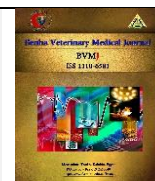




Official Journal Issued by  
Faculty of  
Veterinary Medicine

## Benha Veterinary Medical Journal

Journal homepage: <https://bvmj.journals.ekb.eg/>



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### Original Paper

## Isolation and identification of fungi from subclinical mastitis milk.

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### ARTICLE INFO

#### Keywords

*Sub clinical mastitis*  
*Bovine raw milk*  
*Candida albicans*  
*Penicillium spp*  
*Aspergillus fumigatus*  
*Lactoscan*

Received 19/01/2022

Accepted 12/06/2022

Available On-Line

01/10/2022

### ABSTRACT

Subclinical mastitis causes severe repercussions and economic loss for the dairy industry due to decreased milk quality and quantity. The goal of the research was to isolate and identify the mold and yeast from subclinical mastitis buffalo milk samples after its chemical examination, milk samples were collected from Menoufia and Qalyubia governorates. A total of 160 buffalo milk samples were evaluated for subclinical mastitis using the California mastitis test as a field test. Seventy-five samples tested were positive for subclinical mastitis (46.88 %), indicating that subclinical mastitis is common in these areas. Different mold and yeast from 55 subclinical mastitis milk samples could be isolated (73.33%), with ten samples (18.18%) demonstrating mixed fungal infection, indicating that fungal subclinical mastitis is spreading in these areas. *Candida albicans* (43.64 %), *Penicillium spp* (38.18 %), and *Aspergillus fumigatus* (25.45 %) were shown to be the most common fungi linked with the examined subclinical mastitis milk samples. While low percent was identified for yeast *Rhodotorula* and *Aspergillus flavus* 7.27% and 1.81%, respectively. The chemical examination using lactoscan revealed decreasing in total solids, solids not fat, protein, lactose, and fat % in the examined mastitis milk samples compared with legal standards. The current results pointed to the necessity for field-based screenings of fungal mastitis and the development of safe methods for controlling fungal contamination to provide consumers with high-quality milk and reduce the economic loss caused by fungal mastitis. In addition, it is necessary to produce milk under acceptable hygienic measures to be free from any human health hazards due to mastitis.

## 1. INTRODUCTION

Bovine mastitis is a mammary gland inflammatory disease caused by a variety of infections that has severe repercussions for the dairy industry (Martins *et al.*, 2019). It's one of the most common infectious diseases in dairy cows, and it's becoming a global problem (Ruegg, 2017). Due to low milk quality, drug usage, and veterinary services, mastitis generates substantial economic losses, ending in the eradication of affected animals (Aslanta and Demir, 2016). Antibiotic misuse and overuse in the treatment and prevention cause mastitis microorganisms to acquire resistance (Fessia *et al.*, 2019).

Mastitis is accompanied by physical, chemical, and most commonly bacterial alterations in glandular tissue, so it is the most common and costly disease for dairy industries (Santos *et al.*, 2003). The clinical form of mastitis has apparent signs, but the subclinical form has none. Mastitis causes major financial loss because of lower milk yield, reduced milk quality, and medicine expenditures, lower milk prices, and higher labor costs (Seegers *et al.*, 2003). Mastitis can also impair the dairy product shelf life and cheese manufacturing qualities (Ma *et al.*, 2000; Barbano *et al.*, 2006).

Mastitis has a broad range of causative agents, including fungi (Aalbaek and Stenderup, 1994), viruses, and bacteria, (Wellenberg *et al.*, 2002). Yeast infections contribute to the

importance of the problem (Watts, 1988; Casia dos Santos *et al.*, 2005). Yeast and fungi are common soil flora that can colonize the udder skin in modest numbers (Stantos and Marin, 2005). While natural defense mechanisms are lowered, they are regarded as opportunists and create disease. Mastitis caused by fungi is usually uncommon in dairy herds. According to Kirk and Bartlett, 1986, fungal mastitis affects 2.0 to 7.0 % of dairy cows. The percentage can be higher in tropical regions. Yeasts are the most frequently implicated organisms, particularly those of the genus *Candida*, of which a few species have been collected from infected glands (Costa *et al.*, 1993). There is no doubt that mastitis has both an extreme zoonotic and economic importance constituting multiple hazardous effects on human health and animal production (Al-Majali *et al.*, 2008).

Fungi can be found in a variety of places in nature, including bedding, equipment from the stables, and milking machines. Before the onset of medicines, mycotic mastitis existed in cattle, but, since that day, a growing number of cases have been observed, almost all of which have been linked to preceding antibiotic therapy of suspicious or established mastitis caused by bacteria (Lagneau *et al.*, 1996).

