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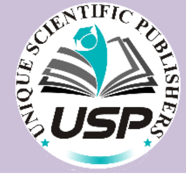
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Phenotypic and Genotypic Investigation of Yeast Species Associated with Bovine Subclinical Mastitis with a Special Reference to their Virulence Characteristics

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ABSTRACT

This study aimed to investigate yeast species associated with subclinical bovine mastitis in dairy farms. It is based on isolation using Sabouraud dextrose agar (SDA) followed by identification of isolates using HiCrome *Candida* agar and ITS-PCR-RFLP. Out of 405 milk samples, 31.6% were positive for yeast isolation on SDA. Based on HiCrome *Candida* agar and ITS-PCR assay, isolates were classified into 90 pure isolates and 38 mixed cultures. Only pure isolates were subjected to species identification and virulence evaluation. By HiCrome *Candida* agar media, the most frequently isolated species were *C. krusei, C. tropicalis,* and *C. albicans* (13.3% for each) while *C. guilliermondii* was the least isolated *Candida species* (1.5%). ITS-PCR-RFLP identified 67 isolates which included non-*albicans Candida* (NAC) species and *Trichosporon assahii.* Neither *C. albicans* nor *C. galabrata* were identified. Twenty-three isolates could not be identified by ITS-PCR-RFLP. Difference between the results of HiCrome *Candida* agar and ITS-PCR-RFLP methods was obvious. Considering slime production, 83.6% were slime producers while 16.4% were not. All strains of *C. krusei,* and *C. tropicalis* were slime producers. Regarding biofilm production, 80.6% of isolates had the ability to form biofilm ranged from strong (6%), moderate (14.9%) to weak (59.7%) while only 19.4% were non-biofilm producers. *In vitro* antifungal susceptibility of isolates displayed different susceptibility and resistance patterns. Conclusively, yeast mastitis in dairy cows demonstrates a growing problem. The diagnosis must rely on accurate laboratory diagnostic tests. The significance of NAC and other yeast genera specifically genus *Trichosporon* and their role in the etiology of mycotic mastitis should be emphasized.

Key words: Yeast, Bovine mastitis, PCR-RFLP, Biofilm formation, Slime production, Antifungal susceptibility.
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INTRODUCTION

Mastitis is the main health problem in bovine dairy herd especially its subclinical form that can cause great harm to dairy herds because of its negative impact on milk production and quality and cow health. Different microorganisms including bacteria and fungi have been implicated as causative agents of bovine mastitis (Krukowski et al. 2006; Khan et al. 2013; Antanaitis et al. 2021). Yeast is the main cause of fungal mammary glands infections and *Candida* is the most common implicated genus (Dworecka-kaszak et al. 2012). *Candida* species are considered opportunistic pathogens that colonize the cow udder. Consequently, the abuse of antibacterial agents and contaminated materials brought in contact with the mammary gland, could favor yeast colonization in cow udders (Santos and Marin 2005; Hussain et al. 2013). In Egypt, there are many predisposing factors that may contribute to the unceasing increase in the prevalence of bovine mycotic mastitis. Among these factors, hot and humid climate, predominant small-scale household rearing systems of cattle, the misuse of excessive antibiotic therapy, and the misdiagnosis and consequent delay of specific antifungal therapy are the most common factors (Abd El-Razik et al. 2011; Juman et al. 2020). In comparison to other mastitis agents, the occurrence of mycotic mastitis was generally very low but it has been significantly increased during the last years. *Candida* spp. that isolated in a high percentage from milk may cause either mastitis or systemic candidiasis with probable decline in the strength of immune system of the animal (Krukowski et al. 2001; Qayyum et al. 2016; Sonmez and Erbas 2017).

Conventional identification of yeasts is based on morphological and physiological criteria. These methods

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are strongly affected by culture conditions giving uncertain results (Kurtzman and Fell 2000). Moreover, phenotypic and biochemical assays are time-consuming and exposed to misdiagnosis, whereas the identification of *Candida* to species level is essential as many non-*albicans Candida* developed drug resistance against antifungal agents (Khalaf et al. 2021). Lately, molecular identification using DNA-based tests have begun to be widely used because of their speed, high sensitivity, and specificity. PCR-RFLP analysis is one of the most efficacious methods for yeast identification. It depends on a comparison of the restriction patterns obtained by digestion of a specific target DNA with restriction endonucleases (Fadda et al. 2013; Taei et al. 2019).

Monitoring the virulence of yeast causing mastitis is very imperative. It can provide a new strategy for the prevention and treatment of fungal bovine mastitis. Amongst the virulence determinants, slime production, ability to form biofilm and antimycotic resistance are the most powerful ones. They are mostly associated with the ability to cause persistent infections, therefore; biofilm production could be a possible explanation for bovine mastitis cases that are not resolved by standard treatment strategies (Cengiz et al. 2006; Pedersen et al. 2021). *C. albicans* in biofilm form is characterized by being resistant to both antifungal drug therapies and host immune defenses (Douglas 2003; Nett and Andes 2006). Many antimycotic drugs have been used for the treatment of yeast mastitis. However, the effectiveness of these drugs was not declared (Zaragoza et al. 2011; Dworecka-Kaszak et al. 2012).

Recently, frequent complaints from the un-effectiveness of the prescribed antibiotic therapy of bovine mastitis cases in several Egyptian dairy farms were received (personal information). Therefore, the present study aimed to investigate yeast species associated with bovine subclinical mastitis based on phenotypic methods of identification. Also, for comparative evaluation, the molecular ITS-PCR-RFLP method was used for accurate and rapid identification of yeast spp. Moreover, virulence factors such as slime production, the capability to form biofilm, and the antifungal susceptibilities of the isolated species were determined.

