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

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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 identi-

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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 $1,000 \times g$ 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 μ L of lysis buffer or 500 μ L 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

Figure 1. Geographical map of Portugal displaying the origin of *Prototheca* isolates. (A) Overview and (B and C) detailed maps of the Viana do Castelo (VC), and Braga and Porto districts. *Prototheca zopfii* (Z) and *Prototheca blaschkeae* (B) strains are color coded according to the clades determined in the phylogenetic analyses (Figure 2). Different shades of gray were set according to the clades determined in the phylogenetic analyses to discern the isolates within clusters, as shown in Figure 2. Adapted from Google Maps. Color version available online.

et al., 1985), located near the 3' end of 18S rDNA, was modified and renamed as 1400Fm (5'-CCTTTG-TACACACCGCCCGTC-3'). This primer was used together with the universal reverse primer NLR 204/21 (5'-ATATGCTTAARTTCAGCGGGT-3; Van der Auwera et al., 1994), located at the 5' end of 28S rDNA. Due to the high GC content of *Prototheca* (Huss et al., 1988), the PCR conditions were adjusted to allow complete melting of the DNA double strands during the denaturation steps. Thirty nanograms of genomic DNA was amplified in 20 μ L of reaction volume containing 5 pmol of each primer, 10 mM dNTP, 4 μ L of 5 $\times$ Phusion GC Buffer, 0.2 μ L of Phusion High-Fidelity DNA Poly-

merase (2 U/ μ L), and 5% dimethyl sulfoxide (DMSO; ThermoScientific) in a BioRad C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). After initial denaturation of 5 min at 98°C, 35 cycles were run with 15 s of denaturation at 98°C, 30 s of annealing at 57°C, and 60 s of extension at 72°C, followed by another 5 min at 72°C after the last cycle. The amplified products were analyzed on a 1.8% (wt/vol) agarose gel stained with GelRed (Biotium Inc., Hayward, CA) and visualized under UV light (Bio-Rad Laboratories). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and cloned with a Zero Blunt Topo PCR Cloning Vector Kit (Invitrogen,

Carlsbad, CA). The insert nucleotide sequences were determined with the universal primers M13 forward and reverse by GATC-Biotech (Constance, Germany).

Phylogenetic Analyses

The ITS sequences determined in our study were manually aligned on a MicroVAX computer (Digital Equipment Corporation, Maynard, MA) with the sequence editor program distributed by Olsen et al. (1992). For the phylogenetic analyses, the sequences were aligned with sequences of 2 trebouxiophycean green algae, *Chlorella vulgaris* and *Closteriopsis acicularis*, which were used as outgroups (Figure 2). To improve the alignment of the ITS data set (ITS1 + 5.8S rDNA + ITS2), secondary structure models were constructed for ITS1 and ITS2 according to the models provided by Coleman and Mai (1997) and Bock et al. (2010). Phylogenetic trees were inferred from all positions of the aligned sequence data by the neighbor-joining (NJ), the maximum parsimony (MP), and the maximum likelihood (ML) method using the PAUP program (Swofford, 2002) for NJ and MP, and randomized accelerated maximum likelihood (RaxML; Stamatakis et al., 2008) for ML. Each 1,000 bootstrap replicates were carried out for NJ, MP, and ML. GenBank Accession Numbers of the ITS sequences determined in this study are listed in Table 1.

ssPCR

Consensus sequences of the *P. zopfii* genotype 2 and *P. blaschkeae* ITS alignments were checked for PCR primer binding sites, which (1) were specific for each species and (2) at the same time generated PCR products of sufficient size difference to be easily discerned on an agarose gel. From several combinations tested, the following primer pairs for *P. zopfii* and *P. blaschkeae*, respectively, proved to be most successful: Pz-ITS-F (5'-TTTCGACCGAACGAAACGA-3') and Pz-ITS-R (5'-AATTCCTGGCATTGGCGACA-3'), as well as Pb-ITS-F (5'-AAGGCCCTGCGTTCTTCGCA-3') and Pb-ITS-R (5'-GCGTGTTCCCGACCGAGAGA-3'). Genomic DNA extracted from *Prototheca* colonies or directly from infected bovine milk was amplified in 20- μ L reactions containing 10 pmol of each specific primer pair, 10 mM dNTP, 4 μ L of 5 $\times$ Phusion GC Buffer, and 0.2 μ L of Phusion High-Fidelity DNA Polymerase (2 U/ μ L). After initial denaturation for 5 min at 98°C, 35 cycles were run with 30 s of denaturation at 98°C, 30 s of annealing at 69°C, and 30 s of extension at 72°C, followed by another 5 min at 72°C after the last cycle. The amplification products were analyzed on a 1.8% (wt/vol) agarose gel as described previously.

