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Performance of methods for identification of yeasts isolated from blotted water: High prevalence of *Candida parapsilosis*

Desempenho dos métodos de identificação de leveduras de água engarrafada: alta prevalência de *Candida parapsilosis*

Kelly Ishida[1*]; Mirian Ueda-Yamaguchi[2]; Tânia Ueda-Nakamura[2]; Benedito Prado Dias Filho[2]; Sueli Fumie Yamada-Ogatta[2]; Celso Vataru Nakamura[3]

Abstract

Yeasts of the *Candida* genus can colonize epithelium and mucosa of the vertebrate organisms; however these can cause infection in a broad range of body sites. *Candida* species also can be found in drinking water and they are considered as a potential indicator of water quality. In this study were evaluated three methods to identify yeasts isolated from blotted water (seminested PCR, culture on CHROMagar *Candida* medium, and Candifast identification system). For this propose, we used 27 isolates from blotted water and compared with 22 clinical isolates from vaginal fluid. Seminested PCR has shown specificity and sensitivity for identification of the *Candida* species. *Candida albicans* and *Candida parapsilosis* were the prevalent species from vaginal fluid and blotted water, respectively. Culture on CHROMagar and Candifast system had low agreement with snPCR (40.9% and 45.5%, respectively) in the yeasts identification from vaginal fluid. On the other hand, CHROMagar *Candida* can be used in the presumptive identification of yeasts isolated from bottled water and it had agreement's percentage of 81.5% with snPCR method.
Keywords: *Candida*. Bottled water. Identification method. Yeast.

Resumo

Leveduras do gênero *Candida* podem colonizar epitélio e mucosa dos organismos vertebrados, entretanto, podem causar infecções em vários lugares do corpo. Espécies de *Candid*a, também, podem ser encontradas em água e são consideradas um potencial indicador da qualidade de água. Neste trabalho, foram avaliados três métodos de identificação de leveduras isoladas de água engarrafada (seminested PCR, cultura no meio CHROMagar *Candida* e sistema de identificação Candifast). Foram utilizados 27 isolados de água engarrafada e comparados com 22 isolados clínicos de fluido vaginal. Seminested

* Corresponding author: Kelly Ishida, Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Prof. Lineu Prestes 1374, 05508-900, Sala 226/247, São Paulo, São Paulo/Brasil; telephone number: +55 (21) 30917294 - E-mail: ishidakelly@usp.br.

[1] Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Prof. Lineu Prestes 1374, 05508-900, Sala 226/247, São Paulo, São Paulo/Brasil.

[2] Laboratório de Inovação Tecnológica no Desenvolvimento de Fármacos e Cosméticos, Departamento de Ciências Básicas da Saúde, Universidade Estadual de Maringá, Av. Colombo 5790, 87020-900, Maringá, Paraná/Brasil.

[3] Departamento de Microbiologia, Universidade Estadual de Londrina, Rodovia Celso Garcia CID, PR 445 Km 380, 86051-990 Londrina, Paraná/Brasil

PCR tem mostrado especificidade e sensibilidade para a identificação das espécies de *Candida*. *Candida albicans* e *Candida parapsilosis* foram as espécies prevalentes do fluido vaginal e da água engarrafada, respectivamente. Cultura em CHROMagar e o sistema Candifast tiveram baixa concordância com snPCR (40,9% e 45,5%, respectivamente) na identificação de leveduras de fluido vaginal. Em contrapartida, CHROMagar *Candida* pode ser usado em identificação presuntiva de leveduras de água engarrafada apresentando concordância de 81,5% com o método snPCR.
Palavras-chaves: *Candida*, água engarrafada. Métodos de identificação. Leveduras.

Introduction

Drinking water is rarely completely free of microorganisms. Typically, this autochthonous flora is present at the source and of little concern to a healthy population. The Food and Drug Administration regulates bottled water solely on the basis of coliform content (ROSENBERG, 2003), whereas the Brazilian directives regulate water from municipal water supplies on the basis of coliform content and heterotrophic plate count, whereas more stringent bottled mineral water regulations prohibit the presence of a group of potentially pathogenic bacteria (*Pseudomonas aeruginosas, Enterococcus faecalis, Enterococcus faecium*, and Clostridia) (ANVISA, 2000, 2004). Besides bacteria as water contaminant, yeasts and filamentous fungi can be present in the bottled water and also in water used in hospital procedure (PIRES-GONÇALVES et al., 2008; YAMAGUCHI et al., 2007). Most of these organisms come from the source water itself and are not problematic for healthy individuals; however they can cause invasive infections, mainly in immunocompromised hosts associated with viral infection, hematological diseases, organ transplants, antibiotic usage, and more intensive and aggressive medical practices (COLOMBO et al., 2006).

