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ISOLATION OF PROMINENT OPPORTUNISTIC YEASTS, *CANDIDA* SPP., FROM COIN-OPERATED WASHING MACHINES

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ABSTRACT

Coin-operated washing machines are convenient household appliances that are widely used in Thailand. The environment in these devices is suitable for fungal colonization. As a result, they may be a potential risk for yeast infection, particularly opportunistic yeasts such as *Candida* spp. Thus, the aim of this study was to isolate *Candida* spp. from thirty coin-operated top load washing machines installed at dormitories near Bansomdejchaopraya Rajabhat University, Bangkok, Thailand. Sterile cotton swabs were used to collect samples from four sites of each device including washing powder drawers, fabric softener drawers, tubs, and lint filters. Samples were inoculated on a surface of Sabouraud dextrose agar (SDA) supplemented with 0.05 g/L chloramphenicol. Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) of ITS1-5.8S-ITS2 regions and *Msp*I as a restriction enzyme were used for *Candida* spp. identification. We identified several species of *Candida* spp. including *Candida parapsilosis* complex, *Candida glabrata*, *Candida tropicalis*, *Candida haemulonii* complex, *Candida albicans* complex, *Candida lusitaniae*, and *Candida guilliermondii* by using PCR-RFLP. This investigation showed that coin-operated washing machines were contaminated with important opportunistic yeasts of *Candida* spp. Therefore, these devices should be maintained and cleaned regularly.

Keywords: *Candida* spp., Coin-operated washing machine, Polymerase chain reaction-restriction fragment length polymorphism, Restriction enzyme

INTRODUCTION

Washing machines are common electric household appliances that people normally use for cleaning their clothes. Coin-operated washing machines are widely used in Thailand, particularly in dormitories, condominiums, or apartments. The operational process of these devices is easy. Users load their laundry into a tub, add powder or liquid detergent and fabric softener, insert coins, and then the washing cycles run automatically. Therefore, many landlords install these devices for their residents. The major function of washing machines is to remove dirt from clothes. Unfortunately, the environment in laundry machines is suitable for fungal growth because these machines frequently come into contact with water and tend experience humid conditions on the inside of the appliance. Fungi can colonize several kinds of household appliances. Previous studies showed that dishwasher machines could harbor many microbial species including bacteria and fungi, such as *Escherichia* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Candida* spp., *Rhodotorula* spp., and *Cryptococcus* spp., and they formed complexes of bacterial-fungal biofilms **(Raghupathi *et al.*, 2018)**.
Candida spp. are normal flora that colonize several parts of the human body, such as the skin, oral cavities, vagina, and gastrointestinal tract. There are several factors that make these yeasts become life-threatening pathogens, such as host immunity, broad spectrum antibiotic usage, immunosuppressive therapy, and hormone therapy **(Jabra-Rizk *et al.*, 2016)**. Candidiasis is a fungal infection caused by *Candida* spp., particularly *Candida albicans*. *Candida* spp. can cause a wide range of infections from superficial to systemic and life-threatening diseases that occur mainly in immunocompromised patients. Candidemia is a blood stream infection (BSI) caused by *Candida* spp. which can occur in hospitalized patients, and which demonstrated a high mortality rate **(Flevari *et al.*, 2013)**. Currently, non-*albicans* *Candida* (NAC) species are also increasingly isolated from a wide variety of clinical specimens, namely *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr*, *Candida guilliermondii*, and *Candida dubliniensis* (**Deorukhkar *et al.*, 2014**). Importantly, some NAC species exhibit a primary resistance to antifungal drugs like *Candida krusei* and fluconazole resistance, while other NAC species, including *C. glabrata*, *C. tropicalis* and *Candida lusitaniae*, often acquire resistance to azole antifungals **(Krcmery and Barnes, 2002)**. Additionally, amphotericin B resistance was also reported in *C. lusitaniae*, *Candida rugosa*, *C. krusei* and *C. guilliermondii* **(Krcmery and Barnes, 2002)**. Therefore, precise *Candida* spp. identification from clinical specimens and antifungal susceptibility testing is important for effective therapy. The aim of this study was to isolate *Candida* spp. from thirty coin-operated top load washing machines and to identify *Candida* species by PCR-RFLP technique. This study provides important information regarding the contamination of common household devices by crucial yeasts such as *Candida* spp., that may lead to serious health problems.

