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

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Abstract

Background: *Prototheca Spp.* belongs to 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 tank milk *Prototheca Spp* prevalence in industrial dairy cattle herds in different area of Iran. And (2). To evaluate the fungal agent prevalence of bulk tank milk of dairy cattle herds in different seasons. **Materials and Methods**: Bulk tank milk for the presence of *Prototheca* and fungal agents from 165 industrial dairy cattle herds complexes were tested. The population of each herd were 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 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 tank milk of industrial dairy cattle herds in different provinces of Iran. **Conclusion**: 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.

Key words: Bulk tank milk, 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 (Kurumisawa *et al*., 2018. Talebkhan Garoussi *et al*., 2012, Blowey and Edmondson, 2010). Fungal agents can cause mastitis, such as yeasts from genera *Candida*

(*Candida albicans*) and *Cryptoccocus (Cryptotoccus neoformans)* which are the most common agents (Bakr et al., 2015, Eldesouky *et al*., 2016, Spanamberg *et al*., 2008).

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 (Kalinska *et al*, 2017. Shahid *et al*, 2016. Blowey, 2010).

Prototheca (P.) Species can cause mastitis (Milanov *et al*., 2016. Osumi *et al*., 2008). *P. Spp.* belongs to family *Chlorellaceae*, although they lost their ability to synthesize chlorophyll and adopted a heterotrophic mode of nutrition and became achlorophyllous algae (Irrgang *et al*., 2015., Krukowski *et al*. 2013 Rapuntean *et al*., 2009., Anderson and Walker. 1988). Currently eight species adopted including: *P. blaschkeae, P. stagnora, P. ulmea, P. wickerhamii, P. zopfii, P. cutis, P. miyajii* and recently *P. tumulicola* (Noriyuki Hirose *et al*., 2018). *P. blaschkeae, P. zopfii, P. cutis* and *P. wickerhamii* are animal pathogens and can cause serious infections in low sanitary conditions or immune system deficiency and also can cause local or systemic diseases for human as a zoonotic agent (Masuda *et al*., 2016. Wawron *et al*., 2013. Satoh *et al*., 2010. Marques., 2008. Lass. Mayr. 2007. Milanov, Suvajdzic. 2006. Roesler *et al*., 2003, 2006). Several *Prototheca* species could be isolated in different parts of the dairy herds. However, *Prototheca* species live and grow on humid places specially 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 (Masuda *et al*., 2016. Anderson, and Walker. 1988). 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 (Marques *et al*., 2008. Pompermayer, and Gaylarde., 2000.).

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 (Jagielski, *et al*., 2011. Ricchi, *et al*., 2010. Osumi, *et al*., 2008).

Prototheca is one of the zoonotic agent and its presence in milk can cause human protothecosis. In milk industry, pasteurization is used for elimination of microbial agents, while *Prototheca* are resistant to pasteurization (Malinowski, 2011. Lassa, Mayr. 2007). Therefore, consuming contaminated milk to *Prototheca* agent can be as a sanitation hazards (Lassa *et al*., 2011. Marques *et al*., 2008. Lass and Mayr. 2007).

First detection of *Prototheca* was in 1894 by Krüger and first detection of *Prototheca* as a mastitis agent was in 1954 in Germany (Pieper *et al*., 2012). However, they were shown that it can be as a mastitis agent across the world. It was reported from USA (Anderson *et al*., 1988), Denmark (Aalbaek *et al*., 1998), Italy (Buzzini, *et al*., 2004), Brazil (Bueno *et al*., 2006), (Osumi *et al*., 2008.) and Canada (Pieper *et al*., 2012), respectively. In the past years, the rates of *Prototheca* mastitis reports were little due to less attention to its role as a mastitis agent but in the last few years, knowledge about this agent increased which led to increase in detection and reports of *Prototheca* mastitis (Dubravka Milanov *et al*., 2016, Rakesh *et al*., 2006). It was shown that *Prototheca* was isolated from the clinical mastitis in Iran (Lavaee *et al*., 2020. Zaini *et al*, 2012).