The majority of fungi have no known human health effect, but a small number of species, primarily within the species of *Candida* genus, are important opportunistic pathogens. *Candida* transmission in host organisms is caused often by yeasts which colonizing the epithelial tissue, however sometimes the *Candida* infection may be caused by exogenous route like contaminated water/food ingestion. Particularly, invasive candidiasis are infections difficult to diagnose and to treat, and are often fatal, presenting mortality rate around 40 % (COLOMBO et al., 2006). Several reasons are involved, as utilization of antifungal drugs in prophylaxis, inappropriate therapy, and emergency of resistant pathogenic strains (SOLL, 2002).

Candida albicans is the yeast species most often isolated from clinical material (PFALLER et al., 2011), and most clinical laboratories approach yeast identification by applying rapid tests such as the property of germ-tube formation to distinguish *C. albicans* from other species. Analysis of microscopic and morphologic characteristics and tests of assimilation and fermentation of sugars are used to identify *Candida* non-*albicans* (CNA), which require more extensive testing for identification (KURTZMAN; FELL, 1998). Due to problems of resistance and presence of one or more etiologic agents in the infection, several methods of the detection and identification have been developed to achieve rapid and accurate techniques (GUNDES; GULENC; BINGOL, 2001). Methods as colorimetric systems, performed on colonies after primary isolation and based on determination of the enzymes expression of the each yeasts, hydrolysis of chromogenic substrates by yeast oxidases and peptidases and carbohydrate assimilation have also been produced commercially (GUNDES; GULENC; BINGOL, 2001). However, the most of the identification methods of yeasts is recommended for clinical isolates. On the other hand, molecular-based methods, DNA probes, DNA fingerprints and, amplification of DNA using PCR method had been developed (AHMAD et al., 2002).

In this context, the aim of this work was to evaluate the performance of the yeasts identification methods using strains isolated from bottled water in comparison with isolates from vaginal fluid. The methods used were culture on CHROMagar *Candida* medium, Candifast identification system and, seminested polymerase chain reaction (snPCR).

Materials and Methods

Fungal samples

Residences and workplaces were randomly selected in Maringá city, Paraná State, Brazil. The bottle mineral water samples were collected in sterilized plastic bottles with sodium thiosulphate (10% w/v, Merck, São Paulo, Brazil). The membrane filter technique was employed for isolation of yeasts from water. A volume of 100 ml of the water was filtered through membrane filters with 0.45 µm pores (Millipore, Massachusetts, USA). The membranes were placed on Sabouraud dextrose agar (SDA, Difco, Maryland, USA) with 50 mg/ml of chloramphenical (Sigma Chemical Co, Missouri, USA), incubated at 37 oC and examined daily during one week. It was obtained 27 yeasts isolated and were kept in sterile distillated water at 4-8 ºC.

The identification of yeasts from bottled water was compared with clinical isolates from vaginal fluid previously obtained (ISHIDA et al., 2013). In this study were used 22 isolates randomly chosen and were kept in sterile distillated water at 4-8 ºC.

Candida albicans ATCC 10231, *Candida glabrata* ATCC 2001, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 28707 were obtained from the American Type Culture Collection (ATCC) and they were used for quality control of the yeasts identification methods.

Identification systems

Culture on chromogenic medium: CHROMagar *Candida* was purchased as powdered medium which contain peptone (10 mg/ml), glucose (20 mg/ml), agar (15 mg/ml), chloramphenicol (0.5 mg/ml) and chromogenic mix (2 mg/ml) (CHROmagar Company, Paris, France). The medium was prepared according to the manufacturer's instructions and dispensed into Petri dishes. The isolates were cultured on medium and incubated at 37 ºC, for 48 h. The presumptive identification of the yeasts was realized according to the colonie's colour as indicated by fabricant (green-*C. albicans*; steel blue-*C. tropicalis* and rose or fuzzi-*C. krusei*).

