

ORIGINAL ARTICLE

Recovery Rate of Fungal Pathogens Isolated from Cases of Bovine and Ovine Mycotic Mastitis

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Abstract

Investigating bovine and ovine mycotic mastitis was the study's goal. A total of 250 milk samples were collected from cows, ewes and does suffering from mastitis, not responding for antibiotic therapy, to determine the prevalence of fungal pathogens associated with mastitis. For the isolated yeasts, antimicrobial susceptibility testing was done through disc diffusion method, and biofilm formation was evaluated phenotypically by cultivation on Congo Red Agar (CRA) medium. Also, *MDR1*, *CDR* and *ERG11* genes were detected by PCR. Out of the collected milk samples, 53 samples (21.2%) were positive for the presence of fungal pathogens. Out of them, 54 fungal pathogens were recovered, including 49 *Candida* species, 2 *A. niger*, in addition to one of *A. fumigatus*, *A. flavus* and *Penicillium* species. The yeast isolates included 18 *C. guilliermondii*, 16 *C. parapsilosis*, 7 *C. tropicalis*, 4 *C. albicans*, and 4 *C. kefyr*. Antimicrobial susceptibility testing of *Candida* isolates showed that all isolates were resistant to nystatin. Regarding to fluconazole, 25 isolates were sensitive, 2 isolates were intermediately sensitive and 22 isolates were resistant. Nine yeast isolates were biofilm former. All tested isolates harbored *MDR1* gene, 4 isolates contained *CDR* gene and only 1 isolate was positive for *ERG11* gene.

Keywords

Biofilm, Mastitis, Molds, Resistance genes, Yeasts

1. Introduction

Mastitis is frequently brought on by a wide range of microorganisms, which results in significant economic consequences and impairment to the dairy processing industry (Bakr et al., 2015). The use of injectable tools, cannulas, or antibiotic preparations (intramammary infusions) during the treatment of animal disorders is frequently linked to mycotic mastitis. Teat injuries may also increase the risk of the development of fungal infection (Frag and El Said, 2012).

Yeasts are typically the cause of mycotic mastitis; however sporadic incidences of *Aspergillus* species-related mastitis, which affect a limited number of animals, have been described (Abd El Razik et al., 2011).

Numerous species of yeasts have been stated to cause mastitis (Staroniewicz et al., 2007). *Candida albicans* and *Cryptococcus neoformans* are definitely the most common causes of mycotic mastitis, but other *Candida* species have also been associated with bovine mastitis (Dworecka-Kaszak et al., 2012).

Recently, efflux pump transporters (*ABC1*, *ABC2*), 14-demethylases, aspartyl proteinases (*SAPPI* and *SAPP2*), ATP-binding cassette (*ABC*) transporters, *Candida* drug resistance gene (*CDR*), major facilitator superfamily transporter (*MFS*), multi-drug resistance gene (*MDR*), and others have been identified in *C. parapsilosis* and *C. krusei*. These elements favor the development and survival of *Candida* species in the mammary glands of cows (Du et al., 2018).

Fluconazole, an antifungal from the azole class, is the most often prescribed medication used to treat most *C. albicans* infections. Azoles block the activity of the *ERG11* gene-encoded enzyme 14-sterol demethylase, which is necessary for the manufacture of the membrane ergosterol, which is unique to fungi (Whaley et al., 2017).

The cells that make up the biofilms organize themselves into structured communities which are submerged in an extracellular matrix. Typically, the composition of biofilm matrix consists of water, phosphate, proteins, carbohydrates, glucose, and hexosamines. The *Candida* species can more easily adhere to the tissue and colonize forming biofilms

which also protect the microbe from the host's defenses. Additionally, because *Candida* biofilms are more resistant to antifungal treatment, their development has significant clinical complications. Consequently, it is thought that biofilm development is crucial to the pathophysiology of candida infections ([Şeker and Erhan, 2011](#)).