RESULTS

ITS Amplification and Sequencing, Phylogenetic Analyses, and Correlation Between Subclades and Geographical Distribution of Infected Herds

The nuclear DNA of *Prototheca* spp. has an exceptional high molar G and C content of about 70 to 75% (Huss et al., 1988). The corresponding high melting temperature may lead to incomplete melting of the DNA during the PCR denaturation steps, thereby explaining the failure of previous attempts to reliably amplify the ITS region of *Prototheca* spp. under standard conditions. We modified the PCR protocol mainly by adding DMSO, which is known to lower the melting temperature of DNA (Escara and Hutton, 1980). Using this protocol with the conserved amplification primers 1400Fm and NLR 204/21, which comprise the complete ITS region, we could successfully amplify and sequence this region from 18 *P. zopfii* genotype 2 and 14 *P. blaschkeae* strains isolated from bovine mastitic milk. The complete ITS length varied from 696 to 744 nucleotides (nt) between strains of *P. zopfii* genotype 2, and from 1,086 to 1,096 nt between *P. blaschkeae* strains. Thus, the intraspecies variability was more pronounced in *P. zopfii* genotype 2, although its ITS was up to 400 nt shorter. The extra nucleotides of the *P. blaschkeae* ITS could mainly be allocated to the extension of helix 1 of ITS1 and helix 4 of ITS2 according to the secondary structure models of Coleman and Mai (1997) and Bock et al. (2010). Under consideration of the secondary structure, the ITS sequences could unambiguously be aligned and all positions used for the phylogenetic analyses. The ML tree depicted in Figure 2 clearly discriminates both species with high statistical support. For the graphical representation of the tree, we preferred a cladogram to a phylogram, as the intraspecies variability, especially in *P. blaschkeae*, is rather low. Nevertheless, within each species some strains cluster together with high or moderate bootstrap support, indicating independent evolution of subclades. It would be interesting to know if the existence of such subclades could be used to trace infection pathways between herds. We therefore tried to correlate, for both species, the occurrence of specific clades obtained by the phylogenetic analyses with the geographical distribution of the herds from which the strains were isolated (Figures 1 and 2). However, no obvious correlation was found. Both *Prototheca* spp. coexist in the same geographical regions and, except for one case, no tendency for the occurrence of a specific subclade only within a restricted area could be deduced. The 3 strains of 1 *P. zopfii* genotype 2 subclade (Z16, Z74A, and Z18) were found in 2 herds only 4 km apart, but the small

Figure 2. Phylogenetic tree inferred from complete internal transcribed space (ITS) sequences. The tree topology is derived from a maximum likelihood (ML) analysis. Bootstrap values are shown at internal nodes for each 1,000 resamplings of neighbor joining/maximum parsimony/maximum likelihood (NJ/MP/MP) if values are higher than 50%. Different clades within *Prototheca zopfii* (Z) and *Prototheca blaschkeae* (B) are color coded (see Figure 1). Different degrees of gray were defined to discern isolates belonging to distinct clusters as shown in Figure 1 [e.g., Z16 and Z74A (dark gray) belong to the same cluster]. The ITS sequences of the related trebouxiphycean green algae *Chlorella vulgaris* and *Closteriopsis acicularis* were used as outgroups, and their GenBank accession numbers are shown. Color version available online.

sample number does not exclude mere coincidence. In contrast, 15 *P. zopfii* genotype 2 strains belonging to the light gray (green) clade with identical or almost identical ITS sequences were identified in herds, which were equally distributed over the whole area under study (60 km north-south vs. 19 km east-west; Figure 1). A similar situation was found for the distribution of *P. blaschkeae* subclades. For example, the strains of the light gray (orange) clade (B84, B66, and B67) were found in more distantly located herds, whereas indi-

vidual cows of other herds in close area were infected by strains belonging to different subclades. Moreover, B80, B84, and B86 belong to different clades, although they were isolated from the same herd. From 1 herd (H1, Table 1), *P. zopfii* genotype 2 as well as *P. blaschkeae* could be isolated, whereas in all other herds from which more than 1 sample was taken, just 1 species was found, although the isolated strains often belonged to different subclades. However, a reliable correlation analysis is hampered by the fact that a possible exchange of

