

## OUTBREAK OF ENDEMIC FORM OF PROTOTHECAL MASTITIS ON A DAIRY FARM

MILANOV DUBRAVKA\*, SUVAJDŽIĆ LJILJANA\*\*, PUŠIĆ I\*, VIDIĆ BRANKA\*  
and ĐORĐEVIĆ-MILIĆ VUKOSAVA\*\*

\*Scientific Veterinary Institute, Department of microbiology,

\*\* Faculty of Medicine, Department of Pharmacy, Novi Sad

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*In this paper an outbreak of mastitis in cows resistant to antibiotic treatment on a Holstein-Friesian cattle farm in Vojvodina (Serbia and Montenegro) was described. Microbiological examination of 73 milk samples from lactating cows revealed the presence of Prototheca zophii in 22 samples (30.13%). Identification of the isolates was performed on the basis of growth features, microscopic appearance, assimilation patterns and antimicrobial susceptibility. Protothecal infection of the mammary gland resulted in clinical symptoms of chronic mastitis in 19 animals. Histopathological findings revealed distinct granulomatous inflammations of the mammary gland interstitium.*

*Key words: bovine mastitis, Prototheca zophii*

### INTRODUCTION

*Prototheca spp.* are ubiquitous unicellular achlorophyllous algae spread worldwide in different environmental habitats (Pore, 1983). They are considered achlorophyll mutants of green algae, although their origin in nature is still a matter of some debate (Huss and Sogin, 1990). They belong to the genus *Prototheca*, family *Chlorellaceae* being the only known plant causative agent of infections in humans and animals (Roesler *et al.*, 2003). Of the five known species of this strain, i.e. *P. zophii*, *P. wickerhamii*, *P. stagnora*, *P. moriformis* and *P. ulmea*, only the first two are considered pathogenic (Pore, 1998). However, their pathogenic potential is considered low. Animal infection caused by *Prototheca spp.* mainly includes cutaneous and systemic infections in dogs and mastitis in dairy cows (Gonzales, 1996; Quin *et al.*, 2002).

Lerche first reported *Prototheca spp.*, as a possible causative agent of mastitis, in 1952. These algae are considered to be environment-born agents (Costa *et al.*, 1998; Gonzales, 1996). High numbers of organisms outside the teat end can eventually overwhelm udder defences. Mastitis occurs in unsanitary milking conditions (Schlestedt *et al.*, 1997), in previous cases of mastitis and antibiotic pre-treatment (Tenhagen *et al.*, 1999), but there are also seasonal cases of mastitis during warm and humid periods of the year (Costa *et al.*, 1998). Cases of such mastitis are mostly sporadic, but endemic forms of the disease were also

reported (Costa *et al.*, 1996; Janosi, 2001b). The *P. zopfii* infection usually results in a chronic subclinical, or mild clinical, inflammatory process in the udder, and is followed by a dramatic loss in milk production and a permanent increase in somatic cell count (Janosi, 2001a). Histological lesions were characterized by interstitial infiltrates of macrophages, plasma cells and lymphocytes; algae were seen in the alveolar lumen and interstitium, as well as in macrophages and neutrophils (Jensen *et al.*, 1998; Corbellini *et al.*, 2001). Prototheca organisms use to persist in the tissue of the mammary gland also during the dry period and antimicrobial treatment proves to be ineffective (Schlenstedt *et al.*, 1997). Spontaneous cures of Protothecal mastitis have not been reported.

In Europe protothecal mastitis was reported in Denmark (Aalbaek *et al.*, 1998), Germany (Schlenstedt *et al.*, 1997), Hungary (Janosi *et al.*, 2001a), Belgium (Swinne *et al.*, 2002), Spain (Abarca *et al.*, 2001) and Poland (Malinowski *et al.*, 2002). In this work a case of chronic protethecal mastitis on a farm in Vojvodina is described. This is the first reported case in Serbia and Montenegro.

