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Study on mycological and molecular detection of yeast and mold isolated from bovine mastitis

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Abstract

Mastitis is an economic food production problem in dairy animals. Bacterial etiology of mastitis is common but mycotic etiology of mastitis is scarce and obscured. The present study aims to use traditional and molecular methods to identify the fungal species that were separated from cattle mastitis, evaluate their susceptibility to antifungals, and investigate the variation between fungi isolated from dairy cows with clinical and subclinical mastitis. Three hundred milk samples were collected from Dakahlia (175) and Kafer Elsheikh (125) governorates. All samples were cultivated on the surface of Malt extract agar, Rice agar medium, Chromogenic agar, and Saburou's dextrose agar. Fungi were identified using phenotypic characteristics. Aspergillus and Candida species were the most isolated fungi. Positive samples for mycotic growth were (103; 59%) from Dakahlia and (55; 44%) from Kafer El Sheikh. In Dakahlia, Aspergillus species was the most common type of isolated mold. Species from clinical (36.3%) and subclinical (22.7%) mastitis while in Kafer Elsheikh were Penicillium (47.5%) and Aspergillus (40 %). The most isolated mould species from clinical mastitis was A. flavus (26%). The isolated yeast from Dakhliea from the clinical milk samples was C. albicans (24%) while in Kafer Elsheikh was C. galabrata (28.6%). PCR results were C. albicans, Cryptococcus neoformans, A. flavus, A. niger and. A. fumigatus. It is recommended that an animal unresponsive to antimycotic druggy must be suspected of mycotic mastitis, detailed mycological investigation should be conducted to establish the role of fungi in mastitis.

Keywords: Mastitis, Fungi, PCR, C. *albicans, Aspergillus.*

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Introduction

Mastitis is the inflammatory response that results from an udder tissue infection, and it is detected in multiple species, specifically in domesticated dairy cattle. This disease is the most common and may be lethal, also it is an economically vital disease related to decreased milk yield, and variations in the quantity and quality of milk, being measured to be one of the costliest to the dairy industry. Thus, bovine mastitis management has been an ongoing topic of field research and numerous attempts are being made to improve unique and successful anti-mastitis drugs (Gomes and Henriques 2016), leads to a significant issue that costs dairy cattle herds an incredible amount of money. According to Eldesouky et al. (2016). The most prevalent etiological agents are bacteria, followed by mycoplasmas, viruses, fungi, and algae. Fungal agents have been frequently identified as the cause of mastitis in cattle in recent years. Among these fungal agents, *Candida* species have been linked to fungal mastitis in cattle (Erbas et al., 2017). Clinical features (clinical and subclinical) are the main basis for categorizing bovine mastitis and cause (non-infection and infectious); the last one accounts for most cases and in several bacterial infections is the most typical herd appearance. Bacterial pathogens are classified into numerous types: Contagious, environmental, and opportunistic (Ndahetuye *et al*., 2019). The appearance of flakes, clots, or watery discharges in milk is indicative of clinical mastitis. Generally, the infected quarters are swollen, hot, and painful, general signs of acute clinical cases can be found (hyperthermia, anorexia, and depression). The severe consequences include agalactia, or cow mortality, which induces early culling (Cobirka *et al.,* 2020). Yeasts and molds can induce fungal mastitis. They can produce various hemolytic, proteolytic, and lipolytic enzymes, these enzymes adhere to the host tissue, cause morphological changes, and contribute to the virulence of fungal pathogens (Sachin *et al.,* 2014), higher morbidity rates from fungal mastitis in cattle have been recorded in recent years, with *Candida albicans* being a common culprit (Awandkar *et al,* 2023). Other *Candida* species were reported inducing fungal mastitis *C. guilliermondii, C. famata, C. tropicalis, C. colliculosa, C. krusei, C. rugosa, C. glabrata, C. parapsilosis, C. incon spicua; Trichosporon* sp., *Rhodotorula glutinis, Saccharomyces fragilis; Pichia kudriavzevii, Cyberlindnera rhodanensis*; mold species were also found *Aspergillus amstelodami, A. fumigatus* and *Geotrichum candidum* (*Dalanezi et al.,* 2018).

The current study is aimed to identification and characterization of the mycotic causes of mastitis in cattle by conventional methods and molecular techniques.

