

Molecular characterization of Polish *Prototheca zopfii* mastitis isolates and first isolation of *Prototheca blaschkeae* in Poland

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Abstract

Bovine mastitis caused by the colorless, yeast-like alga *Prototheca zopfii* is a serious and complex condition that results in heavy economic losses in the dairy industry, both through a substantial reduction in milk production and culling of infected animals. Based on the 18S rDNA sequence analysis, genotype-specific PCR assays have recently been developed to differentiate within the species *P. zopfii* three distinct *P. zopfii* genotypes (1-3), of which *P. zopfii* genotype 3 has been considered a new species *P. blaschkeae* sp. nov. The purpose of this study was to employ the newly-devised molecular approach for the detection of the two *P. zopfii* genotypes and *P. blaschkeae* sp. nov. among bovine mastitis isolates from Poland. This study is the first to provide molecular characterization of Polish *P. zopfii* mastitis isolates. It also gives the first description of bovine mammary protothecosis due to *P. blaschkeae* in Poland, as evidenced by genotypical, microbiological, and electron microscopy findings.

Key words: algae, bovine mastitis, genotyping, *Prototheca blaschkeae*, *Prototheca zopfii*, protothecosis

Introduction

The genus *Prototheca* includes unicellular, yeast-like, colorless algae that propagate asexually by endospore formation. These organisms are ubiquitously distributed in nature and essentially saprophytic, yet some species might occasionally assume a parasitic existence, being the only known plants infectious for humans and animals (Jagielski and Lagneau 2007). Taxonomically, *Prototheca* spp. belong to the class

Trebouxiophyceae, within the *Chlorophyta* lineage, and represent achlorophyllous derivatives of green algae of the genus *Chlorella* (Nedelcu 2002). Five species have been placed in the genus *Prototheca*, namely, *P. zopfii*, *P. wickerhamii*, *P. stagnora*, *P. ulmea*, and *P. moriformis* (Pore 1998). Recently, a sixth species, termed *P. blaschkeae*, has been described (Roesler et al. 2006). Among these species, only *P. zopfii*, *P. wickerhamii*, and *P. blaschkeae* have been associated with human and animal infections, with *P. wicker-*

hamii being almost exclusively isolated from human cases of protothecal infection (Boyd et al. 1995, Torres et al. 2003, Leimann et al. 2004). In animals, the disease has been diagnosed in various species, including dogs (Stenner et al. 2007, Sapiernyński and Jaworska 2008), cats (Kaplan et al. 1976, Dillberger et al. 1988), deer (Frese and Gedek 1968), salmon (Smith 1980) and fruit bats (Mettler 1975). However, the most prevalent form of protothecosis in animals is bovine mastitis, which is characterized by acute to chronic pyogranulomatous inflammation of the mammary gland (Jánosi et al. 2001). Bovine mastitis caused by *P. zopfii* has been extensively recognized as a considerable problem to the dairy industry worldwide (Castagna de Vargas et al. 1998, Jensen et al. 1998, Malinowski et al. 2002, Buzzini et al. 2004). The vast majority of the economic losses from protothecal mastitis are due to milk yield depression and premature culling of dairy cows, which, given the unresponsiveness of the algae to conventional therapy (Marques et al. 2006, Lopes et al. 2008), remains the only effective means of eliminating the disease.

There is a marked phenotypic heterogeneity among *P. zopfii* strains. Based upon biochemical and serological data, the species was categorized into at least three different variants or biotypes (I-III) (Blaschke-Hellmessen et al. 1985, Roesler et al. 2003). More recently, a clear delineation between these three biotypes has been achieved through comparative phylogenetic studies of the 18S rRNA gene sequences (Roesler et al. 2006). Hence, the previous biotype III of *P. zopfii* was reclassified as a new species, *P. blaschkeae* sp. nov., whereas *P. zopfii* biotypes I and II were reclassified as genotypes 1 and 2, respectively.

