Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Simultaneous identification by multiplex PCR of major *Prototheca* spp. isolated from bovine and buffalo intramammary infection and bulk tank

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Significance and Impact of the Study: This work reports PCR assays based on novel *Prototheca* spp. mitochondrial and chloroplastic target sequences. The multiplex PCR protocol described in this study is useful for rapid simultaneous detection of *P. zopfii*, *P. wickerhamii* and *P. blaschkeae.*

Keywords
bovine, buffalo, mastitis, multiplex, PCR, *Prototheca*.

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2014/0848: received 24 April 2014, revised 8 August 2014 and accepted 3 September 2014

doi:10.1111/lam.12326

Abstract

Bovine mastitis caused by *Prototheca* spp. infection is increasing worldwide, therefore becoming more relevant to the dairy industry. Almost all *Prototheca* isolates from bovine mammary protothecosis came from *P. zopfii* genotype 2, with a lower prevalence of infection due to *P. blaschkeae* and rarely to *P. wickerhamii*. In this study, we report the development of two multiplex PCR assays able to discriminate among the three species responsible for bovine intramammary infection (IMI). Our assay is based on the specific amplification of new DNA target from mitochondria and chloroplasts partial sequences, of different *Prototheca* isolates. Both methods were set up using reference strains belonging to all *Prototheca* species and validated by the analysis of 93 isolates from bovine and buffalo IMI and bulk tank milk samples. The investigation involves 70 isolates from North, 13 from Central and 10 from South Italian regions. Isolates from bovine were most commonly identified as *P. zopfii* genotype 2, and only in one case as *P. blaschkeae*, whereas isolates from buffaloes belonged both to *P. zopfii* genotype 2 and *P. wickerhamii*. These findings proved the suitability of our multiplex PCRs as a rapid test to discriminate among pathogenic *Prototheca* strains.

Introduction

Prototheca spp. are algae, assigned to the genus *Prototheca*, family *Chlorelaceae*. They are ubiquitous in nature, living predominantly in aqueous environments containing decomposing plant material (Anderson and Walker 1988; Huerre *et al.* 1993). Within the known *Prototheca* spp., only *Prototheca zopfii*, *P. wickerhamii* and *P. blaschkeae* have been associated with disease in humans and animals (Roesler and Hensel 2003; Pfaller and Diekema 2005; Roesler *et al.* 2006; Zaitz *et al.* 2006; Thompson *et al.* 2009). Even in human, protothecosis is mainly caused by *P. wickerhamii* (Lass-Flörl and Mayr 2007), in veterinary medicine, *P. zopfii* is reported as causative agents of protothecosis in dogs and bovine (Migaki *et al.* 1982; Hodges *et al.* 1985; Corbellini *et al.* 2001; Hosaka and Hosaka

2004). In the past, the genus *Prothoteca* was considered a rare pathogen in dairy cattle and associated with infection in the presence of predisposing factor, such as poor environmental conditions and insufficient milking hygiene (Jánosi *et al.* 2001); however, cases of clinical and chronic mastitis are recognized increasingly to become endemic worldwide (Roesler and Hensel 2003; Osumi *et al.* 2008).

Bovine IMIs are mainly caused by *P. zopfii* infection whereas is rarely associated with *P. wickerhamii* infection (Marques *et al.* 2006). In buffalo herds, association between IMI and *Prototheca* spp. was less investigated, even it was likely related to both *P. zopfii* and *P. wickerhamii* infection (Ali *et al.* 2008; Asfour and El-Metwally 2010).

Almost all *Prototheca* isolates from bovine milk in Italy, Germany, Portugal, Poland, Japan and China were *P. zopfii* genotype 2, suggesting that it is the principal causative agent (Möller *et al.* 2007; Marques *et al.* 2008; Osumi *et al.* 2008; Kishimoto *et al.* 2010; Ricchi *et al.* 2010; Gao *et al.* 2012). However, other reported the involvement of *P. blaschkeae* in bovine mastitis (Marques *et al.* 2008; Jagielski *et al.* 2011; Ricchi *et al.* 2013).

Many molecular assays based on rDNAs have been proposed for the detection on *Prototheca* spp. (reviewed in Cremonesi *et al.* 2012); however, no one has been designed for the identification of *Prototheca* spp. associated with IMI in a single PCR.

Aim of our work was to develop multiplex PCR methods based on specific amplification of mitochondrial and chloroplast sequences, recently obtained by the authors. The assays were able to identify three bovine *Prototheca* spp. associated with IMI in a single reaction. These new assays were validated identifying bovine and buffaloes *Prototheca* isolates from different Italian regions.

