AgroLife Scientific Journal - Volume 8, Number 1, 2019
ISSN 2285-5718; ISSN CD-ROM 2285-5726; ISSN ONLINE 2286-0126; ISSN-L 2285-5718

Candida INFECTION IN HUMANS AND ANIMALS

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Abstract

In recent years, both in the field of veterinary and human medicine, there has been an increase in the incidence of systemic mycosis infections, in particular, caused by Candida species. The mycoses of the genus Candida are opportunistic pathogens and can colonize the mucosa of the gastrointestinal tract, mouth, esophagus and vagina; causing infections of the mucous membranes and of the deep tissues. These fungal pathogens cause candidiasis in both humans and animals, in fact Candida is the main cause of candidiasis in immunocompromised patients. The most isolated species humans and animals are represented by, Candida albicans for a 70-75% of all cases of candidiasis. In recent decades however seems that also non-albicans Candida (NAC) infections increase significantly with an average, in both species, of 30-60% cases with candidaemia. This is rare disease. A fundamental aspect is the rapid and accurate identification of Candida sp., with increasingly advanced methods, to allow establishing an effective antifungal therapy and reducing the mycosis disease rates.

Key words*: Candida spp., candidiasis, identification.*

INTRODUCTION

The term *Candida* comes from the latincandido which means white (Williams et al., 2000). The genus *Candida* belongs to the kingdom of the Fungi (Neppelenbroek et al., 2014) and in particular is part of the phylum of Deuteromycota.

The genus *Candida* includes about 196-200 species (www.antimicrobe.org). It has been observed that *Candida* species are the most common causes of opportunistic mycoses found worldwide (Sardi et al., 2013). In fact, in recent years there has been an increase in infections caused by *Candida* species (Williams et al., 2000), in particular from *Candida albicans* because it is the most common species (www.antimicrobe.org; Ogata et al., 2015) although in recently there has been an increase in infections of non-*albicans Candida* species. Indeed, has been observed that *C. parapsilosis, C. tropicalis* are the most yeast strains isolated in the clinical setting (Deorukhkar et al., 2014; Sardi et al., 2013; Cordeiro et al., 2017).

Candida species can colonize different parts of the body such as the skin, mucous membranes, the oropharyngeal tract as mouth and esophagus, the gastrointestinal tract and the genital apparatus (Sardi et al., 2013). *Candida* lives as a commensal belonging to the human and animal microbiota (Cordeiro et al., 2017).

These microorganisms can become potential pathogenic agent when the mechanisms of host defense or anatomical barriers are compromised causing different types of candidiasis (Cordeiro et al., 2017). Invasive candidiasis has been widely studied to immunecompromised patients (Delaoye and Calandra, 2014; Sardi et al., 2013; Sachin et al., 2014), representing 70-90% of all fungal infections in hospitals (Delaoye and Calandra, 2014). In animals, instead, the rate of colonization varies based on animal species and breeding conditions, although the gastrointestinal tract is the most colonized. Therefore, *Candida* species are also important in veterinary medicine because can infect many animal species such as horses, pigs, sheep, birds, cats and dogs (Cordeiro et al., 2017; Edelmann et al., 2005). The pathogenicity of *Candida* is facilitated by virulence factors such as the secretion of hydrolytic enzymes and the formation of biofilms. The hydrolytic enzymes most important in the virulence of the *Candida* species are proteases, phospholipases,

hemolysins and extracellular lipases fundamental in the colonization and progression of infection of the host tissue (Sardi et al., 2013; Deorukhkar et al., 2014; Rodrigues et al., 2003).

Proteases degrade epithelial barrier and mucosa barrier proteins such as collagen, keratin and mucin. Moreover, the proteases help *Candida* to resist cellular and humoral immunity by degrading antibodies, complement and cytokines (Deorukhkar et al., 2014). Phospholipases are found in the ends of the hyphae. Hydrolyze one or more ester bonds in the glycerophospholipids of the host tissues causing the rupture of cell membranes and allowing the fungal cell to penetrate into the cytoplasm (Deorukhkar et al., 2014; Arantes et al., 2016).

