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BOVINE SUBCLINICAL MASTITIS ASSOCIATED WITH *PROTOTHECA SPP.*

Maletić M.¹, Đukić B.², Nedić S.¹, Stanišić Lj.¹, Stanimirović Z.¹,
Stevanović J.¹, Vakanjac S.¹

Abstract

Mastitis is the most common disease in dairy cattle and presents the most costly disorder to the dairy industry. Subclinical mastitis is inflammation of the mammary gland that does not create visible changes in the milk or the udder, thus, subclinically infected cows will produce less milk, and the quality of the milk will be reduced. In addition, infected cows can be a source of infection to other animals in the herd. Among other unspecified causative agents of mastitis, algae *Prototheca spp.* is not included in routine diagnosis in laboratories. However, *Prototheca spp.* infections usually result in a chronic subclinical or mild clinical, inflammatory process in the udder, and were followed by a dramatic loss in milk production and a permanent increase in somatic cell count. This type of infection is rare, but the incidence of reported cases is increasing worldwide. In our investigation, 605 milk samples were obtained from dairy cows in a total of 5 herds with a history of increasing somatic cell counts, subclinical and mild clinical signs of udder infection, and/or unsuccessful response to the usual therapy. Microbiological isolation of algae on Blood agar and Sabouraud agar at 25°C and 37°C, respectively, showed that 39 (6.45%) samples were positive on *Prototheca spp.* which was confirmed microscopically. The SCC of *Prototheca spp.* positive milk samples were from 5×10^5 /mL up to 13×10^6 /mL. Considering the wide distribution of these algae as saprophytes in the environment, further investigation should include detail microbiological and molecular techniques in order to reveal genotypes involved prevent infections and decrease economic losses.

Keywords: *Prototheca spp.*, mastitis, SCC, dairy cattle

Introduction

Mastitis remains a major challenge to the worldwide dairy industry despite the widespread implementation of mastitis control strategies (Bradley, 2002). In addition to economic losses, mastitis milk is a source of infection for humans and animals, while residues of antibiotics, which are used in local and parenteral therapy, can be dangerous to human health. The most common causative agents of mastitis are bacteria and fungi. Recently, more attention is given to algae as possible causative agents of clinical and subclinical mastitis. Unicellular algae of the genus *Prototheca*, for now are considered as the only algae with pathogenic potential that can cause infections in animals and humans (Tsuji et al., 2006; Lass-Flörl and Mayr, 2007; Marques et al., 2008; Thompson et al., 2009; Ahrholdt et al., 2012; Min et al., 2013; Nguyen and Rosen, 2015). Chronic subclinical mastitis in dairy cattle is the most common clinical manifestation of udder infection caused by algae *Prototheca spp.* and was recorded worldwide (Spalton et al., 1985; Costa et al., 1996; Baumgärtner, 1997; Jensen et al., 1998; Buzzini et al., 2004; Bueno et al., 2006; Roesler et al., 2006; Milanov et al., 2006; Osumi et al. 2008; Ahrholdt et al., 2012). Bovine prototheca mastitis is serious economic problem, primarily because the treatment is uncertain, difficult

