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RESEARCH ARTICLE

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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

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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 (Cry. 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 tit 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%), *Ulucaladium* spp., *Scopolariopsis* 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. krusei*) 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 zopfii* (*Pro. zopfii*) and *Prototheca wickerhamii* (*Pro. wickerhamii*) were isolated form 14 (15.21%) and 2 (2.17%) samples, respectively; whereas in dairy farm No. 2, *Pro. zopfii* 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

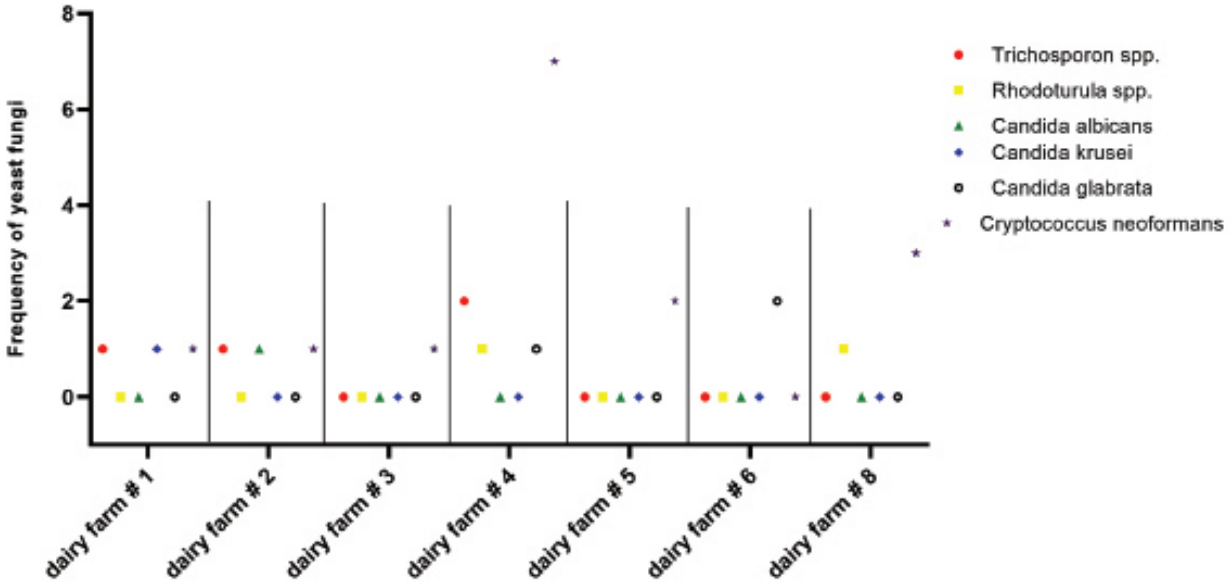

Figure 1.
The frequency of yeast fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.
* Three dairy farms had no yeast fungi contamination.

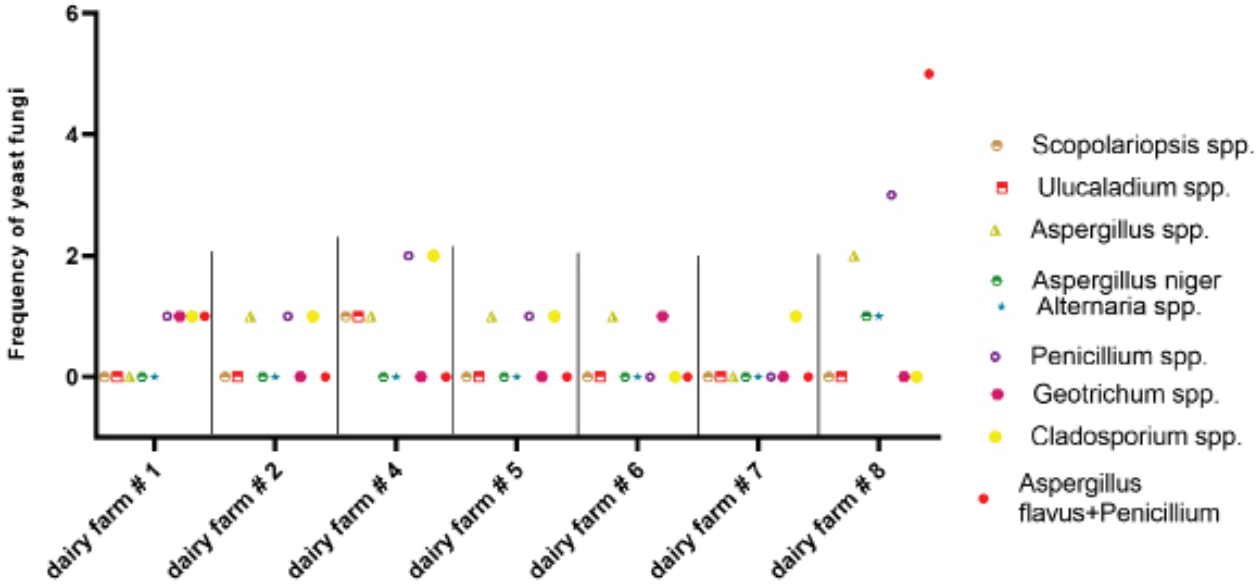

Figure 2.
The frequency of mold fungi isolated from dairy cows with clinical and subclinical mastitis in different dairy farms in suburb of Mashhad, Iran.
* Three dairy farms had no mold fungi contamination.

