High prevalence of *Prototheca* spp. and isolation of fungal species in milk samples from cows suffering from mastitis in Mashhad city, northeast Iran

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**ABSTRACT**

The aim of this study was to investigate the fungi and algae isolated from milk samples in dairy cows with clinical and subclinical mastitis from dairy farms around Mashhad, Iran. A total of 503 milk samples were obtained from 10 industrial dairy farms. All samples were simultaneously cultured on the surface of Blood agar, Macconkey agar, and Sabouraud dextrose agar supplemented with chloramphenicol. Fungi and algae were identified using phenotypic characteristics. In the examined samples, the bacterial contamination (338 out of 503 samples; 67.20%) was the most dominant followed by algae (93 out of 503 samples; 18.5%), filamentous fungi (32 out of 503 samples; 6.4%) and yeast fungi (26 out of 503 samples; 5.2%). *Penicillium* spp. (2.8%), *Aspergillus* spp. (2.6%), *Cladosporium* spp. (1.2%), *Geotrichum* spp. (0.4%), *Ulucaladium* spp., *Scopolariopsis* spp. and *Alternaria* spp. (0.2% each), *Cryptococcus neofermenes* (3%), *Candida* spp. (1%), *Trichosporon* spp. (0.8%) and *Rhodoturula* spp. (0.4%) were the filamentous and yeast fungi isolated from milk samples. Of the 93 algae isolated from 503 milk samples (18.5%), 83 (16.5%) samples were positive for *Prototheca zopfii* and 10 (2%) samples for *Prototheca wickerhamii*. According to the results of this study, yeast fungi, filamentous fungi and algae, especially *Prototheca* spp. are important contaminant factors in milk and contribute to clinical and subclinical bovine mastitis. Therefore, proper sanitation practices and management of dairy herds and judicious use of antibiotics is essential to control the fungal and algal contamination both in the environment and the breast.

**Keywords**

*Mastitis, Cattle, Fungi, Algae, Prototheca* spp.

**Abbreviations**

*spp.*: Species  
SCC: Somatic cell count  
CMT: California Mastitis Test
**Introduction**

Bovine mastitis has been defined as an inflammation of the mammary gland usually as a consequence of microbial infection. Mastitis in cattle is a serious problem which causes considerable economic losses in dairy cattle herds. The most common etiological factors are bacteria followed by mycoplasmas, viruses, fungi and algae [1].

Mastitis is usually transmitted through contaminated milking machines and milker's hands or other materials. Treatment is possible with long-acting antibiotics, but milk from such cows is not marketable until drug residues have left the cow's system. Antibiotics may be administered systemically, or they may be applied locally by upward force through the teat canal. Antibiotic therapy, without identifying the mastitis-causing organisms, is frequently the veterinarian and dairy farmer's first choice of treatment for diseased cows. As a result, cases of mastitis (including fungal mastitis) that are refractory to any type of treatment occur frequently [2-4].

Most common isolated fungi in cases of mycotic mastitis are the yeasts from genera Candida and Cryptococcus, precisely two species Candida albicans (C. albicans) and Cryptococcus neoformans (C. neoformans). Candida is a commensal of mucocutaneous areas, particularly of the intestinal and genital tracts. Yeast mastitis can emerge like clinical, subclinical, chronic and sometimes acute mastitis. The ways of transmission is almost always connected with administration of medicaments in udder or with surgical or other procedure on it and so on. There are no differences between clinical manifestation of bacterial mastitis and yeast mastitis. Only by microbiological examination one can determine yeast mastitis. Prototheca is a genus of algae in the family Chlorellaceae. All species within this genus, even though classified as green algae, have forfeited their photosynthetic ability and have switched to parasitism [1, 5, 6].

