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Prevalence of *Prototheca* and Fungal Contamination of Bulk Milk Tank of Industrial Dairy Cattle Herds in Iran

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Abstract

BACKGROUND: *Prototheca* spp. belongs to the family *Chlorellaceae* and can cause mastitis in dairy cattle herds. Several *Prototheca* species could be isolated in different parts of the dairy herds.

OBJECTIVES: The objectives were to determine: (1) the investigation status of bulk milk tank *Prototheca Spp* prevalence in industrial dairy cattle herds in different area of Iran. And (2). To evaluate the fungal agent prevalence of bulk milk tank of dairy cattle herds in different seasons.

METHODS: Bulk milk tank for the presence of *Prototheca* and fungal agents from 165 industrial dairy cattle herds complexes were tested. The population of each herd was about 850–3500 Holstein dairy cows. The samples were taken from 32 cities and 16 provinces of Iran, respectively. In total, almost 66800 Holstein dairy cows were represented. The population of lactating cows included about 25800. Samples were cultured on sabouraud glucose agar (SGA) medium with chloramphenicol (100 mg/L).

RESULTS: Only 8 (4.84%) out of 165 samples were isolated for *Prototheca* spp. It had been isolated in different seasons. They were 2, 4, 1, and 1 samples in spring, summer, autumn and winter, respectively. *Candida*, *Fusarium*, and *Trichosporon* spp. were isolated from 137, 2 and 1 samples, respectively. According to the obtained results, *Prototheca* and fungal agents are present in bulk milk tanks of industrial dairy cattle herds in different provinces of Iran.

CONCLUSIONS: It is concluded that exposure to *Prototheca* and different fungal agents could be common in the dairy cattle herds in Iran. The results are important as dairy cattle health and human sanitation hazards.

KEYWORDS: Bulk milk tank, Dairy cattle herds, Fungi, Iran, *Prototheca*

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Introduction

There are several infectious agents that can cause mastitis in cows, such as bacterial agents, fungal and algae agents (Blowey & Edmondson, 2010; Talebkhan Garoussi, Khosravi & Pandamoz, 2012; Kurumisawa *et al.*, 2018). Fungal agents can cause mastitis, such as yeasts from genera *Candida* (*Candida albicans*) and *Cryptoccocus (Cryptotoccus neoformans)* which are the most common agents (Spanamberg *et al.*, 2008; Bakr *et al.*, 2015; Eldesouky *et al.*, 2016).

Mastitis is one of the main diseases in industrial dairy cattle herds. It causes major economic losses from reduced milk production and quality, milk wastage, elimination and treatment costs to reproductive disorders. Despite many efforts to control and prevent mastitis and accomplishment of udder health programs, it is still considered the most expensive disease in dairy industry (Blowey & Edmondson, 2010; Shahid *et al.*, 2016; Kalinska, Gołębiewski & Wójcik, 2017).

Prototheca species can cause mastitis (Osumi *et al.*, 2008; Milanov *et al.*, 2016). *Prototheca spp.* belong to the family *Chlorellaceae*. They lost their ability to synthesize chlorophyll and adopted a heterotrophic mode of nutrition and became achlorophyllous algae (Anderson & Walker, 1988; Rapuntean *et al.*, 2009; Krukowski *et al.* 2013; Irrgang *et al.*, 2015). *P. blaschkeae, P. zopfii, P. cutis* and *P. wickerhamii* are animal pathogens and can cause severe infections in low sanitary conditions or immune system deficiency and also can cause local or systemic diseases for humans as a zoonotic agent (Roesler, Scholz & Hensel, 2003; Milanov & Suvajdžić, 2006; Lass-Florl & Mayr, 2007; Marques, 2008; Satoh *et al.*, 2010; Wawron *et al.*, 2013; Masuda *et al.*, 2016). Several *Prototheca* species could be isolated in different parts of the dairy herds. However, *Prototheca* species live and grow in humid places, especially wet places contaminated to agronomy and animal dung, sewage, stagnant spate, soil, mud, corrupt feed, excess animal feed, dirty trough and cow and calf excretion (Anderson & Walker, 1988; Masuda *et al.*, 2016). Moreover, they can bide in several materials such as steel, glass, plastic, and polypropylene and, after transportation to a humid place, duplicate and form biofilm (Pompermayer & Gaylarde, 2000; Marques *et al.*, 2008).