Since, *Prototheca* and fungal agents as important agents in causing mastitis and milk contamination, can be isolated from bulk tank milk of industrial dairy cattle herds in Iran. The aims of the present study were to determine: (1) the investigation status of bulk tank milk *Prototheca Spp* prevalence in different seasons in industrial dairy cattle herds in different area of Iran. And (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 area 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 about almost 10000 Kg/cow/year. For this study, we included only farm of the commercial industrial stratum which processed a bulk tank milk. The industrial dairy cattle herds are the major producer of milk in different area and farmers deliver milk production to the local dairy industries or export milk to somewhere else in Iran. Samples of bulk tank milk 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 (Fig. 1).

In total, almost 66800 Holstein dairy cows 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 lactating cows averaged about 1150. All of the cows were Holstein breed. About more than 75% of the dairy cows had free-stall system. They were housed in intensive system with cubical bedding. They were typically fed alfalfa, corn silage and concentrate in various proportions using totally mixed ration (TMR). The cows milked 3 times a day using milking machine. All of the female 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 in March 2019 to March 2020. The owners of the herds did not inform for the aims of this study. They were collected in 500 ml volume in 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

The vicinity of the flame, 0.01 ml of each milk sample were cultivated on a Sabouraud Glucose Agar (SGA) medium with chloramphenicol (100 mg / l) (Difco Laboratories,USA). Cultivated samples were incubated aerobically in 37̊c for 7 days (Corbellini *et al.*, 2001).

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

Staining

In lactophenol cotton blue staining, one drop is placed on a slide, then a very small part of *Prototheca* colony was picked up with an ans loop and carefully placed on drop of staining solution on the slide. Then a lamel was placed on mixture of stain and sample and examination was done with a magnification of 10 and 40. Organisms which were suspended in the stain were killed due to the presence of phenol. On the other hand, high concentration of phenol deactivates lytic cellular enzymes thus the cells do not lyse. The cotton blue is an acid dye that stains 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 SAS version 9.2.

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%), respectively. *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) and 5 provinces, respectively (Fig. 1) (Table 1). *Prorotheca Spp*. were isolated in all four seasons. However, there were no significant differences among the different seasons ($P<0.05$) (Table 1). Table 2 shows the different alga and fungal infection of bulk tank milk of dairy cattle herds. *Prototheca Spp.* was not isolated from bulk tank milk of dairy cattle herds in other provinces (Fig 1).

Table 1. Distribution of *Prototheca Spp* contamination of bulk tank milk 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 tank milk 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. &	0	1	0	0	1

Rhodotorula					
No Growth	0	14	0	0	14
Total	32	58	13	62	165

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

Figure 1: Distribution of milk sample collection for-*Prototheca* contamination of bulk tank milk 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.

Discussion

Prototheca Spp can play an important role in causing contamination of bulk tank milk 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, the most (95.15%) of the bulk tanks milk 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 the nature and also other resources such as food materials. Humid areas, particularly containing manure and decomposing plant material, are its natural habitat (Inoue *et al*., 2018. Rapuntean *et al*., 2009). As the diet of dairy cattle is based on feed of 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 as the same way of contagious mastitis agents (Anderson & Walker. 1988).

In this survey, it was shown that *Prototheca Spp.* could be isolated purely or in combination with other fungi (Table 2). We found that yeast was the common opportunistic pathogen in bulk tank milk samples. However, *Candida* was isolated from 68.48% (113 out of 165) of bulk tank milk samples and the other mycotic agents were isolated from others (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 (Garoussi *et al*., 2017. Talebkhan Garoussi *et al*., 2012). On the other hand, they can be isolated as the common agents from any bulk tank milk sample.

One of the main reason why this factor in less attention and known and the lack of accurate information about its incidence in livestock, that's why, its evaluation in culture and also specific

Prototheca cultivation is uncommon (Alves *et al*., 2017). The other reason is unfamiliarity of farm veterinarians with the epidemiological and clinical aspects of *Prototheca* mastitis (Jagielski *et al*., 2019a. Dubravka *et al*., 2016). This issue may even cause the herd to become involved to *Prototheca* mastitis and this lack of awareness can lead to the identification of this factor when herd is involved with this alga (Dubravka Milanov *et al*., 2016).