Yeasts are a collection of unicellular microbes in the udder and teat skin of dairy cattle and are constantly present in their normal environment. Yeasts are opportunistic infections that infiltrate the udders of cows. The usage and

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abuse of antibacterial medications, as well as the use of contaminated antibiotic solutions, syringes, and a variety of different things that come into contact also with the udder, may promote the colonization of yeast inside cow mammary glands (Costa *et al.*, 1993; Stantos and Marin, 2005).

Many types include *Aspergillus fumigatus.*, *Aspergillus terreus.*, *Candida spp.*, *Cephalosporium spp.*, *Cryptococcus neoformans.*, *Coccidioides spp.*, *Geotrichum candidum*, *Rhizopus spp.*, *Histoplasma spp.*, *Torulopsis spp.*, *Mucor spp.*, and *Trichosporon spp* had been identified as causes of mycotic mastitis (Aalbaek *et al.*, 1994; Krukowski *et al.*, 2000). Other fungi were identified from healthy glands' milk, including *Cryptococcus spp.*, *Rhodotorula spp.*, *Trichosporum cutaneum*, *Aureobasidium pullulans*, and *pichiaohmeri* (Lagneau *et al.*, 1996; Costa *et al.*, 1993).

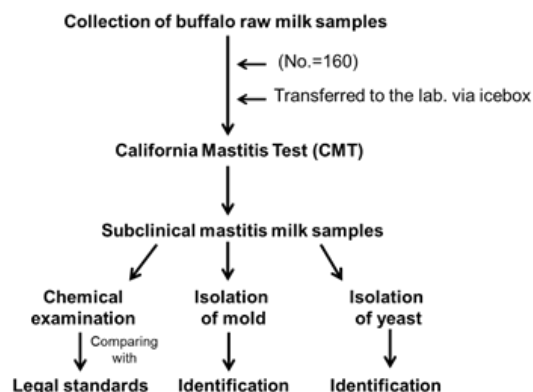
Due to a lack of surveillance, emphasizing the significance of implementing surveillance systems, there are rarely published data on the incidence of foodborne disease caused by fungi, a system like this might provide systematic baseline data on the presence of significant foodborne pathogens in the supply food chain, as well as the prevalence of foodborne illness, allowing for the priority of pathogen control intervention approaches (FAO, 2005). Because there is no food safety surveillance system, there is a major absence of basic epidemiological information, which inhibits prioritization as well as informed implementation of regulations targeted at decreasing the incidence of public health costs incidence of foodborne diseases. According to the World Health Organization (WHO), foodborne infections caused 600,652,361 sickness cases and 418,608 deaths globally in 2010.

Non-heat-treated milk and raw-milk products have been linked in large epidemiological reports as major causes of sickness (El-Ziney and Al-Turki, 2007; Eberlein, 2007 and Keba *et al.*, 2020). As a result, the zoonotic risk posed by this milk should be evaluated (Abera *et al.*, 2016). Therefore, this work monitors the bovine milk quality as well as the detection of fungal pathogens. The data reported here could be used to guide future food safety measures aimed at minimizing the number of cases of foodborne disease caused by fungal infection through dairy products, as well as data on the serious economic consequences of fungal mastitis. The purpose of the research was to isolate fungi and yeast from subclinical mastitis milk samples and further identify them.

## 2. MATERIAL AND METHODS

### 2.1. Collection of milk samples:

About 160 buffalo raw milk samples were collected from Menoufia and Qalyubia Governorates (study area) using sterile milk sampling protocol by Kirk (Kirk, 2000) as follows; First, the udder was cleansed and dried with cotton after the sterile tube was labeled. Then, the tip of each teat was sterilized with 70% alcohol, starting with the farthest teat, and working toward the nearest, and 1-2 streams of milk were removed from each teat. Finally, milk samples were obtained from the nearest one, and 75% of the sterile sample tube was filled with them. It was then transferred to the laboratory via icebox and stored at 4°C for less than 72 hours before being processed further as illustrated in the following flowchart:



### 2.2. California Mastitis Test (CMT):

The milk samples were taken for testing mastitis using the California mastitis test. Milk and reagent are combined in equal parts. After gently spinning the CMT paddle, the gel degree formation was evaluated. Scores represented four categories: 0, negative (-) or trace ( $\pm$ ); 1, positive (+); 2, positive (++); and 3, positive (+++) [Marshall, 1994]. Negative (-) and trace ( $\pm$ ) reactions were considered as "negative," and different intensities of positive reactions (+, ++, +++) were considered as "positive" (Seifu and Tafesse, 2010).

### 2.3. Chemical examination of subclinical mastitis milk samples:

Total solids, solid not fat (SNF), proteins, lactose, Fat, freezing point, and salts in the positive CMT buffalo milk samples were determined using Lactoscan SA standard Milk Analyzers - Basic Models obtained from Narodnibuditeli str., Nova Zagora, 8900, Bulgaria, Milkotronic Ltd 4.