MATERIALS AND METHODS

Sample Collection and Yeast Isolation

Thirty coin-operated top load washing machines, all from dormitories surrounding Bansomdejchaopraya Rajabhat University, Bangkok, Thailand, were sampled. For each washing machine, four parts were investigated by swabbing, including detergent drawers, fabric softener drawers, tubs, and lint filters. Two swabs were applied to each part of the washing machines, which were then inoculated on Sabouraud dextrose agar (SDA) plates supplemented with 0.05 g/L chloramphenicol and then incubated at 25ºC for 7 days After incubation, colonies that showed yeast characteristics (round colonies, convex, white to cream-colored, and smooth, wrinkled, raised or folded) were transferred to new SDA plates which were incubated at 25ºC for 48-72 h for further analysis.

DNA Extraction

DNA extraction from selected yeast colonies was performed by heating in NaOH as described by Dilhari *et al*. with brief modification **(Dilhari *et al.*, 2017)**. Yeasts were transferred in a 1.5 ml microcentrifuge tube containing 100 µl of sterile distilled water. The sample was spun at 8,000 rpm, at 4ºC for 1 min. The supernatant was removed, and the pellet was suspended in 100 µl of 20 mM NaOH. Next, the sample was boiled at 95ºC for 45 min and then centrifuged at 12,000 rpm, at 4ºC for 10 min. The supernatant containing genomic DNA was then transferred to a new microcentrifuge tube for use as a template for PCR-RFLP analysis.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

PCR reactions were done by using universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') to amplify the ITS1-5.8S-ITS2 region. PCR conditions were performed in a final volume of 25 µl, with 12.5 µl of 2X PCR Master Mix (Promega, USA), 0.5 µl of 10 µM forward and reverse primer, 1 µl DNA template and 10.5 µl of distilled water. The PCR reaction was performed under conditions as follows: initial denaturation at 95ºC for 3 min, 35 cycles of denaturation at 95ºC for 30 sec, annealing at 56ºC for 45 sec, and extension at 72ºC for 45 sec, with a final extension at 72ºC for 5 min. The PCR product was observed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and then the gels were visualized using the Bio-Rad gel documentation system.
An aliquot of 20 µl of PCR product was digested with 0.5 µl of the restriction enzyme *Msp*I (20,000 units/ml) (New England BioLabs, Ipswich, MA, USA), 2.5 µl of enzyme buffer, 2 µl of distilled water. After incubation at 37ºC for 2 h, the digested PCR products were analyzed by electrophoresis on 3% agarose gels, stained with ethidium bromide and visualized by the gel documentation system (Bio-Rad).

Prediction of PCR Product Fragment Cutting by Restriction Enzyme; *Msp*I by *In Silico* Analysis

We performed bioinformatics analyses of the *Msp*I-RFLP method. The ITS region sequences of each *Candida* spp. and *Trichosporon* spp. were used to predict the products of the digests using *Msp*I by BioEdit v7.2.5.

Species Identification of *C. haemulonii* complex by DNA Sequencing

PCR products of yeast samples identified as *C. haemulonii* complex via PCR-RFLP method were purified and bidirectionally sequenced by 1st BASE DNA Sequencing (Apical Scientific Sdn Bhd, Malaysia). The retrieved sequence files were edited by BioEdit v7.2.5 and then compared with existing sequences in GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers of the nucleotide sequences deposited in GenBank were MZ261915-MZ261921.

Statistical Analysis

The yeast numbers between each collection size were compared by one-way ANOVA using GraphPad Prism 9 (GraphPad Software). A p-value < 0.05 was considered as statistically significant.