and time consuming. The incidence of prototheca mastitis is constantly increasing, mostly from geographic areas with relatively high humidity and/or temperature, e.g. from climatic conditions which are known to be advantageous for the rapid multiplication of this pathogen in the environment. Today, bovine mastitis with this etiology gets more attention, not only because of the economic losses, but also because of the impact on public health (Jánosi et al., 2001; Roesler and Hensel, 2003). The genus *Prototheca* consists of five accepted species: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora*, *Prototheca ulmea* and *Prototheca blaschkeae* (Roesler et al., 2006; Ricchi et al., 2010). The first case of *Prototheca spp.* udder infection was described in 1952 (Lerche, 1952). Thereafter, for many years in all the cases of prototheca mastitis was identified only *P. zopfii* (Jensen et al., 1998; Jánosi et al., 2001; Roesler et al., 2003; Möller et al., 2007; Osumi et al., 2008) which makes the *P. zopfii* the most common cause of prototheca mastitis (Ahrholdt et al., 2012). Today, advanced monitoring and microbiological surveillance techniques revealed that mastitis in cows can be also caused by other species of the genus *Prototheca*. As a causative agent of mastitis, *P. blaschkeae* was first established in Portugal (Thompson et al., 2009), then in Poland (Jagielski et al., 2011), Germany (Ahrholdt and Roesler, 2011; Ahrholdt et al., 2012) and Italy (Capra et al., 2014), while *P. wickerhamii* was proven as a cause of mastitis in buffaloes (Capra et al., 2014). In most cases of prototheca mastitis in cattle (over 90% of cases), were isolated *P. zopfii* genotype 2 (Jagielski et al., 2007; Roesler et al., 2006; Möller et al., 2007; Kishimoto et al., 2010; Gao et al., 2012; Onozaki et al., 2013). Because bovine mastitis caused by *P. zopfii* is unaffected by conventional antimicrobial therapy, the best method for controlling this type of mastitis is through early diagnosis and elimination of the infected cows (Tortorano et al., 2008).

Material and Methods

In accordance with CMT (California mastitis test) carried out on 5 farms (A, B, C, D, E) in Serbia, there were collected 605 positive milk samples from Holstein-Friesian cows. Survey was conducted in period from September 2015 to February 2016. All cows were kept in similar zootechnical and hygiene conditions in tied breeding systems. In addition, all the cows were fed with appropriate diets depending on the breeding categories, with minimal fluctuation in raw material composition. On farms, milking was performed twice per day. Samples of milk were collected from each quarter before morning milking and stored in sterile vials. After teat and cleaning (with 70% ethanol), first streams of foremilk were discharged and then 10 ml of milk was collected aseptically from each teat into sterile vials. Milk samples were stored in a refrigerator at 4°C until microbiological analysis and microscopic somatic cell count (SCC) in laboratory (within 2 hours). SCC is performed according to standard method SRPS EN ISO 13366-2:2008. To isolate *Prototheca spp.* from each intense homogenized sample, one microbiological loop (0,1mL) was sowed on Sabouraud dextrose agar (Torlak, Serbia) and 8% sheep blood agar medium (Torlak, Serbia), incubated at 37°C for 24-48h in aerobic conditions. Identification of *Prototheca spp.* was made after examination of cultural features on Sabouraud dextrose (white to yellowish matte colonies, Figure 1) and sheep blood agar medium (small, grey, opaque, non-hemolytic colonies up to 1 mm in diameter). Morphological features (spores and sporangia) were examined using Gimza stain and light microscopy (Figure 2).

Results and Discussion

Few hours after the infection of the udder with pathogenic microorganisms, the number of SCC in milk increases in response to activation of inflammatory processes. The International Dairy Federation recommended classification of cow milk as subclinical mastitis or non-mastitis (normal) using a SCC threshold of 500.000 cells/mL. In Europe, the ECC directive 92/46 (1992) stated that milk with SCC over 400,000 cells/mL cannot be used for human consumption. Starting from this recommendation, milk samples are classified in two categories, normal (values below 400.000 cells/mL) and milk originating from cows with subclinical mastitis (values above the limit of 400.000 cells/mL).

From the 605 milk samples selected on the basis of a positive CMT, increased number of SCC (above 500,000 cells/mL) were observed in 420 samples. *Prototheca spp.* was isolated in 39 samples based on the macroscopic appearances of colonies on Sabouraud dextrose and sheep blood agar and morphological features. The number of SCC in protothecal milk of cows with subclinical mastitis ranged from 500.000 to over 5.000.000 cells/mL, while in clinical mastitis cases obtained values of SCC were between 521.000 and 13.367.000 cells/mL. Cows with clinically diagnosed prototheca mastitis had altered milk with flakes or clots, but without disturbance of the general condition.

