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Molecular characterization of *Prototheca* strains isolated from Italian dairy herds

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ABSTRACT

One hundred sixty-one *Prototheca* spp. strains isolated from composite milk and barn-surrounding environmental samples (bedding, feces, drinking, or washing water, surface swabs) of 24 Italian dairy herds were characterized by genotype-specific PCR analysis. Overall, 97.2% of strains isolated from composite milk samples were characterized as *Prototheca zopfii* genotype 2, confirming its role as the main mastitis pathogen, whereas *Prototheca blaschkeae* was only sporadically isolated (2.8%). Regarding environmental sampling, 84.9% of isolates belonged to *P. zopfii* genotype 2, 13.2% to *P. blaschkeae*, and 1.9% to *P. zopfii* genotype 1. The data herein contradict previous hypotheses about the supposed exclusive role of *P. zopfii* genotype 2 as the causative agent of protothecal mastitis and, on the contrary, confirm the hypothesis that such pathology could be caused by *P. blaschkeae* in a few instances.
Key words: *Prototheca zopfii* genotype, *Prototheca blaschkeae*, genotype-specific PCR analysis, milk

INTRODUCTION

The genus *Prototheca* includes unicellular yeast-like, colorless microalgae (phylogenetically related to *Chlorella*), belonging, to date, to 5 accepted species (*Prototheca zopfii*, *Prototheca blaschkeae*, *Prototheca stagnora*, *Prototheca whickeramii*, and *Prototheca ulmea*; Pore, 1998; Roesler et al., 2006). The presence of a sixth species (*Prototheca moriformis*) is still under debate (Ueno et al., 2003), and recently Satoh et al. (2010) proposed a new species, *Prototheca cutis* sp. nov. These microorganisms exhibit exclusively an asexual reproduction by cytoplasm splitting and the consequent formation from 2 to 16 daughter cells. *Prototheca zopfii*, *P. stagnora*, *P. whickeramii*, and *P. ulmea* are commonly associated with some natural habitats (especially wet environments containing rotting organic matter; Pore, 1998). On the other hand, *P. blaschkeae*, *P. whickeramii*, and *P. cutis* have been associated with human protothecosis (Pore, 1998; Roesler et al., 2006, Lass-Flörl and Mayr, 2007; Satoh et al., 2010).

Although infections caused by *P. zopfii* have been observed in small animals (Ribeiro et al., 2009), mastitis in dairy cows represents the most frequent (and often dramatic) form of protothecosis in animals (Janosi et al., 2001). Mammary gland infections caused by *P. zopfii* are rarely associated with clinical signs. Accordingly, the detection of individual cases of protothecal mastitis in some herds can indicate a serious problem affecting a significant percentage of cows. Cattle appear to be susceptible to infections in all stages of lactation, including the dry period (Furuoka et al., 1989). Some authors report a particular susceptibility to the natural infection in the first few weeks of lactation (Janosi et al., 2001). In cows, the infection may be restricted to the udder or disseminated to the lymph nodes (Mc Donald et al., 1984).

Protothecal mastitis is rapidly becoming a global problem (Lagneau, 1996; Castagna de Vargas et al., 1998; Janosi et al., 2001; Buzzini et al., 2004; Scaccabarozzi et al., 2008). Studies carried out over the last 10 yr report that only a few antibiotic drugs (e.g., polyenes) or antiseptics (e.g., sodium hypochlorite, iodine compounds, indole-3-acetic acid + horseradish peroxidase, bovine lactoferrin hydrolysate, synthetic flavonoid derivatives) exhibit in vitro activity against these yeast-like microalgae (Segal et al., 1976; Casal and Gutierrez, 1983; Shahan and Pore, 1991; Marques et al., 2006; Kawai et al., 2007; Buzzini et al., 2008a,b; Tortorano et al., 2008; Cunha et al., 2010; Salerno et al., 2010). On the contrary, their effect in vivo has never been demonstrated.

In recent years, based on the results of a PCR assay carried out on *P. zopfii* isolates, Roesler et al. (2006) described the existence of 2 distinct *P. zopfii* genotypes.