Materials and Methods

Clinical examination:

The clinical picture of animals under investigation was subcutaneous udder edema, enlargement, swelling, induration of the udder, hardening of the udder and supra mammary lymph nodes, anorexia, fever, and reduced milk production have been observed in affected dairy animals, the udder seems painful, tender, and heated. Mammary secretions from a gland affected by fungal mastitis could show watery or pale yellow and gelatinous with strands of cloudy material. In cryptococcal mastitis, a grayish-white, fibrous, and mucous fluid was observed and was carried out according to Radostitis *et al*., (2007).

Sampling:

Three hundred samples of dairy cows' milk were hygienically collected from different localities in both Dakahlia and Kafer Elsheikh governorates in Egypt (175 from Dakahlia and 125 from Kafer Elsheikh governorate) during the period from December 2020 up to December 2021. 70% alcohol was used to disinfect and wash the teat ends before collecting milk samples. Twenty milliliters of the milk samples were collected into individual sterile screw-capped bottles and each bottle was assigned a

serial number once the initial drops were thawed and discarded. During transit to the lab, samples were stored in an ice box. Milk samples were taken from cows that were sub-clinically mastitic and from cows that suffered and exhibited clinical indications of mastitis. Samples from cows were not responsive to various antibiotic treatments. Discomfort, swelling, warmth, and an unusual appearance in the milk (bloody milk, watery discharges, clots, pus) were all indicators of clinical mastitis. Subclinical mastitis primarily manifests as an increase in somatic

cell count and a decrease in milk output. California mastitis test (screening test) was performed on milk samples to look for signs of subclinical mastitis. Schalm et al., 1971 state that CMT scores are -ve, trace +ve, ++ ve, and +++ve.

Mycological examination of milk samples:

Preparation of milk samples:

Milk samples were centrifuged for five minutes at 3000 rpm. The supernatant fluid including the cream layer was discarded and the sediment was obtained for culturing according to Schalm *et al*., (1971)**.**

Cultivation of milk samples: ***(NMC,1999)***

the sediment was cultured on two plates of Sabouraud's dextrose agar and one was incubated aerobically for 5–7 days at 37◦C and the other at 25◦C.

Identification of mold isolates: ***(According to Pitt and Hocking, 2009)***

Macroscopical examination:

A hand magnifying lens was used to do the macroscopical assessment of mold colonies, which included measuring the colonies' diameter, growth rate, color, texture, basal and surface mycelia, colony reversal, and texture.

Microscopical examination:

A triangle section was taken from the edge of the seven-day-old colony and placed onto a cleaned glass slide. The colony piece was filled with one or two drops of 70% alcohol (for Deuteromycetes) or one or two drops of distilled water (for Zygomycetes) using two mycological needles. After the alcohol had evaporated, one drop of Lactophenol cotton blue stain was added, and the slide was cleaned. The prepared slides were examined under low power and an oil immersion lens to remove any excess water and air bubbles, compress the hyphae and other structures to facilitate microscopic inspection, and define the morphological structures of the mold growth to the conidial stage, head, vesicle, sterigmata, conidiophore, and conidia. Additionally, observation and records were made of scleroti, Hulle cells, and other hyphal oddities as well as the ascosporic stage (cleistothecia and asci).

Identification of yeast isolates:

Variety of yeast colonies were separated, moved to Sabouraud's dextrose slope agar, and then incubated for 48 hours at 37 ◦C degrees. Identification of the inoculated tubes was done following Barnett et al. (1990).

The morphological examination:

It was done by looking at the isolates' morphological characteristics under a microscope and-a macroscopic lens (Finegold and Martin, 1982).

Direct microscopic appearance:

To carry it out, a little portion of the colony was placed on a slide with one drop of lactophenol cotton blue, covered with coverslips, and checked for the presence of yeast cells under high power magnification.

Gram's stain:

Smears from isolated colonies were made, stained with Gram's stain, and then inspected under a microscope to check for the presence of big, spherical, Gram-positive yeast cells.

Microscopical examination on rice agar media:

On the surface of corn meal agar plates, by platinum loop 3 lines of isolated colony were streaked then with cover slips on. After 48 hours of incubation at 25◦C, the plates were inspected.

directly under the microscope to direct the shape and size of blastospores and the absence of pseudohyphae, chlamydospores, and arthrospores.