RESULTS

Yeast Isolation

In this study, the total collection sites from thirty coin-operated top load washing machines numbered 140 positions including 30 positions of each detergent drawer, fabric softener drawer, and tub, and 50 positions of lint filters. We isolated 341 yeasts from these home appliances.

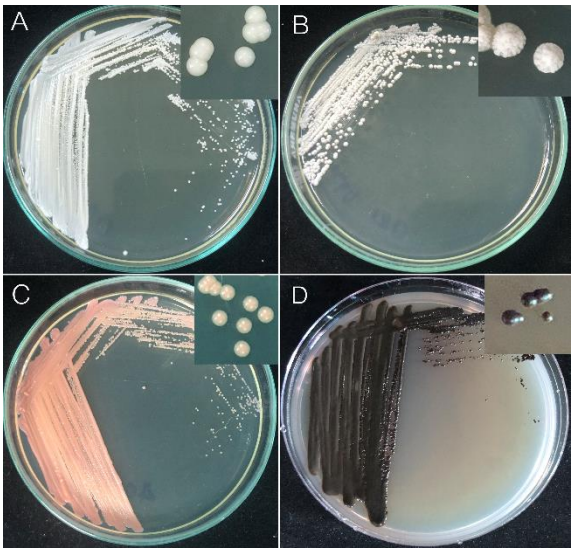

Figure 1 Colony morphology of yeasts isolated from coin-operated top load washing machines. A) round, convex, smooth, creamy or white colonies; B) white to cream-colored, wrinkled, raised or folded colonies; C) round, smooth, mucoid, slightly orange, coral red to salmon-colored colonies, and D) smooth, brown to black colonies.

There were four major types of colony morphology in this investigation (Figure 1): A) round, convex, smooth, creamy or white colonies; B) white to cream-colored, wrinkled, raised or folded colonies; C) round, smooth, mucoid, slightly orange, coral red to salmon-colored colonies, and D) smooth, brown to black colonies. Thus, we selected colonies with type A and B colony morphologies, corresponding to *Candida* and *Trichosporon* species for PCR-RFLP analysis. Colonies with types C and D colony morphologies were not investigated since they correspond to *Rhodotorula* and *Exophiala* species, respectively.

PCR-RFLP

The size of the PCR product of the ITS1-5.8S-ITS2 region for *Candida* spp. and suspected yeasts and of RFLP fragments were predicted. The DNA sequences of the ITS1-2 region deposited in GenBank were used for computerization to determine the length of the RFLP product after digesting with *Msp*I (Table 1).

Table 1 PCR product size of the ITS1-5.8S-ITS2 region and *Msp*I-RFLP analysis.

Yeasts	Size of ITS region	Size of RFLP fragments	GenBank accession number
Candida albicans complex			
C. albicans	537	298, 239	MH545917.1
C. dubliniensis	541	297, 244	MK394123.1
C. africana	536	297, 239	NR_138276.1
C. haemulonii complex			
C. haemulonii	374	-	MN172335.1
C. duobushaemulonii	388	-	KY102063.1
C. haemulonii var. *vulnera*	374	-	MK394151.1
C. auris	398	-	MF817727.1
C. glabrata complex			
C. grabrata	879	561, 318	MH545922.1
C. nivariensis	760	236, 206, 318	MH545923.1
C. bracarensis	803	253, 550	MH545924.1
C. parapsilosis complex			
C. parapsilosis	520	-	MH545914.1
C. orthopsilosis	508	-	MK394126.1
C. metapsilosis	531	-	MK394127.1
C. tropicalis	525	340, 185	MH545915.1
C. guilliermondii	609	370, 155, 84	MT635314.1
C. lusitaniae	382	264, 118	MH545926.1
Trichosporon asahii	541	282, 259	MK267768.1
T. inkin	541	281, 260	JX463242.1

