J. Dairy Sci. 98:3001–3009
http://dx.doi.org/10.3168/jds.2014-9271

Internal transcribed spacer sequence-based rapid molecular identification of *Prototheca zopfii* and *Prototheca blaschkeae* directly from milk of infected cows

S. Marques,*†[1] V. A. R. Huss,‡ K. Pfisterer,‡ C. Grosse,‡ and G. Thompson*†
*Centro de Investigação em Biodiversidade e Recursos Genéticos Universidade do Porto (CIBIO/UP), InBio Laboratório Associado Campus Agrário de Vairão, Rua Padre Armando Quintas, n 7, 4485-661 Vairão, Portugal
†Laboratório de Doenças Infecciosas do Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Rua de Jorge Viterbo Ferreira, n 228, 4050-313 Porto, Portugal
‡Department of Biology, Molecular Plant Physiology, University of Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany

ABSTRACT

The increasing incidence of rare mastitis-causing pathogens has urged the implementation of fast and efficient diagnostic and control measures. *Prototheca* algae are known to be associated with diseases in humans and animals. In the latter, the most prevalent form of protothecosis is bovine mastitis with *Prototheca zopfii* and *Prototheca blaschkeae* representing the most common pathogenic species. These nonphotosynthetic and colorless green algae are ubiquitous in different environments and are widely resistant against harmful conditions and antimicrobials. Hence, the association of *Prototheca* with bovine mastitis represents a herd problem, requiring fast and easy identification of the infectious agent. The purpose of this study was to develop a reliable and rapid method, based on the internal transcribed spacer (ITS) sequences of ribosomal DNA, for molecular identification and discrimination between *P. zopfii* and *P. blaschkeae* in bovine mastitic milk. The complete ITS sequences of 32 *Prototheca* isolates showed substantial interspecies but moderate intraspecies variability facilitating the design of species-specific PCR amplification primers. The species-specific PCR was successfully applied to the identification of *P. zopfii* and *P. blaschkeae* directly from milk samples. The intraspecific ITS phylogeny was compared for each species with the geographical distribution of the respective *Prototheca* isolates, but no significant correlation was found.

Key words: *Prototheca* spp. bovine mastitis, internal transcribed spacer (ITS), rapid molecular identification, species-specific PCR (ssPCR), geographical distribution

INTRODUCTION

Members of the genus *Prototheca* are ubiquitous nonphotosynthetic green algae that are associated with pathologies in humans and animals (DiPersio, 2001; Möller et al., 2007; Marques et al., 2008). Presently, 6 species are assigned to the genus *Prototheca*, with *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca blaschkeae*, and *Prototheca cutis* being identified as pathogenic (Jánosi et al., 2001; Roesler et al., 2006; Satoh et al., 2010). *Prototheca zopfii* and *P. blaschkeae* have been mostly associated with clinical or subclinical cases of bovine mastitis (Möller et al., 2007; Marques et al., 2008; Jagielski et al., 2011). *Prototheca zopfii* was classified into 2 genotypes, genotype 1 and 2, after biochemical, serological, and genetic analyses. Genotype 2 was assigned to be the most prevalent responsible for bovine protothecal mastitis, whereas genotype 1 was considered to be not pathogenic (Roesler et al., 2006; Möller et al., 2007). *Prototheca blaschkeae* was primarily isolated from a human onychomycosis (Roesler et al., 2006), and then for the first time from bovine mastitis in Portugal (Marques et al., 2008, 2010; Thompson et al., 2009); meanwhile, *P. blaschkeae* incidences have been reported globally (Jagielski et al., 2010; Gao et al., 2012; Sobukawa et al., 2012; Ricchi et al., 2013).

Prototheca bovine mastitis is increasing worldwide and is currently recognized as endemic. Moreover, this pathology can lead to significant economic losses for the dairy herds and poses a public health problem (Jánosi et al., 2001; Malinowski et al., 2002; Onozaki et al., 2013). No therapy exists to eradicate this infection, and drying off the affected quarter and isolation or culling the infected animals are the only effective counteractions presently applied (Roesler and Hensel, 2003).

Identification of *Prototheca* species is generally performed by phenotypic and genotypic methods. Whereas phenotypic methods often fail in the correct identifi-

Received December 21, 2014.
Accepted January 23, 2015.
[1]Corresponding author: saramarques@mail.icav.up.pt

cation, molecular methods such as PCR amplification with subsequent sequencing of 18S or 28S ribosomal DNA (**rDNA**) or RFLP analysis can reliably identify *Prototheca* spp. (Ueno et al., 2003, 2005; Möller et al., 2007; Marques et al., 2008). Moreover, a real-time PCR was developed to identify different genotypes of *P. zopfii* (Ricchi et al., 2011). Still, these methods are rather time-consuming and some are costly. Recently, Cremonesi et al. (2012) developed an identification method to differentiate *P. zopfii* genotype 2 from other microalgae. Although this method is rapid, easy, and highly reproducible, it has some limitations, such as temperature and pH sensibility and limitation of the DNA fragment size.