Results and discussion

The PCR-based methods described in the present paper provide an option for a rapid identification of *Prototheca* spp. possibly involved in bovine mastitis and other animal diseases, with particular reference to *P. zopfii* genotype 2, *P. blaschkeae* and *P. wickerhamii*.

Our assays were designed to obtain distinct PCR products by two alternative multiplex specific reactions of the three main *Prototheca* species associated with IMI. Sequences obtained from partial chloroplastic and mitochondrial amplicons were used to obtain two sets of specific primers combination that clearly identify the three infective strains *P. zopfii* genotype 2, *P. blaschkeae* and *P. wickerhamii*. Two multiplex PCRs were optimized using a panel of the six reference strains previously reported (*P. blaschkeae*, *P. wickerhamii*, *P. zopfii* genotype 1 and 2, *P. ulmea* and *P. stagnora*), resulting in specific bands for *P. zopfii* genotype 2, *P. blaschkeae* and *P. wickerhamii* (Fig. 1). Both multiplex PCR assays showed the same capability and sensitivity for pathogenic *Prototheca* spp. detection, therefore each of them can be used separately.

Different species showed amplification profiles with a common band at 216 bp present in all *Prototheca* spp., and a second specific amplicon was then obtained for each strain associated with IMI: a band at 508 bp (multiplex 1 and 2) for *P. zopfii* genotype 2, 379 bp (multiplex 1) or 255 bp (multiplex 2) for *P. blaschkeae* and 115 bp (multiplex 1 and 2) for *P. wickerhamii*.

To test the sensitivity of the assay, we performed a serial dilution (from 80 to 0·08 pg μl^{-1}) of DNA extracted from *P. zopfii* genotype 2, *P. blaschkeae* and *P. wickerhamii* representing pathogenic associated species, and *P. zopfii* genotype 1.

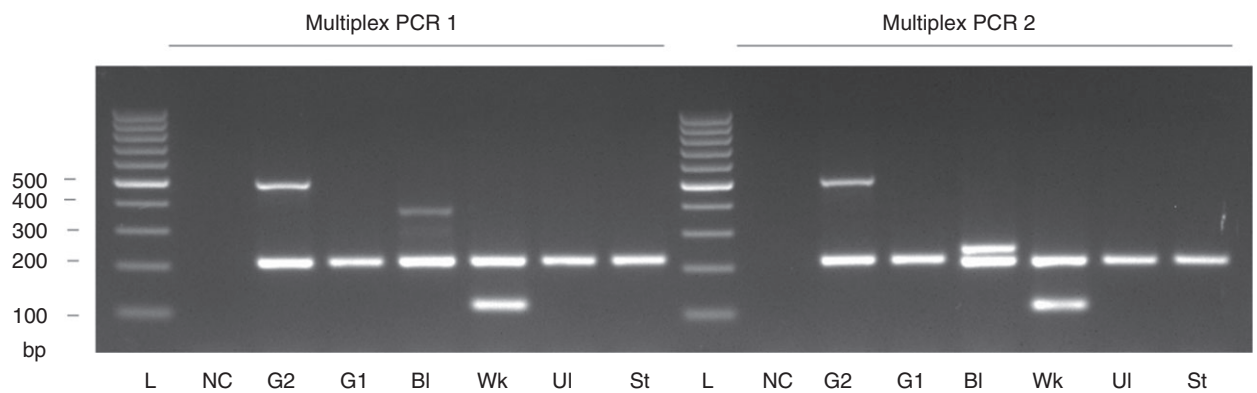

Figure 1 Multiplex PCR assays, using both primer combination, for different *Prototheca* species: Bl, *P. blaschkeae* SAG2064; Wk, *P. wickerhamii* 1357; G2, *P. zopfii genotype* 2 SAG2021; G1, *P. zopfii genotype* 1 SAG2063; Ul, *P. ulmea* ATCC 16528; St, *P. stagnora* ATCC 50112; NC, negative control.

Table 1 Distribution of *Prototheca* spp. isolated from herds in different Italian regions

	North Italy n° isolate (specie)/n° herds	Central Italy n° isolate (specie)/n° herds	South Italy n° isolate (specie)/n° herds
IMI (Bovine)	10 (*P. zopfii* genotype 2)/7		
Bulk talk milk (Bovine)	60 (*P. zopfii* genotype 2)/16	13 (*P. zopfii* genotype 2)/7	1 (*P. zopfii* genotype 2)/1 1 (*P. blaschkeae)*/1
Bulk talk milk (Buffalo)			4 (*P. zopfii* genotype 2)/4 4 (*P. wickerhamii)*/4