MATERIALS AND METHODS

Collection of Milk Samples

From 405-quarters cow's milk samples were collected from cows suffering from subclinical mastitis (according to California Mastitis Test and somatic cell count (José et al. 2018) with a history of resistance to antibiotic treatment in several dairy farms. Quarter milk samples were collected in sterile vials under aseptic conditions and brought to the laboratory in an ice container for processing as soon as possible. The collection of samples was performed in accordance with rules accepted by the local commission for ethics in animal experimentation and investigation.

Isolation and Identification of Yeasts

The samples were plated onto the Sabouraud dextrose agar (SDA) [HIGHMEDIUM, India] contained 0.05mg/ml Chloramphenicol and incubated at 37°C for 48-72h. The cultures were identified at first for morphological characteristics of the grown colonies on SDA by macroscopical examination. Secondly, the micro-morphology of the isolates was examined in wet preparation to detect the formation of chlamydoconidium, germinal tube development in human serum, and pseudohyphae on rice agar medium. Furthermore, microscopical examination of isolates was performed after staining by the Indian ink stain and Gram's stain. HiCrome *Candida* Differential Agar (HIMEDIUM, India) culture was used for further identification of the isolated species of yeast according to Du et al. (2018).

DNA Extraction

The method of Zhang et al. (2010) was followed with some modifications. One or two loops of yeast colonies were relocated from chromogenic agar plates into a micro-centrifuge tube containing 200µl of pure water. The mixture was mixed and centrifuged at 8000 x g for 1min to wash the pellet. After careful removal of the supernatant, 200 µl of lysis solution (1% Triton X100, 0.5% Tween 20, 10 mM Tris-HCl (pH 8.0), and 1mM EDTA, BioBasic, Canada) were added to the resulting pellet, and vortexed well. A boiling step was followed for 10min. The suspension was centrifuged for 10min, to sediment the cell debris. The supernatant was collected, transferred to a clean tube and stored at –20ºC. At the time of PCR, 5µl was used as template DNA.

Molecular Identification using ITS-PCR-RFLP Method

The internal transcribed spacer (ITS) rDNA region was amplified according to Mirhendi et al. (2006). PCR was carried out in a final volume of 30µl. Each reaction included 5µl of template DNA, 30pmol of each primer (ITS1: 5′ -TCCGTA GGT GAA CCT GCG G-3′, ITS4: 5′ -TCC TCCGCT TAT TGA TAT GC-3′), 1x Dream Taq PCR master mix (Thermo Scientific, Lithuania), and nuclease-free water to complete reaction volume. PCR cycling condition was set as follows: initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and polymerization at 72°C for 45s, with final extension step at 72°C for 10min. PCR products were screened using 1.5% agarose gel electrophoresis at 70 volts until complete separation of bands. The size of amplification products of each isolate was recorded. An aliquot of 10µl of each ITS-amplicon was digested according to manufacturing instructions with the restriction enzyme *Msp*I (Thermo Scientific, Lithuania). Patterns of digestion fragments were screened using 3% agarose gel electrophoresis. Identification of the isolate to the species level was achieved according to both its ITS-amplicon size and its MspI-RFLP pattern as described by Mohammadi et al. (2013).

DNA Sequencing

Some isolates (No. = 8) were subjected for sequencing using their ITS-PCR products after being purified using the EZ-10 spin column DNA gel extraction kit (Bio Basic, Canada) according to the manufacturer's recommendations. Eluted DNA was sequenced in an automated ABI 3730 DNA sequencer (Applied Biosystems, USA). ABI sequence files were analyzed using MEGA5 (The Biodesign Institute, Tempe, AZ, USA,

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Tamura et al. 2011) by cutting out 5′ and 3′ regions of high background noise. Consensus sequences were identified using NCBI's Nucleotide BLAST.

Determination of Virulence Determinants

Detection of Slime by Congo Red Agar (CRA) Method

Molecularly identified isolates (n=67) were evaluated for their ability to produce slime by the Congo red agar method (Türkyılmaz and Kaynarca 2010) according to the "Congo red phenomenon". Isolates that produced dark rough red colonies were regarded as slime positive, whereas those appeared as smooth pink or white colonies were considered as slime negative.

Determination of Biofilm Formation

Molecularly identified isolates were evaluated for biofilm formation using the microtiter plate test according to Dubravka et al. (2010). Each strain was tested 4 times and biofilm production quantities were reported as the arithmetic means of absorbance values of the 4 replicate tests. Cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains were interpreted as follows: non-biofilm producers (OD ≤ ODc); weak biofilm producers (ODc<OD ≤ 2 × ODc); moderate biofilm producers (2 × ODc<OD ≤ 4 ×ODc) and strong biofilm producers (4 × ODc<OD).

Antifungal Susceptibility Testing

The antifungal susceptibility of 30 representative isolates was determined by the agar disc diffusion method according to the guideline of the Clinical Laboratory Standards Institute for antifungal susceptibility (Fothergill 2012). Eight antimicrobial agents were used including miconazole (MIC; 50µg/disk), ketoconazole (KT; 15µg/disk), amphotericin B (AP; 100units/disk), itraconazole (IT; 10µg/disk), nystatin (NS; 100units/disk), fluconazole (FLC; 25µg/disk), fusidic acid (FC; 10µg/disk) and voriconazole (VRC; 1µg/disk). Results were recorded according to antifungal zone diameters.

RESULTS

Isolation and Identification

As summarized in Table 1, 31.6% out of 405 collected quarter milk samples were positive for primary yeast isolation on SDA. Out of these 128 isolates, 93% revealed single pure culture while 7% were mixed yeast cultures. All the yeast isolates were subjected to conventional tests as shown in Fig. 1 (a-f).