Therefore, the aim of this study is to detect the recovery rate of fungal pathogens associated with bovine and ovine mycotic mastitis, resistance, virulence and molecular characterization of *Candida* species.

2. Materials and Methods

2.1. Ethical Approval

The Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) accepted this work and assigned it an approval number (Vet CU 01122022580) for animal sampling and handling for isolating the tested fungi.

2.2. Collection of Samples

A total of 250 mastitic milk samples were collected under complete aseptic conditions from clinically diseased cattle, sheep and goats (120 bovine and 130 ovine milk samples), not responding to treatment with antibiotics, for mycological examination. From January to October 2021, milk samples were collected from a variety of dairy farms in the Governorates of Beni-Suef and Fayoum. As soon as possible, the samples were carried in an ice box to the Faculty of Veterinary Medicine at Beni-Suef University's Bacteriology, Mycology, and Immunology laboratory.

2.3. Isolation and Identification of Fungi

Ten ml of milk was centrifuged at 3000rpm/15min, then the sediment was cultivated on Sabouraud's Dextrose Agar (SDA), which contained chloramphenicol (0.05g/l), then incubated aerobically at 25°C for 5 days ([Arther et al., 2004](#)).

Identification of isolated molds depended upon macroscopic examination (colors, consistency of growth on SDA and external general colors of their Petri dishes) ([Jawetz et al., 2004](#)) and microscopic examination by lactophenol cotton blue (LPCB) staining to observe the shape and structure of hyphae and spores ([Winn et al., 2006](#)). Yeasts were identified according to colony morphology, Gram staining as well as germ tube and chlamydospores production on new milk medium ([Pincus et al., 2007](#)).

2.4. Antimicrobial Susceptibility of the Isolated Yeasts

As per the M60 protocol's instructions, the standard disc diffusion technique was used to 2 antifungal drugs, including fluconazole and nystatin, 0.5McFarland standard turbidity isolate suspensions were made, and Mueller Hinton agar plates with chloramphenicol (0.05g/l) were inoculated. On the plates, fluconazole (25mg) and nystatin (100mg) antifungal discs were placed. According to the M60 procedure, the tested isolates were classified as sensitive, intermediately sensitive or resistant ([CLSI, 2020](#)).

2.5. Biofilm Formation of the Identified Yeast Isolates

According to [Sharma et al., \(2017\)](#), Congo Red Agar (CRA) medium was made with brain heart infusion broth 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo red 8 g/l. This medium was used to identify the production of biofilms by isolated yeasts. Prior to being added to the autoclaved brain heart infusion agar with sucrose, which is cooled at 55°C, Congo red stain was first made as a concentrated aqueous solution separately from the other medium constituents and autoclaved at 121°C for 15 min. The yeasts were inoculated onto CRA plates, and then incubated for 48 hour at 37°C. Positive biofilm production is indicated by black colonies with a dry crystalline consistency.

2.6. Detection of Resistance Genes of Yeast Isolates

Five *Candida* isolates (2 *C. albicans*, 2 *C. guilliermondii* and 1 *C. parapsilosis*), including the species that mostly associated with bovine and ovine mycotic mastitis phenotypically resistant to fluconazole, were chosen for genotypic characterization using PCR to identify the presence of several fluconazole-resistant genes, such as *CDR*, *MDR1*, and *ERG11* genes using their particular forward and reverse primers, as indicated in [Table \(1\)](#). DNA used as a positive control was taken from a *Candida* field isolate that tested positive for RLQP (Reference laboratory for veterinary quality control on poultry production, Dokki, Giza, Egypt). A PCR mixture devoid of the DNA template served as a negative control, on the other hand.

Table 1. Oligonucleotide primers used for amplification of fluconazole-resistance genes.

