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 endosporulation. 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-
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’-GACATGGCGAGGATTTGACAGA-3’) and PZGT 1/r (5’-GCCAAGGGCCCCCGGAAG-3’) for P. zopfii genotype 1, Proto18-4f and PZGT 2/r (5’-GTCCGCGGGGCAAAAGC-3’) for P. zopfii genotype 2, and PZGT 3-1K/f (5’-CAGGTTGCAGTTCCGGAG-3’) and PZGT 3/r (5’-GTTGCGCGGCACTGCT-3’) for P. blaschkeae. Furthermore, the following primer pairs served as internal amplification controls: Proto18-4f and Proto18-4r (5’-AGGATGGCTAACCCACGA-3’) for P. zopfii genotypes 1 and 2, and PZGT 3-1K/f and PZGT 3-1K/r.
PZGT 3-IK/r (5’-GAATTACCGCGGCTGCTGG-3’) for *P. blaschkeae*.

Each amplification reaction was carried out in a final volume of 25 μL, with the Mastermix 1.1 x ReddyMix™ (ABgene; 1.5 mM MgCl2, 1.25 U Taq DNA polymerase), 20 μM 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 μm 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-type products, all of the isolates yiel-
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).

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 2063T; R2, reference strain *P. zopfii* genotype 2, SAG 2021T; R3, reference strain *P. blaschkeae*, SAG 2064T; M, molecular weight marker in base pairs (Quantitas DNA Marker 100 bp – 1 kb, Biozym); NC, negative control (no DNA).

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...
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.

References


