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ScienceDirect
Veterinary Microbiology 120 (2007) 370–374
veterinary microbiology
www.elsevier.com/locate/vetmic

Short communication

Prototheca zopfii genotype 2—The causative agent of bovine protothecal mastitis?

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Received 7 July 2006; received in revised form 25 October 2006; accepted 31 October 2006

Abstract

In order to clarify the epidemiology of bovine protothecal mastitis, 30 *Prototheca zopfii* mastitis isolates were genetically investigated. Based on the 18S rDNA, which allows a differentiation of the former species *P. zopfii* in two distinct *P. zopfii* genotypes and *Prototheca blaschkeae* sp. nov., newly developed genotype-specific PCR-assays as well as RFLP-assays were applied.

All mastitis isolates investigated could be assigned to *P. zopfii* genotype 2 suggesting that this genotype is the aetiological agent of bovine *Prototheca* mastitis.

Keywords: *Prototheca zopfii*; *P. zopfii* genotype 2; Subspecies; Biotypes; Genotypes; Bovine mastitis; Epidemiology; 18S rDNA; PCR; RFLP

1. Introduction

The only known plants that cause infectious diseases in humans and animals are the colourless algae *Prototheca* (*P.*) *zopfii* and *Prototheca wickerhamii*. In 1952, *P. zopfii* was first identified as a pathogen of bovine mastitis associated with reduced milk production characterized by thin watery secretion with white flakes (Lerche, 1952). While in the past only sporadic cases of protothecal mastitis have been observed, this form of mastitis now occurs endemic in the most countries of the world (Hodges et al., 1985; Costa et al., 1996; Aalbaek et al., 1998; Janosi et al., 2001).

Algae of the genus *Prototheca* are closely related to the green algae *Chlorella*, but lack chlorophyll. The taxonomic status of the genus *Prototheca* has been evolving during the last decades and currently four species are assigned to this genus: *P. zopfii*, *P. wickerhamii*, *Prototheca stagnora*, and *Prototheca ulmea*. A fifth species, *Prototheca moriformis* is not generally accepted (Krüger, 1894; Arnold and Ahearn, 1972; Pore, 1985; Pore, 1986; Ueno et al., 2003). Preliminary studies showed that *P. zopfii* can be differentiated biochemically and serologically into at least three different biotypes (Blaschke-Hellmessen

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0378-1135/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.vetmic.2006.10.039

et al., 1985; Schmalreck et al., 1998; Roesler et al., 2003). As all mastitis isolates investigated could be assigned to biotype 2 of *P. zopfii*, it has been discussed that infections of cattle are predominantly caused by this biotype of *P. zopfii* (Blaschke-Hellmessen et al., 1985; Baumgärtner, 1997).

Most recent phylogenetic investigations based on the 18S rDNA unequivocally revealed discriminating molecular characteristics between the three different *P. zopfii* biotypes. Therefore, the previous biotype 3 of *P. zopfii* was re-classified as a new species (*Prototheca blaschkeae* sp. nov.), whereas, *P. zopfii* biotypes 1 and 2 were re-classified as genotypes 1 and 2 of *P. zopfii* (Roesler et al., 2006).

This study is a approach to elucidate the epidemiology of bovine *Prototheca* mastitis by molecular characterization (genotyping) of German *P. zopfii* mastitis isolates.

2. Materials and methods

Thirty *P. zopfii* mastitis isolates which originated from 17 different German dairy herds (from the federal states Saxony [5 strains], Brandenburg [4], Lower Saxony [1], and Baden–Württemberg [20]; the herds had no contact to each other) were investigated by genotype (biotype)-specific PCRs as well as by genotype-specific restriction endonucleases. The type strains SAG 2063^T (GenBank no. AY973040), SAG 2021^T (GenBank no. AY940456), and SAG 2064^T (GenBank no. AY973041) of the three *P. zopfii* genotypes (deposited at the Stammsammlung für Algenkulturen (SAG), University of Göttingen, Göttingen, Germany) served as controls. The genotype 1 type strain SAG 2063^T was isolated from cattle liquid manure from a dairy herd without any history of protothecal mastitis, the type strain of *P. zopfii* genotype 2 (SAG 2021^T) was isolated from a clinical case of a severe acute mastitis in a lactating cow and is, furthermore, used as ELISA antigen for the serological diagnosis of bovine protothecal mastitis (Roesler et al., 2001; Roesler and Hensel, 2003). The genotype 3 (*P. blaschkeae*) type strain SAG 2064^T originated from a human onychomycosis.

Preparation of genomic DNA was carried out by ultra-sonication and the DNeasy® Plant kit (Qiagen Inc., Chatsworth, CA, USA). Based on the results of the alignment of the 18S rDNA sequences which show differences of 1.6% between genotype 3 and the genotypes 1 and 2 and 0.9% between genotype 1 and genotype 2 (GenBank no. AY973040, AY940456, and AY973041) the following genotype 2-specific primer pairs were used for genotype-specific PCR (Roesler et al., 2006): Proto18-4f (5′-GACATGGCGAGGATTGACAGA-3′) and PZ GT 1/r (5′-GCCAAGGCCCCCCGAAG-3′) for genotype 1, Proto18-4f (5′-GACATGGC GAGGATTGACAGA-3′) and PZ GT 2/r (5′-GTCGGCGGGGCAAAAGC-3′) for genotype 2, and PZGT 3-IK/f (5′-CAGGGTTCGATTCCGGAGAG-3′) and PZ GT 3/r (5′-GTTGGCCCGGCATCGCT-3′) for genotype 3. The genotype-specific primer pairs were evaluated for their specifity against the two other genotypes and the three other known *Prototheca* species (*P. wickerhamii* GenBank no. X74003, *P. stagnora* AB096930, *P. ulmea* AB096929). All of the tested oligonucleotides were specific for their *P. zopfii* genotype, with exception of the genotype 3-specific primer pair that also recognizes the apathogenic species *P. stagnora.*

The following oligonucleotides served as internal amplification controls: Proto18-4f (5′-GACATGGCGAGGATTGACAGA-3′) and Proto18-4r (5′-AGGATGGCTAACCC-ACACGA-3′) for genotype 1 and 2; PZGT 3-IK/f (5′-CAGGGTTCGATTCCGGAGAG-3′) and PZGT 3-IK/r (5′-GAATTACCGCGGCTGCTGG-3′) for genotype 3. PCR amplification (50 μl/reaction) was carried out with the Mastermix 1.1xReddyMix™ (ABgene Ltd., Hamburg; 0.025 units/μl DNA Polymerase, 1.5 mM $MgCl_2$) in a Tpersonal® thermal cycler (Whatman Biometra Ldt., Göttingen, Germany). PCR conditions were 35 cycles with 30 s denaturation at 94 °C, 30 s annealing at 58 °C (63 °C for genotype 3), and 40 s extension at 72 °C.

In addition, also based on the results of the 18S rDNA alignment the following genotype-specific endonucleases were chosen for RFLP analysis: *Kpn*21 (genotype 1), *Sma*I (2), and *Bcn*I (3) (Fermentas Ldt., Burlington, Can). The RFLP analysis was performed using non-specific amplicons of the *P. zopfii* 18S rDNA. The target sequences of the specific restriction enzymes differed from the target sequences of the above described genotype-specific oligonucleotides. The RFLP analysis was performed with purified 18S rDNA fragments which were amplified using the oligonucleotides Proto18-2f (5′-CGCGCAAA