Identification using HiCrome *Candida* Agar-medium

Identification of the 128 yeast isolates was performed based on HiCrome *Candida* agar-medium. According to culture appearance, 70.3% of the isolates showed a single colony cultures while 29.7% showed mixed colony cultures. Table 2 summarized the species of isolated yeast, their colony characteristics on HiCrome *Candida* agar medium, and their prevalence. Fig. 2a to Fig. 2i displayed the characteristic colony morphology of different *Candida* spp. on HiCrome *Candida* agar. Mixed *Candida* spp. infections were also detected as appeared in Fig. 3a to Fig. 3c.

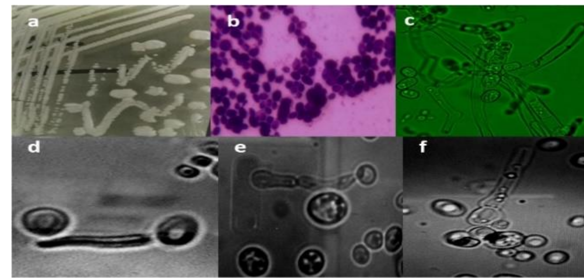

Fig. 1: Characteristic morphology of some yeast spp. a: Growth appearance of yeasts isolated from subclinical mastitis cases on SDA. b: Appearance of Gram-positive staining of the isolated yeast colonies under light microscope X100. c: Wet preparation of *C. krusei* showing the characteristic pseudohyphae with blastoconidia forming cross-matchstick appearance under light microscope 100X. (d, e, f): Wet preparations showed true germ tube formation (d; *C. albicans*) and early pseudo-hyphal extension with constriction that may be falsely interpreted as germ tubes (e and f; *C. tropicalis*) of different yeasts when grown in human serum under light microscope 100X.

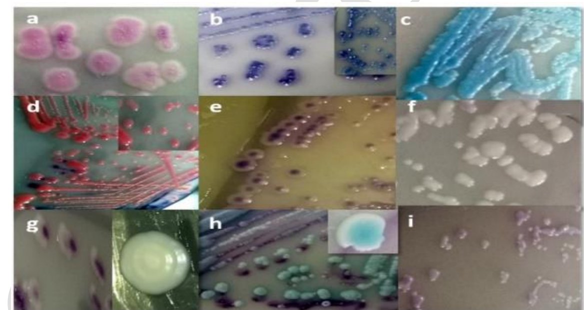

Fig. 2: The characteristic colony morphology of different yeast spp. on HiCrome *Candida* agar medium. a: pale pink colonies of *C. krusei.* b: metallic blue colonies of *C. tropicalis.* c: light green colonies of *C. albicans.* d: rose pink colonies of *C. parapsilosis.* e: lilac colonies of *C. galabrata* f: white colonies of *C. kefyr*. g: white to light pink colonies of *C. famata* on HiCrome *Candida* agar (left) versus its button-like colony on SDA (right). h: greenish pasty colonies of *C. rugosa*. i: purple colonies of *C. guilliermondii.*

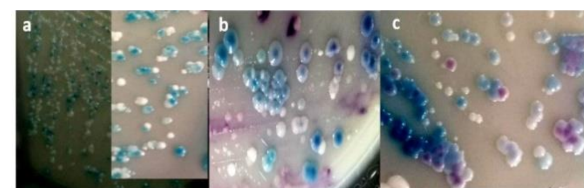

Fig. 3: Mixed infection of different *Candida* spp. as displayed on HiCrome *Candida* agar medium a: Mixed infection of *C. albicans* (green colonies) and *C. kefyr* (white colonies) b: Mixed infection of *C. albicans* (green colonies) and *C. tropicalis* (metalic blue colonies). c: Mixed infection of *C. albicans* (green colonies), *C. galabrata* (pink colonies) and *C. tropicalis* (metalic blue colonies).

Table 1: Percentages of yeasts associated with bovine mastitis and number of pure and mixed culture using different methods.

Total No. of samples	405	
No of yeast positive samples (%)	128 (31.6)	
Methods	Purity of culture	
	Pure culture No (%)	Mixed culture No. (%)
SDA	119 (93)	9 (7)
HiCrome Candida agar	90 (70.3)	38 (29.7)
ITS-PCR	90 (70.3)	38 (29.7)

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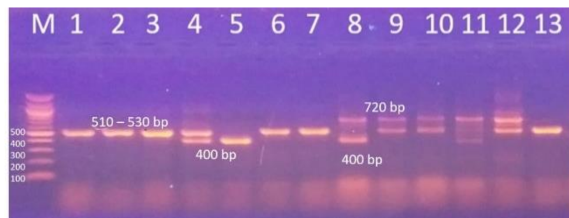

Fig. 4: ITS-PCR products of representative Yeast isolates showing both single and multiple amplicons. M: 100 bp ladder DNA marker, Lanes 1-3, 5-7, 13: single amplicons, Lanes 4, 8-12: multiple amplicons due to mixed infection.

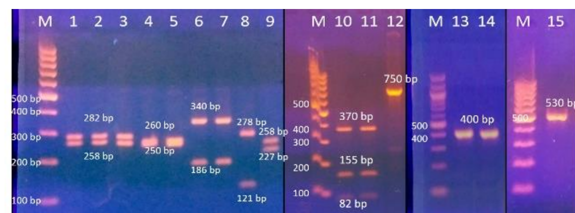

Fig. 5: Agarose gel electrophoresis of ITS-PCR products of different Yeast isolates after digestion with *MspI*. M: 100 bp ladder DNA marker, 1-3: *T. asahii* (258&282 bp), lanes 4-5: *C. krusei*, Lanes 6-7: *C. tropicalis*, Lane 8: *C. rugosa*, Lane 9: *C. norvegenesis*, Lanes 10-11: *C. guilliermondii*, Lane 12: group II (750 bp): not identified, Lanes 13-14: group I (400 bp): not identified, Lane 15: *C. parapsillosis* complex.

