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
Introduction

There are several infectious agents that can cause mastitis in cows, such as bacterial agents, fungal and algae agents (Blowey & Edmondson, 2010; Talebkhani Garoussi, Khosravi & Pandamoz, 2012; Kurumisawa et al., 2018). Fungal agents can cause mastitis, such as yeasts from genera Candida (Candida albicans) and Cryptococcus (Cryptococcus 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; Irgang 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 & Su vajdžić, 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 hide 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):

\[
N = \frac{1.96^2 \times P_{exp} \times (1 - P_{exp})}{d^2}
\]

Where \( P_{exp} \) = expected prevalence; \( d \) = desired absolute precision; 1.962 = multiplier for level of confidence 95%. Therefore, substituting these values in the above formula:

\[
N: \text{minimum required sample size} \\
P_{exp}: \text{expected prevalence} \\
d: \text{desired accuracy}
\]

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 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 Protothec 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 de-activated 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.

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**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>
<thead>
<tr>
<th><em>Prototheca</em> spp.</th>
<th>Season</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spring (%)</td>
<td>Summer (%)</td>
</tr>
<tr>
<td>+</td>
<td>2(1.21)</td>
<td>4(2.42)</td>
</tr>
<tr>
<td>-</td>
<td>30(18.18)</td>
<td>54(32.72)</td>
</tr>
</tbody>
</table>

**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.

**Table 1.** Distribution of *Prototheca Spp* contamination of bulk milk tank of industrial dairy cattle herds in different seasons in Iran.
Table 2. Frequency of *Prototheca* spp. and fungal isolates from bulk milk tank of dairy cattle herds of industrial dairy cattle herds in Iran.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prototheca</em></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Yeast Fungi</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Geotrichum candidum</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Trichosporon beigelii</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Candida Spp.</em></td>
<td>22</td>
<td>33</td>
<td>8</td>
<td>50</td>
<td>113</td>
</tr>
<tr>
<td>Mold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Geotrichum candidum &amp; Candida Spp.</em></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td><em>Candida Spp. &amp; Rhodotorula Spp.</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Prototheca &amp; Trichosporon beigelii</em></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Prototheca &amp; Candida Spp.</em></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>Prototheca, Candida Spp. &amp; Geotrichum candidum</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Geotrichum candidum, Candida Spp. &amp; Rhodotorula</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>No Growth</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>58</td>
<td>13</td>
<td>62</td>
<td>165</td>
</tr>
</tbody>
</table>

*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 (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 (Talebkhān Garoussi, Khoṣravi & 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). 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). 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, Bozzo 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; Türkyilmaz & 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 (Talebkhān Garoussi, Khoṣravi & 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* genotypes in 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 *Prototheca* and fungal infection.

**Acknowledgments**

This research was financially supported by the vice chancellor of research, Faculty of Veterinary Medicine, University of Tehran, Iran.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**References**


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Iranian Journal of Veterinary Medicine


بررسی شیوع پروتوتکا و آلودگی قارچی مخازن شیر دامپروری‌های صنعتی ایران

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چکیده

زمینه مطالعه: Prototheca. این گونه در خانواده Chlorellaceae و در کلاسیک‌های خود فاقد عصاره خوشه‌ای است که می‌تواند باعث بروز ورم پستان در گاوهای شیری شود. گونه‌های متعددی از Prototheca می‌توانند از قسمت‌های مختلف گله‌های شیری جداسازی شوند.

هدف: اهداف این بررسی برای 1) بررسی وضعیت شیوع Prototheca Spp در گاوداری‌های شیری مناطق مختلف ایران است 2) ارزیابی شیوع عوامل قارچی در شیر مخازن گاوداری‌های شیری در فصول مختلف سال ایران.

روش: کلاروکل ۱۰۰ میلی گرم/لیتر در صبای سپاروگلوکز آگار به تعداد ۶۶۸۰۰ نمونه گاوهای شیری تحت آزمایش قرار گرفت. نتایج: فقط از ۸ نمونه از ۶۶۸۰۰ نمونه مثبت پروتوتکا و عوامل قارچی شده شد، به‌طورکلی، نتایج پروتوتکا و عوامل قارچی در شیر مخازن گاوداری‌های صنعتی ایران حضور داشتند.

نتیجه‌گیری نهایی: بررسی‌های انجام شده نشان داد که عوامل قارچی و پروتوتکا در شیر گاوهای صنعتی ایران حضور داشتند و همچنین عوارض ممکن ندارند.

واژه‌کلیدی: شیر مخازن، گله‌های شیری، قارچ، ایران، پروتوتکا