Outbreak of clinical *Prototheca zopfii* mastitis in a herd of dairy cows

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**Introduction**

*Prototheca zopfii* is an achlorophyllous unicellular alga, assigned to the genus *Prototheca*, family *Chlorelaceae*. During the last decade, *Prototheca* taxonomy underwent several variations, and five species are currently assigned to the genus: *Prototheca zopfii*, *P. wickerhamii*, *P. stagnora*, *P. ulmea*, and most recently, *P. blaschkeae* (Roesler et al., 2006; Marques et al., 2008). The new species *P. blaschkeae* was identified in 2006 from a previous case of human onychomycosis, which has previously been defined as biotype III of *P. zopfii* (Marques et al., 2008). Species *P. moriformis* has not been generally accepted, and it is still the subject of molecular research (Roesler et al., 2003; Möller et al., 2005). Pathogenic potential of species *P. zopfii* and *P. wickerhamii* is well established. They are associated with local and systemic infections in humans, cutaneous protothecosis in dogs and cats, systemic protothecosis in dogs and mastitis of dairy cows.

*P. zopfii* predominantly occurs as the causative agent of mastitis in cows, whilst *P. wickerhamii* and *P. blaschkeae* are isolated quite rarely (Costa et al., 1998; Janosi et al., 2001; Marques et al., 2008). Prototheca mastitis is described in numerous reports worldwide, showing increasing incidence and prevalence during the last decade. First isolation of *P. zopfii* on a dairy farm in Vojvodina dates back to 2006 (Milanov et al., 2006). *P. zopfii* is widely distributed in the natural environment, and can be isolated from a variety of sources, including soil, plants, drinking and marine water, and faeces of domestic or wild animals (Pore et al., 1983; Roesler et al., 2001). Mammary gland infection commonly develops as a result of exposure to the pathogenic organism in the environment, but disease can be transmitted from cow to cow or via the milking equipment (Anderson and Walker, 1988; Roesler et al., 2001; Lopes et al., 2008).

The occurrence of Prototheca mastitis is associated with predisposing factors such as poor environmental conditions, inadequate milking hygiene, and prolonged antimicrobial treatment. Increased incidence of infection is reported during warm and humid seasons that are advantageous for the rapid multiplication of this pathogen in the environment (Schlenstedt et al., 1997; Tenhagen et al., 1999; Costa et al., 1998). Prototheca mastitis results in significantly reduced milk production, and the somatic cell count in milk can reach even several millions (Janosi et al., 2001a). Chronic course of the infection results in the proliferation of granulomatous tissue and irreversible defects of mammary gland parenchyma. Effective therapy of *prototheca mastitis* has not yet been developed. Timely identification of infected cows and their removal from the herd can reduce the risk of spreading infection to other animals and contamination of the environment (Lopes et al., 2008). The classical diagnosis of prototheca mastitis is still based upon the isolation of the agent from milk, thus accurate and reliable identification of *P. zopfii* in clinical microbiology laboratories is of utmost importance (Roesler et al., 2003, Milanov 2006a).
Material and methods

Microbiology examination: Milk samples were collected from 315 Holstein-Friesian cows from a dairy farm using aseptic techniques. The samples were microbiologically processed by cultivation 50µL- aliquots onto the Columbia agar plates supplemented with 5% sheep blood (Oxoid, Basingstoke, UK), MacConkey agar (Oxoid, Basingstoke, UK) and Sabouraud dextrose agar (Torlak, Serbia). Plates were incubated during 72h at 37°C under aerobic conditions, and microbial growth was mentored daily.

