

Molecular identification of *Prototheca zopfii* genotype 2 mastitis isolates and their influence on the milk somatic cell count

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ABSTRACT

Algae from the genus *Prototheca* are the only plant-like microorganisms which can cause inflammation and alterations in the mammary gland. *Prototheca* mastitis is usually recognized as a chronic and symptomless disease with reduced milk production and a very high somatic cell count. Molecular identification of *Prototheca* spp. is helpful for the differentiation of pathogenic from non-pathogenic strains which are probably milk contaminants. Genotype-specific PCR assays, based on the 18S rDNA gene sequences, have recently been developed to differentiate three genotypes of *Prototheca zopfii*, of which *Prototheca zopfii* genotype 3 was reclassified in a new species: *Prototheca blaschkeae*. *P. zopfii* genotype 2 is characterized as the main causative agent of *Prototheca* mastitis that leads to significant economic losses in primary milk production. The purpose of this study was to give a molecular characterization of *Prototheca* strains isolated in cases of subclinical and clinical mastitis, as well as to determine the influence of these pathogenic algae on the milk somatic cell count. After microbiological examination, algae from the genus *Prototheca* were isolated in pure cultures from 1.8% of all tested milk samples, and all 13 (100%) isolates were determined as *Prototheca zopfii* genotype 2 by a genotype-specific PCR. This study has provided the first molecular identification of *Prototheca zopfii* genotype 2 in the Republic of Serbia. In the case of subclinical *Prototheca* mastitis, the somatic cell count was $4,175,244 \pm 1,233,685/\text{mL}$ of milk. A distinctly higher somatic cell count ($P < 0.05$) was found in the quarters infected by *Prototheca zopfii* genotype 2 than in the quarters infected by *Staphylococcus aureus*, which is the most common mastitis causative agent worldwide. The results from this study support previous observations that *P. zopfii* genotype 2 is the main causative agent of *Prototheca* mastitis which leads to a significant increase in the somatic cell count in the milk.

Key words: *Prototheca* mastitis, *Prototheca zopfii* genotype 2, genotype-specific PCR, somatic cell count

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Introduction

Udder health of cows in intensive cattle farming is significant for the hygiene and quality of produced milk, but also because of the profitability of production. Mastitis is an inflammation of the mammary gland occurring as a response to microorganism invasion and is characterized by physical, chemical and microbiological changes in the milk and pathological changes in the parenchyma of the mammary gland (SHARMA et al., 2011). Studies show that more than 137 different species of microorganisms are associated with the etiology of mastitis (BAČIĆ, 2009). Algae from the genus *Prototheca* are the only plant-like microorganisms which can cause inflammation and alterations in the mammary gland (JAGIELSKI et al., 2011). *Prototheca* mastitis is rapidly becoming a problem worldwide (BUZZINI et al., 2004; SCACCABAROZZI et al., 2008; RICCHI et al., 2010) and in the Republic of Serbia it was diagnosed and described first by MILANOV et al. (2006).

Members of the genus *Prototheca* are unicellular, colorless algae that reproduce asexually by endosporeulation. Considering the fact that during the phylogeny lost chlorophyll these algae do not have photosynthetic ability, while species such as *Prototheca zopfii* (*P. zopfii*), *Prototheca wickerhamii* (*P. wickerhamii*) and *Prototheca blaschkeae* (*P. blaschkeae*) have become pathogenic for humans and animals (MARQUES, 2010). Infections in animals are usually caused by *P. zopfii* while human infections are mostly associated with *P. wickerhamii* (ROESLER and HENSEL, 2003). Previously, *P. zopfii* differentiated biochemically and serologically into three different biotypes, but at the last classification based on molecular characteristics, *P. zopfii* biotype 1 and biotype 2 were reclassified as *P. zopfii* genotype 1 and genotype 2, while *P. zopfii* biotype 3 was reclassified in a new species (*P. blaschkeae*) (ROESLER et al., 2006).