Prototheca species (spp.) are colorless algae that can cause mastitis in dairy cattle. They are widespread in housing areas, pens and pastures used by dairy cattle. Most infections are clinical and remain as chronic infections. Prototheca spp. are often associated with wet areas containing decaying manure and plant matter [7, 8]. Prototheca infections are thought to occur when the teats of cows are exposed to high populations of algae in environmental sites during the milking intervals. Spread during milking time is not significant. However, new Prototheca infections can occur in situations where a high percentage of cows are infected with Prototheca and milking techniques are poor. Most mammary infections with Prototheca are clinical with the milk being grossly abnormal but without severe systemic signs such as off feed, depression or a high fever. Non-clinical outbreaks have been marked by normal milk with many quarters or cows with somatic cell count (SCC) greater than 1,000,000. In addition, most cows with protothecal infections will have reduced milk production [8].

Studies on fungal and algal infections of the mammary gland in cows are increasingly common due to their growing incidence. Therefore, in the present study we evaluated the isolation and frequency of fungal and algal species in dairy cows with clinical and subclinical mastitis in Mashhad, northeast of Iran.

**Results**

Out of 503 samples (232 from clinical cases and 271 from subclinical cases), 381 samples (75.74%) were positive for fungal, algal and bacterial contamination. In the examined samples, the bacterial contamination (338 out of 503 samples; 67.20%) was the most dominant followed by algae (93 out of 503 samples; 18.5%), filamentous fungi (32 out of 503 samples; 6.4%) and yeast fungi (26 out of 503 samples; 5.2%). There was no significant difference in bacterial/fungal/algal contamination between the clinical and subclinical mastitis cows (p > 0.05).

Overall, Penicillium spp. (2.8%), Aspergillus spp. (2.6%), Cladosporium spp. (1.2%), Geotrichum spp. (0.4%), Ululacadium spp., Scoparius spp. and Alternaria spp. (0.2% each), Cryptococcus neoformans (3%), Candida spp. (1%), Trichosporon spp. (0.8%) and Rhodoturula spp. (0.4%) were the filamentous and yeast fungi isolated from milk samples.

The culture-positive results obtained for Candida spp. showed that C. albicans, Candida krusei (C. kru-sei) and Candida glabrata (C. glabrata) were isolated from 1 (3.8%), 1 (3.8%) and 3 (11.5%) out of 26 samples, respectively.

Algal contamination was observed in only 3 dairy farms (30%). In dairy farm No. 1, Prototheca zopfi (Pro. zopfi) and Prototheca wickerhamii (Pro. wick-erhamii) were isolated form 14 (15.21%) and 2 (2.17%) samples, respectively; whereas in dairy farm No. 2, Pro. zopfi and Pro. wickerhamii were isolated from 17 (20.73%) and 1 (1.21%) samples and in dairy farm No. 4, they were isolated from 52 (25.49%) and 7 (3.43%) samples, respectively (Table 1).

Figures 1 and 2 and Table 1 show the frequency of fungal and algal isolates in bovine mastitis of dairy farms in suburb of Mashhad, Iran. Two dairy farms had no fungal and algal contamination. Dairy farm number 4 had the most frequency for yeast and algal contamination and dairy farm number 8 had the most frequency for mold contamination.

From 381 culture-positive milk samples, mixed
and single cultures were found in 98 samples (24.72%) and 283 samples (74.27%), respectively. Table 2 show the microorganisms isolated from mixed cultures in examined samples.

### Discussion

Conditions decreasing the resistance and susceptibility of cow udders to inflammations can be e.g. prolonged intra-udder antibiotics administration, increased incidence of udder mycosis results from mineral-vitamin deficiencies, antioxidant deficiencies, imbalanced diet, poor environmental conditions and even weather changes [10]. Our observations concerning higher incidence of mycotic mastitis in cows treated with antibiotics confirm the earlier results of other authors [11-13].

In general, fungi are normal flora of the soil and

![Figure 1](image1.png)

**Figure 1.**
The frequency of yeast fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.

*Three dairy farms had no yeast fungi contamination.*

![Figure 2](image2.png)

**Figure 2.**
The frequency of mold fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.

*Three dairy farms had no mold fungi contamination.*
Table 1
The frequency of algae isolated in examined samples in different dairy farms in suburb of Mashhad, Iran.