Microscopical examination on (CHROM Candida agar media HIMEDIA-INDIA*): (Baradkar et al., 2010)*

Detection of lipolytic activity:

Fungi were grown on Peptone agar medium supplemented with 1% individually sterilized Tween 20 added to media to test for lipase activity. After the incubation period, the colony was surrounded by a precipitate that was visible because the enzyme had formed calcium ions from the lauric acid it had released. This indicated that there was positive lipase activity according to Sunitha *et al*., (2013).

Detection of hemolytic activity:

A blood plate assay was used to measure hemolysin activity per Manns et al.'s 1994 description. To prepare the media, 100 milliliters of SDA were combined with 7 milliliters of recently drawn sheep blood, and then 3% (w/v) of glucose was added. A standard inoculum containing 10 yeast cells (milliliters of saline) for both the test and control *Candida* isolates (Streptococcus) {10} was added to the medium. After that, the plate was incubated for 48 hours at 37ºC (5%) CO_2.

Detection of proteolytic activity:

EI-Fadaly et al. (2015) reported that proteolytic activity was assessed on a modified Czapek agarose (MCA) medium. Spot inoculation of all plates was done using tested fungal strains. Following inoculation at 25 °C, 10% copper sulfate was added to the plates. The growth and clear zone diameters were measured to record the results.

PCR-based molecular identification.

DNA extraction from the samples was carried out according to the instructions (INTRON kit cat. No. 17154 Korea).

PCR amplification:

Table (1): Primers used in PCR reactions for detecting fungi in milk samples:

Gene	Primer	Primer Design	Reference
ITS	Forward ITS1	5'- TCCGTAGGTGAACCTGCGG-3'	Mirhendi etal., 2007
	Reverse ITS4	5'- TCCTCCGCTTTATTGATATG-3'	

DNA samples were tested in 50µl, reaction volumes in a 0.2 ml, Eppendorf tube, containing25 µl PCR mix, which included one primer, two target DNA segments, 10x buffer, 10m M d NTPs combination, Taq polymerase, and enough sterile molecular water to fill a final volume of 50µl. Table (1) specific fragments of the expected length were examined in PCR results using a 1.5% agarose gel electrophoresis stained with Ethidium bromide (Table 2).

Antifungal sensitivity test

Filter paper discs, Whatman (No.1) of 5mm, impregnated with four antifungals had been supplied from (Himedia, India) Ultra Griseofulvin, Lamifen,

Table (2): PCR thermal profile for amplification of ITS region:

Steps	Temperature	Time	No. of cycles
Initial denaturation	94ºC	4min	One cycle
Denaturation	94ºC	1min	35 cycle
Annealing	56ºC	1min	
Extension	72ºC	1min	
Final extension	72ºC	10min	One cycle

Itrapex, Diflucan 10 spore suspensions from pure culture isolates. Sabouraud's Dextrose Agar media.

Disk Diffusion Technique*: (NCCLS,2002)*

Preparation of paper discs of commercial antifungal, filter paper discs, Whatman of 5 mm, were impregnated for 10 minutes with four antifungals (Ultra Griseofulvin, Lamifen, Itrapex, Diflucan). The antifungal sensitivity test was conducted using the disc diffusion test. In vitro, isolates antifungal susceptibility was determined using the National Committee for Clinical Laboratory Standards' recommendations (NCCLS2002). On Sabouroud's dextrose agar, the isolated fungi were incubated at 37 °C for yeast and 25 °C for mold. The pure culture of each isolate was spread out over the surface of Sabouroud's dextrose agar plates, after being thoroughly mixed with nine milliliters of sodium chloride solution. Any extra fluid was then suctioned off. Spread out across the inoculation plate's surface were antifungal discs. Plates were cultured for five days at 25 °C (mold) and 37 °C (yeast) for 24hr. Each disc's inhibitory zone diameter was measured in millimeters and evaluated.

Results

California mastitis test (CMT)

A CMT (screening test) was performed on milk samples to look for signs of subclinical mastitis. CMT scores -ve, trace +ve (14), ++ ve (10) and +++ve.(5), table (3).