Hemolysins are proteins produced by microorganisms to destroy red blood cells (Sardi et al., 2013). The haemolytic capacity allows fungi of the genus *Candida* to acquire iron from the host tissues, which is then used by the fungus for metabolism, growth and invasion during host infection (Rossoni et al., 2013).

Extracellular lipases have putative roles which consist of the digestion of lipids for nutrient acquisition, adhesion to host cells and tissues (Sardi et al., 2013).

In addition to these enzymes, another factor that can induce pathogenicity is biofilm. Biofilms are biological communities and can grow on any wet surface (Sardi et al., 2013). The knowledge of these virulence factors is fundamental to understand the pathogenesis of candidiasis (Deorukhkar et al., 2014).

Today, the molecular techniques are routinely used as rapid, reliable, easy to perform methods in the identification of yeasts that are clinically isolated (Mohammadi et al., 2013).

Furthermore, molecular methods can lead to the administration of an adequate specific antifungal treatment of candidiasis (Khlif et al., 2009).

The aim of this work is report the epidemiological identification and distribution of different cases of candidiasis diagnosed in humans and domestic animals with spontaneous disease in a scientific collaboration work between University of Agronomic Sciences and Veterinary Medicine of Bucharest and University of Perugia in the last years.

MATERIALS AND METHODS

Biological samples

Three samples were provided from a clinical microbiology laboratory of a human hospital, obtained from human blood cultures that presented yeast fungal disease, while another sample was provided by the veterinary hospital. This last sample it was examined at histopathological level with specific histochemical staining. The sample taken was a skin biopsy of a male cat's ear canal. For this reason, the four samples follow different procedures.

Isolation and identification of fungal strains

The samples were obtained from human blood cultures and re-isolated on Sabouraud agar medium (Acumedia, USA) containing 0.02% chloramphenicol and incubated at 30°C for 24-48 hours. After that a single colony is taken from each distinct isolate and inoculated into the respective liquid media to obtain the biomass. Three fungal strains have been isolated. These isolates were first identified bymicroscopic and macroscopic observations. *C. albicans* ATCC 10231, *C. parapsilosis* ATCC20019 and two clinically *Candida strains such* as *C. guilliermondii* MI 40 and *C. krusei* MI 41 were used as control strains where required.

Molecular identification

The extraction of fungal DNA was performed using the E.Z.N.A.® FUNGAL DNA KIT, (Omega Bio-Tek, USA).

The molecular identification of the fungal isolates was performed by the PCR-RFLP method. 5.8S-ITS region has been amplified using two universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

The PCR mixture contained 5 µL of 10x reaction buffer, 1.5 mM of $MgCl_2$, 0.2 mMdNTP, 2.5 U of DNA Taq polymerase, 0.5 µmol of each primer and 10-30 ng of fungal DNA extracted in a final volume of 50 µL. The PCR conditions were follows: initial denaturation at 94°C for 5 minutes, followed by

35 denaturation cycles at 94°C for 1 minute, seconds, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, with a final extension phase at 72°C for 10 minutes.
Then, the amplicons were subjected to enzymatic digestion. An aliquot of 10 µL of each ITS amplicon was digested with the restriction enzyme *Msp*I (Thermo Fisher Scientific, USA).
Afterwards, the PCR amplicons and restriction fragments were fractionated by 2% agarose gel electrophoresis and observed at the UV transilluminator. All fragment sizes were approximated using the DNA ladder (GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific, USA).

Haemolytic activity of fungal isolates
Fungal isolates were streaked on the surface of Columbia blood agar plates (Oxoid) supplemented with sheep's blood and incubated at 30°C. After 48 h of incubation, the blood agarplates were examined for haemolytic reaction.