Gene	Sequence	(bp)	Reference
<i>CDR</i>	GTGGTGTTCGGTGGTGAAAGAAA	503	Henry et al. 2000
	CCTGGTGTGGATCGTTCACATTCA		
<i>MDR1</i>	GAGTCGTAGCTACATTGCCATTAAAC	590	Wang et al. 2015
	GGTGATTCTAATGGTCTCCATAATGT		
<i>ERG11</i>	CAAGAAGATCATACTCAAT	1641	Wang et al. 2015
	AGAACACTGAATCGAAAG		

3. Results

3.1. Prevalence and recovery rate of fungal pathogens

A total of 250 mastitic milk samples were collected from cows, ewes and does suffering from chronic mastitis that not responding for antibiotic treatment. Fifty three samples (21.2%) were positive for mycotic mastitis. Bovine mycotic mastitis recoded 12.5% and ovine mycotic mastitis recorded 29.2%.

Recovered isolates included 54 fungal pathogens, out of them 49 *Candida* species (90.7%), 2 *A. niger* (3.7%), 1 *A. fumigatus* (1.9%), 1 *A. flavus* (1.9%) and 1 *Penicillium* species (1.9%). *Candida* species included 18 *C. guilliermondii* (36.7%), 16 *C. parapsilosis* (32.7%), 7 *C. tropicalis* (14.3%), 4 *C. albicans* (8.2%) and 4 *C. kefir* (8.2%). Only one bovine sample was mixed with yeast and mold isolates (*C. kefir* and *A. fumigatus*) (Tables, 2-3).

Table 2. Prevalence of mycotic mastitis and recovery rates of the isolated yeasts and molds.

	No. of samples	Positive mycotic mastitis		Yeasts		Molds	
		No.	%	No.	%	No.	%
Bovine	120	15	12.5%	14	11.7%	2	1.7%
Ovine	130	38	29.2%	35	26.9%	3	2.3%
Total	250	53	21.2%	49	19.6%	5	2%

Table 3. Recovery rates of the isolated fungal species and percentage of biofilm former isolates.

Strain	Bovine		Ovine		Total		Biofilm former	
	No.	%	No.	%	No.	%	No.	%
<i>C. guilliermondii</i>	4	26.7	14	36.8	18	34	1	5.6
<i>C. parapsilosis</i>	2	13.3	14	36.8	16	30.2	4	25
<i>C. tropicalis</i>	3	20	4	10.5	7	13.2	1	14.3
<i>C. albicans</i>	3	20	1	2.6	4	7.6	3	75
<i>C. kefir</i>	2	13.3	2	5.3	4	7.6	0	0
<i>A. niger</i>	0	0	2	5.3	2	3.8	--	--
<i>A. fumigatus</i>	1	6.7	0	0	1	1.9	--	--
<i>A. flavus</i>	1	6.7	0	0	1	1.9	--	--
<i>Penicillium spp.</i>	0	0	1	2.6	1	1.9	--	--

3.2. Antimicrobial Susceptibility of the Recovered Candida Isolates

The antimicrobial susceptibility testing of *Candida* isolates ($n = 49$) showed that all isolates were resistant to nystatin.

3.4. Biofilm Formation of Candida Isolates on CRA Medium

Out of the total tested *Candida* isolates ($n=49$), 9 *Candida* isolates (18.4%) were grown as black colonies with a dry

crystalline consistency on CRA medium and described as biofilm formers. While, 40 *Candida* isolates (81.6%) were grown as red colonies and described as non-biofilm formers, as illustrated in Table (3) and Fig. (2).

Regarding fluconazole, 25 isolates (51.02%) were sensitive, 2 isolates (4.1%) were intermediately sensitive and 22 isolates (44.9%) were resistant (Table, 4 and Fig. 1).

Table 4. Antimicrobial susceptibility of different *Candida* isolates ($n = 49$).

	<i>C. albicans</i>		<i>C. parapsilosis</i>		<i>C. guilliermondii</i>		<i>C. tropicalis</i>		<i>C. kefir</i>		Total	
	No	%	No	%	No	%	No	%	No	%	No	%
S	1	2.04	10	20.4	11	22.5	2	4.1	1	2.04	25	51.02
I	--	--	1	2.04	1	2.04	--	--	--	--	2	4.1
R	3	6.12	5	10.2	6	12.3	5	10.2	3	6.1	22	44.9

S: Sensitive, I: Intermediate, R: Resistant.