P. zopfii genotype 2 was characterized as the main causative agent of *Prototheca* mastitis in Germany, Italy, Japan, Portugal and Poland, after molecular characterization of mastitis isolates (ROESLER et al., 2006; MÖLLER et al., 2007; MARQUES et al., 2008; OSUMI et al., 2008; RICCHI et al., 2010; JAGIELSKI et al., 2011). Sporadic cases of mastitis caused by *P. blaschkeae* have also been confirmed, but less frequently (MARQUES et al., 2008; RICCHI et al., 2010; JAGIELSKI et al., 2011). The presence of these pathogenic algae on a farm poses the risk of mastitis, while bad hygiene conditions and poor milking hygiene only contribute to the spread of infection in the herd (ROESLER and HENSEL, 2003). Dairy cows are susceptible to infections in all stages of lactation, including the dry period (RICCHI et al., 2010). Despite low virulence, pathogenic algae can cause an expressed mammary gland immune response, which results in very high somatic cell content elevation (SCC) (over $10^6/\text{mL}$) as well as irreversible changes in the mammary gland tissue (JANOSI et al., 2001). *Prototheca* mastitis is usually recognized as a chronic and symptom-less disease, with reduced milk production and very high SCC. However,

Prototheca mastitis can appear in a clinically visible form characterized by a thin watery secretion containing white flakes and lumps (MARQUES, 2010). Successful therapy and spontaneous recovery of infected animals have not been reported (WAWRON et al., 2013). Therefore, it is necessary to exclude infected cows from production in order to reduce the risk of spread of the infection to other animals and contamination of the environment (LOPES et al., 2008).

Timely detection of mastitis caused by algae from the genus *Prototheca* and their identification is extremely important from the aspect of epidemiology and farm management. The aim of this study was to provide molecular characterization of *Prototheca* strains isolated in cases of subclinical and clinical mastitis, as well as to determine the influence of these pathogenic algae on the SCC in milk.

Materials and methods

Milk sampling. Quarter milk samples were collected aseptically from cows in which the California mastitis test indicated increased SCC, or from cows with clinical mastitis. A total of 726 milk samples were collected from 193 caws.

Microbiological examination. For isolation of *Prototheca* spp. strains, 0.1 mL milk of each sample was streaked onto Columbia agar plates, supplemented with 5% sheep blood and Sabouraud agar with chloramphenicol (0.05 g/L), following incubation for 72 h at 37 °C under aerobic conditions, and microbial growth was monitored daily. After incubation, the morphology of the isolated colonies was evaluated. Typical colonies were chosen for making Gram-stained microscopic preparations. The preparations were examined using light microscopy with immersion ($\times 100$). Identification of other mastitis causative agents isolated on blood agar was performed according to the standard procedures (KATIĆ, 2007a). The API Staph-Ident system (BioMérieux, France) was used for identification of *Staphylococcus aureus* (*S. aureus*) isolates. In order to obtain single colonies needed for DNA extraction, isolates of *Prototheca* spp. were subcultured on Trypticase soy agar at 37 °C for 48 h.

DNA extraction. Extraction of genomic DNA was carried out with a DNeasy Plant Mini kit (Qiagen®, Germany) following the manufacturer's instructions. The extracted DNA was used as a template for PCR amplification

PCR assay. After DNA extraction, 13 *Prototheca* spp. strains were analyzed by a modified genotype-specific PCR procedure (ROESLER et al., 2006). The genotype-specific primers used in this study are listed in Table 1, and were synthesized by Invitrogen (United States). All PCR reactions were performed in 50 μ L reaction volumes containing 25 μ L Dream Taq Master Mix (2X) containing 2 \times Dream Taq buffer, 4 mM MgCl₂ and 0.4 mM of each of the 4 dNTPs (Thermo Scientific, Lithuania), 0.8 μ M of each primer, 5 μ L DNA, and nuclease free water to 50 μ L. DNA amplification was performed in a

FlexCycler (AnalyticJena, Germany). The PCR program specific for *P. zopfii* genotype 1 and 2 was: (1) initial denaturing step at 94 °C for 4.5 min; (2) 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and 40 s at 72 °C; and (3) a final extension step at 72 °C for 5 min. The PCR program specific for *P. blaschkeae* was: (1) initial denaturing step at 94 °C for 4.5 min; (2) 35 cycles of 30 s at 94 °C, 30 s at 63 °C, and 40 s at 72 °C; and (3) a final extension step at 72 °C for 5 min. A negative control was included in all PCR reactions. Amplification products were analyzed by electrophoresis on 1.6% (wt/vol) agarose gel (TopVision agarose Thermo Scientific, Lithuania), after staining with ethidium bromide. A molecular size marker (Gene Ruler 100 bp DNA ladder, ThermoScientific, Lithuania) was used as the molecular weight marker.