Candifast system: The system contains two rows, each containing eight wells; one is for identification of the most yeast species and other for determination of the susceptibility of yeasts to seven antifungal drugs. The row for identification includes a well with phenol red, actidione and glucose followed by seven wells containing phenol red with sugars (glucose, galactose, trehalose, maltose, cellobiose, raffinose and lactose). Identification is based on the susceptibility of the isolates being tested to actidione, which is seen by the change in indicator color to yellow or to fuschia, and then either the fermentation of seven sugars which is seen by the change the color of the indicator to yellow due to acidification of the medium, or the urease activity, which produces an alkalinization of the medium making the indicator change its color to fuschia in each well. Positive result is characterized by change of medium color and turbidity, and negative result when the well is limpid and orange-red. The results interpretations were performed according fabricant (International Microbio, Signes, France).

Molecular method: Genomic DNA extraction was performed according Ausubel et al. (1999) with some modifications. A single colony on SDA was subcultured in the broth Sabouraud dextrose at 37 ºC for 48 h. The cells were treated with lysis´s buffer TENTS (10 mM Tris HCl pH 8.0 with 2%

v/v Triton X-100, 1% SDS, 100 mM NaCl and 1 mM EDTA) and DNA purification was performed by extraction in phenol:chloroform:isoamilic acid (25:24:1,v/v). After that, the seminested Polimerase Chain Reaction (snPCR) for *Candida* species identification was performed using oligonucleotide primers directed against 3' end of 5.8S and 5' end of 28S rDNA region, including the intervening internally transcribed spacer (ITS2), as describe by Ahmad et al. (2002). The first step, PCR is carried out with universal oligonucleotide primers for detection of *Candida* genus (forward primer CTSF 5'-TCGCATCGATGAAGAACGCAGC-3' and reverse primer CTSR 5'-TCTTTTCCTCCGCTTATTGATATGC-3'). The product of this amplification was submitted to a second PCR using CTSR and species-specific oligonucleotide primers of *C. albicans* (CADET, 5'-ATTGCTTGCGGCGGTAACGTCC-3'), *C. glabrata* (CGDET, 5'-TAGGTTTTACCAACTCGGTGTT-3'), *C. parapsilosis* (CPDET, 5'-TCTTTTCCTCCGC TTATTGATATGC-3'), and *C. tropicalis* (CTDET, 5'-ATTTTGCTAGTGGCC-3'). PCR cycling was carried out in thermocycler under the following conditions: denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. An initial denaturation step at 94 °C for 3 min and a final extension step at 72 °C for 10 min were also included.

Results

The identification of yeasts isolated from bottled water and vaginal fluid was performed by CHROMagar *Candida* medium, Candifast system, and snPCR method (Table 1 and 2). The specie more frequent in bottled water was *C. parapsilosis* by three identification methods: culture on CHROMagar (n=18, 66.7%), Candifast (n=19, 70.4%), and snPCR (n=14, 51.8%) (Table 1). Seven isolates were not identified by Candifast system; however they were identified as *Candida* non-*parapsilosis* by other methods in six isolates (7M, 17M, 20M, 22M, 31M, and 39S). snPCR and CHROMagar were concordant in 81.5% of the yeasts identification from bottled water; and the agreement between Candifast and Chromagar, Candifast and snPCR were 66.7% and 51.8%, respectively (Table 3).

In fluid vaginal specimen, *C. albicans* was identified in 12 (54.5%), 13 (59.1%), and 14 (63.7%) isolates by culture on CHROMagar, Candifast system, and snPCR, respectively. The isolates 111, 150, 187, 191, 278, and 307 were identified as *C. albicans* by snPCR, however they were identified as CNA by other methods. On the other hand, four isolates (1, 2, 8, 52, and 128) were identified as *Candida* sp. (non-*albicans*, non-*glabrata*, non-*parapsilosis*, and non-*tropicalis*) by molecular method, but by other methods they were identified as *C. albicans* (Table 2). In the Table 3 we can be observed low agreement in the identification of *Candida* species between snPCR and Candifast (45.5%) and CHROMagar (40.9%). However, Candifast and CHROMagar had agreement of 86.0% in the identification of yeasts from vaginal fluid.

Table 1 - Identification of yeasts isolated from bottled water by Candifast system, culture on CHROMagar *Candida* medium, and seminested PCR method.