## MATERIAL AND METHODS

### *Cattle farm*

The investigation was carried out on a farm with a closed housing system, situated in the part of Vojvodina characterized by continental climate. A total of 102 diary Holstein-Friesian cows were investigated, 73 of which were in lactation and 29 were dried off. Milking was performed under the standard regimen, twice a day, with an average milk yield of 5700 liters. Udder papillae were disinfected before and after milking using chloride based solutions. Most of the year the cows were held in corals, but during the winter animals were tied in a stall barn. They were fed silage, dry beet pulp, brewer's grain containing 16% protein and green crop.

In the past few years the farm experienced significant economic losses due to culling of cows with chronic granulomatous mastitis resistant to antibiotic treatment. In the period 2002-2004 some 2320 intramammary injectors were spent for mastitis therapy, and 92 cows were eliminated from the herd. According to anamnestic records, the majority of animals had painful episodes of acute mastitis, followed by udder swelling and watery milk secretion with flakes, but without systemic symptoms of infection. The inflammatory process was mostly present in two quarters (rarely three or all four). In the course of the disease, the quarters became enlarged, painless and hard, with a slight drop in milk yield.

### *Milk samples*

Milk samples from 73 lactating cows were collected aseptically in sterile sampling tubes. Milk was sent to the Laboratory for Clinical Bacteriology at the Scientific Veterinary Institute in Novi Sad. Inoculation was performed within 2 hours.

### Microbiological examination

Aliquots of 0.1 mL milk were streaked onto blood agar (with 8% sheep blood), MacConkey agar and Sabouraud dextrose agar plates ("Torlak", Belgrade, Serbia and Montenegro). Streaked plates were incubated under aerobic conditions for three days as following: blood and MacConkey agar at 37°C, and Sabouraud dextrose agar at 27°C. The microbial growth was monitored daily.

Of the colonies grown on Sabouraud dextrose agar after 48 hours of incubation, microscopic smears were made, which were Gram and methylene-blue stained. The preparations were examined using light microscopy with immersion (100x).

Assimilation patterns of the isolates were examined using API20C AUX (Bio Merieux, Marcy l'Etoile-France). Antimicrobial susceptibility was tested by disk diffusion method on Müeller-Hinton's agar ("Torlak", Belgrade, Serbia and Montenegro) to the following agents: nystatin (100 i.u.), amphotericin B (10 µg), polymyxin B (300 i.u.), penicillin (10 µg), streptomycin (30 µg), amoxicillin (25 µg), ceftriaxon (30 µg), tetracycline (30 µg), kanamycin (30 µg), enrofloxacin (5 µg), linkomycin (10 µg), novobiocin (30 µg), neomycin (10 µg), gentamicin (30 µg).

The sample of changed tissue from the mammary gland was fixed in 10% formaldehyde solution. Using standard technique, tissues were paraffin embedded and sectioned at 5 µm. The tissues were stained with haematoxylin-eosin (HE) for pathohistologic examination.

## RESULTS

Out of 73 milk samples, only in 6 samples bacteria implicated in the etiology of mastitis were isolated: *Streptococcus uberis* (2 samples), *Arcanobacterium pyogenes* (1 sample) and coagulase negative *Staphylococcus sp.* (3 samples). Yeast-like microorganisms were isolated in pure culture in 22 milk samples (33.13%). After 48 hours of incubation distinct colonies were observed in all used media. On Sabouraud dextrose agar, colonies were white, 1-3 mm in diameter, round, irregular margined and with a granular surface when observed with a microscope (magnification 10x) (Fig. 1). The colonies grown on blood agar were small, gray-whitish in color, rounded rough and opaque. On MacConkey agar distinctly small, lactose-negative colonies were formed. Subcultures of the isolates were made on all media used, and colonies were visible 24 hours after incubation at 27°C and 37°C.

Microscopy of the methylene-blue stained smear revealed oval formations, different in diameter, in which numerous vacuoles could be seen. Larger formations (>10 µm) have formed intensively stained endospores (Fig. 2). Their shape reminds of a rosette or a morula. In Gram stained preparations empty sporangia were stained Gram negative, while spores were Gram positive (Fig. 3).