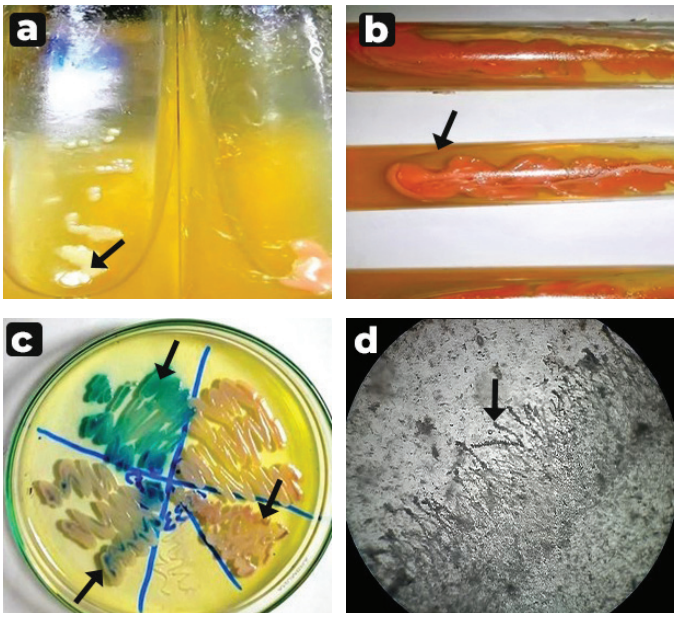

Fig (1): Macroscopic and microscopic criteria of fungi in mastitis milk (a) Candida spp. culture on SDA revealed pasty creamy colonies, (b) Rhodotorula appeared as light orange on Sabroud's dextrose agar medium, (c) CHROM Candida agar revealed *C. albicans* (Light green), *C. glabrata* (Dark pink) *C. krusei* (Pale pink). (d) *Candida albicans* on RAT that showed pseudohyphae

Macroscopic appearance of colonies:

The colonies' characteristics were explained by considering the size, consistency, surface color, rate, and pattern of growth. *Candida spp.* culture on SDA revealed pasty creamy colonies in (Figure 1a), *Candida parapsilosis* showed wrinkled colonies on SDA, *Candida kruzei* showed flat creamy colonies on SDA, *Candida tropicalis* showed rough, heaped colonies on SDA and *Rhodotorula* appeared as light orange on Sabouraud dextrose agar medium. (Figure 1b)

Microscopical examination on Rice agar media:

Table(3): Interpretation of California Mastitis test (CMT) results for evaluating the degree of mastitis (Schalm et al., 1971)

Results	No. of cell/ml	Degree of reaction
Trace	500.000	Traces flakes formation
Weak positive(+)	1 million	Distinct precipitation with no gel formation
Distinct positive(++)	2 million	Milk thickens with some gel formation
Strong positive(+++)	4 million	Gel mass formation tend to adhere to the bottom of the cup

Table (4): Prevalence of fungal species in milk samples collected from Dakhliea and Kafrelsheikh governorates

Governorate	Total No. samples	No. of +ve samples	% of +ve samples	No. of -ve samples number	% of -ve samples
Dakhliea	175	103	59%	72	41%
Kafrelsheikh	125	55	44%	70	56%

Candida glabrata on RAT exhibited only small blastospores without pseudo hyphae. *Candida albicans* on RAT showed chlamydoconidium and pseudo hyphae (Figure 1d), *Candida parapsilosis* showed giant cell pseudo hyphae and blastospores, *Candida krusei* on RAT showed pseudo hyphae with elongate blastospores.

Microscopical examination on CHROM Candida agar media:

On CHROM Candida agar: *Light green C. albicans, dark pink C. glabrata, and pale pink C. krusei* are discernable. (Figure 1c)

Prevalence of fungal species from Dakahlia and Kafr ELsheikh governorates:

Out of 300 examined samples collected from dairy cows, 158 were positive for fungal isolation., the rate of isolation of fungi was 59% in Dakahlia governorate while in Kafer Elsheikh governorate was 44% (Table 4).