Samples	Candifast	CHROMagar	snPCR
1S	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
7M	NI[a]	*C. krusei*	*C. glabrata*
13M	*C. parapsilosis*	*C. parapsilosis*	*Candida* sp.
17M	NI[a]	*C. krusei*	*C. glabrata*
20M	NI[a]	*C. krusei*	*C. glabrata*
22M	NI[a]	*C. glabrata*	*C. glabrata*
25M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
31M	NI[a]	*C. glabrata*	*C. glabrata*
32M	*C .parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
33M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
33'M	Non *Candida*	NI[a]	Non-*Candida*
34M	*C. parapsilosis*	*C. glabrata*	*C. glabrata*
36M	*C. parapsilosis*	*C. parapsilosis*	*C .parapsilosis*
37M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
39S	NI[a]	*C. tropicalis*	*Candida* sp.
39M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
42S	*C. parapsilosis*	*C. glabrata*	*C. glabrata*
42M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
42'M	*C. parapsilosis*	*C. parapsilosis*	*Candida* sp.
46S	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
47S	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
47M	NI[a]	*C. parapsilosis*	*C. parapsilosis*
49M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
50M	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
51S	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*
58S	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
60M	*C. parapsilosis*	*C. parapsilosis*	*Candida* sp.

Fonte: autores
[a] Not identified

Table 2 - Identification of yeasts isolated from vaginal fluid by Candifast system, culture on CHROMagar *Candida* medium, and seminested PCR method.

Samples	Candifast	CHROMagar	snPCR
1	*C. albicans*	*C. albicans*	*Candida* sp.
2	*C. albicans*	*C. albicans*	*Candida* sp.
8	*C. albicans*	*C. albicans*	*Candida* sp.
52	*C. albicans*	*C. albicans*	*Candida* sp.
111	*C. krusei*	*C. krusei*	*C. albicans*
128	*C. albicans*	*C. albicans*	*Candida* sp.
144	*C. parapsilosis*	*C. parapsilosis*	*C. parapsilosis*
145	*C. albicans*	*C. albicans*	*C. albicans*
150	*C. albicans*	*C. albicans*	Non-*Candida*
174	*C. albicans*	*C. albicans*	*C. albicans*
187	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*
191	*C. parapsilosis*	*C. tropicalis*	*C. albicans*
197	Non-*Candida*	*C. tropicalis*	*C. albicans*
233	*C. albicans*	NI [a]	*C. albicans*
235	*C. tropicalis*	*C. tropicalis*	*C. albicans*
278	*C. parapsilosis*	*C. parapsilosis*	*C. albicans*
299	*C. tropicalis*	*C. tropicalis*	*C. tropicalis*
307	*C. tropicalis*	*C. tropicalis*	*C. albicans*
351	*C. albicans*	*C. albicans*	*C. albicans*
367	*C. albicans*	*C. albicans*	*C. albicans*
405	*C. albicans*	*C. albicans*	*C. albicans*
502	*C. albicans*	*C. albicans*	*C. albicans*

Fonte: autores
[a] Not identified

Table 3 - Agreement percentages between identification methods of yeasts isolated from vaginal fluid and blotted water by culture on CHROMagar *Candida* medium, Candifast system, and seminested PCR method.

	Strains from:	
	Vaginal fluid	Blotted water
	n (%)	n (%)
CHROMagar / Candifast	19 (86.0)	18 (66.7)
CHROMagar / snPCR	9 (40.9)	22 (81.5)
snPCR / Candifast	10 (45.5)	14 (51.8)

Fonte: autores.
n: number of isolates

Discussion

Yeasts are a significant component of the microbiota of the most natural aquatic ecosystems and can also occur in drinking water as a result of their ability to survive treatment practices and become incorporated into biofilms (BRINKMAN et al., 2003). Researchers had verified a strong correlation between presence of yeasts with fecal pollution indicators (total coliforms, fecal coliforms and enterococcus) isolated from water. In addition, *Candida* species are the most prevalent yeast in the water (17.9%) (ARVANITIDOU et al., 1999; PIRES-GONÇALVES et al., 2008). With continuing concern regarding problems of water pollution, in particular drinking water, it is appropriate to consider yeast *Candida* genus as a potential indicator of water quality (BUCK; BUBUCIS, 1978; YAMAGUCHI et al., 2007). Furthermore, some researchers had show that bottled water is a potential transmission route for bacteria and fungi both in hospitals and the community in the examined region and may pose a health hazard mainly for the immunocompromised host (ARVANITIDOU et al., 1999; PIRES-GONÇALVES et al., 2008; SCOARIS et al., 2008; YAMAGUCHI et al., 2007). Amongst fungi, *Fusarium* spp. have been the most abundant genus found, whereas *C. parapsilosis* the predominant yeast species (PIRES-GONÇALVES et al., 2008; YAMAGUCHI et al., 2007).