Gram-stained microscopic preparations were made from 48h-old cultures grown on Sabouraud dextrose agar and examined using light microscopy (x1000). Suspension of P. zopfii isolate prepared in Tryptone Soya Broth (Oxoid, Basingstoke, UK) was used for scanning electron microscopy. A 100-µL volume of suspension was spread onto a stainless steel coupon and incubated at 37°C during two days. After incubation, the coupon was washed with sterile saline and fixed by immersing overnight into the 4% glutaraldehyde solution (glutaraldehyde solution, Centrohem) at 5°C. After immersion, the coupon was washed and successively dehydrated by ethanol with a graded series: 30%, 50%, 60%, 70%, 90% and 95-96 %. The coupon was air-dried, sputter-coated with gold (Sputter Coater SCD 005, BALTEC SCAN, WD=50mm, 90s, 30mA) and examined using scanning electron microscope JMS SEM 6460 LV, (acceleration voltage 25 KV, at WD 20 to 8 mm).

Biochemical examination was performed using BBL Crystal® tests (Becton Dickinson, Detroit, MI, USA): Crystal Enteric/Nonfermenter’ and BBL Crystal Gram positive’. The test panels were incubated in a humid chamber at 37°C for 48h. Repeated sampling was performed in all cows with positive result for P. zopfii, and milk samples were collected from each mammary quarter. In all samples with positive finding of P. zopfii, the CFU/mL was determined using the standard colony count technique in decimal serial dilution series on Sabouraud dextrose agar.

Antibiotic and antimycotic susceptibility testing

Susceptibility of P. zopfii isolates was examined by the disc diffusion method using Mueller-Hinton agar (Oxoid, Basingstoke, UK). The following antibiotics and antifungal agents were tested: amoxicillin + clavulanic acid (20/10 µg), ampicillin (10 µg), cefpodoxime (10 µg), cefazidime (30 µg), ceftotaxime (30 µg); ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulphonamides (300 µg), tetracycline (30 µg), trimethoprim (5 µg), trimethoprim + sulfamethoxazole (1,25/23,75 µg), kanamycin (30 µg), amphotericin B (10 µg), ketoconazole (15 µg), and nystatin (50 µg). The plates were incubated for 48h at 37°C.

Results and discussion

Within the framework of regular mastitis survey program, milk samples were obtained from 315 lactating Holstein-Friesian cows originating from one dairy farm in Vojvodina Province. P. zopfii was isolated from milk samples of eight cows. Repeated sampling was performed in all infected cows, collecting milk samples from each quarter individually. The finding was confirmed by isolating P. zopfii from milk samples obtained from one mammary quarter in 5 cows, from two quarters in 2 cows and from three quarters in 1 cow. The animals did not manifest any signs of systemic infection. All milk samples from which P. zopfii was isolated demonstrated visible changes such as watery consistency, greyish colour and apparent small flakes. Significant increase of somatic cell count was established in all milk samples, ranging from 1,000,000 to 3,000,000/mL. Standard colony count technique revealed the amount of P. zopfii ranging between 10³ and 10⁵ CFU/mL.
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Growth of *P. zopfii* colonies was observed in all nutritive media used in this experiment. Colonial growth was first noticed on Sabouraud dextrose agar, as early as after 24 h incubation at 37°C, revealing very small, white colonies that were smaller than yeast colonies of the genus *Candida* isolated from other samples (Fig. 1). After 48h incubation period, the colonies were similar in size to the *Candida* colonies; however, the differences in colour and colonial appearance were apparent. *Prototheca* colonies appeared white, granulated and with irregular margins, whereas *Candida* colonies were yellowish-white in colour, with smooth surface and clear margins (Fig. 1). Colonial growth on blood and MacConkey agar was visible after 48h of incubation. On blood agar, *P. zopfii* formed small, grey, opaque, non-haemolytic colonies up to 1 mm in diameter, whereas colonies grown on MacConkey agar were very small and lactose-negative. *In subcultures*, the growth was noticed after 24 hours of incubation in all media used in this experiment.

![Figure 1. Colonies *P. zopfii* (left plate) and *Candida* sp. (right plate) on Sabouraud dextrose agar incubation at 37°C for 24 h (photo left) and 48 h (photo right)](image)

Light microscopy of Gram stained preparations as well as scanning electron microscopy revealed characteristic structure of the *P. zopfii* sporangia (Gram-positive spores and Gram-negative sporangia) (Fig 2). *Prototheca* spp. are monocellular organisms, oval or spherical in shape, 7-16μm in diameter. The sporangia contain 2-16 or more daughter cells (sporangiospores), which, following the characteristics cell-wall breakage, further develop the endosporulating cells.