### 2.4. Isolation of fungi:

Baiyewu *et al.*, (2007) described the procedure for fungi isolation. In Petri dishes, 100  $\mu$ l of positive CMT milk sample was distributed on potato dextrose agar containing streptomycin (to limit bacteria development) and incubated at 28°C for 5 days. The inoculated plates were checked after 3 days during the incubation period. The pure cultures of yeast and mold were obtained on slopes for identification.

### 2.5. Identification of fungi:

#### Identification of mold:

According to fungi morphological characters, mold colonies were picked up with their surrounding medium under aseptic conditions and transferred to Sabouraud Dextrose agar slopes and malt extract agar then incubated at 25°C for seven days for further identification. The fungus was identified using Fawole and Oso's (1995) method. A drop of lactophenol cotton blue stain was deposited on a clean slide, and a little amount of the mycelium from the fungal cultures was extracted and inserted in the drop of the stain with the use of a mounted needle. With the help of the two mounted needles, the mycelium was spread evenly across the slide, and a cover slip was gently lowered on top of it. After then, the slide was viewed under a microscope. The observation was carried out using a microscope with a high-power objective ( $\times 40$ ). The fungi's morphological properties, such as hyphae type and a sexual reproductive organization, were studied.

#### Identification of isolated yeasts:

The identification of yeast genera was carried out according to the methods recommended by Viljoen *et al.*, (1993). The procedure was divided into two parts. First, six tests from the simplified identification method's master key were used to perform preliminary characterization of isolates. The urease reaction, growth in the presence of 0.1 % cycloheximide, and assimilation of nitrate, erythritol, mannitol, and cellobiose are all examples of these processes.

#### 2.6. Data analysis:

A statistical study was carried out with the SPSS program. The results showed the mean of three reading with standard error according to Feldman *et al.* (2003).

### 3. RESULTS AND DISCUSSION

Bovine mastitis is one of the most common infections afflicting dairy animals around the world. It could result in a variety of physical, chemical, and microbiological alterations in milk, rendering it unfit for human consumption or causing dairy processing to fail. In many mastitis dairy animals' cases do not seem to respond to therapy as the problem of antibiotic resistance worsens. On the other hand, many dairy buffalos presently produce colored milk with a decrease in milk yield. As a result, our research focused on the potential origins of colored milk caused by fungi or yeast. Our study according to the flow chart illustrates the following results, using the California mastitis test (CMT test), 75 samples (46.88 %) from 160 buffalo raw milk samples were proven to be positive for bovine subclinical mastitis. Mold and yeast were recovered from 55 samples (73.33 %) examined subclinical mastitis milk samples, but no fungal isolates were found in 20 samples (26.66 %) samples. Mixed fungal infection was found in 10 samples from the 55 mastitis cases (18.18 %). The data represented in table (1) showed the chemical profile of subclinical mastitis buffalo milk samples: Fat, solid not fat (SNF), proteins, lactose, freezing point, salt, and total solids. The average findings for the positive 75 buffalo subclinical mastitis milk were  $8.52 \pm 0.76$ ,  $6.90 \pm 0.53$ ,  $2.68 \pm 0.29$ ,  $3.56 \pm 0.33$ ,  $-0.46 \pm 0.02$ ,  $0.65 \pm 0.01$  and  $15.43 \pm 1.2$ , respectively. Differences in fat concentration was found to be according to the levels of mastitis observed. Protein, lactose, freezing point, and salt percent in the examined buffalo milk samples showed low percentage than Egyptian legal standards, which stated legal standards of protein 3.5%, lactose 4.5%, freezing point - 0.550 and salt 0.8% (Egyptian Standard, 2005).

Some milk samples showed normal chemical composition although it was positive CMT. Many factors are associated with the presence of mastitis, even in the absence of bacterial growth; these include traumatic damages (lacerations, cuts, injuries), chemical irritants, and external conditions that affect the teats or the udder in general, increasing the somatic cell count.

The identified fungal isolates as in table (2) and table (3), revealed the high prevalence (73.33 %) of mycotic subclinical mastitis, (33.33%) due to *Candida albicans* and yeast *Rhodotorula* (8.89%) followed by fungi that were classified into the following genera *Penicillium spp.*, and *Aspergillus fumigatus* (3.33 % and 22.22 %, respectively) were shown to be the most common fungi linked with subclinical mastitis. A low percentage was identified as

*Aspergillus flavus* (2.22%). This investigation was in parallel with (Zhou *et al.*, 2013).