Table 1. Primers used in genotype-specific PCR analysis for *Prototheca* spp. mastitis isolates

Genotype	Primer	Sequence (5'-3')
Genotype 1	Proto 18-4f	GACATGGCGAGGATTGACAGA
	PZGT 1/r1	GCCAAGGCCCCCGAAG
Genotype 2	Proto 18-4f	GACATGGCGAGGATTGACAGA
	PZGT 2/r1	GTCGGCGGGGCAAAAGC
Genotype 3	PZGT 3-IK/f	CAGGGTTCGATTCCGGAGAG
	PZGT 3/r1	GTTGGCCCGGCATCGCT

Somatic cells counting. SCC in milk samples was determined using the microscopic method according to the standard SRPS EN ISO 13366-1:2010 - part 1.

Statistical analysis. Statistical analysis of the results was conducted using the software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego Ca, USA, www.graphpad.com). Results of SCC were described by descriptive statistics (mean, maximum, minimum and standard deviation). The Student t-test was used for testing the differences between SCC in milk samples from cows with *Prototheca* mastitis and mastitis caused by *S. aureus*. Values of $P < 0.05$ were considered significant.

Results

The present research was conducted to determine the cause of the increased SCC in the bulk milk. During sampling, it was found that 46 (5.96%) dried udder quarters had resulted from unsuccessful antibiotics treatment of chronic mastitis. Bad hygiene conditions, primarily unclean and humid accommodation for the cows, were also observed during the sampling. These findings indicated a problem with subclinical mastitis in the herd. The causes of mastitis were isolated from 86 milk samples (11.85% of all tested milk samples). Coagulase negative staphylococci were proven in 32 samples (4.41%), *Corynebacterium bovis* in 27 samples (3.72%), *S. aureus* in 9 samples (1.24%),

Streptococcus uberis and *Escherichia coli* in 2 samples (0.28%) while yeast was isolated from only 1 sample (0.14%). Algae from the genus *Prototheca* were observed on the blood agar as small round grey colonies, with a characteristic yeast-like appearance, while on the Sabouraud agar with 0.05 g/L chloramphenicol this microorganism formed white colonies 1-3 mm in diameter, with a granular surface. Gram-stained microscopic preparations demonstrated blue, sometimes red, oval-shaped sporangia containing endospores. As such, algae from the genus *Prototheca* were isolated in pure cultures from 13 milk samples (1.8% of all tested milk samples).

In order to identify isolates of *Prototheca* spp. which were isolated in cases of bovine mastitis, three variable regions of the 18S rDNA genes were amplified by PCR. All 13 isolates were identified as *P. zopfii* genotype 2, since the amplicon (165 bp) specific for *P. zopfii* genotype 2 (Fig. 1) was observed. The PCR specific for *P. zopfii* genotype 1 was always negative, as was the PCR specific for *P. blaschkeae*.

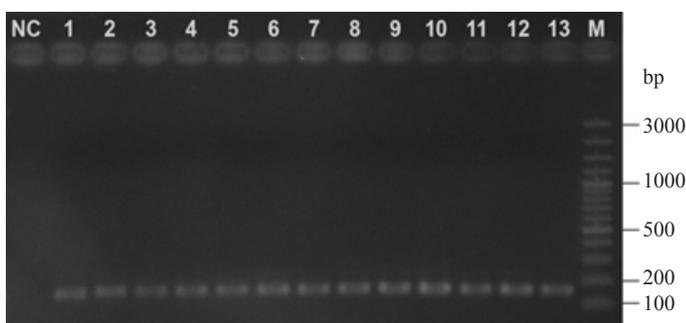


Fig. 1. Results of genotype-specific PCR analysis for *Prototheca* spp. mastitis isolates. NC: negative control; Lines 1-13: *P. zopfii* genotype 2 mastitis isolates; M: molecular weight marker.

Molecular identification of *P. zopfii* genotype 2 and the results of SCC in milk from infected quarters were sufficient for diagnosis of *Prototheca* mastitis. This pathogenic alga caused expressed immune response in the mammary gland in all infected cows which may be seen from the results of SCC in the milk. In the case of subclinical *Prototheca* mastitis, the SCC ranged from 2,150,842 to 5,308,737/mL of milk, while in milk from quarters subclinically affected by *S. aureus* it ranged between 477,895 and 2,204,210/mL. A statistically significant difference ($P < 0.05$) was established between SCC in milk samples from cows with *Prototheca* mastitis and mastitis caused by *S. aureus* (Fig. 2).