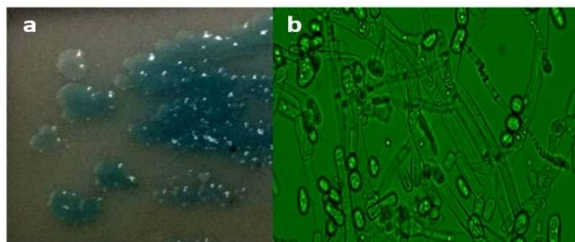

Fig. 6: Characteristic colonies morphology and wet preparation of *T. asahii*. a: Green Colony appearance of *T. asahii* that lead to its misidentification as C. *albicans* on HiCrome *Candida* agar. b: Wet preparation of *T. asahii showing the* characteristic development of hyaline, septated hyphae that fragment into oval or rectangular arthroconidia (light microscope 100 X).

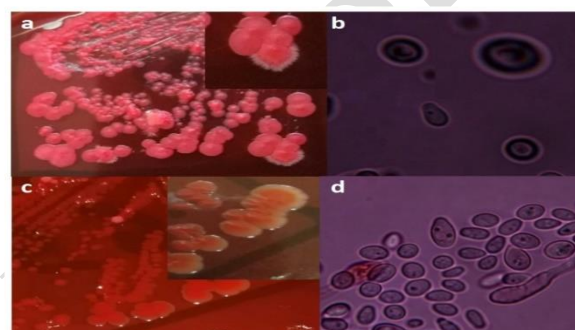

Fig. 7: The characteristics morphology of some yeast spp. on Congo red agar and after staining with Indian ink a: Rough red colonies with filamentous pseudo-hyphal growth of *T. asahii* (resemble *C. albicans*) on red background of Congo red agar. b: Wet preparation of slime producing *Candida* spp. stained with Indian ink showed exopolysaccharide layer around the cell wall as examined under light microscope (X100). C: Smooth red colonies of non-slime producer *Candida* species (*C. rugosa*) Congo red agar media. d: wet preparation of non-slime producer *Candida* species stained with Indian ink showed the absence of exopolysaccharide layer around the cell wall examined under light microscope X100.

Table 2: Isolated Yeast species and their colony characteristics on HiCrome Candida agar-medium

Species of yeast	Colony characteristics	No.	%*
Single Candida infection (No.=90)			
C.krusei	Large, flat, spreading, pale pink colonies with matt surfaces and the characteristic fringe.	17	13.3
C. tropicalis	Metallic blue colored colonies.	17	13.3
C. albicans	Light green colonies.	17	13.3
C. parapsilosis	Rose pink smooth large colonies.	13	10.2
C. galabrata	Glossy pale pink to lilac colonies.	9	7
C. kefyr	White to light violet colonies.	7	5.5
C. famata	White to light pink colonies.	4	3.1
C. rugosa	Pale white to greenish pasty colonies	4	3.1
C. guilliermondii	Small pink to purple colonies.	2	1.5
Mixed Candida infection (No.=38)			
C. galabrata+ C. tropicals.	Lilac and metallic blue colored colonies	10	7.8
C. famata+ C. parapsilosis.	Light violet and rosy colonies.	10	7.8
C. albicans+ C. kefyr.	Green and white colonies.	8	6.3
C. albicans+ C. tropicals.	Green and metallic blue colonies.	5	3.9
C. albicans+ C. tropicalis+ C. galabrata.	Green, metallic blue and lilac colonies.	5	3.9

*Percentages were calculated in proportion to total number of isolates (128).

Molecular Identification using ITS-PCR-RFLP

Based on ITS-PCR results, out of the 128 isolates, 90 produced a single positive PCR product while 38 isolates produced multiple PCR products as displayed in Table 1 and Fig. 4. Only the 90 isolates that produced single band were subjected for species identification by its RFLP. Considering the digestion patterns, 67 out of the 90 pure isolates produced previously reported digestion patterns and therefore were clearly identified to the species level. On the other hand, 23 isolates yielded new digestion pattern and could not be identified to the species level according to previously reported PCR-RFLP results. They were classified according to their amplicon size and digestion products into 2 groups named: I (n=22; 24.4%), П (n=1; 1.1%). Table 3 summarized the results of the ITS-PCR-RFLP method. It also declared the numbers and percentages of each identified species. Fig. 5 showed the digestion pattern of representative isolates.

Comparison between Identification by using HiCrome *Candida* Agar Media and ITS-PCR-RFLP Method

The difference between the identification of yeast isolates on species level between HiCrome *Candida* agar medium and PCR-RFLP methods was obvious. Out of the 90 studied isolates, the PCR-RFLP results of 47 isolates confirmed their HiCrome results. On the other side, 43 isolates showed deviation between their PCR-RFLP and HiCrome *Candida* agar results. Table 4 showed the original identification of these 43 isolates by HiCrome *Candida* agar method versus their molecular identification by ITS-PCR-RFLP. Fig. 6a and Fig. 6b showed the characteristic colony morphology of *T. asahii* on the HiCrome agar medium which was very similar to the reported morphology of *C. albicans* on HiCrome *Candida* agar.

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Table 3: Identification of pure yeast isolates using ITS-PCR-RFLP method and their ITS-product size and *MspI* digestion patterns.