A total of 259 yeast isolates were identified via PCR-RFLP analysis by amplifying the ITS1-5.8S-ITS2 region, and the PCR products were digested with *Msp*I (Figure 2-3). We isolated several *Candida* species that showed PCR products ranging from 374 to 879 bp. After digestion with *Msp*I, *Candida* species were identified as follow: *C. parapsilosis* complex, *C. glabrata*, *C. tropicalis*, *C. haemulonii* complex, *C. albicans* complex, *C. lusitaniae* and *C. guilliermondii.* Additionally, we also isolated yeasts showing approximately the PCR product 540 bp and two fragments (~280, 260 bp) after cutting by *Msp*I. According to colony morphology, yeasts in this group showed as white to cream-colored, wrinkled, with raised or folded colonies. Thus, from PCR-RFLP and colony characteristics, these yeasts were identified as *Trichosporon* spp.

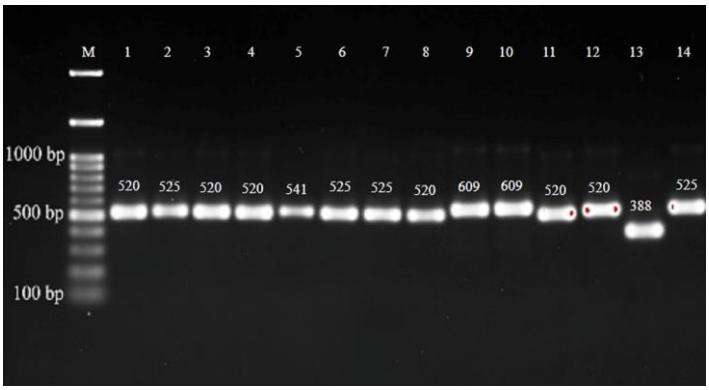

Figure 2 PCR products of yeasts isolated from coin-operated top load washing machines. Lane 1: *C. parapsilosis* complex. Lane 2: *C. tropicalis*. Lanes 3-4: *C. parapsilosis* complex. Lane 5: *Trichosporon* spp. Lanes 6-7: *C. tropicalis*. Lane 8: *C. parapsilosis* complex. Lane 9-10: *C. guilliermondii*. Lanes 11-12: *C. parapsilosis* complex. Lane 13: *C. haemulonii* complex. Lane 14: *C. tropicalis*. M: Molecular size marker, 100 bp.

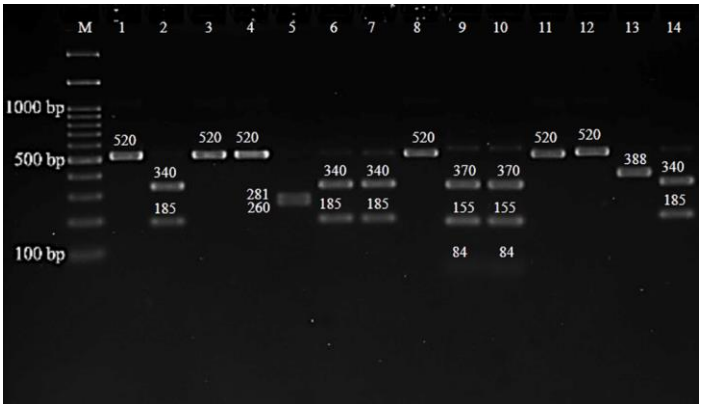

Figure 3 PCR-RFLP fragments of yeast isolated from coin-operated top load washing machines. Lane 1: *C. parapsilosis* complex. Lane 2: *C. tropicalis*. Lanes 3-4: *C. parapsilosis* complex. Lane 5: *Trichosporon* spp. Lanes 6-7: *C. tropicalis*. Lane 8: *C. parapsilosis* complex. Lanes 9-10: *C. guilliermondii*. Lanes 11-12: *C. parapsilosis* complex. Lane 13: *C. haemulonii* complex. Lane 14: *C. tropicalis*. M: Molecular size marker, 100 bp