Histopathological examination of animal samples
A skin biopsy from a cat suspected of having a fungal infection was detected at University of the Department of Veterinary Medicine of Perugia (DMV-UNIPG). The sample was fixed in neutron buffered 10% formalin and embedded in paraffin for histopathological investigations.
The sample was dehydrated by effecting an increasing series of alcohol till absolute alcohol and then dipped in xylene. Then, the sample was included in paraffin to obtain the paraffin block containing the tissue sample to be analyzed.
Subsequently the block was cut to the microtome to create sections of 3-5 µm, position them on the appropriate slides. Finally, 4 types of colors have been made which are hematossilin-eosin (H&E), PAS, Grocott and Mucicarminio.
These colors, observed by pathologist to identify the fungal genus and to make a correct and precise diagnosis.

RESULTS AND DISCUSSIONS

Identification of fungal isolates
Three fungal strains (denominate C1, C2 and C3) supposed to belong to *Candida* species were isolated and characterized using standard conventional methods (macroscopic and microscopic observations). The C1 isolate showed colonies that had a cream color conducive to yellow and the cells were short-ellipsoidal, while the C2 and C3 isolates had white circular colonies and the cells were spherical-ovoid.
Subsequently, a molecular analysis such as RFLP have been carried out to identify different fungi using universal primers. PCR-RFLP of 5.8S-ITS region using the restriction enzyme *Msp*I allowed to identify the yeast strainsat specie level, according to technique previously described by Mohammadi et al., 2013.The size of allamplified PCR products was estimated beetwen 540-600 bp (Figure 1 and Table 1).

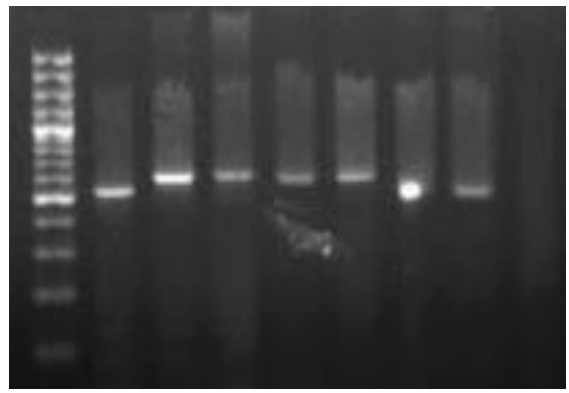

Figure 1. PCR products with primers ITS1 and ITS4;
1 - *C. albicans* ATCC 10231 (reference strain);
2 – *C. parapsilosis* ATCC 20019 (reference strain);
3- *C. guilliermondii* MI 40 (reference strain);
4 - *C. krusei* MI 41 (reference strain); 5 - C1 isolate;
6 - C2 isolate; 7- C3 isolate; C-negative control
L– GeneRuler 100bp Plus DNA Ladder

In this study, after the digestion of the ITS products with *Msp*I were visualized a single band (250 bp) for the isolates C2 and C3, a single band (520 bp) for *C. parapsilosis* ATCC 20019 (referance strain); two-band patterns (240 bp +300 bp) for *C. albicans* ATCC 10231 (referance strain); three-band patterns (90 bp + 150 bp+ 380 bp) for *C. guilliermondii* MI 40

(reference strain), *C. krusei* MI 41 (reference strain) and isolate C1 (Figure 2 and Table 1).

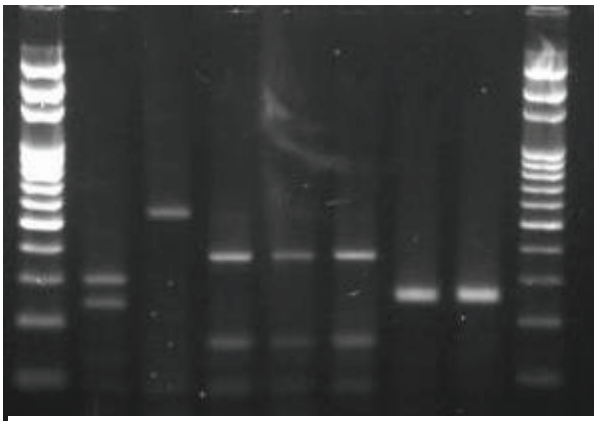

Figure 2. Restriction fragments of ribosomal DNA for fungal strains after digestion with *Msp*I:
1 - *C. albicans* ATCC 10231 (reference strain);
2 - *C. parapsilosis* ATCC 20019 (reference strain);
3 - *C. guilliermondii* MI 40; 4 - *C. krusei* MI 41;
5 - C1 isolate; 6 - C2 isolate; 7- C3 isolate;
L– GeneRuler 100bp Plus DNA Ladder