The isolates assimilated glucose and glycerol, did not utilize arabinose, xylose, galactose, sorbitol, celobiose, lactose, maltose, saccharose, trehalose and raffinose. All the isolates were sensitive to nystatin and amphotericin B,

intermediate sensitive on polymyxin B, gentamicin and neomycin, but were resistant to kanamycin, enrofloxacin, ceftriaxon, streptomycin, amoxycillin, tetracycline, penicillin, lincomycin and novobiocin. Histological examination of mammary gland tissue revealed granulomatous interstitial mastitis associated with cell proliferation of macrophages, plasma cells and lymphocytes (Fig. 4).

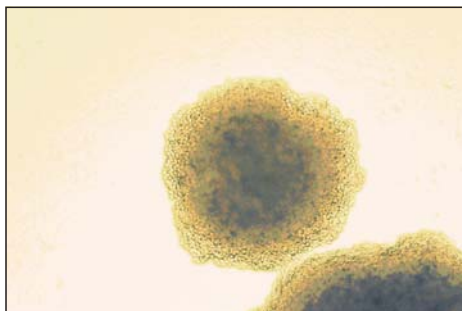


Figure 1. Colonies of *Prototheca zopfii* grow on Sabouraud dextrose agar for 48h at 37°C, light microscopy, (10x)

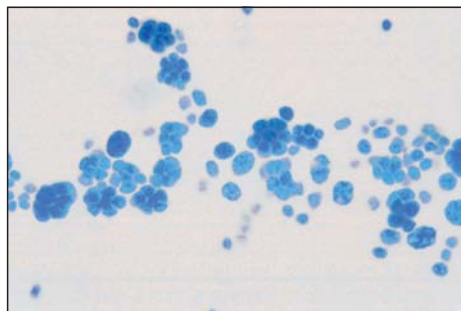


Figure 2. Methylene – blue stained smears, 48 hours culture *Prototheca zopfii*, (100x)

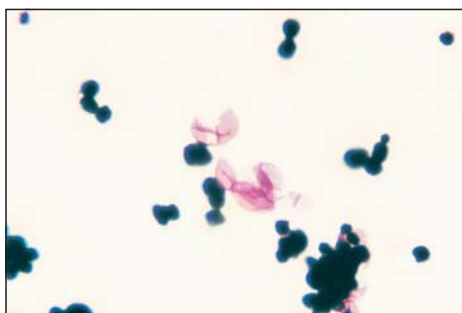


Figure 3. Gram stained, 48 hours culture *Prototheca zopfii*, (100x)

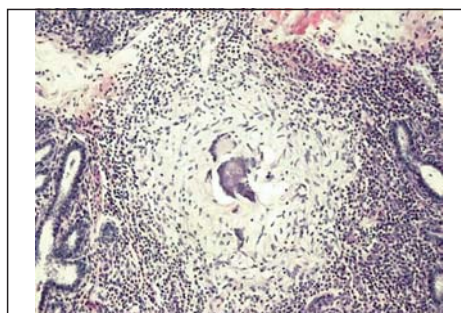


Figure 4. Granulomatous interstitial mastitis associated with cell proliferation of macrophages, plasma cells and lymphocytes (20x)

#### DISCUSSION

Yeast-like micro-organisms were isolated in 22 milk samples (30.13%). Nineteen samples originated from animals with clinical mastitis and three samples from cows with sub-clinical mastitis. All clinically manifest forms of mastitis were characterized by substantial enlargement, painlessness and hard consistency of the infected udder quarter. Identification of *Prototheca zopfii* was based upon its morphology and microscopic appearance, assimilation patterns and

antimicrobial susceptibility. In primary isolation visible colonies were formed after 48 hours, and in subculture after 24 hours. This is described as a growth characteristic (DiPersio, 2001). If media inhibitory to normal flora are not used, slower-growing colonies of protothecae may be overgrown by bacteria (DiPersio, 2001) or they may be overlooked in routine diagnosis, especially if the quality of sampling routines is restricted (Tenhagen *et al.*, 1999). The most abundant growth was observed on Sabouraud dextrose agar, and the colonies were large, different in diameter, rough and whitish in color. These growth characteristics may mislead the diagnosis and confuse them with yeasts, hence microscopy of the isolates is necessary. The colonies are pasty, and they can be readily picked off the agar and easily suspended in saline.