The major clinical mastitis can cause by *P. zopfii* (genotype 2), *P. blaschkeae* and *P. wickerhamii*. They have been isolated only from milk samples which obtained from sporadic mastitis cases (Roesler *et al.*, 2006; Marques *et al.*, 2008). These species can cause clinical mastitis (Osumi, *et al.*, 2008; Ricchi, *et al.*, 2010; Jagielski, *et al.*, 2011).

Prototheca is one of the zoonotic agents, and its presence in milk can cause human protothecosis. In the milk industry, pasteurization is used to eliminate microbial agents, while *Prototheca* are resistant to pasteurization (Lass-Florl & Mayr, 2007). Therefore, consuming contaminated milk to *Prototheca* agent can be a sanitation hazard (Lass & Mayr, 2007; Marques *et al.*, 2008).

Prototheca was first detected in 1894 by Krüger, and the first detection of *Prototheca* as a mastitis agent was in 1954 in Germany (Pieper *et al.*, 2012). However, it was shown that it could be a mastitis agent worldwide as it was reported in the USA (Anderson & Walker, 1988), Denmark (Aalbaek *et al.*, 1998), Italy (Buzzini, *et al.*, 2004), Brazil (Bueno *et al.*, 2006), Japan (Osumi *et al.*, 2008), and Canada (Pieper *et al.*, 2012). In the past years, the rates of *Prototheca* mastitis reports were little due to less attention to its role as a mastitis agent, but recently, the knowledge about this agent has increased, which led to an increase in detection and reports of *Prototheca* mastitis (Milanov *et al.*, 2016, Ranjan *et al.*, 2006). According to the reports, *Prototheca* was isolated from clinical mastitis in Iran (Lavaee *et al.*, 2019; Zaini *et al.*, 2012).

Prototheca and fungal agents are important agents in causing mastitis, and milk contamination can be isolated from bulk milk tanks of industrial dairy cattle herds in Iran. The aims of the present study were to determine: 1) the investigation status of bulk milk tank *Prototheca Spp* prevalence in different seasons in industrial dairy cattle herds in different areas of Iran; 2) To evaluate the fungal agent prevalence of dairy cattle herds.

Materials and Methods

Geographical Regions, Study Population and Management of the Herds

In total, samples were collected randomly from 165 industrial dairy cattle herds in different areas of Iran. Samples were taken true randomly using a lottery mechanism in the dairy cattle herds. The approximate minimum required sample size was calculated based on the following formula, which according to the confidence level (95%), desired absolute precision 5% (5%) and expected prevalence (10%) will be as follows (Thrusfield and Christley, 2018):

$$N = \frac{1.96^2 \times Pexp \times (1 - Pexp)}{d^2}$$

Where Pexp = expected prevalence; d = desired absolute precision; 1.962 = multiplier for level of confidence 95%. Therefore, substituting these values in the above formula:

N: minimum required sample size

$Pexp$: expected prevalence

d: desired accuracy

The minimum required sample size was 138 (Thrusfield and Christley, 2018). The commercial-industrial herds use more advanced technology with average milk production of about almost 10000 Kg/cow/year. We included only the commercial-industrial stratum farms that processed a bulk milk tank for this study. The industrial dairy cattle herds are the major milk producer in different areas. Farmers deliver milk production to the local dairy industries or export milk somewhere else in Iran. Samples of bulk milk tanks were obtained from 165 industrial dairy cattle herd complexes selected according to a proportional geographical distribution in various parts of Iran. The samples were taken from dairy cattle herds in 32 cities and 16 provinces, respectively (Figure 1).

In total, almost 66800 Holstein dairy cows were represented. The lactating cow population included about 25800. The minimum and maximum of lactating cows in the herds were about 850 and 3000, respectively. The milk production of cows averaged about 11500 per lactating period. All of the cows were Holstein's breed. Almost more than 75% of the dairy cows had a free-stall system. They were housed in an intensive system with cubical bedding. They were typically fed alfalfa, corn silage, and concentrate in various proportions using a mixed ration (TMR). The cows were milked 3 times a day using a milking machine. All of the cows were vaccinated against brucellosis. The animals were immunized against foot and mouth disease and Clostridial diseases. All of the herds used artificial insemination. Nutrition and reproduction management of the herds were controlled using computerized herd health management.