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Moreover, Möller et al. (2007) postulated the nonpathogenic nature of both *P. zopfii* genotype 1 and *P. blaschkeae* (previously defined as *P. zopfii* genotype 3). In this background, the same authors suggested that *P. zopfii* genotype 2 could be the exclusive causative agent of bovine protothecal mastitis. Accordingly, *P. zopfii* genotype 2 was preliminarily designated as *P. zopfii* ssp. *bovimastidogenes*.

However, further studies only partially confirmed this assumption. A large-scale screening survey carried out on *Prototheca* spp. Japanese strains (isolated from bovine mastitis and surrounding cow barns) found that all isolates from mastitic milk belonged to *P. zopfii* genotype 2 (Osumi et al., 2008), whereas Marques et al. (2008) reported a few bovine mastitis associated with *P. blaschkeae*. Accordingly, these results present a complicated scenario about the occurrence of protothecal mastitis caused by *P. zopfii* genotype 2 or by *P. blaschkeae*.

The management measures applied to protothecal mastitis outbreaks are directed to control the possible sources of infection. Because of the double nature of the infection, both contagious and environmental, these measures mainly involve separate milking of infected cows, culling infected animals, and controlling environmental contamination. Accordingly, the discrimination between pathogenic and nonpathogenic species is critical in evaluating the possible sources of infection.

The aim of the study was to give a molecular characterization of *Prototheca* strains isolated from composite milk of cows and from environmental barn-surrounding samples, to yield a profile of the occurrence of *P. zopfii* (genotype 1 and 2) and *P. blaschkeae* in Italian dairy herds.

MATERIALS AND METHODS

Sample Collection

The number of herds (24) was chosen to represent dairies participating in a somatic cell count control project (Milk Quality Premium, Modena and Piacenza, Italy) with the problem of high SCC in the bulk tank. Samples used in this study derived from dairy herds with contagious and environmental mastitis; 18 were positive also for *Prototheca* and 6 negative for *Prototheca*.

Samples were collected from cows with clinical mastitis or high SCC and that were not responding to mastitis therapy. Infection status was defined according to the procedures recommended by the National Mastitis Council (NMC, 1999), including *Prototheca*. In all cases of positive diagnosis of protothecal infection, further analysis was performed on all lactating cows of the herd. As a negative control, all animals of 6 uninfected herds were sampled. The total of composite milk samples from both infected and uninfected herds was 3,208.

Moreover, 411 environmental samples were collected from the same herds and screened for the presence of *Prototheca* spp. To prevent cross-contamination, all environmental samples from different sites (in both the lactating and dry cow areas) were aseptically collected in sterile vials or bags, stored at 4°C in a cool box, and transported within a few hours to the laboratory for microbiological analysis. Environmental samples included cow drinking water, wash water from the milk transport system and refrigeration tank, cow resting areas (bedding material), cattle feces (direct rectal sampling by single-use sleeves), and swabs samples collected from liners and in the barns.

Isolation of Prototheca spp. Strains

All microbiological substrates were from Difco (Franklin Lakes, NJ), whereas all chemicals were from Sigma (St. Louis, MO) and Fermentas (Glen Burnie, MD).

Direct streaking of 0.01 mL of milk on Petri dishes containing *Prototheca* isolation medium (**PIM**) agar (Pore, 1973), blood agar, and Gassner agar was used for isolating *Prototheca* spp. strains from milk samples.

The isolation of *Prototheca* spp. strains from environmental samples was carried out by a preliminary enrichment on liquid PIM, followed by streaking on Petri dishes containing PIM agar. Colonies exhibiting a yeast-like morphology were picked and sub-cultured on yeast extract-peptone-glucose (**YEPG**) agar (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L, agar 20 g/L; pH 6.5). All *Prototheca* spp. isolates were identified by auxonographical and biochemical methods (Pore, 1998; Roesler et al., 2003, 2006; Osumi et al., 2008).

All strains used in the present study are deposited either in the Industrial Yeasts Collection DBVPG of the University of Perugia (Italy; www.agr.unipg.it/dbvpg) or in the collection of the Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna, Piacenza, Italy.