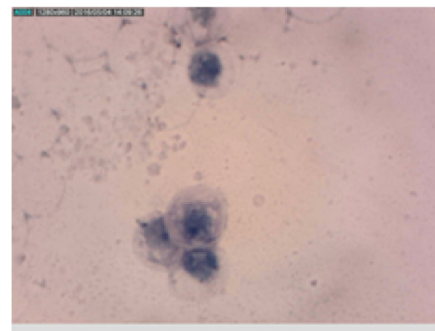


Figure 1. Cultural features of *Prototheca spp.* Figure 2. Morphological features of on Sabouraud dextrose agar *Prototheca spp.* (1000x) on microscopic slide Gimza stain

Table 1. Number of clinical and subclinical mastitis caused by *Prototheca spp.* in investigated farms in Serbia

Farms	SCC>500.000	No. of isolated <i>Prototheca spp.</i>	Subclinical mastitis	Clinical mastitis
A	70	-	-	-
B	98	17	13	4
C	128	12	10	2
D	83	10	9	1
E	41	-	-	-
Total	420	39	32	7

All samples of milk (70) originating from farm A and samples of milk (41) originating from farm E with positive CMT, after microbiological processing there were no *Prototheca spp.* isolated. From farm B, in 98 of 148 milk samples, somatic cell count were over 500,000 cells/ml, of which *Prototheca spp.* were found in 4 and 13 milk samples from cows with clinical and subclinical mastitis, respectively. From farm C, in 128 of 142 milk samples,

somatic cell count were over 500,000 cells/ml, of which *Prototheca* spp. were found in 2 and 10 milk samples from cows with clinical and subclinical mastitis, respectively. From farm D, in 83 of 93 milk samples, somatic cell count were over 500,000 cells/ml, of which *Prototheca* spp. were found in 1 and 9 milk samples from cows with clinical and subclinical mastitis, respectively.

Among the various mastitis types, prototheca mastitis is rapidly becoming a global problem (Jánosi et al., 2001). Although prototheca mastitis is often recognized as a symptomless infection that progresses slowly, acute clinical mastitis associated with reduced milk production, thin watery secretion with white flakes and resistant to conventional therapy may occur in outbreaks (Jánosi et al., 2001; Roesler and Hensel, 2003). In the literature there are more data on the medium and temperature conditions for the *Prototheca* spp. isolation. The majority of researchers agree that algae of the genus *Prototheca* can be successfully isolated on Sabouraud dextrose agar at 37°C during incubation time of 48-72 hours (Roesler et al., 2001; Janos et al., 2001; Marques, 2010b; Gao et al., 2012). However, Onozaki et al. (2013) suggest that the temperature of 25°C is more efficient for growth of *Prototheca* spp. while Zaini et al. (2012) suggest that the incubation period of 48-72h at a temperature of 27°C is most suitable for the isolation of the algae. The results obtained in our investigation are consistent with the results of several studies (Roesler et al., 2001; Janos et al., 2001; Marques, 2010a; Gao et al., 2012) where the temperature and incubation conditions were equivalent (Sabouraud dextrose agar, 48-72h/37°C).

Milk samples from cows with prototheca subclinical mastitis had significantly increased SCC (up to 5.000.000/ml). Increased SCC in the cumulative milk samples from the dairy farms have negative effects on the price of milk and usage of milk for human consumption. In our survey, the SCC in milk samples taken from udder quarters with prototheca subclinical mastitis ranged from 500.000 to over 5.000.000 cells/mL. The obtained data of our examination are in accordance with the results of Malinowski et al. (2002) and Jagielski et al. (2011), where in cases of prototheca subclinical mastitis determined SCC ranged from 591.000 to 3.072.000 cells/mL and 434.000 to 1.325.000 cells/ml, respectively. In the cases of prototheca clinical mastitis, SCC in milk obtained in our study were between 521.000 to 13.367.0 cells/mL, while in most of the milk samples SCC were above 7.118.000 cells/ml, which were in accordance with previous investigations (Malinowski et al., 2002; Jagielski et al., 2011).

Conclusion

Due to the potentially pathogenic effects of *Prototheca* spp. on human and animal health, economic losses and ineffective therapy treatment, these microorganisms should be routinely monitored and included in a regular control of mastitis on dairy farms.

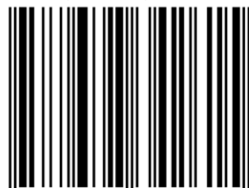
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