Sample Collection

A bulk milk sample was obtained from each herd from March 2019 to March 2020. The owners of the herds were not informed of the aims of this study. They were collected in 500 mL volume in a sterile bottle after finishing the whole milking process (Bauman *et al.*, 2018). No preservative was used. The samples were put on ice and transported directly to the laboratory of Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Sample transfer took a maximum of 16 hours.

Culture, Isolation, and Purification of Grown Microorganisms

Beside the flame, 0.01 mL of each milk sample was cultivated on a Sabouraud Glucose Agar (SGA) medium with chloramphenicol (100 mg/L) (Difco Laboratories, USA). Cultivated samples were incubated aerobically at 37°C for 7 days (Corbellini *et al.*, 2001).

All grown colonies were purified on SGA culture media for isolation and *Prototheca* spp. and mycotic flora identification. Isolation of purified colonies was based on morphological features, and staining was performed using the lactophenol cotton blue method. Protothecal colonies had a creamy appearance with white to gray-white color. Isolates were stored in TSB medium enriched with 0.6% yeast extract and 20% glycerol at -20°C. Other fungal colonies and yeast species such as *Candida* spp., *Geotrichum* spp., *Fusarium* spp., and *Trichosporon*

spp. were also purified, isolated, and identified using the lactophenol cotton blue method.

Staining

In lactophenol cotton blue staining, one drop was placed on a slide, then a very small part of *Prototheca* colony was picked up by an inoculation loop and carefully placed on the drop of staining solution on the slide. Then a lamel was placed on the mixture of stain and sample, and examination was done with a magnification of 10 and 40. Organisms suspended in the stain were killed due to the presence of phenol. On the other hand, a high phenol concentration deactivated lytic cellular enzymes; thus, the cells did not lyse. Cotton blue was an acid dye that stained the chitin present in the cell wall. It is important not to take too much from the sample to make microscopic observation easy.

Statistical Analysis

The data were analyzed using the x2 statistic method using SAAS version 9.2.

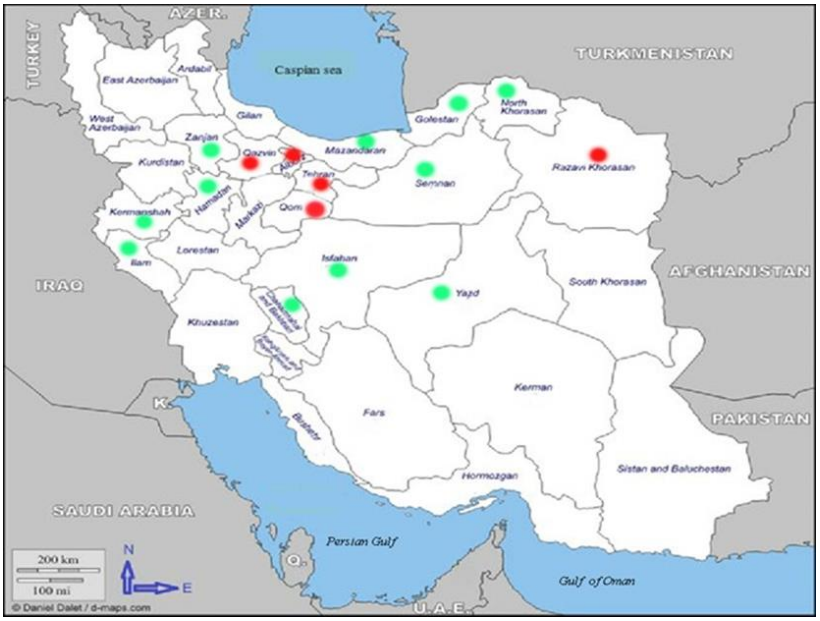

Figure 1. Distribution of milk sample collection for *Prototheca* contamination of bulk milk tank of industrial dairy cattle herds in Iran.
Green circles show negative samples as *Prototheca Spp* isolates.
Red circles show positive samples as *Prototheca Spp* isolates.