Yeast Isolated from Each Position of Coin-Operated Washing Machines

Our investigation demonstrated that yeast could be found in every part of coin-operated washing machines (Table 2). *Candida* spp. was the most prevalent genus isolated from this study. *C. parapsilosis* complex was the most dominant with 133 total isolates followed by *C. glabrata*, *C. tropicalis*, *C. haemulonii* complex, *C. albicans* complex, *C. lusitaniae*, and *C. guilliermondii*, respectively. In terms of collected positions, lint filters had a greater abundance of *Candida* spp. colonization followed by fabric softener drawers, tubs, and detergent drawers, respectively. Additionally, we isolated a high number of yeasts that showed PCR products and RFLP fragments identical to *Trichosporon* spp. (Figure 2-3 and Table 2). Seventeen yeasts could not be identified by the PCR-RFLP conditions used in this study. They showed PCR products ranging from 400 to 900 bp and some strains showed multiple RFLP fragments or were not cut by *Msp*I (data not shown). Additionally, the average number of yeasts isolated from each position was compared. We found that the number of yeasts collected from lint filter was statistically significantly different from detergent drawers, fabric softener drawers, and tubs (*P-value* < 0.05) (Figure 4).

Table 2 Total number of yeasts isolated from each position of thirty coin-operated top load washing machines.

Yeasts	Total number of yeasts isolated from each position				Total isolates
	Lint filters	Detergent drawers	Fabric softener drawers	Tubs	
C. parapsilosis complex	90	4	24	15	133
C. glabrata	18	-	-	1	19
C. tropicalis	9	-	-	1	10
C. haemulonii complex	1	-	6	-	7
C. albicans complex	2	-	-	1	3
C. lusitaniae	1	-	1	1	3
C. guilliermondii	2	-	-	-	2
Trichosporon spp.	57	-	2	6	65
Unidentified yeasts	13	2	1	1	17
Total isolates	193	6	34	26	259

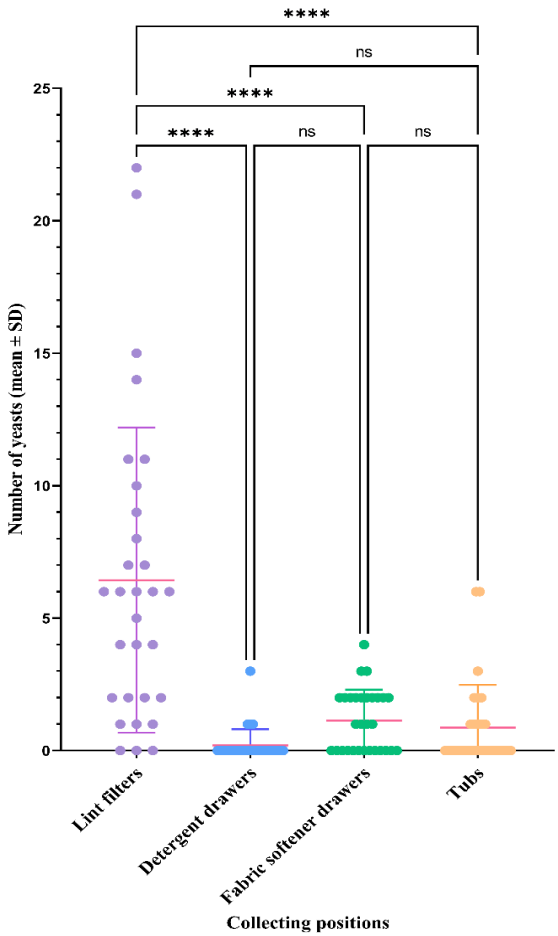

Figure 4 Number of yeasts (mean ± sd) isolated from each part of thirty coin-operated top load washing machines. **** indicates statistically significant differences (*P-value* < 0.05). ns = not significant.