The present investigation was designed as a pilot study to apply the newly-devised molecular approach for the detection of the two different *P. zopfii* genotypes and *P. blaschkeae* among bovine mastitis isolates from Poland. This study is thus the first to provide molecular characterization of Polish *P. zopfii* mastitis isolates. It also gives the first description of the isolation of *P. blaschkeae* in Poland.

Materials and Methods

Algae isolates

The study included nine *Prototheca* sp. isolates from the collection of the Department of Pathophysiology of Reproduction and Mammary Gland of the National Veterinary Research Institute (Bydgoszcz, Poland). The isolates were originally recovered from milk samples of nine cows with mastitis belonging to three dairy farms in the north-west of Poland (Greater Poland Province) between 2003 and

2009. For microbiological analyses, quarter milk samples were collected aseptically and inoculated in 0.1-mL aliquots onto Sabouraud dextrose agar (SDA, Difco Laboratories) plates. Following an incubation at 37°C for 48 to 72 h, the plates were examined for the presence of *Prototheca* colonies. The suspected colonies were subcultured, by streaking onto new SDA plates, incubated at 37°C for 48 h, and subjected to confirmatory tests. These included microscopical inspection of lactophenol cotton blue (LCB)-stained culture smears and carbohydrate assimilation profiling with the API 20C AUX system (BioMérieux).

The reference strains SAG 2063^T (GenBank no. AY973040), SAG 2021^T (GenBank no. AY940456), and SAG 2064^T (GenBank no. AY973041), representing *P. zopfii* genotype I, *P. zopfii* genotype II, and *P. blaschkeae*, respectively (deposited in the Stammsammlung für Algenkulturen (SAG) at the University of Göttingen, Göttingen, Germany) served as controls for genotyping assays.

Isolation of genomic DNA

Prototheca cultures grown on SDA at 37°C for 48 h were used for DNA extraction. The cells from 2-4 colonies were resuspended in 500 µl of distilled water and disrupted by sonication. The obtained homogenate was further processed with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. (The procedure is based on the selective binding of the DNA to a silica gel membrane in the presence of high concentrations of chaotropic salts and subsequent elution of the DNA off the membrane by a low-salt buffer). The purified DNA was used as a template (undiluted) for PCR amplification.

Genotype-specific typing

The determination of *P. zopfii* genotypes (1 & 2) and *P. blaschkeae* was performed by 18S rDNA-based genotype specific PCR assay, as described previously (Roesler et al 2006). The following oligonucleotide pairs were used in PCR procedure: Proto18-4f (5'-GACATGGCGAGGATTGACAGA-3') and PZGT 1/r (5'-GCCAAGGCCCCCGAAG-3') for *P. zopfii* genotype 1, Proto18-4f and PZGT 2/r (5'-GTCGGCGGGGCAAAAGC-3') for *P. zopfii* genotype 2, and PZGT 3-IK/f (5'-CAGGGTTCGAT-TCCGGAGAG-3') and PZGT 3/r (5'-GTTGGCCCCGGCATCGCT-3') for *P. blaschkeae*. Furthermore, the following primer pairs served as internal amplification controls: Proto18-4f and Proto18-4r (5'-AGGATGGCTAACCCACACGA-3') for *P. zopfii* genotypes 1 and 2, and PZGT 3-IK/f and

PZGT 3-IK/r (5'-GAATTACCGCGGCTGCTGG-3') for *P. blaschkeae*.

Each amplification reaction was carried out in a final volume of 25 μ L, with the Mastermix 1.1 x ReddyMix™ (ABgene; 1.5 mM MgCl₂, 1.25 U Taq DNA polymerase), 20 μ M of each primer, 0.2 mM of each dNTP, and *ca* 50 ng of genomic DNA. PCR conditions were as follows: an initial denaturation of 95°C for 5 min, 37 cycles of 95°C for 30 sec (denaturation), 58°C (63°C for genotype III) for 30 sec (annealing), and 72°C for 40 sec; and a final extension of 72°C for 7 min.

The amplified PCR products were resolved electrophoretically in 2% agarose gels, and visualized under UV, by using GelRed (Biotium) dye incorporated into the gel. The gel images were captured with the ChemiDoc XRS system (BioRad) and analysed by using the Quantity One (version 4.6.3.) software (BioRad).