No. of examined isolates	Obtained digestion patterns	No.	Yeast species	ITS-PCR product size	*Msp I* digestion fragments	No.	%*
90	Previously reported patterns	67	*C. krusei*	510	250, 260	16	17.8
			C. tropicalis	526	186, 340	9	10
			C. albicans	-	-	0	0
			C. parapsilosis	510/530	510/530	13	14.4
			C. galabrata	881	320, 561	0	0
			C. kefyr	720	720	8	8.9
			C. famata	639	639	4	4.4
			C. rugosa	399	121, 278	8	8.9
			C. guilliermondii	607	82, 155, 370	2	2.2
			C. norvegenesis'	493	8, 227, 258	1	1.1
			T. asahii	540	258, 282	6	6.7
	New patterns	23	Group I Not identified	400	400	22	24.4
			Group II Not identified	750	750	1	1.1

*Percentages were calculated in proportion to total number of pure isolates (90).

Table 4: Comparison between identification of *Yeast species* using both HiCrome candida agar and ITS-PCR-RFLP.

Identification by Hi Crome Candida agar	No.	Identification by ITS-PCR-RFLP	No.
C. krusei	17	*C. Krusei*	13
		C. norvegenesis	1
		Group I (Not identified)	3
C. tropicalis	17	*C. tropicalis*	7
		C. krusei	1
		C. kefyr	1
		Group I (Not identified)	8
C. albicans	17	*C. albicans*	-
		C. krusei	1
		C. tropicalis	2
		C. rugosa	3
		C. kefyr	1
		Group I (Not identified)	4
		T. asahii	6
C. parapsilosis complex	13	*C. parapsilosis complex*	13
C. galabrata	9	*C. rugosa*	3
		Not identified	6
C. kefyr	7	*C. kefyr*	6
		Group II (Not identified)	1
C. famata	4	*C. famata*	4
C. rugosa	4	*C. rugosa*	2
		C. krusei	1
		Group I (Not identified)	1
C. guilliermondii	2	*C. guilliermondii*	2

Confirmation of Some Yeast Isolates by Sequencing

PCR products of the ITS region of some isolates were sequenced and the phylogenetic closest neighbors were determined. It included the 4 isolates that were molecularly identified as *T. asahii* and the other 4 isolates that couldn't be identified including 3 from group I and 1 of group II. The species of *T. asahii* was confirmed by blast search and the sequences of 3 of them were successfully submitted to GenBank with accession numbers MT953196, MT953197 and MT953618. Isolates of group I were not similar and submitted to GenBank database as *Yarrowia lipolytica*, *Wickerhaiella pararugosa* and *Candida catenulate* with the following accession numbers: MT953447, MT953577, MT953617, respectively while the isolate of group II was identified as *Kluyveromyces lactis* withMT966794.

Evaluation of Virulence Determinants

Slime Production and Biofilm Forming Ability

As clarified in Table 5, 83.6% out of 67 isolates were slime producers (Fig. 7a and 7b) while 16.4% were non-slime producers (Fig. 7c and 7d). A total of 80.6% of isolates were able to produce biofilm ranged from strong (6%), moderate (14.9%) to weak (59.7%) biofilm producers. Only 19.4% of the isolates were recorded as non-biofilm producers. Table 5 enumerated the number of biofilm producers amongst each species.

In Vitro Antifungal Susceptibility

In vitro antifungal susceptibilities of 30 yeast isolates against 8 antifungal agents were presented in Table 6. It included the susceptibility of randomly selected 6 isolates of each of the subsequent species: *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr,* and *T. asahii*. Isolates were recorded as either sensitive or resistant to each antifungal agent. None of the examined isolates was found to have an intermediate resistance.

DISCUSSION

Mastitis is one of the major economic diseases that distressing dairy cattle. In recent years, fungi have been frequently reported as a cause of mastitis (Erbaş et al. 2017; Mohammed and Yassein 2020). Several species of the yeasts of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporon* have been associated with mastitis in dairy cows (Akdouche et al. 2018). *Candida* is generally the most often isolated genus. Variation in both the prevalence and species identified was reported (Krukowski et al. 2006). The present study aimed to rapidly and accurately identify yeast species that might be associated with bovine mastitis. Out of 405 subclinical milk samples, 31.6% were positive for primary yeast isolation. Different rates of yeast isolation from the milk of cow suffering from mastitis were reported. Lower prevalence (29.54, 29.35, 23.44, and 22.1%) of yeast mastitis infections were displayed by Akdouche et al. (2018), Costa et al. (2012), Du et al. (2018), Spanamberg et al. (2008) and Mbuk et al. (2016) in Algeria, Brazil, China, Brazil and Nigeria, respectively. Slightly higher yeasts and yeast-like fungi isolation rates were reported by Khalaf et al. (2021), Asfour et al. (2009), Bekele et al. (2019) and Zhou et al. (2013) with percentages of 47.2, 40.8, 38.18 and 35.6%, in Egypt, Ethiopia and China, respectively. High prevalence was debated by Costa et al. (2012). It has been attributed to insufficient milkers training, repetitive intra-mammary infusion, and poor teat hygiene prior to the intra-mammary infusion. Some authors ascribed differences in the distribution of yeast species to both animal species and geographical variations (Seker and Özenç 2011; Gohar et al. 2020).

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Table 5: Slime production and biofilm forming ability among the identified *Yeast species*.