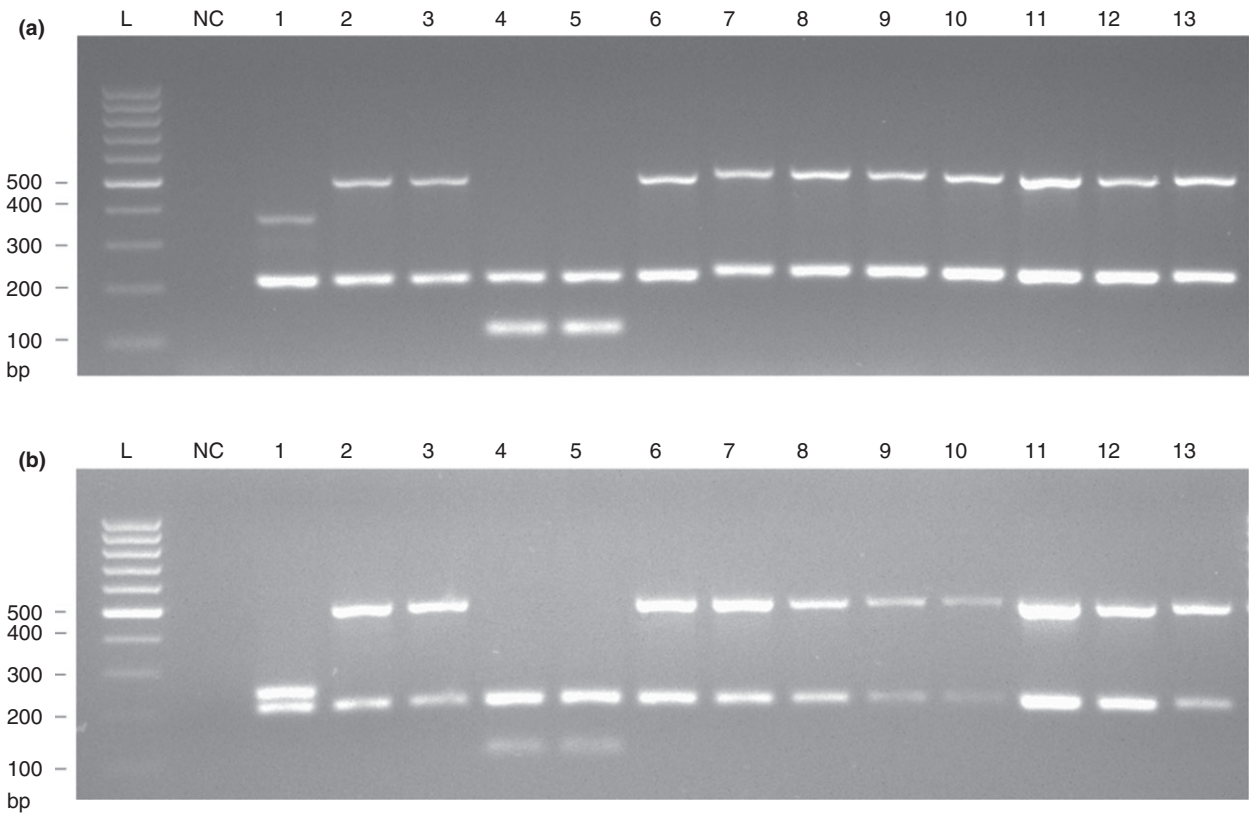

Figure 2 (a) Multiplex PCR 1 and (b) multiplex PCR 2 on different *Prototheca* spp. isolated in field (1–5 from south Italy; 6–9 from centre Italy; 10–13 from north Italy). Lane 1 identified as *P. blaschkeae*, lanes 2, 3, 6, 7, 8, 9, 10, 11, 12, 13 identified as *P. zopfii genotype* 2, lanes 4 and 5 identified as *P. wickerhamii*, NC negative control.

Both multiplex PCR methods showed high sensitivity with detection until 0·8 pg μl^{-1} of DNA in solution (Figure S1) and specificity (data not shown).

Both multiplex PCR assays were then validated on 93 *Prototheca* spp. isolates from bovine IMI and essentially underlined the high prevalence of *P. zopfii* genotype 2, except for one sample identified as *P. blaschkeae* (Table 1). The identification of all isolates was also confirmed by the PCR–single strain conformational polymorphism SSCP typing methodology (Cremonesi *et al.* 2012) (data not shown).