*Candida* is a yeast pathogen that thrives in a variety of environment. On its usual host, the yeast has evolved to be a successful commensal (Bradley, 2002). The isolation rate of fungal pathogenic organisms (33.33%) was greater than that of other aerobic pathogenic bacteria; these samples came from cases that had been treated with medications for more than two weeks. Excessive and unpredictable use of antibiotics, corticosteroids, and immunosuppressive medicines for chronic conditions are the leading causes of mastitis caused by yeast (Krukowski *et al.*, 2000).

California mastitis test (CMT) gives sharp discrimination between normal and subclinical mastitis milk samples, and it is considered the most important screening field test in predicting camel udder infection status comparatively to somatic cell count (SCC) (Sargeant *et al.*, 2001). The mean fat composition of the different mastitis infected milk showed a statistically significant variation ( $P < 0.05$ ) in chemical composition. According to Andrews *et al.* (2003), who described mastitis as a cause for a drop in fat composition, the current study's fat concentration resulted in a decrease in fat composition. Coulon *et al.* (2003) discovered that an increase in somatic cell count in milk caused by mastitis changed the fatty acid composition of raw milk.

The average percentage of protein was below the Egyptian legal standards, (Egyptian Standard, 2005), which were similar to those found by Romero *et al.* (2018). The decrease in protein could be related to injury to the mammary secretory tissue and the disruption of the blood-milk permeability barrier, which reduces protein transfer from interstitial tissue to milk (Hortet and seegers, 1998). Mastitis also has a significant impact on ionic concentrations. Sodium and chloride levels are elevated. Potassium, on the other hand, which is generally the most abundant mineral in milk, is decreasing. Other authors have validated that these increases in salt and chloride, as well as a drop in potassium levels, can be used to assess udder health (Mahran *et al.*, 1992; Vijayalakshmi *et al.*, 2001; Bruckmaier *et al.*, 2004). Damage to the ductal and secretory epithelium, opening of the "tight junctions" between secretory cells, and increased permeability of blood capillaries are all symptoms of intermammary infection. As a result, sodium and chloride (which are abundant in extracellular fluid) rush into the alveolus lumen, lowering potassium levels to maintain osmolality.

Table 1 Chemical examination of different parameters in examined subclinical mastitis milk (N=75).

Parameter	Minimum	maximum	Mean $\pm$ SE
Fat%	3.42	24.11	8.52 $\pm$ 0.76
SNF%	3.51	9.35	6.90 $\pm$ 0.53
Protein %	1.01	4.13	2.68 $\pm$ 0.29
Lactose %	1.19	4.90	3.56 $\pm$ 0.33
FP( $^{\circ}$ c)	-0.75	-0.25	-0.46 $\pm$ 0.02
Salt %	0.42	0.83	0.65 $\pm$ 0.01
T.S %	8.70	27.62	15.43 $\pm$ 1.2

Table 2 Incidence of fungal growth in subclinical mastitis milk.

Item	Total subclinical mastitis samples	Number of positive fungal samples	Number of negative fungal samples
Number	75	55	20
Percent%	100%	73.33%	26.66%

Item	Number of positive fungal samples	Single fungal isolates	Mixed fungal isolates
Number	55	45	10
Percent %	100	81.82%	18.18%

Table 3 Serotypes of fungal causing mastitis.

Single Fungal isolates	Number	percent
<i>Candida albicans</i>	15	33.33
<i>Penicillium</i>	15	33.33
<i>Aspergillus fumigatus</i>	10	22.22
Yeast <i>Rhodotorula</i>	4	8.89
<i>Aspergillus flavus</i>	1	2.22
Total	45	100%
Mixed Fungal isolates	Number	percent
<i>Penicillium</i> & <i>Candida albicans</i>	6	60
<i>Aspergillus fumigatus</i> & <i>Candida albicans</i>	3	30
<i>Aspergillus fumigatus</i> & <i>Penicillium</i>	1	10
Total	10	100

## 5. CONCLUSION

In buffalos, the prevalence of subclinical mastitis caused by mycotic mastitis has increased. The etiological agents vary from yeast and mold. The principal pathogen implicated was the yeast genus (*Candida albicans*) followed by different genera from mold as *Penicillium spp.*, and *Aspergillus fumigatus* were shown to be the most common mold. A low percentage was identified as *Aspergillus flavus*. The chemical examination of the subclinical mastitis milk showed decrease in protein, lactose, freezing point, and salt percent within the normal range. This research looked at the need for field-based fungal mastitis screenings as well as the development of safe strategies for controlling fungal contamination. Furthermore, to avoid any human health risks associated with mastitis, milk must be produced under appropriate hygienic conditions. To assure safe milk and milk products for consumers, we recommended efficient therapy for ill animals and efficient heat treatment for milk samples. Recent studies have focused on using nanotechnology to combat fungus in milk.

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