Scanning electron microscopy (SEM)

A detailed morphological examination of two *Prototheca* species (*P. zopfii* and *P. blaschkeae*) was made by means of scanning electron microscopy (SEM). Briefly, cells from four independent colonies, grown on SDA, were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.0] for 16 h at room temperature (RT). The samples were then washed in cacodylate buffer three times over the course of 30 min and post-fixed in 1% osmium tetroxide for 2 h at RT. The samples were then rinsed in distilled water three times over the course of 30 min before dehydrating with an ascending series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, 96%, and 100%),

allowing *ca* 10 min at each step. Finally, the specimens were critical point dried, mounted onto stubs using carbon dots, sputter coated with gold-palladium, and analyzed in a LEO 1430 VP (Carl Zeiss) SEM.

Results

For the purpose of this study, all *Prototheca* strains were revitalized by plating onto SDA and incubated at 37°C for 48 h. The algae grew as creamy-white colonies, with a characteristic yeast-like appearance and odour.

On the basis of carbon source assimilation profiles, indicating the ability to metabolize glucose and glycerol but not galactose, trehalose, inositol and sucrose, all of the isolates were confirmed to be *P. zopfii*. Likewise, staining of *Prototheca* cells with LCB revealed morphological features typical of *P. zopfii* species, with spherical to oval sporangia containing four or more sporangiospores of *ca* 5 μ m in diameter (Fig. 1A). Only one isolate (No. 6) exhibited clearly different cell morphology. Its sporangia showed a more compact and regular arrangement of the spores, which were about twice the size of those of the other isolates (Fig. 1B). These findings were further corroborated by electron microscopical studies (Fig. 2).

To determine the genotypes of *P. zopfii* isolates associated with bovine mastitis, three variable regions of the 18S rDNA genes were amplified by PCR. The results of the *P. zopfii* genotype 2- and *P. blaschkeae*-specific PCR assays are shown in Fig. 3. For all but one isolate, the *P. zopfii* genotype 2-specific amplicon (165 bp) was observed (Fig. 3A). One isolate (No. 6) produced a pattern containing the *P. blaschkeae*-specific band (126 bp) (Fig. 3B). Apart from the genotype-specific products, all of the isolates yield-