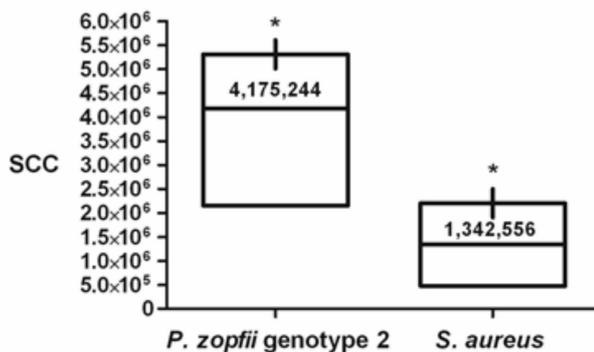


Fig. 2. Somatic cell count in case of subclinical mastitis caused by *P. zopfii* genotype 2 and subclinical mastitis caused by *S. aureus*. * $P < 0.05$

Discussion

Prototheca mastitis is rapidly becoming a problem worldwide (BUZZINI et al., 2004; SCACCABAROZZI et al., 2008; RICCHI et al., 2010) and in the Republic of Serbia it was first diagnosed and described by MILANOV et al. (2006) but without molecular characterization of *Prototheca* strains. Genotype-specific PCR assays, based on the 18S rDNA gene sequences, have recently been developed to differentiate three genotypes of *Prototheca zopfii*, of which *Prototheca zopfii* genotype 3 was reclassified in a new species *Prototheca blaschkeae* (ROESLER et al., 2006). This study represents the first molecular determination of algae from the genus *Prototheca* isolated from bovine mastitis in the Republic of Serbia.

After microbiological examination, algae from the genus *Prototheca* were isolated in pure cultures from 1.8% of all tested milk samples, and all 13 (100%) isolates were determined as *P. zopfii* genotype 2 by genotype-specific PCR. The results of this study are in agreement with the findings of MÖLLER et al. (2007) from Germany and OSUMI et al. (2008) from Japan, which also identified only *P. zopfii* genotype 2 from bovine mastitis. In contrast to the results obtained in our study, the findings of MARQUES et al. (2008) from Portugal, RICCHI et al. (2010) from Italy and JAGIELSKI et al. (2011) from Poland indicate *P. blaschkeae* as the causative agent of mastitis instead of *P. zopfii* genotype 2. RICCHI et al. (2010) pointed out that the mastitis caused by *P. blaschkeae* occurs less frequently, while *P. zopfii* genotype 2 is major *Prototheca* mastitis causative agent. The same authors confirmed the status of *P. zopfii* genotype 1 as an environmental organism, with no involvement in pathology of mammary glands. Therefore, molecular identification of *Prototheca* spp. is helpful for differentiation of pathogenic from non-pathogenic strains which are probably milk contaminants (MÖLLER et al., 2007).

Although *P. zopfii* genotype 2 was isolated only from 1.8% milk samples (in 4.66% of all examined cows), this finding is not negligible, considering that infected cows are a source of infection for other animals in the herd (MILANOV et al., 2006). *P. zopfii* genotype 2 can survive for a long time in the udder, even during the dry period, because the udder's antimicrobial defense mechanism cannot overcome and eliminate this pathogen (RICCHI et al., 2010). Despite low virulence, *P. zopfii* genotype 2 may cause serious problems in the herd of dairy cows and induces endemic infections. Consequently, individual cases of *Prototheca* mastitis in a herd also require alertness (WAWRON et al., 2013).

According to previous studies, *Prototheca* mastitis is usually subclinical or clinical, with mild symptoms (JANOSI et al., 2001; MARQUES, 2010; WAWRON et al., 2013). These data are supported by the findings of this study where only two cows had expressed clinical mastitis without visible changes to the udder, but with milk alterations (watery appearance and the presence of white flakes), while other cows had a subclinical form of *Prototheca* mastitis characterized by decreased milk production and SCC elevation.