Results

In total, 165 samples were collected. They were taken during spring (32, 19.4%), summer (58, 35.15%), autumn (13, 7.9%) and winter (62, 37.5%).

Prototheca spp. was isolated from 8 (4.84%) samples which were taken from 8 different dairy cattle herds in 5 cities (Mashhad, Saveh, Varamin, Abyek, and Karaj) (Figure 1) (Table 1). There were no significant differences among the samples collected in different seasons ($P<0.05$) (Table 1). Table 2 shows the different alga and fungal infections of bulk milk tanks of dairy cattle herds. *Prototheca* spp. was not isolated from the bulk milk tank of dairy cattle herds in other provinces (Figure 1).

Table 1. Distribution of *Prototheca Spp* contamination of bulk milk tank of industrial dairy cattle herds in different seasons in Iran.

***Prototheca* spp.**	**Season**				**Total (%)**
	Spring (%)	Summer (%)	Autumn (%)	Winter (%)	
+	2(1.21)	4(2.42)	1(0.6)	1(0.6)	8(4.84)
-	30(18.18)	54(32.72)	12(7.27)	61(36.96)	157(95.15)

Total	32(19.39)	58(35.15)	13(7.87)	62(37.57)	165

Table 2. Frequency of *Prototheca* spp. and fungal isolates from bulk milk tank of dairy cattle herds of industrial dairy cattle herds in Iran.

Agent	Spring	Summer	Autumn	Winter	Total
Prototheca	0	2	0	0	2
Yeast Fungi					
Geotrichum candidum	6	0	0	2	8
Trichosporon beigelii	0	1	0	0	1
Candida Spp.	22	33	8	50	113
Mold					
Fusarium	2	0	0	0	2
Mix agents contamination					
Geotrichum candidum & Candida Spp.	0	4	4	9	17
Candida Spp. & Rhodotorula Spp.	0	1	0	0	1
Prototheca & Trichosporon beigelii	1	0	0	0	1
Prototheca & Candida Spp.	1	1	1	1	4
Prototheca, Candida Spp. & Geotrichum candidum	0	1	0	0	1
Geotrichum candidum, Candida Spp. & Rhodotorula	0	1	0	0	1
No Growth	0	14	0	0	14
Total	32	58	13	62	165

Candida spp. was the most (113, 68, 48%) yeast isolated in purity. However, this agent was isolated in a mixed bulk milk tank contamination (Table 2). Fungal agents were isolated from 95.15% of the bulk milk tank of dairy cattle herds.

Discussion

Prototheca spp. can play an essential role in causing contamination of bulk milk tank of dairy cattle herds complexes and possibly bovine mastitis. *Prototheca* was isolated from 8(4.84%) dairy farms in 5 (15.62%) cities and 5 (31.25%) provinces, respectively (Table 1). However, most (95.15%) of the bulk milk tanks were contaminated by mycotic agents. This alga was detected in different seasons of the year. It was more isolated in summer 4 (2.42%) than in the other seasons. Probably several environmental conditions can affect the growth and reproduction of this agent (Corbellini *et al.*, 2001). This agent is widely distributed in nature and other resources such as food materials. Humid areas, mainly containing manure and decomposing plant material, are its natural habitat (Rapuntean *et al.*, 2009; Inoue *et al.*, 2018). As the diet of dairy cattle is plant origin, the algae may be a transient flora in the gastrointestinal tract of dairy cows. Therefore, it can be excreted intact (Marques *et al.*, 2008). As a consequence of recent global climate changes, high temperatures, often combined with high humidity, enhance the multiplication of the algae in the environment (Wawron *et al.*, 2013). At a dairy farm, *Prototheca* was isolated from drinking water, bovine feces and sewage water (Osumi *et al.*, 2008). *Prototheca* was also isolated from milking machine liners and milking cups (Krukowski *et al.*, 2013). Therefore, this agent may be transferred to healthy cows in the same way as contagious mastitis agents (Anderson & Walker. 1988).