Species Identification of *C. haemulonii* complex by DNA Sequencing

To identify species in the *C. haemulonii* complex, the nucleotide sequences of seven PCR products of the ITS1-5.8S-ITS2 region of the samples identified as *C. haemulonii* complex by PCR-RFLP technique were sought in the NCBI database. The results from BLASTn were classified and a cutoff ≥ 97% sequence-based identities to the query sequence was considered significant. Two species of *C. haemulonii* complex were identified. There were *C. haemulonii* (2 isolates; GenBank accession number MZ261915 and MZ261916), *C. haemulonii* var *vulnera* (4 isolates; GenBank accession number MZ261917-MZ261920) and *C. duobushaemulonii* (1 isolate; GenBank accession number MZ261921). All of them were isolated from fabric softener drawers except *C. haemulonii* var. *vulnera* GenBank accession number MZ261918 which was found in the lint filter.

DISCUSSION

Household appliances have been reported as suitable places for microorganism colonization. Kulesza *et al*. isolated different kinds of yeasts from dishwashers **(Kulesza *et al.*, 2021)**. Previous studies found several fungal genera from washing machines, both filamentous fungi and yeasts, such as *Fusarium oxysporum*, *Fusarium solani* species complex, *Cladosporium* spp., *Mucor* spp., *Trichoderma* spp., *C. parapsilosis*, *Rhodotorula* spp., and *Exophiala phaeomuriformis* **(Babič *et al.*, 2015; Tischner *et al.*, 2019)**. In this study, we showed the diversity of yeasts accumulating in coin-operated top load washing machines, especially *Candida* spp. The most prevalent yeast isolated from these devices was *C. parapsilosis* complex followed by *C. glabrata*, *C. tropicalis*, *C. haemulonii* complex, *C. albicans* complex, *C. lusitaniae*, and *C. guilliermondii*, respectively. Actually, we were able to isolate numerous filamentous fungi from our investigation; for instance, *Aspergillus* spp., *Fusarium* spp., *Acremonium* spp., *Trichoderma* spp., *Cladosporium* spp., and *Scopulariopsis* spp. (data not shown). We emphasized important opportunistic human pathogenic yeasts that are *Candida* species. In our study, we did not have any information about frequency of use, age of each washing machine, type of detergents, or fabric softener usage. Dögen *et al*. also showed the presence of *C. parapsilosis* in laundry machines and found no difference between the age of machines, frequency of use, powder or liquid detergents, and fabric softener usage **(Dögen *et al.*, 2017)**.

Previous studies normally isolated fungi from detergent and softener drawers, door rubber seals, and tubs. The samples from our study were collected from detergent drawers, fabric softener drawers, tubs, and lint filters. Coin-operated washing machines in this study were of the top loaded type. We found that lint filters showed a high number of yeasts followed by fabric softener drawers, tubs, and

detergent drawers, respectively. We isolated several *Candida* species in lint filters, namely *C. parapsilosis* complex, *C. glabrata*, *C. tropicalis*, *C. albicans* complex, *C. lusitaniae*, C. *haemulonii* var. *vulnera*, and *C. guilliermondii*. This part was used for trapping lint or pieces of wet paper so it could be the favored place for microorganism accumulation. Additionally, lint filters are often not effectively maintained and cleaned by users. Previous investigation demonstrated predominantly filamentous fungi in washing powder drawers and *C. parapsilosis* in fabric softener drawers **(Babič *et al.*, 2015)**. Moreover, coin-operated washing machines are employed by several users, so that cross contamination during the washing process may occur. Secondhand clothes harbored many microorganisms, both bacteria and fungi such as *Staphylococcus* spp., *Enterobacter* spp., *Bacillus subtilis*, *Aspergillus flavus*, *Aspergillus niger*, *C. albicans*, *C. tropicalis*, *Penicillium* spp., *Rhodotorula rubra*, and *Trichophyton rubrum* **(Agbulu *et al.*, 2015; Al-Easawi and Emran, 2017)**. Dermatophytes could be isolated from socks from patients with tinea pedis and onychomycosis, namely *T. rubrum* and *Trichophyton mentagrophytes* **(Bonifaz *et al.*, 2013)**.