Epidemiological studies on bovine protothecal infection in different countries, for example Germany (Möller *et al.* 2007), Belgium (Aouay *et al.* 2008), Italy (Ricchi *et al.* 2010), Brazil (Salerno *et al.* 2010), Japan (Osumi *et al.* 2008) and Poland (Jagielski *et al.* 2011), using other approaches, showed similar results with the predominance of the *P. zopfii* genotype 2 in the aetiology of bovine mammary protothecosis, with few cases of *P. blaschkeae* associated with bovine IMI. Interesting, four of the 8 isolates from buffalo bulk tank milk collected in south Italy were identified as *P. wickerhamii*, (Fig. 2). The presence of

P. wickerhamii isolate from buffaloes mastitis was previously described (Asfour and El-Metwally 2010), but, to the best of our knowledge, this is the first study reporting the presence of this *Prototheca* species in Italian buffalo herds.

In veterinary diagnostic, *Prototheca* spp. isolates are currently characterized using molecular methods. At first, the sequencing of rDNA was used for molecular species identification, but more rapid methods such as genotype-specific PCR (Roesler *et al.* 2006) or RFLP analysis (Möller *et al.* 2007) were developed. Nevertheless, these assays were able to distinguish between *P. blaschkeae* and *P. zopfii* (genotype 1 and 2), but unable to differentiate among other *Prototheca* species. Even nested PCR (Onozaki *et al.* 2009) and duplex PCR (Gao *et al.* 2012) methods, although allowing the detection of *Prototheca* spp. directly from milk, were aimed to the identification of the only *P. zopfii* species. Further methods, one based on 2-step quantitative PCR followed by DNA resolution melting analysis (Ricchi *et al.* 2011) and another on SSCP (Cremonesi *et al.* 2012) overwhelmed this problem and permitted the identification of all species and genotypes. However, these assays were more technically demanding than a simple PCR. More recently, Hirose *et al.* (2013) described a new PCR assay, based on ITS1 and ITS2 amplification resulting in amplicons differing in size species-dependent manner, suggesting that the variation might be useful for differentiation of *Prototheca* spp. Nevertheless, the method has limited value as it focused on *P. wickerhamii* subtyping, while an extensive analysis about the test's ability to distinguish among other pathogenic *Prototheca* spp. has not been reported. Notably, all molecular methods previously described were based on the amplification of 18S rDNA, while our assays are the first developed one targeting mitochondrial and chloroplast DNA sequences.

In conclusion, the use of two multiplex PCRs based on the amplification of mitochondrial and chloroplast DNA target sequences provided a rapid and specific test for major *Prototheca* spp. associated with IMI. Using these methods, a survey of isolates from herds located in different Italian regions was carried out. The results confirmed the *P. zopfii* genotype 2 as the main species associated with IMI and provided evidence that *P. wickerhamii* is also present in buffalo herds.

Materials and methods

Source of samples

Reference strains belonging to both pathogenic (*P. zopfii* genotype 2 SAG2021; *P. wickerhamii* 1357 (provided by the Institute of Agricultural Biology and Biotechnology-Italian National Research Council bacterial collection); *P. blaschkeae* SAG2064) and nonpathogenic species (*P. zopfii* genotype 1, SAG2063; *P. stagnora*, ATCC 16528; *P. ulme*a, ATCC 50112) were used in this study. For assay validation, 93 isolates were retrieved from 10 IMI composite milk samples, 75 bovine bulk tank milk and eight buffalo bulk tank milk samples originating from 40 dairy farms of which 23 located in Lombardia (north Italy), seven in Emilia Romagna (centre Italy) and 10 in Campania (south Italy) Italian regions between 2011 and 2013.

Algae cultivation and microbiological analyses

The isolation of *Prototheca* spp. was performed by plating 0·1-ml aliquots of bulk tank milk or by streaking aseptically collected mastitic milk samples onto Sabouraud dextrose agar (Biogenetics, Padova, Italy) plates and incubating the plates under aerobic conditions at 37°C for 48 h. The colonies grown after 48 h with the typical aspect were confirmed by microscopic examination (after