Candida species	No.	Slime production		Biofilm forming ability			
		Positive No. (%)	Negative No. (%)	Strong No. (%)	Moderate No. (%)	Weak No. (%)	Non- No. (%)
C. krusei	16	16 (100)	-	-	2(12.5)	13(81.25)	1(6.25)
C. tropicalis	9	9 (100)	-	1(11.1)	2(22.2)	4(44.5)	2(22.2)
C. parapsilosis complex	13	10 (76.9)	3 (23.1)	2(15.4)	2(15.4)	6(46.1)	3(23.1)
C. Kefyr	8	7 (87.5)	1 (12.5)	-	-	3(37.5)	5(62.5)
C. famata	4	3 (75)	1 (25)	1(12.5)	-	3(75)	-
C. rugosa	8	6 (75)	2 (25)	-	1(12.5)	5(62.5)	2(25)
C. guilliermondii	2	1 (50)	1 (50)	-	1(50)	1(50)	-
C. norvegenesis	1	1(100)	-	-	-	1(100)	-
T. asahii	6	3 (50)	3 (50)		2(33.3)	4 (66.7)	-
Total	67	56 (83.6)	11(16.4)	4 (6)	10(14.9)	40(59.7)	13(19.4)
				54 (80.6)			

Table 6: Antifungal susceptibilities of representative *Yeast species*.

Species	No	Antifungal discs*															
		MIC		KT		AP		IT		NS		FLC		FC		VRC	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
C. krusei	6	2	4	6	-	6	-	6	-	6	-	6	-	-	6	6	-
C. tropicalis	6	6	-	4	2	6	-	6	-	6	-	4	2	2	4	6	-
C. kefyr	6	6		6	-	6	-	6	-	6	-	6	-	6	-	6	-
C. parapsilosis complex	6	2	4	4	2	6	-	6	-	6	-	6	-	-	6	6	-
T. asahii	6	6	-	6	-	6	-	6	-	6	-	6	-	4	2	6	-
Total No.	30	22	8	26	4	30	-	30	-	30	-	28	2	12	18	30	-
%		73.3	26.7	86.7	13.3	100	-	100	-	100	-	93.3	6.7	40	60	100	-

*MIC (miconazole, 50μg/disk), KT (ketoconazole, 15μg/disk), AP (amphotericin B, 100units/disk), IT (itraconazole, 10μg/disk), NS (nystatin, 100units/disk), FLC (fluconazole, 25μg/disk), FC (fusidic acid, 10μg/disk) and VRC (voriconazole, 1μg/disk).

The traditional methods used for yeasts differentiation in veterinary laboratories are so exhausting and consuming a long time (Imran et al. 2020). So, reducing time is the most important argument required for their identification. Therefore, HiCrome *Candida* agar was used for faster standing on the species level of yeasts associated with bovine mastitis. Based on the appearance of culture morphology on HiCrome *Candida* agar media, about 70.3% was a single yeast infection and 29.7% was a mixed infection. This was confirmed by further identification by ITS-PCR. Mixed yeast infection of *C. albicanis* and *C. krusei* in bovine mastitis was also reported (Asfour et al. 2009).

With the developments of molecular biology, many DNA-based techniques have been developed for the identification of yeast species (Mirhendi et al. 2006; Fadda et al. 2013; Mohammadi et al. 2013; Erbas et al. 2017). In our study, a one-enzyme PCR-RFLP assay developed by Mirhendi et al. (2006) was used for identification of yeast isolates. It's based on the use of fungal-specific universal primer pair ITS1 and ITS4 to amplify the internal transcribed spacer region (ITS) of all yeast species followed by restriction fragment length polymorphism for species identification. Molecular identification of yeast isolates revealed that 67 out of the 90 pure isolates produced known reported digestion patterns and therefore were clearly identified to the species level while 23 isolates produced new digestion patterns and so couldn't be identified to the species level.

Because of HiCrome *Candida* agar medium was not originally developed for the identification of *Trichosporon spp*. Therefore, some isolates molecularly identified as *T. asahii* were subjected for sequence analysis and BLAST search of the ITS gene sequences. They were confirmed as *T. asahii* and their sequences were submitted to GenBank with the following accession numbers MT953196, MT953197, and MT953618.Also, representative of isolates displayed new patterns were sequenced. The three isolates representative of the group I, were not similar and were identified as; *Yarrowia lipolytica*, *Wickerhaiella pararugosa*, *Candida catenulate,* and their sequences were uploaded in the GenBank with accession numbers as MT953447, MT953577, MT953617, respectively. Finally, the isolate of the group II was identified as *Kluyveromyces lactis* with accession number MT966794.

In our study, HiCrome *Candida* agar enabled the growth of *T. asahii* with colony characteristics resembling those of *C. albicans.* This was observed by PCR-RFLP and confirmed by the sequencing results. Fadda et al. (2013) reported the preliminary identification of the genus *Trichosporon*, by API to be problematic. On the contrary, they reported that the RFLP technique gave characteristic restriction profiles for each species; moreover, sequencing allowed not only successful identification of certain spp. of *Trichosporon* where API could not, but also correct identification of misidentified isolates. Nadăş et al. (2014) and Khalaf et al. (2021) held a comparison between phenotypic methods (Chromatic *Candida* and API 20 CAUX) and a PCR-RFLP analysis for the identification of *Candida* strains. They found that the Chromatic *Candida* test is designed for the isolation and identification of some *Candida* species while other species were difficult to be distinguished by this method; this was attributed to the close similarity of the colonies morphology of some *Candida* species and therefore, they recommended the genotypic method for the identification of *Candida* spp. that agreed with our results.