Table 1. RFLP analysis of 5.8S-ITS region with *Msp*I

Candida isolates	PCR size(pb)	Restriction fragments (pb)
C. albicans ATCC 10231	540	240+300
C. parapsilosis ATCC 20019	600	520
C. guilliermondii MI 40	600	90+150+380
C. krusei MI 41	600	90+150+380
C1	600	90+150+380
C2	540	250
C3	540	250

The isolates C1 and *C. krusei* MI 41 presented RFLP type appropriate to *C. guilliermondii,* according to Mohammadi et al. (2013). Initial, the reference strain *C. Krusei* MI 41 have been mis-identified as *C. krusei.* By digestion of the ITS region with *Msp*I, *C. krusei* MI 41 have been reidentified as *C. guilliermondii* MI 41.
The isolates C2 and C3 isolates gave RFLP type appropriate to *C. krusei,* according to Mohammadi et al. (2013).

Haemolytic activity

Haemolytic activity was tested because among non-*albicans Candida* species, 86% showed haemolytic activity (Rossoni et al., 2013).
In the present study, we performed the haemolysis test to evaluate the production of hemolysin, an important virulence factor for *Candida* yeast. This enzyme degrades host erythrocytes to release iron for use in the growth and metabolism of these fungi in systemic infections (Rossoni et al., 2013; Pakshir et al., 2013).
Finally, we wanted to observe their hemolytic capacity, so the hemolysis test was done of all five inoculated *Candida* strains, including the two reference strains. From this test it is deduced that all 7 *Candida* species have not hemolytic activity observed by the lack of halo around the smears made on the specific culture medium.

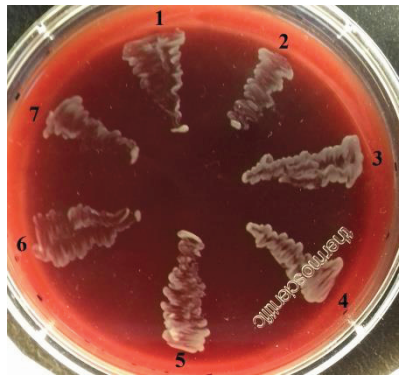

Figure 3. Determination of haemolytic activity of fungal strains:
1 - *C. albicans* ATCC 10231 (reference strain);
2 -*C. parapsilosis* ATCC 20019 (reference strain);
3- *C. guilliermondii* MI 40 (reference strain);
4 - *C. krusei* MI 41 (reference strain); 5 - C1 isolate;
6 - C2 isolate; 7- C3 isolate

Histopathological investigation of animal samples

In addition, a different process was used for the animal samples. In fact, for the animal samples a histopathological investigation was performed. This is an effective method to provide a definitive diagnosis of a fungal infection. The histopathological examination of the samples is very important to define the invasion of tissues and vessels (Guarner et al., 2011).
In our study to identify present of fungal we made some specific staining that allow to detect *Candida*. These staining are represented by Hematoxylin-Eosine (H&E), PAS (periodic acid-Schiff), Grocott and Mucicarmine. Hematoxylin-Eosin is a routine staining method that allows the pathologist to evaluate the presence of fungi (Figure 4A), but it is not sufficient to make a correct diagnosis. For this reason, if a fungal infection is suspected, it is also advisable to do the above-mentioned

histochemical staining, because when the fungi are scattered there is the probability that are neglected in the observation of the sample. Morphological evaluation can be difficult, when histoplasmas and blastomyces present cytoplasmic artifacts (Palliola et al., 1979). In this regard, in addition to the routine staining, were effectuated the other staining to identify *Candida*.