Prevalence of mold genera isolated from mastitic milk samples collected from Dakahlia and Kafer Elsheikh governorates:

Aspergillus species were identified with percentages of (36.3%) and (22.7%), respectively, as the most common isolated mold species from clinical and subclinical mastitic samples. Penicillium species, with percentages of (6%) and (10.6%) in both clinical and subclinical mastitis samples, while the least species that had been isolated from clinical mastitic samples were *Fusarium* species in percentages of (9%) followed by *Monelia species* (3%). While those species were not isolated from subclinical milk samples, I have isolated five mold genera from the milk samples taken from clinically mastitic cases in Kafer Elsheikh governorate in different percentages, which were *Alternaria, Aspergillus, Geotrichum, Mucor,* and *Penicillium* species. *Penicillium and Aspergillus* were the most predominant isolated mold genera in a percentage of 47.5% and 40 % respectively, followed *by Geotrichum, Alternaria,* and *Mucor* in low percentages that were 7.5% for *Geotrichum* species and 2.5% for both *Alternaria* and *Mucor* species, while *Aspergillus species* was the only isolated mold genera from subclinical mastitic milk samples in the percentage of only 2.5% (table 5)

Prevalence of mold species isolated from samples of mastitic milk collected from Dakahlia and Kafer Elsheikh governorates:

A.flavus, A.niger, and *A.fumigatous* were the most common aspergillus isolates from clinical mastitis cases with percentages of (45%, 7.6%, 6%) respectively, while, *F. proliferatum and F. verticilloides* were detected in 4.5% examined samples. While in case of subclinical cases, the species of mold that is most isolated were *A.flavus,* and *A.niger* in percentages of (18 % and 4.5 %) respectively, followed by *P. rugulusum* and *P. Decumbens* in percentages of (3 % and 1.5 %). In Kafer Elsheikh *A. flavus* was isolated from clinical samples (26%), *P. citriniume* (20.5%), *P. decumbens* (14.7%), including *P.viridicarum* and *P. glabrum* (2.9% for both), *A.fumigatus* , *A. niger* and *P. italicum* were the same percentage of (8.8%) while the least isolated strain was *P.terreus* in a percentage of (5.8%) while in case of the subclinical samples, only *Aspergillus* species were isolated in the percentage of (2.9%) (table 6).

Table (5): Prevalence of mould genera isolated from milk samples collected from Dakhliea and Kafrelsheikh governorates.

Mould species	Dakhliea				Kafrelsheikh				
	No. of samples	% of species	Clinical samples	Subclinical samples	Mould species	No. of samples	% of species	Clinical samples	Subclinical samples
Aspergillus species.	39	59.09%	24	15	*Alternaria Spp*	1	2.5٪	1	----
Fusarium species	6	9.09%	4	2	*Aspergillus Spp*	16	40%	15	1
Geotrichum candidum	8	12.12%	2	6	*Geotrichum SPP*	3	7.5%	3	-----
Monelia species	2	3.03%	2	----	*Mucor Spp*	1	2.5%	1	-----
Penicillium species	11	16.67%	6	5	*Penecellium Spp*	19	47.5%	19	-----
Total	66	100%	38	28	Total	40	100%	39	1

Table (6): Isolated moulds species from milk samples collected from Dakhliea and Kafrelsheikh governorates.

Dakhliea					Kafrelsheikh				
Mould species	No of samples	% of species	Clinical samples	Subclinical samples	Mould species	No. of samples	% of species	Clinical samples	Subclinical samples
A. flavus	30	45.45%	18	12	*A. flavus*	9	26%	8	1
A. niger	5	7.6%	2	3	*A. fumigatus*	3	8.8%	3	----
A. fumigatus	4	6.06%	4	------	*A. niger*	3	8.8%	3	----
F. proliferatum	3	4.5%	2	1	*p. terreus*	2	5.8%	2	-----
F. Verticilloides	٣	٤,٥%	2	1	*P. citrinum*	7	20.5%	7	-----
P. oxalicum	4	6.06٪	2	2	*P.glabrum*	1	2.9%	1	-----
P. rugulusum	3	4.5%	1	2	*P.italicum*	3	8.8%	3	-----
P. Decumbens	4	6.06%	3	1	*P.viridicarum*	1	2.9%	1	------
Total	٥٦	84.73%	٣٤	٢٢	Total	34	84.5%	33	1

Prevalence of yeast species isolated from milk samples collected from Dakahlia and Kafer Elsheikh governorate:

There were four isolated species including *C. albicans, C. guilliermondii, C. kefyr, C. tropicalis*. *C. albicans* was the most common yeast strain isolated from the clinically mastitic samples in a percentage of (24%) followed by *C. tropicalis* (21.6%), *Rhodotorula* (16.2%), *Saccharomyces* (10.8%), respectively, and the least isolated strains were *C. guilliermondii, C. kefyr* (8.1%) *Torulopsis* (5.4%) followed by equal isolates of *Debaryomyces*, *Trichosporon* detected as 2.7% for each of them. While in the case of subclinical samples, no yeast species were isolated. There were three isolated species in Kafer Elsheikh including *C. galabrata*, *C. famata* and *C. kruseii. C. galabrata* takes most of the isolated yeast strains from the clinically mastitic samples in a percentage of (28.6%) followed by *C. famata* (23.8%), *Rhodotorula* (14%) followed by *Cr.neoformans* (9.5%) and strains with the least isolation were *C. kruseii*, *Debromyces*, *Saccharomyces, Torulopsis* and *Trichosporon* were the same percentage of (4.8%), while in case of subclinical samples, no yeast species were isolated (table 7).

Molecular characterization of some fungal isolates by (PCR):

PCR was applied to six Aspergillus spp. (2 *A. flavus*, 2 *A. niger,* and 2 *A. fumigatus*) as well as three yeast isolates (2 *C. albicans and 1 C. neoformans*) using internal transcribed spacer (ITS1 and ITS4). Result illustrated in (Figure 2) revealed positivity of all isolates to PCR using ITS1 and ITS4 primers and the amplified product size is 570 bp.

Detection of antifungal sensitivity of some isolates:

The antifungal sensitivity of three isolates (*C. parapsilosis*, *C. tropicalis* and *Geotrichum canddidum)*. Lamifen was the most effective anti-fungal against *C. tropicalis* and *C. parapsilosis,* while it is the second in its effect against *Geotrichum canddidum* after Diflucan, while Itrapex was the least used antifungal in its effect against the tested strains

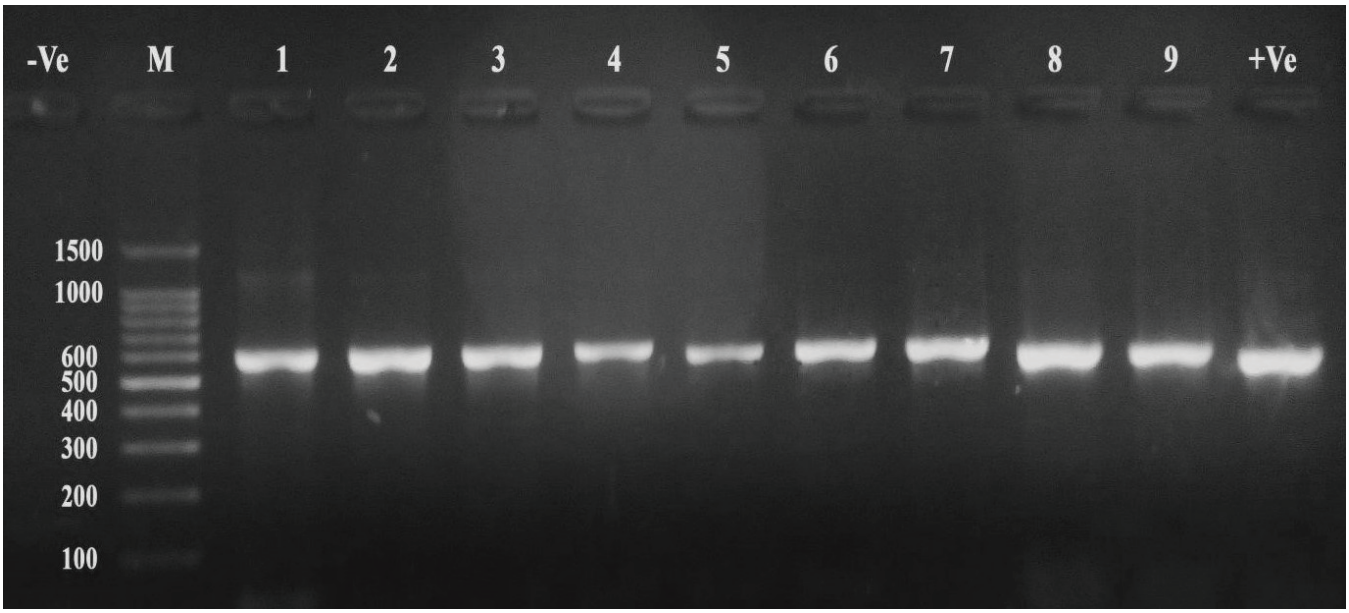

Fig (2): PCR results for 9 fungal isolates. Amplification of 570bp fragment of ITS region from all tested isolates.