Fig. 1. Antimicrobial susceptibility testing of *Candida* showing sensitivity to fluconazole and complete resistance to nystatin.



Fig. 2 Cultivation of *Candida* isolates on CRA medium. **Right side:** *Candida* colonies appeared black with dry crystalline consistency (biofilm positive). **Left side:** *Candida* colonies seemed to be red on CRA medium (biofilm negative).

3.5. Detection of antimicrobial resistance genes of *Candida* isolates

Five *Candida* isolates including 2 *C. albicans* and 2 *C. guilliermondii* and 1 *C. parapsilosis* were tested using PCR for the detection of *CDR*, *MDR1* and *ERG11* genes. All tested

isolates harbored *MDR1* gene (100%), 4 isolates contained *CDR* gene (80%) and only 1 isolate (20%) was positive for *ERG11* gene (Table 5 and Figs. 3, 4).

Table 5. Fluconazole-resistance genes of *Candida* isolates.

Gene	<i>C. albicans</i>		<i>C. guilliermondii</i>		<i>C. tropicalis</i>	(%)
	1	2	3	4	5	
<i>CDR</i>	+	+	+	+	+	100
<i>MDR1</i>	+	+	-	+	+	80
<i>ERG11</i>	-	+	-	-	-	20

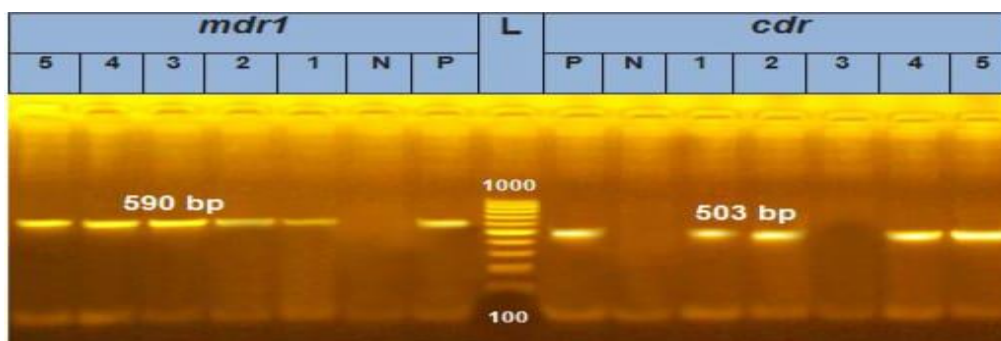


Fig. 3. PCR amplification of the *CDR* and *MDR1* genes at 503 bp and 590 bp, respectively. **On the right side:** lanes (1, 2, 4 and 5) showed positive amplification of *CDR* gene. **On the left side:** lanes (1-5) showed positive amplification of *MDR1* gene. L = ladder; P = Positive control; N = Negative control.



Fig. 4. PCR amplification of the *ERG11* gene at 1641 bp. Lane (2) showed positive amplification of *ERG11* gene. L = ladder; P = Positive control; N = Negative control.

4. Discussion

Udder infection is one of the most important diseases affecting dairy cattle, ewes and does that caused by invasion of the udder tissue with different types of microbes especially bacteria and fungi. Considering that mycotic mastitis is incurable or difficult to treat, it had lost popularity (Mousa et al., 2021).