PAS and Grocott allow rapid visualization of *Candida* in organ sections (Palliola et al., 1979). In the histological sample, the characteristic morphological structures of *Candida* appear to be colored in a different way because with PAS take on a purplish red color (Figure 4B) while with Grocott take on a brown-black color (Figure 4 C, D). Beyond the specific color given by the coloring, in both, the pathologist can distinguish different fungal elements as the hyphae, pseudohyphae and blastoconidia (Figure 4). In literature *Candida* can be found in superficial epithelium such as hyphae, pseudohyphae or blastoconids and from data observed in literature and these structures are often composed by oval to round, 3-6 μm diameter, pale-stained, thin-walled blastospores and blastoconidia arranged in short chains (pseudohyphae), and slender, 3-4 μm wide, septate, parallel-walled hyphae (Kumar et al., 2009).

In our study, both PAS and Grocott staining confirmed the initial hypothesis that it was *Candida* demonstrated by a positive result, in which all the *Candida* specific morphological structures can be observed. In addition, can be observed that the tissue is covered by a stratified epithelium that rests on a lamina propria expanded by a diffuse infiltration of macrophages, neutrophils, lymphocytes and plasma cells. Moreover, within the cytoplasm of macrophages, are evident many rounds yeast-like bodies of variable size with bistratified reflecting wall and a not visible cytoplasm, associated with the pseudohyphae confirmed by PAS and Grocott.

In addition to the two staining mentioned, which resulted positive for Candida, the Mucicarmine staining was also made. In cases where was made the Mucircamine staining, were not found hyphae, pseudohyphae and blastoconidia. In our case, the two stainings that allow us to detect and make a correct diagnosis are the PAS and the Grocott where there is a better visualization of the hyphae, pseudohyphae or bastoconidia.

Substantially our results have been confirmed of the data found in the literature, which support and confirm our initial hypothesis. Finally, the histopathological examen must be supported by other investigations such as molecular or microbiological techniques such as cultures for the identification of *Candida* species.

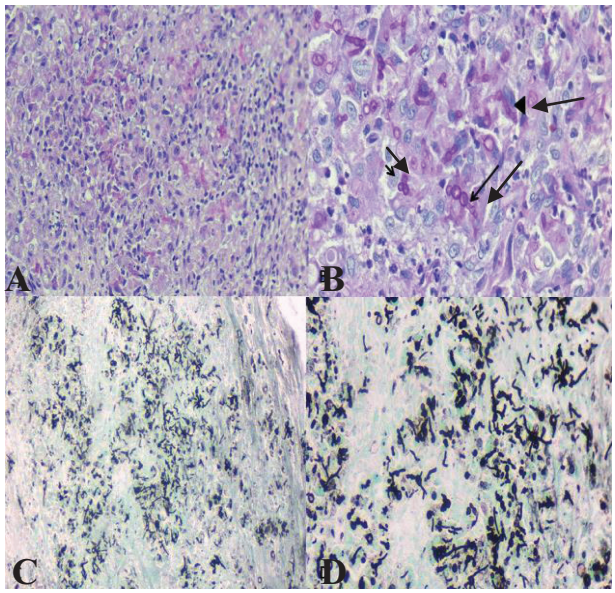

Figure 4. Histochemical stainings.
A) H&E staining 20x; B) PAS staining 40x. Pseudohyphae composed by oval to round a short chains (arrow); blastoconidia that have a round shape (short arrow) and hyphae that are represented by septa (arrow head); C-D) Grocott staining 10x and 20x

CONCLUSIONS

In our study, three *Candida* strains have been isolated and identified by ITS-RFLP patterns obtained with *Msp*I as being *C. gluilliermondii* (1 isolate) and *C. krusei* (2 isolates). No *C. albicans* or *C. parapsilosis* have been isolated. The haemolytic activity of *Candida* isolates was not reported. The histopathological examination with the PAS and the Grocott staining sconfirmed the presence of *Candida* in animal samples. According with these results can be deduced that *Candida* sp. were presented in human and animal samples. Therefore, rapid and reliable methods are needed to detect this yeast in a short time, in such a way as to administer an adequate therapy and avoid a worsening of the patients.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the ERASMUS programme. We would like Dr. Ana Maria Tănase from Faculty of Biology, University of Bucharest for her unconditional support.

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