<table>
<thead>
<tr>
<th>Dairy farm</th>
<th>Number of samples</th>
<th>Pro. zopfii</th>
<th>Pro. wickerhamii</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (%)</td>
<td>Number (%)</td>
<td>Number (%)</td>
<td>Number (%)</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>14 (15.21)</td>
<td>2 (2.17)</td>
<td>16 (17.39)</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>17 (20.73)</td>
<td>1 (1.21)</td>
<td>18 (21.95)</td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>4</td>
<td>204</td>
<td>52 (25.49)</td>
<td>7 (3.43)</td>
<td>59 (28.92)</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>8</td>
<td>44</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>503</td>
<td>83 (16.50)</td>
<td>10 (1.98)</td>
<td>93 (18.48)</td>
</tr>
</tbody>
</table>

Table 2
The frequency of the microorganisms isolated from mixed cultures in examined samples

<table>
<thead>
<tr>
<th>Microorganisms in mixed cultures</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillium spp. + bacteria</td>
<td>4 (0.79)</td>
</tr>
<tr>
<td>Geotrichum spp. + bacteria</td>
<td>3 (0.59)</td>
</tr>
<tr>
<td>Cladosporium spp. + bacteria</td>
<td>2 (0.39)</td>
</tr>
<tr>
<td>Aspergillus flavus + penicillium spp. + bacteria</td>
<td>4 (0.79)</td>
</tr>
<tr>
<td>Cladosporium spp. + bacteria + Pro. zopfii</td>
<td>5 (0.99)</td>
</tr>
<tr>
<td>Aspergillus niger+ Cry. neoformans + bacteria</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Trichosporon spp. + bacteria</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Rhodotorula spp. + bacteria</td>
<td>3 (0.59)</td>
</tr>
<tr>
<td>C. glabrata + bacteria</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Cry. neoformans+ bacteria</td>
<td>2 (0.39)</td>
</tr>
<tr>
<td>Cry. neoformans + bacteria+ Pro. zopfii</td>
<td>8 (1.59)</td>
</tr>
<tr>
<td>Cry. neoformans + penicillium spp. + bacteria</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Rhodotorula spp. + bacteria + Pro. zopfii</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Pro. zopfii + Cry. neoformans</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Pro. zopfii + bacteria + C. albicans</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td></td>
<td>2 (0.39)</td>
</tr>
<tr>
<td>Pro. zopfii + C. glabrata + bacteria</td>
<td>1 (0.19)</td>
</tr>
<tr>
<td>Pro. zopfii+ bacteria</td>
<td>51 (10.13)</td>
</tr>
<tr>
<td>Pro. wickerhamii + bacteria</td>
<td>6 (1.19)</td>
</tr>
<tr>
<td>Total</td>
<td>98 (19.48)</td>
</tr>
</tbody>
</table>

rarely cause mastitis but sometimes can occur in epizootic proportions [14], especially in farms with poor environmental and hygienic conditions as well as the reduction in animal’s defense mechanisms [14, 15]. Poor quality of materials used as bedding (e.g. straw) with high humidity can be the source of fungi causing mastitis in cattle [14]. Fungi are also the reason of udder inflammation when udder is washed with water but is not dried [16].

Earlier studies conducted from 1982 to 1992 showed that about 6% of mastitis cases were caused by yeasts [17]; so that an important increase in the number of udder infections caused by Candida spp. and other yeasts was reported in various countries in recent years [15, 18-20].

The current study showed 11.53% fungal contamination (including 6.4% mold and 5.2% yeast) in milk samples from cattle with clinical and subclinical mastitis in the examined dairy farms.

In different studies, fungal contamination in cattle mastitis is variable. In this regard, in Tehran, Iran a comprehensive study was reported on the isolation of fungi by Talebkhan Garoussi et al. on milk of healthy, clinical and subclinical mastitis of dairy cows. They isolated different fungi from cows with clinical (14%), subclinical (18%) mastitis and healthy animals (15%)
then incubated aerobically at 37°C for 24-48 h for bacteria growth until processing (no longer than 24 h after collection). All samples were collected from cows with clinical and subclinical mastitis [23]. Several studies have been conducted on the isolation of fungi in other countries with rates of 17.3% in Brazil, 24.24% in Poland, 64% in India, and 6% in Serbia [15, 24-26].

