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Comparative identification of *Candida* species isolated from animals using phenotypic and PCR-RFLP methods

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Abstract

The aim of this study was to identify 58 *Candida* sp. strains isolated from animals using the Chromatic Candida test, the API 20 C AUX system, and polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP). The Chromatic Candida test was able to identify only *C. albicans* and *C. krusei*. The API 20 C AUX system and PCR-RFLP had similar specificity for the identification of *Candida* strains. In case of both methods, *Candida albicans* was the most frequently isolated species - 22 (37.93%) strains, followed by *Candida krusei* - 17 (29.31%) strains, *Candida famata* - 10 (17.24%) strains, *Candida parapsilosis* - five (8.62%) strains, and *Candida kefyr* - four (6.89%) strains. PCR-RFLP represents a reliable, quick and relatively inexpensive genotyping method, recommended for rapid identification of *Candida* spp.

Key words: *Candida*, identification, PCR-RFLP, Chromatic Candida, test API 20 C AUX system, Romania.

Introduction

The genus *Candida* includes over 50 species of yeasts, many of which are associated with infections in humans and animals. *Candida albicans*, the only phospholipase-producing species (4, 11), is worldwide the most frequently isolated from both humans and animals. However, the past decades have demonstrated an increase in the number of non-albicans species – *Candida krusei, Candida parapsilosis, Candida famata, Candida kefyr*, etc. (19, 22). Moreover, such non-albicans species are less susceptible to azole derivatives (2, 14).

Rapid and precise identification of both opportunistic and pathogenic *Candida* genus/species isolated from animals becomes very important because antifungal therapy needs to be specifically targeted for a species, whilst the resistance to antifungal drugs should also be considered (13, 16).

The diverse methods available for *Candida* identification, starting with the assimilation tests, usually require 2-4 d to complete. The procedure initially involves microscopic and macroscopic examination of 24-48 h culture using classic microbiological tests. The next step is the determination of biochemical characteristics using a chromogenic medium such as Chromatic Candida culture medium (6, 10), or a commercial test such as API 20 C AUX (8, 15).

Molecular methods, such as PCR, have developed apace in the past decades to become a modern and reliable alternative to the phenotypic systems (1, 12, 15). Among the diversity of molecular methods, restriction fragment length polymorphism (RFLP) recommends itself to the present study because it relies on differences in homologous DNA sequences. The DNA sequence is digested by a restriction enzyme and the resulting fragments are separated by gel

electrophoresis (20). PCR-RFLP is widely used for the identification of bacteria (3) and viruses (7).

The aim of this study was to perform a comparative identification of *Candida* species isolated from animals from the Northern Romania using phenotypic and PCR-RFLP methods.

Material and Methods

Candida spp. isolates. Between July 2010 and February 2012, 58 *Candida* spp. strains were isolated from different sources, such as: milk from cows with mastitis, ear secretions and pharyngeal exudates from dogs, and faeces from parrots, pigeons, and chickens.

All the tests were performed within the facilities of the Faculty of Veterinary Medicine, in Cluj-Napoca, Romania. Initial identification of the genus *Candida* was performed using classical microbiological methods. The strains were cultured on Sabouraud's dextrose agar (SDA) at 32°C for 48 h and then identified by microscopic examination. Afterwards, the isolates were subcultured on Chromatic Candida (Liofilchem, Italy) and incubated at 35°C for 48 h to observe the growth and colour of the colonies. The same strains were also tested using the API 20 C AUX system (bioMérieux, France), according to the producer's specifications.

Candida isolates and additionally six standard ATCC strains (*C. albicans* 90028, *C. albicans* 10231, *C. krusei* 14243, *C. famata* 62894, *C. parapsilosis* 22019, and *C. kefyr* 66028) were evaluated for their genetic profile using PCR-RFLP.