In the API 20C AUX test isolates assimilated only glucose and glycerol. This is reported as one of the characteristics of biotype II *Protothecae zopfii* which is commonly isolated in bovine mastitis and human enteropathia (Roesler *et al.*, 2003). Susceptibility to nystatin, amphotericin B., polymyxin B, gentamicin and neomycin corresponds with the literature data (McDonald *et al.*, 1984; Malinowski *et al.*, 2002).

Pathohistological examination revealed large, partly merged granulomas with central necrosis, like small granuloma. Around the necrotic zone epithelioid and giant Langhans' type cells were observed. However, the periphery of the epithelial cells there was a thick lymphocyte infiltrate. Granulomatous inflammation was typical tissue response on protothecal infection (Cheville *et al.*, 1984; Costa *et al.*, 1996; Jensen *et al.*, 1998). The organism was not detectable in hematoxiline-eosine stained tissue preparations, since it is hematoxylinophilic. For their visualization staining with *periodic acid-Schiff* (PAS) or *Gomori methenamine silver* strain is recommended (DiPersio, 2001).

Data obtained from the dairy farm where mastitis was diagnosed indicated numerous predisposing factors for occurrence of protothecal mastitis. For example, at this particular farm mastitis occurs in spring and summer, i.e. in the warm periods of the year with a lot of rain, making a favourable environment for growth of *Prototheca spp* (Costa *et al.*, 1998). In the majority of infected animals clinical manifestation of mastitis was noticed at the beginning of the lactation period, when there is an inclination to protothecal mastitis due to energetic imbalance. A similar case was described by Janosi *et al.* (2001b). Anamnestic records on cows with chronic mastitis reported on a previous long-term antibiotic treatment, which is an important risk factor for the onset of protothecal mastitis (Tenhagen *et al.*, 1999).

*Prototheca spp.* can be transmitted from cow to cow during milking (Anderson and Walker, 1988). In this farm the infected animals were not left to be the last in the milking process, so it is likely that their milk was a source of infection to other animals. It was also noticed that prototheca-positive animals occurred in "waves", i.e. the organism was spreading among animals that were close to each other.

Based upon the presented data we are of the opinion that by isolating *P. zopfii* we proved its aetiologic importance and pointed out the endemic character of mastitis on this farm. We believe that inappropriate farming conditions, poor

hygiene and prolonged antibiotic therapy contributed to this situation. We also think that more attention should be given to diagnosis of *Prototheca spp.* in routine practice, especially in cases of sub-clinical and clinical mastitis that are resistant to antibiotic treatment.

Address for correspondence:  
 Mr Dubravka Milanov  
 Department of microbiology  
 Scientific Veterinary Institute  
 Rumenački put 20, 21000 Novi Sad  
 Serbia and Montenegro  
 e-mail: beba@niv.ns.ac.yu

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## POJAVA ENDEMIČNE FORME PROTOTEKALNOG MASTITISA NA FARMI MUZNIH KRAVA

MILANOV DUBRAVKA, SUVAJDŽIĆ LJLJANA, PUŠIĆ I, VIDIĆ BRANKA  
i ĐORĐEVIĆ-MILIĆ VUKOSAVA

### SADRŽAJ

U ovom radu je opisana pojava mastitisa rezistentnih na antibiotski tretman, na jednoj farmi krava Holštajn-Frizijske rase u Vojvodini (SCG). Mikrobiološkim pregledom 73 uzorka mleka od krava u laktaciji, iz 22 uzorka (30,13%) izolovana je *Prototheca zopfii*. Identifikacija izolata izvršena je na osnovu karakteristika rasta, mikroskopskog izgleda, biohemijskih osobina i antimikrobne osetljivosti. Prototekalne infekcije mlečne žlezde su kod 19 jedinki rezultirale klinički manifestnim mastitisom hroničnog toka, a patohistološki izrazitom granulomatoznom proliferacijom intersticijuma mlečne žlezde.