In this study, out of the collected 250 mastitic milk samples, 53 samples were positive for fungi with the percentage of 21.2%, bovine mycotic mastitis recorded 12.5% and ovine mycotic mastitis recorded 29.2%. Zhou et al., (2013) recorded a higher percentage of 35.6% from cases of bovine mastitis, but very higher percentage of 92.5% were recorded by Bakr et al., (2015). Also, Dworecka-Kaszak et al., (2012) recovered 55 (83.3%) fungal isolates out of 66 mastitic milk samples collected from cows. On the other hand, very lower percentages of 2, 9.4 and 14.5 % were recorded by Bekele et al., (2019), Hassan et al., (2014), and Mousa et al., (2021), respectively. The differences in

prevalence of mastitis between studies may be due to the wide variety between the number of the collected samples as well as other factors including climate, sanitation, the use of antibiotics in continuous as intramammary infusion therapy and environmental bacterial mastitis, which can result in necrotic tissues in the udder and mycotic infections (Hassan et al., 2014).

In our study, yeasts were more prevalent than molds with a percentage of 19.6% for yeasts against 2% for molds, similar results were recorded by Zhou et al. (2013), but Farag and ELsaid (2012) recorded different results, where molds were higher than yeasts.

In this study, the most predominant fungal species was *C. guilliermondii* (36.7%), followed by *C. parapsilosis* (32.7%), *C. tropicalis* (14.3%), *C. albicans* (8.2%), *C. kefyr* (8.2%), *A. niger* (3.7%), *A. fumigatus* (1.9%), *A. flavus* (1.9%) and *Penicillium* species (1.9%). Most of issued papers mentioned that *C. albicans* had taken the upper hand in mycotic mastitis,

but in our study, *C. guilliermondii* and *C. parapsilosis* recorded the highest percentages followed by *C. tropicalis*, *C. albicans* and *C. kefyr*. Similar results were documented by Dworecka-Kaszak et al., (2012). Contrarily, *C. albicans* was the primary cause of mycotic mastitis in investigations by Pachauri et al., (2013), Bakr et al., (2015) and Mousa et al., (2021).

The antimicrobial susceptibility testing of *Candida* isolates showed that all isolates were resistant to nystatin. But 25 isolates (51.02%) were sensitive, 2 isolates (4.1 %) were intermediately sensitive and 22 isolates (44.9%) were resistant to fluconazole. Similar results of fluconazole resistance were recorded by Khan et al., (2018), but complete resistance for *C. krusei* was reported by Du et al., (2018). On the other hand, only 4% resistance to nystatin, 34.2% resistance to fluconazole were recorded by Mohamadi et al., (2014).

Because biofilm-associated microorganisms exhibit an intrinsic resistance to antimicrobials, disinfectants and clearance by host defense mechanisms, biofilms have been recognized as a significant virulence component in the pathogenesis of diseases (Şeker and Erhan, 2011).

Out of the total tested *Candida* isolates in this study, 9 isolates (18.4%) were positive for biofilm formation that was lower than results recorded by Sharma et al., (2017), Şeker and Erhan, (2011) and SAV and Öztürk, (2022).

Changes in sterol biosynthesis, mutations in the drug target enzyme and sterol 14a-demethylase, overexpression of the *ERG11* gene, mutations in the genes encoding membrane transport proteins of the ABC transporter (*CDR1/CDR2*) or the major facilitator (*MDR1*) super families and changes in sterol biosynthesis are some of the mechanisms that can lead to fluconazole resistance. Where it has been demonstrated that *C. albicans* develops fluconazole resistance when *CDR1*, *CDR2* and *MDR1* expression levels are increased (Khosravi Rad et al., 2016), that is agreed with our results where *MDR1* and *CDR* genes were detected by 100% and 80%, respectively. One isolate (20%) was positive for *ERG11* gene, similar results were recorded by Mohamed et al., (2022) who detected an overexpression of *ERG11* gene by a percentage of 27.8%.

5. Conclusion

The present study focused on the recovery rate of fungal pathogens associated with bovine and ovine mycotic mastitis, where *Candida* species other than *C. albicans* were more prevalent, with detection of their susceptibility to fluconazole and nystatin, biofilm formation and molecular characterization of some resistance genes (*MDR1*, *CDR* and *ERG11*). We recommend that, taking into account the mycotic mastitis during dealing with udder infections.

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