In this study, we amplified the ITS1-5.8S-ITS2 regions with the specific primers ITS1 and ITS4, and the PCR products were digested with the restriction enzyme *Msp*I. We also predicted RFLP fragments of ITS1-5.8S-ITS2 regions of *Candida* spp. and suspected yeasts by *in silico* analysis to ensure support our results (Table 1). Howerver, we could not identify 17 yeasts. Using only *Msp*I as a restriction enzyme, it was not possible to clearly distinguish some cryptic species in the *Candida* genus, particularly in the *C. albicans* complex, *C. haemulonii* complex, and *C. parapsilosis* complex **(Fontecha *et al.*, 2019)**. This is the limitation of PCR-RFLP, which is insufficient to distinguish intra-species variations in both length and nucleotide sequence differences, since restriction enzymes recognize and cut only a few specific sites in the DNA. Therefore, alternative techniques are required for species identfification, such as DNA barcode, ITS or other genes sequencing, and MALDI-TOF Mass Spectrometry. However, many researchers have also used PCR-RFLP to identify *Candida* spp. **(Mirhendi *et al.*, 2006; Mohammadi *et al.*, 2013;Ortiz *et al.*, 2017; Fontecha *et al.*, 2019)**. Additionally, PCR products and PCR-RFLP fragment patterns of *C. auris* were similar to *C. haemulonii*, *C. duobushaemulonii* and *C. haemulonii* var. *vulnera* (Table 1). Therefore, the sequencing of the ITS1-5.8S-ITS2 region was performed to identify the species in the *C. haemulonii* complex. From our investigation, *C. auris* was not found.

Among *Candida* spp., *C. albicans* is a significant cause of invasive fungal infections, particularly in immunocompromised patients **(Firacative, 2020)**. There are several risk factors that support invasive fungal infections caused by *Candida* spp., such as intravenous or intracranial catheters, the use of broad-spectrum antibiotics and immunosuppressive drugs, and disease-induced immunosuppression **(Firacative, 2020)**. Currently, an increasing incidence of infection caused by non-*albicans Candida* (NAC) has been reported **(Pappas *et al.*, 2018)**. Candidemia caused by *C. tropicalis*, *C. glabrata* and *C. parapsilosis* has been reported in cancer patients **(Wu et al., 2017)**. Furthermore, NAC are also the causative agents of vulvovaginal candidiasis (VVC). For instance, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. rugosa*, *C. dubliniensis*, *C. guilliermondii*, and other NAC species may show resistance to antifungal drugs **(Das *et al.*, 2019; Yassin *et al.*, 2020)**. Additionally, we isolated a high number of yeasts that appeared as white to cream-colored, wrinkled, raised, or folded colonies, and showed PCR products and PCR-RFLP fragments after cutting by *Msp*I as *Trichosporon* spp. Even though PCR products and RFLP fragments of the ITS1-5.8S-ITS2 regions for *Trichosporon* spp. and *Candida albicans* are difficult to differentiate (Table 1), colony morphology is useful to distinguish these fungi. *Trichosporon* spp. are yeast-like fungi that are ubiquitous in the environment. White piedra, trichosporonosis, and onychomycosis are diseases caused by various species of *Trichosporon* spp. such as *Trichosporon asahii*, *Trichosporon inkin*, *Trichosporon cutaneum*, *Trichosporon mucoides*, and *Trichosporon ovoides* **(Colombo *et al.*, 2011; Montoya and González, 2014)**.

CONCLUSION

This investigation revealed that coin-operated washing machines are potential reservoirs of opportunistic yeasts. For this reason, these devices should be maintained and cleaned regularly, for instance, washing interior parts or lint filters and opening the washing machine door after use.

Disclosure of Interest: The authors have no conflict of interest**.**

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