The most convenient way of genotyping *Prototheca* isolates with minimal effort seems to be a PCR approach with genotype-specific primers. Such an approach was developed for *P. zopfii* biotypes 1 and 2 and *P. blaschkeae* by Roesler et al. (2006) and Jagielski et al. (2011) based on 18S rDNA sequences. However, the conserved 18S rDNA offers little choice for selection of genotype-specific primers. The primer pairs applied yielded PCR products of 150, 165, and 126 bp, respectively, too similar to be reliably distinguished on conventional gels without additional controls. In an attempt to improve molecular identification of *Prototheca*, recently Capra et al. (2014) were able to amplify chloroplast and mitochondrial target sequences by a multiplex PCR approach.

In contrast to the conserved 18S and 28S rDNA, the ribosomal internal transcribed spacer (**ITS**) region is much more variable and therefore useful for differentiation of strains (Coleman and Mai, 1997). The ITS analysis is now widely applied for phylogenetic studies of closely related *Chlorella*-like algae (Bock et al., 2010; Krienitz et al., 2011). Attempts to consistently amplify the complete ITS region (ITS1, 5.8S rDNA, and ITS2) of *Prototheca* were unsuccessful in the past in our hands. Only recently were the first *Prototheca* ITS sequences published by Hirose et al. (2013) for *P. wickerhamii*.

The aim of the current study was to modify PCR conditions for the successful amplification of ITS sequences from *P. zopfii* genotype 2 and *P. blaschkeae* to determine the genotypic variability and study the correlation of geographical distribution versus phylogeny within 32 strains isolated from milk of infected cows in the north of Portugal. The species-specific (**ss**) differences of the ITS sequences should then be used to develop a rapid and simple molecular diagnostic tool, based on ssPCR primers, to conclusively identify both *Prototheca* species directly from milk samples.

MATERIALS AND METHODS

Prototheca Isolates

The 32 *Prototheca* isolates used in our study belong to a major collection of several milk pathogens retrieved between 2002 and 2009 from the milk of cows with mastitis originating from different dairy herds from the north of Portugal (Figure 1). Eighteen strains were identified as *P. zopfii* genotype 2 and 14 as *P. blaschkeae* as described in a previous study (Marques et al., 2008). In addition, 20 samples of bovine mastitic milk (11 infected by *P. zopfii* genotype 2 and 9 by *P. blaschkeae*) and 4 samples of noninfected bovine milk from different dairy herds from the north and center of Portugal were used for DNA isolation. The 11 samples containing *P. zopfii* genotype 2 and the noninfected milk samples were collected during 2014; the 9 samples with *P. blaschkeae* were from 2007 to 2009. Noninfected milk samples were defined as milk samples retrieved from cows without mastitis or any other infection and with less than 100,000 cells/mL (Guidelines of the National Mastitis Council: Smith et al., 2001). All milk samples were spread on 5% sheep blood agar (bioMérieux, Marcy l'Etoile, France) and incubated for 24 to 72 h at 37°C. Colonies resembling *Prototheca* were transferred and spread on Sabouraud Dextrose agar medium (Merck Laboratories, Darmstadt, Germany), incubated for another 48 to 72 h at 37°C, and identified microscopically thereafter.

Genomic DNA Extraction, ITS Amplification, and Sequencing

Prototheca genomic DNA preparations were essentially performed as described by Cremonesi et al. (2006, 2012), except that the centrifugation speed of all steps was increased to 1,000 × *g* at 4°C (centrifugation for 15 to 20 min for the lysis step and 5 min for the elution step); washing time was increased to 1 min. . Briefly, this protocol makes use of lysing the pellet of cultured cells or centrifuged milk samples with a guanidine thiocyanate containing lysis buffer, in which few colonies were suspended in 500 μL of lysis buffer or 500 μL of milk; samples were washed twice with sterilized saline solution and then resuspended in lysis buffer. Then, free DNA was adsorbed to silica contained in a binding solution, washed with ethanol/isopropanol, and desorbed by an elution buffer. The DNA content was then measured in a NanoDrop ND-1000 UV-visible Spectrophotometer (ThermoScientific, Waltham, MA).

For amplification of the complete ITS region the conserved eukaryote-specific primer 1400 forward (Elwood