Regarding the comparison between the results of both, HiCrome *Candida* agar medium and PCR-RFLP methods, the difference was obvious as cleared in Table 4. Out of 90 isolates, 43 showed deviation between their PCR-RFLP results and HiCrome results. These discrepancies may be attributed to the physical individual variation in

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discrimination of colors of the isolated colonies which is the cornerstone of species identification on the HiCrome agar. These differences were reported also by many authors who used different methods for confirmation of yeast species. Jain et al. (2012) recorded that chromogenic agar was certainly the more efficient method over Vitek 2 for identification of the most commonly isolated species but both methods were disabled for identification of some species. Meanwhile, Fadda et al. (2013) mentioned that traditional yeasts identification by morphological and physiological criteria was strongly influenced by culture conditions and hence could give uncertain results. On the contrary, they reported the PCR-RFLP analysis to be one of the most successful methods for yeast identification.

Consequent to the previous findings, we have depended on the accurate results of the PCR-RFLP assay and sequencing technique. It was noticed that non-*albicans Candida* (NAC) species were the most frequently isolated *Candida* species among mastitis-causing yeast in this study. Also, other yeast genera such as *Trichosporon* spp. were involved in bovine mastitis. This was agreed with Zhou et al. (2013), Erbaş et al. (2017) and Akdouche et al. (2018). In the current study, the most identified yeast species were *C. krusei* and *C. parapsilosis* complex (17.8 and 14.4%, respectively) followed by *C. tropicalis*, *C. kefyr*, *C. rugosa,* and *T. asahii* (10, 8.9, 8.9, and 6.7%, respectively). These results resembled that of Du et al. (2018) and Namvar et al. (2020) who reported that *Candida* spp. other than *C. albicans* played a pathogenic role in cow's mycotic mastitis. *C. krusei* was the predominant species isolated and *C. parapsilosis* was the second most frequent *Candida* in their study. Nearly similar results of *C. krusei* (18.18; 17.4 and 19.8%) were also reported by Ruz-Peres et al. (2010), Erbas et al. (2017) and Namvar et al. (2020), respectively. Considering *C. parapsilosis* complex, many studies have described their isolation from milk samples with varied frequencies from 0.57 up to 25.4% and reached to 33.4 % in recent study, depending on the sanitary condition and environmental factors (Zaragoza et al. 2011; Sartori et al. 2014; ErbaŞ et al. 2017; Akdouche et al. 2018; Hussein et al. 2019). In our study, the highest isolation rates of both *C. krusei* and *C. parapsilosis* complex among NAC species could be attributed to the inadequate sanitary practices of milking procedures and the intrinsic natural resistance of *C. krusei* to fluconazole (Freydiere et al. 2001). Moreover, the relative cell-surface hydrophobicity (CSH) of *C. parapsilosis* adherence to host tissues and plastic surfaces such as milkers or other prosthetic materials is another cause of the high rate of isolation (Panagoda et al. 2001; Kuhn 2002; Trofa et al. 2008). Silage was also reported as a source of lactate assimilating yeasts known to cause mastitis especially *C. krusei* (Elad et al. 1995).

Noteworthy, neither *C. albicans* nor *C. glabrata* were identified among the identified yeast isolates as single yeast infection. Likewise, similar results were also recorded in previous studies (Zaragoza et al. 2011; ErbaŞ et al. 2017; Du et al. 2018; Namvar et al. 2020); they couldn't isolate *C. albicans* from milk samples collected from cows with subclinical mastitis. This could be explained to some extent to the presence of a greater number of bacteria competing for substrates in subclinical mastitis. Also, some bacterial metabolites are known as yeast antagonists and consequently could restrict some yeast development (Moretti et al. 1998).

Back to our results, *C. tropicalis*, *C. kefyr* and *C. rugosa* were also isolated by different rates (10, 8.9, and 8.9%, respectively) from cow's mastitic milk. Erbaş et al. (2017) isolated the same NAC spp. but with higher frequencies. Also, Hussein et al. (2019) recorded higher rate of *C. tropicalis* isolation (22.7%) from mastitic cattle. Meanwhile lower isolation rates were obtained by Türkyılmaz and Kaynarca (2010). The lowest yeast species isolations in the current study were *C. famata*, *C. guilliermondii* and *C. norvegenesis* (4.4, 2.2, and 1.1%, respectively). Similar results were identified by Türkyılmaz and Kaynarca (2010) for *C. guilliermondii* (4.9%) and *C. famata* (2.4%). Recently, Akdouche et al. (2018) reported *C. guilliermondii* (12.5%) and *C. famata* (1.7%) in their study.

Considering *T. asahii,* it was isolated with a percentage of 6.7%. Spanamberg et al. (2008) also identified *Trichosporon* (4.4%) from cows suffered from subclinical mastitis. Türkyılmaz and Kaynarca (2010) and Akdouche et al. (2018) identified only 2.4, and 1.13%, respectively of *T. asahii* from cows suffered subclinical mastitis. Moreover, Zhou et al. (2013), Bekele et al. (2019) and Lavaee et al. (2020) recorded *Trichosporon* spp. in percentages of 5.9, 3.2, and 0.8%, respectively from mycotic mastitis in dairy cows. A higher isolation rate of *Trichosporon* spp. (19.7%) was reported by Fadda et al. (2013). Spanamberg et al. (2018) reported the presence of *Trichosporon* spp. in raw milk to be of great concern as their enzymatic activities may alter milk quality and constituents. Also, it creates a hazard where some species of this genus have been reported as both human and animal's pathogens.

Based on the sequencing results and regardless of their isolation rate, *Diutina (Candida) catenulate, Wickerhamiella (Candida) pararugosa, Yarrawia (Candida) lipolytica* were confirmed by sequencing to be isolated. Similarly, Akdouche et al. (2018) isolated *C. lipolytica* form mastitic cow's milk with a low percentage (1.13%) while Czernomysy-Furowicz et al. (2008) isolated *C. catenulate* with a very low percentage (0.81%). A higher percentage of *Yarrawia lipolytica* (10.9%) was recorded lately by Bekele et al. (2019) from mastitic dairy cows. Therefore, the involvement of these species in bovine mastitis needs to be more investigated.