Prototheca species can cause an important individual mastitis in dairy cattle herds which usually doesn't respond to current therapies (Park et al., 2019. Jagielski, *et al*., 2017, Marques. *et al*., 2008, Janosi, *et al*., 2001). 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 with culture method (Jagielski *et al*., 2019b). In US stated that from total 787 individual dairy farm bulk tank milk samples examined, 3.6% samples contained *Prototheca Spp*. (Pore *et al*., 1987). It was isolated 187 out of 2508 quarter milk samples which shows 7.5% quarter infection in Korea (Ho-sung 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, Suvajdzic. 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.

It was detected 7.4% *Prototheca* infection from mammary gland of dairy cows using PCR (Ricchi et al., 2010). However, *Prototheca Spp* was isolated from dairy cows, bulk tank milk of dairy cattle herds and the related environment (Ricchi *et al*., 2013). it shows that environment plays an important role. However, PCR may be helpful for isolation of *Prototheca* from bulk tank milk samples which must follow it in future studies in Iran (Jagielski *et al*., 2018). Differences in infections may be due to the geographical differences such as: climate, weather, rainfall, altitude and other factors may effect on 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 individual 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 tank milk contamination of dairy cattle herds (Gonçalves *et al*., 2015. Wawron *et al*., 2013. Pieper. *et al*., 2012).

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

In this study, *Prototheca* is isolated from bulk tank milk of dairy cattle herds in 5 provinces which are the major producer of livestock and dairy production 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 tank milk 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 the development of *Prototheca* infection of the mammary gland (Kano *et al*., 2018. Pieper *et al*.2012). 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 tank milk and sanitation hazards for human healthy (Fernandez *et al.*, 2019).

It was concluded that *Porototheca* and the fungal agents (mainly *Candida*) were isolated as the mycoflora of bulk tank milk 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 important as a sanitation hazards. This study was also important from the point of view of veterinarian studies and humans' health since bulk tank milk contamination in dairy cattle herds can effect on human general health. More studies must be carried out in the field of dairy cattle herds *Protothca* and fungal infection.

Acknowledgment

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 کهمی تواند باعث بروز ورم پستان در گاوهای شیری شود. گونه های متعددی از *Prototheca* را میتوان از قسمتهای مختلف گله های شیری جداسازی نمود. **اهداف:** اهداف این بررسی جهت 1) بررسی وضعیت شیوع *Prototheca Spp* در گاوداری های شیری مناطق مختلف ایران است 2) ارزیابی شیوع عوامل قارچی در شیر مخازن گاوداری های شیری در فصول مختلف سال ایران. **مواد و روش کار:** مخازن شیر 165 دامپروری صنعتی 32 شهر از 16 استان ایران به منظور وجود پروتوتکا و عوامل قارچی مورد آزمایش قرار گرفت. بطورکلی، تقریباً 66800 راس گاو نژاد هلشتاین تحت نظر قرار گرفتند. تعداد گاوهای شیری تقریباً 25800 راس بود. نمونه هادر محیط سابوروگلوکز آگار همراه با کلرامفیکل(100 میلی گرم/لیتر)کشت داده شدند. **نتایج:** فقط از 8 نمونه از 165(4/84 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: 2، 4، 1 و 1 نمونه بود. کاندیدا، فوزاریوم و تریکوسپورن به ترتیب از: 137 ، 2 و 1 نمونه جداسازی شد. بر اساس اطلاعات بدست آمده پروتوتکا و عوامل مختلف قارچی در شیر مخازن دامپروریهای صنعتی ایران حضور دارند. **نتیجه گیری نهایی:** نتیجه گرفته می شود که تماس گاوهای شیری ایران و شیر مخازن گله های شیری ایران با پروتوتکا و عوامل قارچی شایع است. نتایج از نظر بهداشت گاوهای شیری و مخاطرات بهداشتی انسان حائز اهمیت است.

کلمات کلیدی: شیر مخازن، گله های شیری، قارچ، ایران، پروتوتکا.