In this survey, it was shown that *Prototheca* spp. could be isolated purely or combined with other fungi (Table 2). We found that yeast was the common opportunistic pathogen in bulk milk tank samples. *Candida* was isolated from 68.48% (113 out of 165) of bulk milk tank samples (Table 2). Each fungal infection can arise from a saprophytic organic matter, commonly moldy hay or straw, or moist feeds such as beet pulp, corn silage, and wet grains. However, most of these organisms are opportunists

with different sources, including the skin of the udder, hands of milking man, milking machines, treatment instruments, floor, straw, feed, dust, drug mixtures, and sanitation solutions (Talebkhan Garoussi, Khosravi & Pandamoz, 2012; Garoussi *et al.*, 2017). On the other hand, they can be isolated as the common agents from any bulk milk tank sample.

Prototheca culture is not a common process in a laboratory. So it may get less attention. (Alves *et al.*, 2017). The other reason is the unfamiliarity of farm veterinarians with the epidemiological and clinical aspects of *Prototheca* mastitis (Milanov *et al.*, 2016; Jagielski *et al.*, 2019[a]). This issue may even cause the herd to become involved in *Prototheca* mastitis, and this lack of awareness can lead to the identification of this factor when the herd is involved with this alga (Milanov *et al.*, 2016).

Prototheca species can cause important individual mastitis in dairy cattle herds, which usually don't respond to current therapies (Jánosi *et al.*, 2001; Marques *et al.*, 2008; Jagielski *et al.*, 2017; Park *et al.*, 2019). Therefore, prevention and health programs must be fully conducted in dairy cattle herds.

It was reported that the milk *Prototheca* contamination was less than 0.5% in Poland (Krukowski, 2006; Krukowski *et al.*, 2009). It was isolated from 8/172 milk samples of 7 dairy farms using a culture method (Jagielski *et al.*, 2019[b]). In the US, it was stated that from total 787 individual dairy farm bulk milk tank samples examined, 3.6% samples contained *Prototheca* spp. (Pore *et al.*, 1987). In Korea, 187 isolates were identified out of 2508 quarter milk samples which shows 7.5% quarter infection (Park *et al.*, 2019).

It was reported that the rate of *Prototheca* milk contamination was 9% in herds with clinical mastitis and usual therapy in Brazil (Corbellini *et al.*, 2001). *Prototheca* was isolated from 22 out of 73 milk samples in a dairy herd with chronic mastitis in Serbia (Milanov & Suvajdžić, 2006). In 2014, Bozo *et al.* reported 9% contamination rate of milk samples of individual cows of 5 herds to *Prototheca* in Italy (Bozzo *et al.*, 2014). It seems that these higher relative rates are due to clinical appearance and the herd level of Protothecosis.

Prototheca infection was detected from 7.4%the mammary glands of dairy cows using PCR (Ricchi *et al.*, 2010). However, *Prototheca* spp. were isolated from dairy cows, bulk milk tanks of dairy cattle herds, and the related environment (Ricchi *et al.*, 2013). It shows that the environment plays an important role. However, PCR may be helpful for the isolation of *Prototheca* from bulk milk tank samples which must follow this infection in future studies in Iran (Jagielski *et al.*, 2018). Differences in infections may be due to geographical differences such as climate, weather, rainfall, altitude, and other factors that may affect the growth, production, and sustainability of *Prototheca*. In this study, it was shown that *Prototheca* was more isolated in summer samples than the others.

Causal relationships between various factors in each case are still difficult to establish. Poor hygiene practices, excessive antibiotic use, and inadequate milking hygiene are most commonly considered the major risk factors for mastitis development and bulk milk tank contamination of dairy cattle herds (Pieper *et al.*, 2012; Wawron *et al.*, 2013; Gonçalves *et al.*, 2015).

It was shown that fungal mastitis increased in recent years across the world (Rui 2020). According to the reports, fungal agents have been isolated from 17.3% and 64% samples in Brazil and India, respectively (Dos Santos & Marine. 2005; Krukowski *et al.*, 2006; Türkyılmaz & Kaynarca, 2010; Zaragoza *et al.*, 2011). It may be due to increased bacterial mastitis agents control programs in dairy cattle herds. Therefore, fungal agents can be grown. Fungal infection rate in cows with clinical, subclinical and healthy cows in Iran were 14%, 18% and 15%, respectively (Talebkhan Garoussi, Khosravi & Pandamoz, 2012). However, the contamination rate in cows was stated at 12.07% with clinical mastitis in Tabriz, Iran (Rasouli, 2016). While, it was reported 7.5% in clinical and subclinical mastitis in Urmia, Iran (Batavani *et al.*, 2002).