Candida albicans is more frequent species isolated from clinical material, however CNA species have increased in the last years (PFALLER et al., 2011). In this study, *C. albicans* was prevalent in vaginal fluid samples; however in bottled water the species more isolated was *C. parapsilosis.* Identification methods of yeasts isolated from vaginal fluid showed low agreement between the molecular method with methods based on yeast's metabolism (culture on CHROMagar *Candida* medium and Candifast system). In addition, isolates identified as CNA species by Candifast and CHROMagar were characterized as *C. albicans* by snPCR method. On the other hand, snPCR was agreement with CHROMagar in 81.5% of yeasts isolated from bottled water. Although it was observed low agreement between identification methods, *C. albicans* and *C. parapsilosis* were the prevalent species in vaginal fluid and bottled water, respectively, by three identification methods.

Traditional typing techniques based on phenotypic characteristics are increasingly challenged by the use of DNA-based techniques, which revolutionized in the better differentiation of microorganisms (BUSCH; NITSCHKO, 1999). Molecular identification techniques for fungi allow for more accurate and rapid identification of *Candida* spp. based on differences within the ribosome DNA (XU et al., 2002). The genotypic technique used in this study for yeast identification can detect members of the genus *Candida* by means of a specific and conserved nucleotide sequence, in region 5.8S and 28S rDNA. This methodology has shown specificity and sensitivity for *Candida* species (AHMAD et al., 2002).

Standard mycological methodology is the gold standard technique to identify *Candida* species; however it does not differentiate some *Candida* species, such as *C. dubliniensis* of *C. albicans.* There are a variety of phenotypic techniques for differentiating, but none of the tests alone is satisfactory in the identification of *C. dubliniensis* (MOMANI; OADDOOMI, 2005). Identification systems, such as Candifast system and culture on CHROMagar *Candida* medium also not get distinguish these *Candida* species. On the other hand, the identification by molecular method could be used to distinguish *C. albicans* and other CNA species using a specific nucleotide sequence to each specie (XU et al., 2002). In our study, four isolates from vaginal fluid were identified as *C. albicans* by culture on CHORMagar and Candifast system; however these strains were identified only as *Candida* genus using snPCR method and excluding *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*.

CHROMagar *Candida* medium is rapid and practical method with high sensitivity and specificity for the *Candida* species identification (*C. albicans*, *C. krusei*, and *C. tropicalis*) in clinical material (ODDS; BERNAERTS, 1994). Althought CHROMagar *Candida* medium is indicated for identification of clinical isolates; in comparison with snPCR had low agreement in the identification the isolates from vaginal fluid. On the other hand, CHROMagar *Candida* medium showed be useful for presumptive identification of yeasts isolated from bottled water. Besides, it allows differentiating of more than one *Candida* species grown on the same culture medium. Researchers emphasized that these chromogenic media were not proposed as substitutes for thorough identification protocols (GULTEKIN; YAZICI; AYDIN, 2005; ODDS; BERNAERTS, 1994).

In this work, Candifast system did not get identify yeasts from bottled water in seven isolates (7M, 17M, 20M, 22M, 31M, 39S and, 39M) and six them were identified as *C. parapsilosis* by snPCR and CHORMagar *Candida* medium. We observed Candifast system interpretation showed confuse to isolates from bottled water, while to yeasts from vaginal fluid the interpretation was easier and fast. In addition, Candifast system identification was developed and recommended by manufacturer to identify yeasts from clinical material.

In summary, our work showed that *C. albicans* and *C. parapsilosis* were prevalent species isolated from vaginal fluid and bottled water, respectively, by three yeast identification methods. Culture on CHROMagar *Candida* medium and Candifast system had low agreement with molecular method (snPCR) in the yeasts identification from vaginal fluid. On the other hand, CHROMagar *Candida* can be used in the presumptive identification of yeasts isolated from bottled water.

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