PCR conditions. For PCR analysis, genomic DNA was extracted by Isolate DNA Kit (Bioline, USA) according to the manufacturer's instructions (DNA isolation from cell culture). The amplification was performed targeting the internal transcribed spacer (ITS) regions ITS1-ITS4 (12) as follows: 25 µL of reaction mixture contained: 4 µL aliquot of isolated DNA; 12.5 µL of PCR buffer (MyTaq Red Mix, Bioline, USA), and 1 µL of each primer: ITS1 (forward 5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (reverse 5'-TCCTCCGCTTATTGATATGC-3'). PCR was carried out in a T100 Thermal Cycler (Bio-Rad, USA) and the amplification profile consisted of one cycle of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with one cycle of final-extension at 72°C for 5 min. Each time PCR was performed including negative and positive control samples.

RFLP analysis. Ten microlitres of PCR amplification products were subjected to RFLP analysis by digestion with 2200 U of restriction enzyme *Bsi*S I (Jena Bioscience, Germany), which was carried out according to the manufacturer's instructions. The enzyme *BsiS* I is an isoschizomer, which recognises the same target sequence as enzymes *Hap* II, *Hpa* II, and *Msp* I, previously used by Mirhendi *et al.* (12) and Shokohi *et al.* (18). The digested fragments were visualised in a 4% agarose gel (1xTAE, pH 8.0) following staining with Midori Green Advanced DNA Stain (Nippon Genetics Europe, Germany).

Results

The Chromatic Candida test was able to distinguish only two species: *C. albicans* (22 strains) which were green coloured, and *C. krusei* (17 strains) which were pink coloured. The remaining 19 strains were white-pink colonies, represented by other *Candida* species amalgamated into a single category.

The accuracy of API 20 C AUX was much higher compared to the Chromatic Candida test. *C. albicans* was most frequently isolated (37.93%), followed by *C. krusei, C. famata, C. parapsilosis*, and *C. kefyr*.

The universal primers ITS1 and ITS4 were used for the amplification of the ITS region, in order to distinguish between the five *Candida* species isolated in this study. For 58 samples and six positive controls, the universal primer pairs enabled a successful amplification of the ITS region. The PCR revealed a unique product of about 450-900 bp for each species.

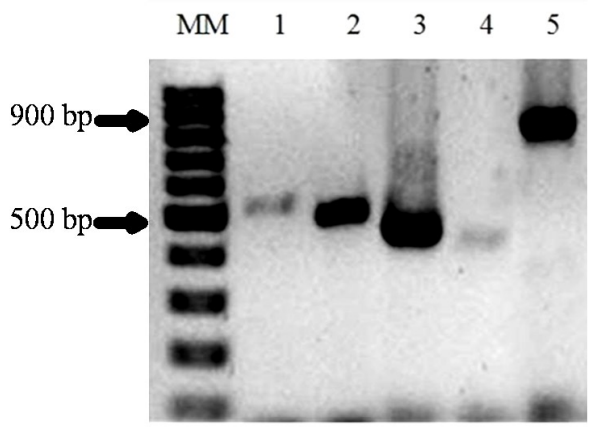

Fig. 1. Amplification profiles of the PCR products targeting the ITS1-ITS4 region. MM – Molecular Marker (100 bp, Thermo Fisher Scientific, formerly Fermentas, Lithuania); 1 - *C. albicans* (535 bp), 2 - *C. parapsilosis* (520 bp), 3 - *C. krusei* (510 bp), 4 - *C. famata* (490 bp), 5 - *C. kefyr* (840 bp)

In case of RFLP, *BsiS* I enzyme cleaves DNA at the level of a 5'...C▼CGG...3', 3'...GGC▼C...5' sequence. For *Candida* spp., the digestion with *BsiS* I enzyme produced two bands for *C. albicans* and *C. krusei,* while for *C. famata* and *C. kefyr* only one band was observed. In case of *C. parapsilosis*, the digestion and PCR products had the same size, since *Msp* I is not able to recognise the site of the ITS region.

The PCR-RFLP technique showed high specificity, so that the identified strains had similar patterns to standard strains.