Table 1
The frequency of algae isolated in examined samples in different dairy farms in suburb of Mashhad, Iran.

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

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

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

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%)

[21]. Rasouli in Tabriz, Iran showed that 12.07% of milk samples of cows with mastitis had fungal contamination [22]. Batavani et al in Urmia, Iran isolated fungi (7.5%) (including 4.16% mold and 3.33% yeast) from milk of dairy 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% P*rototheca 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 tube and transported to the laboratory under ice, and kept at 4°C until processing (no longer than 24 h after collection). All samples were simultaneously cultured on the surface of Blood agar (Merck Co., Darmstadt, Germany), Macconkey agar (Merck Co., Darmstadt, Germany) and Sabouraud dextrose agar (Merck Co., Darmstadt, Germany) supplemented with chloramphenicol. Plates were then incubated aerobically at 37°C for 24-48 h for bacteria growth and 25°C for 2 weeks for fungi and algae. Bacterial media were only studied based on the presence or absence of bacterial growth. Filamentous fungi were identified by standard mycological techniques based upon gross cultural and microscopic morphology. Yeast and algal colonies were identified on the basis of macro- and micromorphological characteristics, and on the basis of physiological characteristics, such as melanin production on Caffeic Acid Ferric Citrate test agar (HiMedia, India), presence of capsule by Nigrosin (Merck Co., Darmstadt, German) stain, urease production on urea agar medium (Merck Co., Darmstadt, Germany), the germ tube test, micromorphological analysis on corn meal Tween 80 agar, growth in CHROMagar candida (CHROMagar Co., Paris, France) and API 20 C AUX system (BioMérieux, France). The data were analyzed using the Chi-square and Fisher Exact tests. A *p*- value less than 0.05 was considered significant.

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Author Contributions

M.L. performed the experiments and drafted the manuscript. S.E. designed and conducted the study, analyzed the data, drafted and reviewed the manuscript. B.K. supervised sample collection.

Conflict of Interest

The authors declare no conflict of interest.

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Abstracts (in Persian)

شیوع بالای گونه های پروتوتکا و جداسازی گونه های قارچی در نمونه های شیر از گاوهای مبتلا به ورم پستان در شهر مشهد، شمال شرق ایران

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چکیده

این مطالعه با هدف بررسی قارچ ها و جلبک های جدا شده از نمونه های شیر اخذ شده از گاوهای مبتلا به ورم پستان بالینی و تحت بالینی در گاوداری های اطراف مشهد انجام شد. ۵۰۳ نمونه ی شیر از ۱۰ گاوداری صنعتی گرفته و هر نمونه همزمان در محیط های بلاد آگار، مک کانکی آگار و سابورو دکستروز آگار حاوی کلرآمفنیکل کشت داده شد. تشخیص قارچ ها و جلبک ها با استفاده از خصوصیات فنوتیپی انجام شد. بیشترین میزان آلودگی مربوط به باکتری ها (۶۷.۲۰٪؛ ۳۳۸ نمونه از ۵۰۳ نمونه) بود و بعد از آن جلبک ها (۱۸.۵ ٪؛ ۹۳ نمونه از ۵۰۳ نمونه)، قارچ های رشته ای (۶.۴ ٪؛ ۳۲ نمونه از ۵۰۳ نمونه) و قارچ های مخمری (۵.۲ ٪؛ ۲۶ نمونه از ۵۰۳ نمونه) قرار داشتند. پنیسیلیوم (۲.۸ ٪)، آسپرژیلوس ها (۲.۶ ٪) ،کلادوسپوریوم (۱.۲ ٪)، ژئوتریکوم (۰.۴ ٪)، اولوکلادیوم، اسکوپولاریوپسیس و آلترناریا (هر کدام ۰.۲٪)، کریپتوکوکوس نئوفرمنس (۳ ٪)، کاندیداها (۱ ٪)، تریکوسپورون (۰.۸ ٪) و رودوتورولا (۰.۴ ٪) قارچ های رشته ای و مخمری جدا شده از نمونه های شیر مورد بررسی بودند. از ۹۳ جلبک جدا شده از ۵۰۳ نمونه شیر (۱۸.۵ ٪)، ۸۳ نمونه (۱۶.۵ ٪) آلوده به پروتوتکا زوپفی و ۱۰ نمونه (۲ ٪) آلوده به پروتوتکا ویکرهامی بودند. با توجه به نتایج حاصل از این تحقیق، قارچ های مخمری،کپکی و جلبک ها بخصوص پروتوتکاها از عوامل مهم آلوده کننده شیر بوده و در ایجاد ورم پستان نقش دارند. بنابراین، شیوه های بهداشتی مناسب و مدیریت گله های شیری و استفاده مدبرانه از آنتی بیوتیک ها برای کنترل آلودگی قارچی و جلبکی محیط و پستان ضروری است.

واژگان کلیدی

ورم پستان، گاو، قارچ ها، جلبک ها، گونه های پروتوتکا