Table 1. List of *Prototheca zopfii* (Z) and *Prototheca blaschkeae* (B) strains isolated in Portugal from bovine mastitic milk, their origin, herd, sample, and strain identification, and GenBank accession number of the internal transcribed space sequence

Herd ID	Sample ID ¹	Strain ID	Accession Number	Village	City
H1	Z1	UP-PT-P1	FR848896	Macieira	Vila do Conde
	B2	UP-PT-P2	HG515045	Macieira	Vila do Conde
H2	Z3	UP-PT-P3	HG515030	Árvore	Vila do Conde
H4	Z7	UP-PT-P7	HG515034	Fajozes	Vila do Conde
H5	Z8	UP-PT-P8	HG515037	Afife	Viana do Castelo
	Z9	UP-PT-P9	HG515033	Afife	Viana do Castelo
H6	Z11	UP-PT-P11	HG515035	Afife	Viana do Castelo
	Z14	UP-PT-P14	HG515036	Afife	Viana do Castelo
	Z16	UP-PT-P16	FR848897	S. Pedro Rates	Póvoa de Varzim
	Z18	UP-PT-P18	FR848898	S. Pedro Rates	Póvoa de Varzim
	Z57	UP-PT-P57	HG515042	S. M. Coronado	Trofa
H22	Z63	UP-PT-P63	HG515044	Covelas	Trofa
H24	Z65A	UP-PT-P65A	HG515038	Arcos	Vila do Conde
H25	B66	UP-PT-P66	HG515046	Rio Mau	Vila do Conde
H26	B67	UP-PT-P67	HG515047	Outeiro Maior	Vila do Conde
H27	Z68	UP-PT-P68	HG515031	Fradelos	Vila Nova de Famalicão
H29	B71	UP-PT-P71	HG515057	Fradelos	Vila Nova de Famalicão
H30	Z74A	UP-PT-P74A	HG515043	Paradela	Barcelos - Braga
H34	Z78	UP-PT-P78	HG515032	Fajozes	Vila do Conde
H36	B80	UP-PT-P80	HG515055	Fajozes	Vila do Conde
	B84	UP-PT-P84	HG515052	Fajozes	Vila do Conde
	B86	UP-PT-P86	HG515051	Fajozes	Vila do Conde
	Z83	UP-PT-P83	HG515040	Fajozes	Vila do Conde
H39	Z96	UP-PT-P96	HG515039	Lavra	Matosinhos
H50	Z106	UP-PT-P106	HG515041	Rio Mau	Vila do Conde
H54	B113	UP-PT-P113	HG515049	Árvore	Vila do Conde
H56	B117	UP-PT-P117	HG515050	Fradelos	Vila Nova de Famalicão
H57	B118A	UP-PT-P118A	HG515056	Vilar do Pinheiro	Vila do Conde
	B118B	UP-PT-P118B	HG515048	Vilar do Pinheiro	Vila do Conde
H58	B119	UP-PT-P119	HG515058	Junqueira	Vila do Conde
	B122	UP-PT-P122	HG515053	Junqueira	Vila do Conde
	B125	UP-PT-P125	HG515054	Junqueira	Vila do Conde

¹The sample ID was used as abbreviation in Figures 1 and 2 for *Prototheca zopfii* (Z) and *Prototheca blaschkeae* (B).

the dairy cows between different herds, resulting from trading between the dairy farmers, could not be taken into account.

Development of ssPCR for Culture-Independent Identification of *P. zopfii* and *P. blaschkeae*

The species-specific differences of the ITS sequences together with their considerable length variation allowed us to design ssPCR primer pairs for *P. zopfii* (Pz-ITS-F and Pz-ITS-R) and *P. blaschkeae* (Pb-ITS-F and Pb-ITS-R) with PCR products of about 490 and 760 bp, respectively. These primers were tested with DNA extracted either from colonies grown on agar plates, or directly from bovine mastitis milk using a slightly modified protocol published by Cremonesi et al. (2006, 2012). We should point out, that in contrast to the amplification of the complete ITS region, no DMSO had to be added for the ssPCR. Figure 3 exemplarily shows an agarose gel with PCR products obtained from milk samples and from cultured *Prototheca* strains that were isolated from these samples and characterized by 18S rDNA and ITS sequencing. The PCR primers clearly

are specific for *P. zopfii* genotype 2 (Figure 3A) and *P. blaschkeae* (Figure 3B), and the size difference of the PCR products can easily be detected on the standard agarose gel. As negative controls, DNA extracted from milk samples of healthy cows were used (lanes 15 and 16) and 1 reaction was carried out without DNA template (lane 7). Moreover, DNA extracted from cultured *P. zopfii* genotype 1 (SAG 2063) and *P. wickerhamii* [isolated from systemic canine protothecosis (Tsuji et al., 2006)] failed to be amplified by both primer pairs as expected (data not shown).