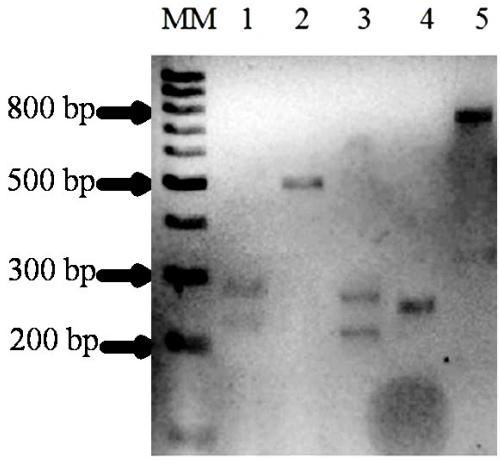

Fig. 2. RFLP pattern of the amplified ITS1-ITS4 region digested with *BsiS* I (*Msp* I). MM – Molecular Marker (100 bp, Thermo Fisher Scientific, formerly Fermentas, Lithuania); 1 - *C. albicans,* 2 - *C. parapsilosis,* 3 - *C. krusei,* 4 - *C. famata,* 5 - *C. kefyr*

The number and percentage of strains Candida spp. identified in northern Romania using the PCR-RFLP technique, were identical to those identified with the API 20 C AUX technique. Twenty-two strains (37.93%) were identified as *C. albicans*, 17 (29.31%) strains as *C. krusei*, 10 (17.24%) strains as *C. famata*, five (8.62%) strains as *C. parapsilosis*, and four (6.89%) strains as *C. kefyr*.

Discussion

Rapid, easy, and inexpensive identification of *Candida* sp. represents an important goal for any respectable microbiological laboratory (9, 12). Our study presents the results of a comparison between two phenotypic methods (Chromatic Candida and API 20 C AUX) and a PCR-RFLP analysis used for the identification of 58 *Candida* strains in northern Romania. The Chromatic Candida test is designed for the isolation and identification of *C. albicans, C. tropicalis*, and *C. krusei*. Other *Candida* species appear white-pink coloured in this medium. *C. famata, C. parapsilosis*, and *C. kefyr* species were not identified. Our results are in agreement with previous findings (6, 10).

The API 20 C AUX test is able to distinguish between all species of *Candida* except *Candida dubliniensis*, and is a more time-consuming but lower-cost alternative method to genotypic identification (17, 21). Nowadays, it is considered as the reference method among phenotypic tests (5), but requires experience at an appropriate working technique and interpretation of results. Our study confirmed that the API is a reliable system; all *Candida* isolates and the reference strains were successfully identified in a similar manner to the PCR-RFLP test.

Regarding the PCR method, the universal primers ITS1 and ITS4 enabled a successful amplification of a fragment from the genomic DNA of the studied strains. Several methods, such as multiplex PCR, nested PCR, DNA probes, and PCR-RFLP have previously been used for the study of *Candida*. With regard to the RFLP method, Williams *et al.* (20) initially used three enzymes for the digestion (*Hae* III, *Dde* I, and *Bfa* I). However, the major advantage of using only one enzyme (*Msp* I) was first demonstrated by Mirhendi *et al.* (12). As in our study, *BsiS* I was used for the digestion of the ITS amplified products. This resulted in a specific pattern for each species. However, there was an exception; in the case of *C. parapsilosis*, the enzyme was not able to recognise the site of the ITS region. The results obtained using the PCR-RFLP technique were identical for standard strains and *Candida* isolates. Furthermore, the results of PCR-RFLP were similar to those obtained using API 20 C AUX, and in agreement with the results obtained by Mirhendi *et al.* (12).

Although molecular methods require trained personnel and specific molecular biology equipment, we recommend the genotypic method for the identification of *Candida* spp. strains by virtue of the similar sensitivity of PCR-RFLP and the API system. The reduced time is the most important argument required for identification, which is important when clinical specimens are to be examined.

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