Table 2 Sequence of primers and their combination for multiplexes PCR experiment

Primer Name	Primer Sequence (5′ 3′)	Primer Mix Conc. μmol l^{-1}	Specificity
N476-F	TCGGAGTTAGCTGGTTCTCC	0·8	All *Prototheca* spp.
N476-R	ATTTTGGGGCCTTAACTGGT	0·8	All *Prototheca* spp.
N2-F	TGTAATAGATATTAGAAACGCAACAAA	0·8	*P. zopfii* genotype 2
N2-R	GCAGCAGTAGGGAATTTTGG	1·6	*P. zopfii* genotype 2 and *P. blaschkeae*
Bl2-F	CTTCGCCTTTGGCCTTCT	0·8	*P. blaschkeae*
Bl3-F	AAGTTTACATTAAGATCATTCGATTCT	0·8	*P. blaschkeae*
Wk3-F	CGGGAATCTTCGGATCATTA	3·2	*P. wickerhamii*
Wk5-R	GGTCAAATGCTTAAAGGCGTA	3·2	*P. wickerhamii*
Primer combination and amplicons size for multiplex 1			
N476-F, N476-R (216 bp); N2-F, N2-R (508 bp); Bl2-F, N2-R (379 bp); Wk3-F, Wk5-R (115 bp)			
Primer combination and amplicons size for multiplex 2			
N476-F, N476-R (216 bp); N2-F, N2-R (508 bp); Bl3-F, N2-R (255 bp); Wk3-F, Wk5-R (115 bp)			

staining with lactophenol cotton blue) showing the typical cell morphology with ovoid or globose sporangia with several sporangiospores arranged as a 'morula' (Pore 1985; Marques *et al.* 2010). Subcultures of individual colonies were obtained for the subsequent analysis. Reference strains were aerobically cultured on Sabouraud dextrose agar at the same incubation conditions.

Genotype-specific multiplex PCR

Genomic DNA was isolated as previous described (Cremonesi *et al.* 2012). Assignment of *Prototheca* isolates to different species (*P. blaschkeae*, *P. wickerhamii* and *P. zopfii* genotype 2) was carried out by multiplexing genotype-specific PCR assays, with primers designed on mitochondrial and chloroplastic sequences recently deposited to NCBI database by the authors (Accession Numbers KF983331, KF983332, KF983333, KF983334, KF983335, KF983336, KF983337, KF983338, KF983339, KF983340, KF983341). Two multiplex PCR assays were developed by using different primers combination (Table 2). Multiplexes PCR were performed by using 1× HotStarTaq Master Mix (Qiagen, Valencia, CA) following manufacture instructions. All primers were combined in a single reaction using the following concentration in solution: N476-F, N476-R, N2-F, Bl2-F or Bl3-F, at 0·8 μmol l^{-1}, N2-R at 1·6 μmol l^{-1}, Wk3 and Wk5 at 3·2 μmol l^{-1}. Two primer combinations were used to specifically amplify different *Prototheca* spp. by two different multiplex reactions. Multiplex 1 combined N476-F with N476-R (amplicon size 216 bp); N2-F with N2-R (amplicon size 508 bp); Bl2-F with N2-R (amplicon size 379 bp); and Wk3-F with Wk5-R (amplicon size 115 bp) primers pair, whereas multiplex 2 combined N476-F with N476-R (amplicon size 216 bp); N2-F with N2-R (amplicon size 508 bp); Bl3-F with N2-R (amplicon size 255 bp); and Wk3-F with Wk5-R primers pair (amplicon size 115 bp). The cycling conditions were 15′ at 94°C, followed by 30 cycles of 1′ at 94°C, 1′ at 60°C and 1′ at 72°C, and finally 7′ at 72°C. The amplified PCR products were separated electrophoretically on 2·5% agarose gels, and visualized under UV, by using GeneRuler 100 bp as DNA ladder (Thermo Fisher Scientific Inc, Waltham, MA).

Multiplex PCR validation

Assay sensitivity was tested using both multiplex primer combinations. DNA extracted from *P. zopfii* genotype 2 SAG2021, *P. wickerhamii* 1357, *P. blaschkeae* SAG2064 and *P. zopfii* genotype 1 SAG2063 were quantified by PicoGreen fluorescent dye (Invitrogen, Carlsbad, California), serial diluted 1 : 10 (from 80 pg μl^{-1} to 0·08 pg μl^{-1}) and amplified by multiplex PCR and products separated electrophoretically as above reported. PCR specificity was tested on genomic DNA isolated from different micro-organism, commonly present in milk sample (*Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae, Staphylococcus epidermidis, Streptococcus uberis, Streptococcus bovis, Mycoplasma bovis, Enterococcus faecalis*).

Acknowledgements

This work was partly supported by grants from Italian Ministry of Health (E87G12000150001 RC IZSLER 11/11) and from Mastfield Project 'Applicazione di sistemi molecolari innovative per il controllo in campo delle mastiti bovine', funded by Regione Lombardia, DG Agricoltura.

Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Multiplex PCR assays sensitivity with DNA extracted from different *Prototheca* species: G1, *P. zopfii genotype* 1 SAG2063; Wk, *P. wickerhamii* 1357; Bl, *P. blaschkeae* SAG2064; G2, *P. zopfii genotype* 2 SAG2021 at different DNA concentrations in solution from 80 pg μl^{-1} to 0·08 pg μl^{-1}