-Ve: Control negative (S. aureus).+Ve: Control positive (A. niger).M: 100bp DNA ladder.

1, 2: A. flavus; 3, 4: A. niger; 5, 6: A. fumigatus; 7, 8: C. albicans and 9: C. neoformans.

and there is no any effect of Ultra griseofulvin on any one of three strains (table 8).

Discussion

Mastitis is a condition that can be caused by a wide range of bacteria. It reduces milk supply, raises the cost of antibiotic treatment, and damages the dairy business. When compared to other mastitis infections, the prevalence of fungal mastitis is typically quite low; however, throughout the past ten years, it has increased dramatically (Yanuartono et al., 2019).

Due to their rising occurrence, fungal mammary gland infections in cows are the subject of more and more investigations; these infections cause 2% to 13% of all mastitis cases in cows. Occasionally, they occur at a significantly greater incidence or are enzootic (Krukowski et al., 2001).

Fungal diseases can spread quickly thanks to management practices used on dairy cows, such as discarding the first few pieces of milk on the ground during milking and treating mastitic animals, and milkers' unwillingness to wash their hands after milking. (Pachauri et al., 2013). According to Noris et al. (2007), there are reports of yeasts, such as Candida species, using nitrogen from antibiotics like penicillin and tetracycline. Antibiotic therapy disrupts udder homeostasis and inhibits T cell and neutrophil activity, which may in turn promote yeast growth.

In the current study, out of three hundred examined samples, for mycotic mastitis, 158 samples were positive. The prevalence in Dakhalia governorate was 59% while it was 44% in Kafer Elsheikh governorate similar findings were reported by (Pachauri *et al.,* 2013) who isolated fungi from 64% of milk samples. While lower rates 25.2% and 7.07% were reported by Abd El-Razik *et al., (2011)* and Wladyaslaw *et al*., (2010). The high prevalence in Dakhalia governorate may be to high amount of antibiotics that farmers used without awareness with its side effect in decreased immune system response which leads to fungal mastitis, difficult muddy yard management and high humidity that led to high fungal population.

In the present study, *A.flavus, A.niger, A.fumigatous, F. proliferatum, F. Verticilloides, P. rugulusum* , *P. Decumbens* and *P.terreus* were mold species isolated from examined samples however , *C. albicans, C. guillierimondii, C. kefyr, C. tropicalis*, *C. galabrata*, *C. famata*, *Rhodotorula, Cr.neoformans, Torulopsis* and *Trichosporon* were the yeast species isolate. Current study showed variations in the isolated yeast and mold causing mycotic mastitis with different studies, this may be attributed to the test method for isolation and identification due to the variation in weather conditions that affect fungal growth or due to the ability of some strains to perform some activities as hemolytic, lipolytic and proteolytic activities that enable the fungal strain to invade the host cell and produce the disease condition.

Aspergillus and *Geotrichum* species were isolated. Similar finding was reported by Samir

et al., 2015. *Aspergillus, Penicillium,* and *Alternaria* were identified from Kafer Elsheikh samples, similar findings were reported by Wladyslaw *et al.,* 2010 and Suhyla and Seyhan, 2011 who isolated *(Aspergillus, Penicillium*, *Epicoccum, Phoma,* and *Alternaria*) from infected mammary secretions. In addition, *A.flavus* ,*A.fumigatus*, *A.niger, A.terreus,* and some *Penicillium* species were isolated from kafer Elsheikh samples. *Aspergillus fumigatus* was among the primary reasons of mycotic mastitis. This finding agreed with Abd El-Razik *et al.,* 2011 and Bakr *et al.,* 2015 as they isolated *Aspergillus fumigatus.* On the contrary, (Enany *et al*., 2007) failed to isolate *A.fumigatus* from milk samples.