DISCUSSION

The worldwide increasing incidences of bovine mastitis caused by *Prototheca* spp., concomitant with considerable economic losses, challenge the development of culture-independent diagnostic methods for rapid and easy identification of the major infectious pathogens, *P. zopfii* genotype 2 and *P. blaschkeae*. Although ribosomal ITS sequences may vary considerably between species, and therefore are widely used for species-specific identification of organisms and interspecies phylogenies

Figure 3. Species-specific PCR (ssPCR) on *Prototheca* isolates and on infected milk samples using species-specific internal transcribed spacer (ITS) PCR primers. Amplification product sizes were about 490 and 760 bp for (A) *Prototheca zopfii* (Z) genotype 2 and (B) *Prototheca blaschkeae* (B), respectively. Lanes 1 and 9, Z1 isolate; lanes 2 and 10, B2 isolate; lane 3, Z130 milk; lane 4, Z130 isolate; lane 5, Z133 milk; lane 6, Z133 isolate; lane 7, negative control without template; lanes 8 and 17, molecular weight standard; lane 11, B113 milk; lane 12, B113 isolate; lane 13, B118B milk; lane 14, B118B isolate; lanes 15 and 16, negative control with milk of healthy cows. Strain abbreviations are from Table 1.

(Coleman and Mai, 1997; Bock et al., 2010; Kamau et al., 2011), previous methods for genotyping *Prototheca* species from infected dairy cows were mainly based on 18S ribosomal DNA (Möller et al., 2007; Marques et al., 2008). However, the conserved nature of this gene was not useful for designing ssPCR primers without additional controls or using more elaborate and expensive techniques (Möller et al., 2007; Ricchi et al., 2011; Cremonesi et al., 2012). In the current study, we modified PCR conditions, considering the exceptional GC-rich DNA base composition of *Prototheca* (Huss et al., 1988), by adding 5% DMSO to the PCR reaction and could now reliably amplify the complete ITS region

of 32 *P. zopfii* genotype 2 and *P. blaschkeae* isolates from infected cows in the north of Portugal. The ITS sequences not only showed considerable interspecific sequence and length variation, but also revealed intraspecific differences leading to subclades within each species in a phylogenetic analysis (Figure 2). It was tempting to see if the distribution of strains belonging to a specific subclade could be correlated to the geographical location of the herds, from which the strains were isolated (Figure 1). However, considering the relatively small sample number and missing information about a possible exchange of individual animals between herds, no obvious correlation could be detected. Nevertheless,

our study shows that the ITS sequences even within the same *Prototheca* species are different enough to principally make use of such a correlation analysis for tracing infection pathways, if the critical parameters are known.

As expected, the ITS sequence alignment allowed us to design 2 ssPCR primer pairs, which can be used to discern *P. zopfii* genotype 2 and *P. blaschkeae* simply on a PCR agarose gel (Figure 3). The PCR product size difference of about 270 bp is sufficient to obviate the need for additional controls. Moreover, this diagnostic tool can also be applied in a culture-independent assay using DNA isolated directly from milk samples of infected cows by a protocol described by Cremonesi et al. (2006, 2012). We verified this possibility by using DNA templates isolated from cultured strains and in parallel from the milk samples, from which the cultures were obtained (Figure 3). In each case, bands of the correct size could be detected, whereas the milk of healthy cows served as a negative control. Thus, the ssPCR primers can be used for rapid and easy identification of pathogenic *Prototheca* species directly from milk samples. Only recently, a paper was published by Capra et al. (2014) with the same objective. They used multiplex PCR with ssPCR primers based on chloroplast and mitochondrial sequence comparisons. In 2 PCR reactions, each 8 primers were combined to identify and distinguish *P. zopfii* genotype 2, *P. blaschkeae*, and *P. wickerhamii* by a combination of PCR products of different size on an agarose gel. Although they could clearly differentiate the *Prototheca* species, our protocol requires only 4 primers and uses a more convenient and widely applied molecular marker.

In summary, we present the first complete ITS sequences of 32 *Prototheca zopfii* genotype 2 and *P. blaschkeae* strains isolated from bovine mastitic milk of infected cows in the north of Portugal. Based on these sequences, ssPCR primers were designed for rapid and easy identification of pathogenic *Prototheca* directly from milk samples.

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