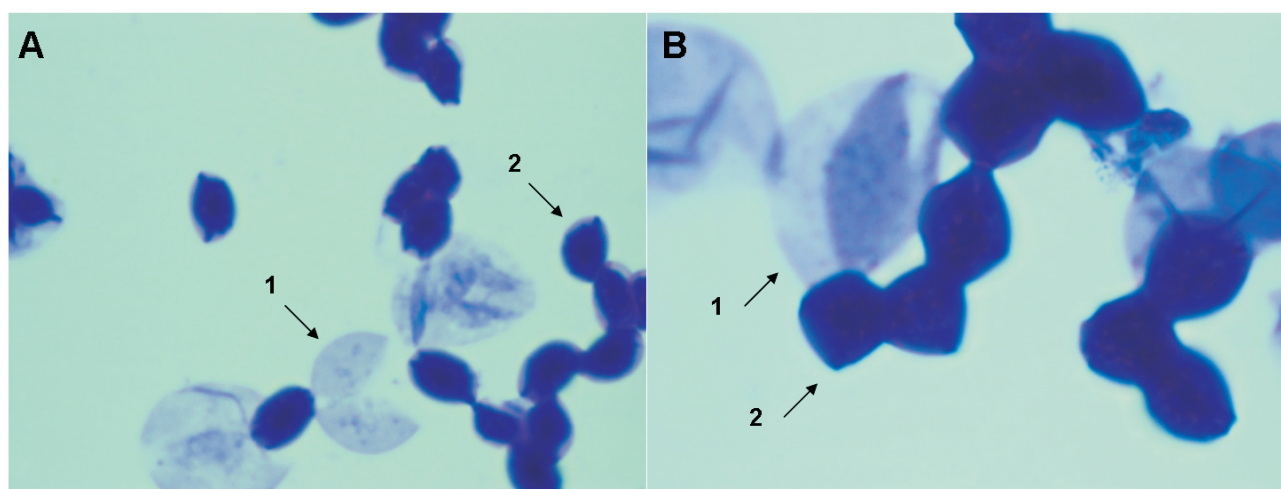


Fig. 1. Light microphotographs of cells of *P. zopfii* genotype 2 (A) and *P. blaschkeae* (B) stained with LCB. Arrows indicate sporangia (1) and released sporangiospores (2). Magnification, X 1,000.

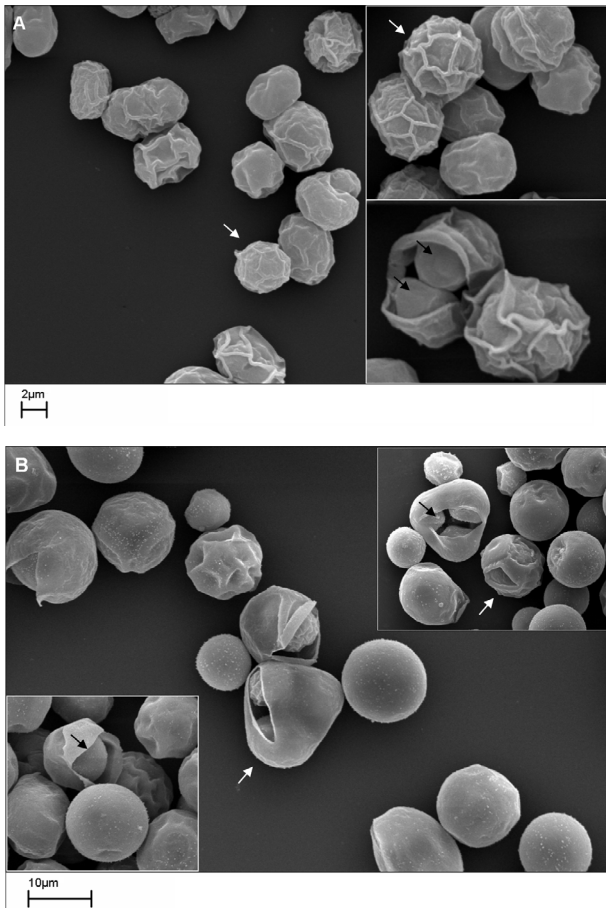


Fig. 2. Scanning electron microphotographs of cells of *P. zopfii* genotype 2 (A) and *P. blaschkeae* (B) in different developmental stages (white and black arrows indicate sporangia and sporangiospores, respectively). The sporangiospores of *P. zopfii* genotype 2 measure ca 5 µm in diameter, whereas those of *P. blaschkeae* are ca 10 µm in size. Magnification, X 4,000 (A); X 2,000 (B).

ded an additional band, of 450 bp (for *P. zopfii* genotypes 1 and 2) or 213 bp (for *P. blaschkeae*) in length, both being internal amplification controls. The PCR specific for *P. zopfii* genotype 1 was always negative, except for an appropriate reference strain (R1, *P. zopfii* genotype 1 SAG 2063^T) (data not shown).

Discussion

This study is the first molecular analysis of Polish isolates of *P. zopfii* based on the 18S rDNA gene sequences, and was performed to explore the relationship of the genotypes with the etiology of protothecal bovine mastitis. Among the isolates tested, all were identified as *P. zopfii* genotype 2, except one, which was assigned to *P. blaschkeae*. Despite the very small sample size (nine isolates), the results from this study suggest that *P. zopfii* genotype 2 may be the leading causative agent of bovine *Prototheca* mastitis in Poland. This is in agreement with the findings of Möller et al. (2007) in Germany and Aouay et al. (2008) in Belgium, and Osumi et al. (2008) in Japan.