The yeast species with the greatest isolation from Dakahlia governorate was C.albicans with percentage of 24%, among all the Candida species, C. albicans is the most isolated. (Costa et al., 2012,

Pachauri et al., 2013). C. albicans was identified at a rate ranging from 2% to 36.9% from milk samples. Approximately 10% of cow cases of mastitis are caused by udder infections generated by Candida. (Dolgun et al., 2022). In addition, the isolation rate of Rhodotorula, Saccharomyces, and Trichosporn species were 16.2%, 10.8%, and 2.7% respectively. These findings are in total contrast to those reported by Du et al. (2018), who identified C. krusei as the primary cause and C. parapsilosis as the secondary cause. There were also sporadic findings of other non-albicans Candida (NAC) species. This investigation did not isolate any Candida albicans.

In the current study with a proportion of 61.8 percent, the species *Candida* was the most identified from Dakhlia. The most isolated species were (*C. albicans, C. guilliermondii, C. kefyr,* and *C. tropicalis*), while *Rhodotorula* and *Trichosporon* were the least isolated. *C. albicans* strains were isolated from the clinically Dakhliea mastitic samples in a percentage of (24%). Eldesouky *et al.,*2016 reported comparable results, and isolated *C. albicans* with a relative percentage (29%). However, the percentage of *C. tropicalis, Rhodotorula*, *and Saccharomyces* was isolated (21.6%), 16.2%), and (10.8%) respectively. While the rate of isolation of *C. guillierimondii, C. kefyr* (8.1% for both), *Torulopsis* (5.4%) (*Debaryomyces*, *Trichosporon* 2.7%.for both.

C. albicans weren't isolate from all the milk samples collected from Kafer Elsheikh governorate. The present research revealed that milk samples contained *Cryptococcus neoformans*

(9.5%). This finding agreed with Abou Elmaged *et al.,* 2011 and Ghodasara *et al.,* 2015 identified *cryptococcus neoformans* from milk samples. On the contrary, *Cryptococcus neoformans* was not isolated from any of the samples that Bakr et al. and Esraa et al. (2015) examined. The *candida* species that was most isolated was *C. albicans.* Similar finding was reported by Esraa *et al.,* (2015). However, no milk sample examined by Spanamberg *et al.,* (2008) or *Zhou et al.,* (2013) revealed the presence of *C. albicans.*

Lamifen was the best and most effective antifungal, followed by Diflucan and then Itrapex while Ultra griseofulvin did not affect any one of the tested strains. The results we obtained aligned with the findings of Mbuk et al. (2016) regarding the ineffectiveness of Ultra griseofulvin on Geotrichum canddidum. Conversely, Goksel et al. (2017) discovered that C. parapsilosis exhibited 80% sensitivity to ketoconazole, 80% intermediate susceptibility to amphotericin and nystatin (100%), 60% miconazole, and 100% resistance to flucytosine and fluconazole (80%). Due to the significant drug resistance of the Candida species to certain antifungals, it is advised that Griseofulvin and Itraconazole antifungal medications not be used in the treatment of NAC species isolated from mycotic mastitis.

Conclusion

Mastitis is a multifactorial disease of serious concern for dairy farmers both in the developing as well as developed nations of the world. Several fungi are implicated in the etiology of mycotic mastitis in dairy animals. *Candida* species, however, account for most mammary gland fungal infections. The routine application of SDA medium and Giemsa stain will undoubtedly help the microbiologist. In the study of mycotic mastitis in dairy animals. It is recommended that an animal unresponsive to antibacterial antibiotic therapy must be suspected of mycotic mastitis, and a detailed mycological investigation should be conducted to establish the role of fungi in mastitis. As fungal mastitis is a global problem, comprehensive and systemic studies research should be done on the creation of affordable, secure, and efficient antifungal medications.

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Conflict of interest

Following the principles of openness and objectivity in research, all the study's authors certify that they have no real or prospective conflicts of interest that could affect, or be interpreted as having the potential to affect, the conclusions and results of the investigation. According to the authors, this study was carried out impartially and objectively, with the only motivation being the expansion of scientific knowledge in the relevant sector.

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