2$, 0.1 μM of ITS4, 0.2 μM of ITS1 primer and 0.1 μM of *C. auris* and *C. haemulonii* specific primers. An Applied Biosystems thermocycler was used for DNA amplification using the following program: 2 min 95 °C (initial step) followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, and a final step of 10 min at 72 °C. PCR amplification products were resolved by electrophoresis using a 1.2% agarose gel.

The usefulness of the new method was evaluated by testing a panel of 50 strains of 20 different species specifically assembled for this work. The species were chosen taking into account species that are indistinguishable from *C. auris* if the currently available methods are used.[4,9] Many of these species are included in a CDC panel distributed to be used in the validation of diagnostic tools.[3] All the strains of the panel were identified by ITS sequencing[15] and were obtained from international collections (e.g. ATCC or CBS) or clinical samples. Our panel included 20 *C. auris*, 5 *C. haemulonii*, 1 *C. pseudohaemulonii*, 1 *C. duobushaemulonii*, 3 *Candida guilliermondii*, 2 *Candida lusitaniae*, 2 *Saccharomyces cerevisiae*, 1 *Rhodotorula glutinis*, 1 *Candida sake*, 1 *Candida albicans*, 1 *Candida glabrata*, 2 *Candida parapsilosis sensu stricto*, 2 *Candida krusei*, 2 *Candida tropicalis*, 1 *Cryptococcus neoformans*, 1 *Candida orthopsilosis*, 1 *Candida nivariensis*, 1 *Candida dubliniensis*, 1 *Candida kefyr* and 1 *Candida famata*. Yeast genomic DNAs were obtained using a phenol-based method.[12]

When *C. auris* or *C. haemulonii* DNA was used, the PCR amplification yielded two bands: a 401 nt ITS internal control band together with a 281 nt or a 118 nt species specific band, respectively. PCR products of different sizes showing the amplification of the ITS internal controls (using ITS1 and ITS4 primers) were obtained when DNA from other species were used (Fig. 2). In this study, we evaluated 50 strains and the results obtained with the proposed single tube PCR protocol were in total agreement with those obtained by ITS sequencing.

Since the first *C. auris* isolation, rDNA ITS region sequencing has been used as the unique unequivocal identification tool.[5,9,13] Later, the introduction of the MALTI-TOF technology in clinical labs added a new identification option.[4] In 2016, the CDC suggested to molecularly characterize or identify by means of MALDI-TOF any clinically resistant yeast and/or any yeast identified as (i) *Candida sake* or *Rhodotorula glutinis* (with non-red colony) by using API20C, (ii) *Candida catenulata* by DB Phoenix and (iii) *C. catenulata*, *C. guilliermondii*, *C. lusitaniae* or *Candida famata* when using MicroScan.[4,9]

Herein we present a fast and inexpensive PCR method able to unequivocally identify the multiresistant species *C. auris* and *C. haemulonii*. As any other method of molecular identification, a possible drawback is the misidentification due to point mutations in the region where the primers hybridize. However, the ITS regions of all the *C. auris* and *C. haemulonii* strains included in this work were sequenced and showed exactly the same nt sequences as the genebank sequences used for the primers design. It must be noted that all the strains used in this work were isolated in different hospitals of Colombia. Geographic clonality was described for *C. auris* strains.[2,9] Thus, these methodology should be tested using *C. auris* from other world's regions to ensure that all the *C. auris* share the exact ITS sequence. Other limitation of this PCR technique is that it is not able to differentiate *C. haemulonii* from *C. haemulonii* var. *vulnera* (a variety of *C. haemulonii* group I).[2]

The proposed PCR would be a useful tool for supporting the decision to isolate a patient, reducing the possibility of *C. auris* outbreaks.

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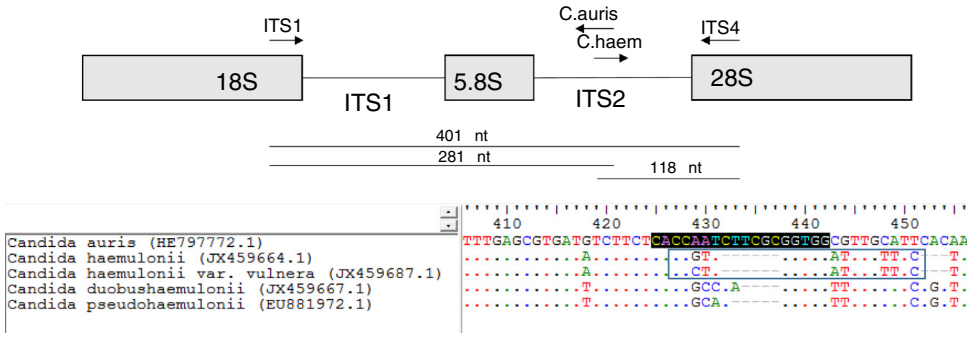

Fig. 1. Representation of fungal nuclear ribosomal DNA (rDNA). Arrows represent the primers used in this work. Lines show the expected PCR band sizes.

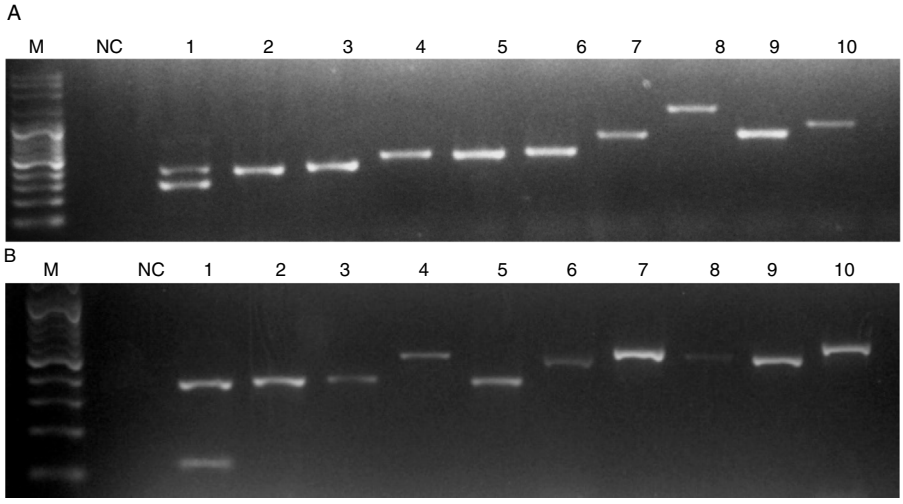

Fig. 2. Electrophoresis of the PCRs resolved in a 1.2% agarose gel. M, molecular size marker. NC, negative control. (A) Lanes: 1, *C. auris*; 2, *C. pseudohaemulonii*; 3, *C. sake*; 4, *Cryptococcus neoformans*; 5, *C. krusei*; 6, *C. dubliniensis*; 7, *Rhodotorula glutinis*; 8, *S. cerevisiae*; 9, *C. famata*; 10, *C. guilliermondii*. (B) Lanes: 1, *C. haemulonii*; 2, *C. pseudohaemulonii*; 3, *C. duobushaemulonii*; 4, *C. orthopsilosis*; 5, *C. lusitaniae*; 6, *C. albicans*; 7, *C. neoformans*; 8, *C. parapsilosis*; 9, *C. tropicalis*; 10, *C. nivariensis*.

Conflict of interest

None declared.

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