In the milk of healthy cows the SCC is lower than 1×10^5 cells/mL (BYTYQI et al., 2010), while an increase in the SCC above 2×10^5 /mL of milk is usually the result of intramammary infection. The strength of the mammary gland immune response depends on the type of pathogen or its virulence (SHARMA et al., 2011). Despite low virulence, *P. zopfii* can cause expressed mammary gland immune response, which results in SCC elevation from 1×10^6 to 1×10^7 cells/mL of milk (JAGIELSKI et al., 2011). This is in accordance with the data obtained in the present study, where SCC was $4,175,244 \pm 1,233,685$ /mL of milk. MALINOWSKI et al. (2002) found that SCC in cases of subclinical *Prototheca* mastitis ranges from 591,000 to 3,072,000/mL of milk, which are slightly lower values than our findings. Distinctly higher SCC ($P < 0.05$) was found in quarters infected by *P. zopfii* genotype 2 than in the quarters infected by *S. aureus*, which is the most common mastitis causative agent worldwide (BAČIĆ, 2009). SCC is a useful indicator of intramammary infection and a very important milk component in assessment of milk quality and hygiene (SHARMA et al., 2011). The increased incidence of subclinical mastitis in a herd leads to SCC elevation in bulk milk. Therefore, any increase in SCC in bulk milk indicates the need to test milk from individual udder quarters in order to identify cows with subclinical mastitis, and it also requires determination of the causative agent of mastitis (KATIĆ, 2007b).

In conclusion, the results from this study support previous observations that *P. zopfii* genotype 2 is the main causative agent of *Prototheca* mastitis which leads to a significant increase in SCC in the milk. In order to determine the prevalence of *P. zopfii* genotype 2 in the Republic of Serbia, as well as to clarify the epidemiology of this pathogenic algae, it is necessary to include a greater number of milk samples from different geographic localities.

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SAŽETAK

Alge roda *Prototheca* mikroorganizmi su nalik na biljke koji mogu uzrokovati upalu i promjene u mliječnoj žlijezdi. Njima uzrokovan mastitis obično je kroničnog tijeka, bez izraženih znakova bolesti, ali dovodi do smanjene proizvodnje mlijeka i do vrlo visokog broja somatskih stanica. Molekularna identifikacija *Prototheca* spp. korisna je za razlikovanje patogenih od nepatogenih sojeva koji su vjerojatno kontaminanti mlijeka. Nedavno su razvijeni genotip-specifični PCR postupci zasnovani na sekvenciji gena 18S rDNK s ciljem da se razlikuju tri genotipa vrste *Prototheca zopfii*, od kojih je *Prototheca zopfii* genotip 3 preimenovan u novu vrstu, *Prototheca blaschkeae*. *Prototheca zopfii* genotip 2 okarakteriziran je kao glavni uzročnik mastitisa uzrokovanog prototekama koji dovodi do značajnih gospodarskih gubitaka u primarnoj proizvodnji mlijeka. Cilj je ovog istraživanja bio da se izvrši molekularna identifikacija *Prototheca* sojeva izdvojenih u slučaju supkliničkih i kliničkih mastitisa, kao i da se utvrdi utjecaj tih patogenih algi na broj somatskih stanica u

mlijeku. Nakon mikrobiološke pretrage, alge iz roda *Prototheca* bile su izdvojene u čistoj kulturi iz 1,8 % pretraženih uzoraka mlijeka i svih 13 (100 %) izolata su pomoću PCR identificirani kao *Prototheca zopfii* genotip 2. Ova studija predstavlja prvu molekularnu identifikaciju *Prototheca zopfii* genotip 2 u Republici Srbiji. U slučaju supkliničkih *Prototheca* mastitisa broj somatskih stanica bio je $4\,175\,244 \pm 1\,233\,685$ / mL mlijeka. Izrazito veći broj somatskih stanica ($P < 0,05$) utvrđen je u četvrtima inficiranim genotipom 2 *Prototheca zopfii* nego u četvrtima inficiranim vrstom *Staphylococcus aureus*, koji je najčešći uzročnik mastitisa u krava diljem svijeta. Rezultati ove studije potvrđuju prethodna promatranja da je *Prototheca zopfii* genotip 2 glavni uzročnik *Prototheca* mastitisa koji dovodi do značajnog porasta broja somatskih stanica u mlijeku.

Ključne riječi: mastitis, *Prototheca zopfii* genotip 2, genotip-specifičan PCR, broj somatskih stanica