Our results are considered to be the first report on the high prevalence of *Prototheca* spp. (18.5%) (including 16.5% *Prototheca zopfii* and 2% *Prototheca wickerhamii*) in examined milk samples in Mashhad, Iran. Poor environmental conditions, inappropriate milking hygiene and prolonged antibiotic therapy can be the reason of increased protothecal mastitis occurrence that can reach even over 30% [27]. Studies carried out by Krukowski et al. (2006), Lassa et al. (2013), Milanov et al. (2014) and Jagielski et al. (2019) revealed that *Prototheca* spp. was responsible for 0.35%, 0.9%, 4.6% and 11.3% of mastitis cases, respectively [15, 18, 20, 28]. Presence of the algal species *Prototheca zopfii* was demonstrated in analyzed samples from cows with mastitis in different papers [18, 27, 29-31]. In the present study, the investigation in examined dairy farms showed that feeding milk cattle containing fungi and algae (e.g. sugarcane bagasse) have increased the occurrence of protothecal mastitis.

According to the results of this study, yeasts and molds and algae, especially *Prototheca*, are important milk contaminants and contribute to the development of clinical and subclinical mastitis in dairy cattle. Compliance with the hygiene and management principles of dairy herds is essential for the control of fungal and algal agents in the environment as well as in the breast in order to prevent the development of clinical and subclinical fungal and algal mastitis and subsequently to prevent its economic losses and protect the community health.

Material and methods

During spring and summer of 2018, a total of 503 milk samples were collected from cows with clinical and subclinical mastitis from 10 industrial dairy cattle herds in suburb of Mashhad, Iran. Cows with clinical and subclinical mastitis were determined by clinical symptoms and using a California Mastitis Test (CMT), respectively [8, 9]. Cows were not taken antibiotic(s) before sampling. Before sampling, the teats were washed, cleaned and disinfected using 95% alcohol. The first two stripping of milk were discarded. 15ml of milk was collected from each animal in sterile plastic tubes and transported to the laboratory under ice, and kept at 4°C. 15ml of milk was collected from each animal in sterile plastic tubes and transported to the laboratory under ice, and kept at 4°C.

ACKNOWLEDGEMENTS

The authors are grateful to the faculty of veterinary medicine, Ferdowsi University of Mashhad for funding this research (Grant no: 3/46511).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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چکیده
این مطالعه با هدف بررسی قارچ‌ها و جلبک‌های جدا شده از نمونه‌های شیر از گاوهای مبتلا به ورم پستان بالینی و گاوداری صنعتی در محیط ۵۰۳ نمونه از گاوهای مبتلا به ورم پستان بالینی و گاوداری صنعتی در مناطق مختلف شهر مشهد انجام شد. تشخیص قارچ‌ها و جلبک‌ها با استفاده از خصوصیات فنوتیپی انجام شد. بیشترین میزان آلودگی به پروتوتکا و ورشکش در نمونه‌های ۴۰.۲٪ (۱۰۳ نمونه) داشته ۱۰.۲٪ (۲۴ نمونه) آلودگی به نرمک داشته و ۲۴.۸٪ (۶۱ نمونه) آلودگی به جلبک و ۱۵.۱٪ (۳۷ نمونه) آلودگی به پروسپرالیس و آلترناریا داشته. بیشترین میزان آلودگی به قارچ‌ها در گروه پاتوبیولوژی دانشکده دامپزشکی دانشگاه فردوسی مشهد بود که ۳۷.۸٪ (۸۸ نمونه) آلودگی را داشته و بیشترین میزان آلودگی به جلبک‌ها در گروه علوم درمانگاهی دانشکده دامپزشکی دانشگاه فردوسی مشهد بود که ۴۷.۷٪ (۱۰۶ نمونه) آلودگی را داشته.

وسیله‌کشی
ورم پستان، گاو، قارچ‌ها، جلبک‌ها، گونه‌های پروتوتکا