An important finding of the study was the identification of *P. blaschkeae* (the former *P. zopfii* genotype 3), which has only recently been implicated in bovine mammary protothecosis (Marques et al. 2008). Prior to that, *P. blaschkeae* had been isolated only from swine farms and one case of human onychomycosis (Roesler et al. 2006). Up to now, there have been very few reports on the association of *P. blaschkeae* with bovine mastitis. Apart from the aforementioned study of Marques et al. (2008) such association was found in a study of Aouay et al. (2008) and that of Thompson et al. (2009). The present investigation is therefore, to our knowledge, the fourth to evidence the role of

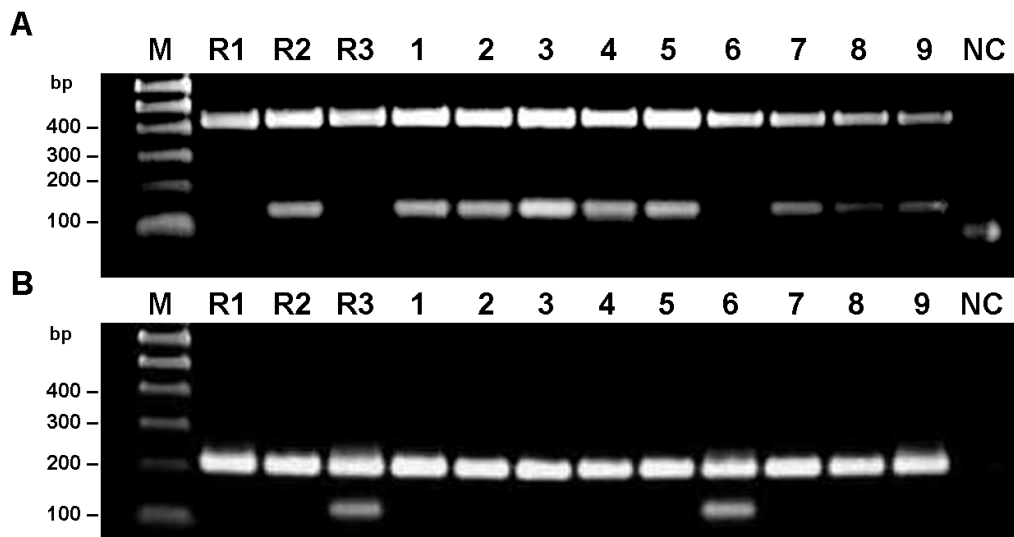


Fig. 3. Results of *P. zopfii* genotype 2- (A) and 3- (B) specific PCR assays. Lanes 1-9, *P. zopfii* bovine mastitis isolates; R1, reference strain *P. zopfii* genotype 1, SAG 2063^T, R2, reference strain *P. zopfii* genotype 2, SAG 2021^T, R3, reference strain *P. blaschkeae*, SAG 2064^T; M, molecular weight marker in base pairs (Quantitas DNA Marker 100 bp – 1 kb, Biozym); NC, negative control (no DNA).

P. blaschkeae in the causation of bovine mastitis. Interestingly, neither Möller et al. (2007) nor Osumi et al. (2008) could isolate *P. blaschkeae* from cases of clinical mastitis in dairy cattle. (In both those studies, all mastitis isolates were identified as *P. zopfii* genotype 2). It is worth mentioning that whereas *P. zopfii* genotype 2 and *P. blaschkeae* appear to be the pathogenic agents of bovine mastitis, *P. zopfii* genotype 1 has never been involved in this condition, although, isolates of *P. zopfii* genotype 1 have consequently been isolated from cow-barn surroundings, such as feces, manures and drinking water (Roesler et al. 2006, Osumi et al. 2008).

In conclusion, the results from this study support previous observations that both *P. zopfii* genotype 2 and *P. blaschkeae* are the causative agents of bovine mastitis, albeit with an important difference in the frequency of isolation (in favor of the former). In order to better recognize the prevalence of *P. zopfii* genotype 2 and *P. blaschkeae* and their contribution to the burden of protothecal bovine mastitis, as well as to clarify the epidemiology of these organisms, especially their reservoir and routes of infection, further investigations involving large-scale sampling and different geographical locations are required.

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