In this study, *Prototheca* was isolated from bulk milk tanks of dairy cattle herds in 5 provinces, the major livestock and dairy producers in Iran. Therefore, livestock densities may play an important role in this infection. *Prototheca* and fungal agents must be considered as a zoonotic disease.

It is believed that the actual prevalence of *Prototheca spp.* in positive herds and animals was even higher than the one determined in every study because the dry cows at the time of the investigation were not tested. Moreover, intermittent shedding of the algae in milk may prevent detection of the agent during one single testing or examination of lactating cows or bulk milk tank of the dairy cattle herds (Morandi *et al.*, 2017). It is reported that unsanitary or repeated intra-mammary infusions and antibiotic treatment of cows with mastitis can contribute to developing *Prototheca* infection of the mammary gland (Pieper *et al.*, 2012; Kano *et al.*, 2018). Excessive usage of antibiotics and consequent elimination of the competitive natural udder flora can give rise to the development of algal infections within the mammary gland(s) and subsequently shedding to the bulk milk tank and sanitation hazards for human health (Fernández *et al.*, 2019).

Conclusion

It was concluded that *Porototheca* and the fungal agents (mainly *Candida*) were isolated as the mycoflora of bulk milk tank samples in Holstein dairy herds in Iran. We suggest that these isolates can be members of the resident or opportunistic mammary gland disorder processes or environmental resources, which can be necessary as a sanitation hazard. This study was also important from the point of view of veterinarian studies and humans' health since bulk milk tank contamination in dairy cattle herds can affect general human health. More studies must be carried out in dairy cattle herds *Protothca* and fungal infection.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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بررسی شیوع پروتوتکا و آلودگی قارچی مخازن شیر دامپروری‌های صنعتی ایران

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زمینه مطالعه: *Prototheca Spp.* متعلق به خانواده Chlorellaceae است که می‌تواند باعث بروز ورم پستان در گاوهای شیری شود. گونه‌های متعددی از *Prototheca* را می‌توان از قسمت‌های مختلف گله‌های شیری جداسازی نمود.

هدف: اهداف این بررسی برای ۱) بررسی وضعیت شیوع *Prototheca Spp.* در گاوداری‌های شیری مناطق مختلف ایران است ۲) ارزیابی شیوع عوامل قارچی در شیر مخازن گاوداری‌های شیری در فصول مختلف سال ایران.

روش کار: مخازن شیر ۱۶۵ دامپروری صنعتی ۳۲ شهر از ۱۶ استان ایران به‌منظور وجود پروتوتکا و عوامل قارچی آزمایش شد. به‌طورکلی، تقریباً ۶۶۸۰۰ راس گاو نژاد هلشتاین تحت نظر قرار گرفتند. تعداد گاوهای شیری تقریباً ۲۵۸۰۰ راس بود. نمونه‌ها در محیط سابوروگلوکز آگار همراه با کلرامفیکل (۱۰۰ میلی‌گرم/لیتر)کشت داده شدند.

نتایج: فقط از ۸ نمونه از ۱۶۵(۴/۸۴ درصد) نمونه شیر مخازن، پوروتوتکا جداسازی شد. پرووتکا در فصول مختلف جداسازی شد. این عامل به ترتیب در فصول بهار، تابستان، پاییز و زمستان به ترتیب: ۲، ۴، ۱ و ۱ نمونه بود. *کاندیدا*، *فوزاریوم* و *تریکوسپورن* به ترتیب از: ۱۳۷، ۲ و ۱ نمونه جداسازی شد. بر اساس اطلاعات به‌دست‌آمده پروتوتکا و عوامل مختلف قارچی در شیر مخازن دامپروری‌های صنعتی ایران حضور دارند.

نتیجه‌گیری نهایی: نتیجه گرفته می‌شود که تماس گاوهای شیری ایران و شیر مخازن گله‌های شیری ایران با پروتوتکا و عوامل قارچی شایع است. نتایج از نظر بهداشت گاوهای شیری و مخاطرات بهداشتی انسان حائز اهمیت است.

واژه‌های کلیدی: شیر مخازن، گله‌های شیری، قارچ، ایران، پروتوتکا

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