![Figure 2. Isolate *P. zopfii* from cow milk: light microscopy (left) and scanning electron microscopy (right)](image)

*Prototheca zopfii* has consistently been divided into three biotypes (Roesler et al., 2003). Classification of the different strains into biotypes of *P. zopfii* was performed auxanographically by means of assimilation of glucose, glycerol and galactose. A strong assimilation activity of galactose and glycerol
within 48h indicated \textit{P. zopfii} biotype I. Strains of biotype I were not able to utilize lysine. \textit{P. zopfii} biotype II did not show assimilation of galactose within 48h, whereas \textit{P. zopfii} biotype III was not able to utilize glycerol at all (Roesler et al., 2003). In a recent study, biotype I and II strains were reclassified as two genotypes of \textit{P. zopfii} by 18S rRNA gene sequence analysis and determination of cellular fatty acids, and biotype III strains were defined as a new species, \textit{P. blaschkeae} (Marques et al., 2008). All \textit{P. zopfii} isolates tested positive for glucose, glycerol and lysine. After 48h incubation, the isolates did not ferment galactose, indicating the biotype II, which is mostly isolated in bovine mastitis and human enteropathia (Janosi et al., 2001; Möller et al., 2007; Jagielski et al., 2011; Roesler et al., 2003). Based on sequence analysis this genotype should be reclassified as a subspecies \\textit{Prototheca zopfii} ssp. \textit{bovimastitogenes} (Möller et al., 2005).

Classical diagnostics of mammary protothecoses still relies upon microbiological examination of milk samples. Accurate identification of the agent may be compromised in routine practice, since the slow-growing organism may easily be missed if incubation is terminated after 24 h (Tenhagen et al., 1999). In prolonged incubation, small \textit{Prototheca} colonies may remain unnoticed, since overgrown (covered) by bacterial colonies from microbiologically contaminated samples (not adhering to principles of asepsis during sampling).

\textit{P. zopfii} isolates form cow milk revealed high resistance to antibiotics and antifungal agents. The most active antibiotics used in this experiment were gentamicin, kanamycin and amphotericin B. Resistance was confirmed to all other antibiotics used in this study. However, though sensitivity to some antibiotics and antimycotic was confirmed in vitro, there are still not drugs with proven clinical effectiveness against protothecal mastitis (Lopes et al., 2008).

Diagnosis of mastitis caused by \textit{P. zopfii} indicates serious problems in the herd (Costa et al., 2004). Infection control is difficult because of wide distribution of the organism in plant matter and faeces of domestic animals. \textit{P. zopfii} was isolated from 20% - 70% samples of faeces of dairy cows originating from herds with no history of Prototheca mastitis (Anderson and Walker 1988; Roesler et al., 2001). High humidity and warmer temperatures enhance multiplication of this alga in the bedding. Poor housing conditions and inadequate milking hygiene are two crucial factors contributing to infection outbreak. Increased incidence of Prototheca mastitis was established during past decade, and is likely associated with massive administration of antibiotics in the treatment of mastitis. Pathogenic potential of \textit{P. zopfii} is relatively small; however, it survives in macrophages and persists in the tissue during the dry off period (Schlenstedt et al., 1997). Firm evidence for spontaneous recovery has not been reported so far. Elimination of infected animals is considered the best method for disease control in the herd (Costa et al., 2004).

\section*{Conclusions}

Prototheca mastitis is becoming more relevant and worrisome because of its increasing prevalence and incidence, unresponsiveness of the infection to therapy and potential danger for human health. Mastitis control programs in dairy herds should encompass the \textit{Prototheca} algae (Schlenstedt et al., 1997), and veterinary microbiology laboratories should ensure accurate and reliable identification in a routine practice.

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References