Because of the paucity of data concerning the virulence factors of yeast species isolated from bovine milk, investigating the ability to produce slime, form biofilm as well as, antifungal sensitivity of the isolated yeast species was performed in this study. According to our knowledge, this is the first study to investigate these criteria among yeast species isolated from animal sources specifically those associated with bovine mastitis in Egypt.

Regarding slime production, our results revealed that a high percentage (about 83.6%) of the identified isolates were slime producers while only (16.4%) were not. Notably, all strains of *C. krusei* and *C. tropicalis* were slime producers. Lower percentage was reported by Türkyılmaz and Kaynarca (2010) who found that only 36.6% of their yeast strains were slime producers. Moreover, they reported that most of non-*albicans Candida* species were slime producers which settled with our results. Krukowski et al. (2010) demonstrated that 22.34% of the *Candida*

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strains were slime producers. Specifically, the majority (83.33%) of *C. krusei* were capable of slime formation while other NAC species (*C. rugosa* and *C. guilliermondii*) did not form slime.

The ability to form biofilm represents a fundamental role in the pathogenicity of *Candida* spp. through evading the host immune systems, resisting antifungal therapy, and enduring the competitive pressure from other pathogens (Lahkar et al. 2017). It is considered as a pointer for the development of the infection (Mohammed and Yassein 2020). In this study, about 80.6% of the examined yeast isolates had the ability to form biofilm ranging from strong (6%), moderate (14.9%) to weak (59.7%) degree, while 19.4% were non-biofilm producers. Notably, 15.4% of *C. parapsilosis* complex, 12.5% of *C. rugosa,* and 11.1% of *C. tropicalis* were strong biofilm producers. The most isolated yeast species that showed moderate biofilm production were *C. guilliermondii* (50%), *T. asahii* (33.3%), *C. tropicalis* (22.2%), *C. parapsilosis* complex (15.4%), *C. krusei* (12.5%) and *C. rugosa* (12.5%). More than half of the yeast isolates were weak biofilm producers (62.2%). A higher percentage (94.1%) of biofilm producers among yeast spp. isolated from animal origins was detected by Cordeiro et al. (2015). In accordance with our results, Brilhante et al. (2014) found that all tested strains of *C. parapsilosis* complex isolated from animals were able to form biofilms and strains from animals produced more biofilm when compared with human isolates. Other studies reported that *C. tropicalis* isolates from animals displayed a greater biofilm formation than *C. albicans* (Chatzimoschou et al. 2011; Estivill et al. 2011). Our result and the limited previous studies on biofilm-forming capability of NAC clearly exposed that these isolates had a high capacity to form biofilms. This could provide resistance to antifungal drugs and could induce mastitis which may be difficult to be treated on the long run. Further, it may represent a potential threat to human and animal health.

Considering the *in vitro* antifungal susceptibility, a detailed antifungal resistance was investigated. On the level of tested isolates (n=30), the most effective antifungal drugs were AP, IT, NS, and VRC as all isolates were 100% sensitive to all of them. On the other hand, the least effective anti-mycotic agents were FC, MIC, KT, and FLC because 60, 26.7, 13.3, and 6.7% of the isolates were resistant to them, respectively. The obvious sensitivity of the tested NAC strains may be attributed to the uncommon use of anti-mycotic preparation in the treatment of bovine mastitis in Egypt. This came in agreement with Asfour et al. (2009) who found high sensitivity rates to the used anti-mycotic drugs in their study. In another study presented by Sonmez and Erbas (2017), they found KT the most effective antifungal agent that can be used for treatment of yeast mastitis and they addressed the development of antifungal resistance in their literature. Natural intrinsic resistances to some antifungals were reported like Gunes et al. (2001) who confirmed natural resistance to fluconazole in both *C. krusei* and *C. glabrata.* Our finding supported this literature because 100 % resistance to fluconazole was noticed in both *C. krusei* and *C. parapsillosis* complex. The differences in the resistance rates between isolates were noticed. Therefore, when yeasts are identified as the cause of mastitis, selection of the appropriate antifungal for treatment is mandatory. Milanov et al. (2014) revealed that the crucial agents in the etiology of bovine mastitis are non-*albicans* species of the genus *Candida*. Tanwani and Yadava (2006) recommended nystatin as intramammary or amphotericin B given intravenously for the treatment of mycotic mastitis. The increase of Imidazole resistance among fungi may be the cause of treatment failure of bovine mycotic mastitis with this preparation (Krukawski and Lisowsko 2007).

Conclusions

Yeast mastitis in dairy cows demonstrates an increasing problem. Therefore, diagnosis must be relied on accurate laboratory identification of the causative agent. The use of the ITS-PCR-RFLP method was confirmed to provide accurate identification of *Candida species*. The importance of NAC and their progressed role in the pathogenesis of mycotic mastitis should be highlighted by studying their virulence factors as a routine work in the veterinary laboratories. The involvement of yeast species from genera other than genus *Candida* in bovine mastitis has to be well investigated.

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Authors' Contributions

Hanaa AE Asfour and Samah F Darwish designed the research; Hanaa AE Asfour, Rasha H Eid and Safaa A. Elwakeel performed the experiments of isolation, identification and virulence determination. Samah F Darwish and Tahani S Behour performed the experiments concerning the molecular identification. Hanaa AE Asfour drafted the paper. Samah F Darwish revised, edited and published the manuscript. All the authors revised the manuscript prior to submission for publication.

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