DNA Extraction

One hundred sixty-one representative *Prototheca* strains were analyzed: 108 from composite milk and 53 from environmental samples (bedding = 18 strains, feces = 3, drinking or washing water = 14, swabs = 18).

A procedure previously used for DNA extraction from yeasts (Turchetti et al., 2008) was modified for *Prototheca* species strains. Disruption of the cell wall was achieved by suspending 3 loopfuls of 48-h cells (grown

Table 1. Genotype-specific PCR primers used in this study

Primer	Target	Sequence
Proto 18–4f	Internal control for PCR	5′-GACATGGCGAGGATTGACAGA-3′
Proto 18–4r	Internal control for PCR	5′-AGCACACCCAATCGGTAGGA-3′
PZGT 3-IK/f	Internal control for PCR	5′-CAGGGTTCGATTCCGGAGAG-3′
PZGT 3-IK/R	Internal control for PCR	5′-GAATTACCGCGGCTGCTGG-3′
PZGT 1/r[1]	Genotype 1-specific PCR	5′-GCCAAGGCCCCCCGAAG-3′
PZGT 2/r[1]	Genotype 2-specific PCR	5′-GTCGGCGGGGCAAAAGC-3′
PZGT 3/r[1]	Genotype 3-specific PCR	5′-GTTGGCCCGGCATCGCT-3′

[1]These specific reverse primers were used in association with specific oligonucleotides (which also acted as part of the internal amplification controls): Proto 18–4f for the primers PZGT 1/r and PZGT 2/r; PZGT 3-IK/f for the primer PZGT 3/r.

in YEPG agar) in 500 μL of lysis buffer (Tris-HCl 50 m*M*, EDTA 50 m*M*, NaCl 250 m*M*, SDS 0.3% wt/vol, pH 8.0) and 500 μL of phenol:chloroform mixture (1:1, pH = 8.0). One hundred fifty microliters (calculated as equivalent volume) of glass beads (diameter = 425–600 μm) was added. After centrifugation (17,000 × *g* for 30 min), 400 μL of supernatant was mixed with an equal volume of ice-cold 99% ethanol. After precipitation, DNA was harvested by centrifugation (12,000 × *g* for 15 min at 4°C), resuspended in 100 μL of pure (analytical grade) water (Fermentas) and incubated (37°C for 30 min) with 4 μL of RNase (Sigma; 93 units/mg of protein). After further precipitation with 11 μL of 3 *M* sodium acetate and 200 μL of 99% ice-cold ethanol, DNA was harvested by centrifugation (12,000 × *g* for 15 min at 4°C) and resuspended in 100 μL of pure (analytical-grade) water.

Genotype-Specific PCR Reaction

After DNA extraction, the 108 *Prototheca* spp. strains were analyzed by a modified genotype-specific PCR procedure (Roesler et al., 2006). Genotype-specific primers are listed in Table 1. All PCR reactions were performed in 25-μL reaction volumes containing 1× PCR buffer, 2 m*M* $MgCl_2$, 250 μ*M* of each of the 4 dNTPs, 0.8 μ*M* of primer, and 0.02 U/μL of *Taq* DNA polymerase (Fermentas).

Amplification of DNA was performed in a T personal Combi Thermal Cycler (Biometra GmbH, Goettingen, Germany). The *P. zopfii* genotype 1- and 2-specific PCR program was (1) initial denaturing step at 94°C for 4.5 min; (2) 30 cycles of 30 s at 94°C, 30 s at 65°C, and 40 s at 72°C; and (3) final extension step at 72°C for 5 min. The *P. blaschkeae*-specific PCR program was (1) initial denaturing step at 94°C for 4.5 min; (2) 35 cycles of 30 s at 94°C, 30 s at 63°C, and 40 s at 72°C; and (3) final extension step at 72°C for 5 min. A negative control was included in all PCR reactions.

Amplification products were analyzed (after staining with ethidium bromide) by electrophoresis on 1.6% (wt/vol) agarose gel. A molecular size marker (Gene Ruler 50 bp DNA ladder, Fermentas) was used for reference.

For comparative purposes, the strains *P. zopfii* genotype 1 SAG 2063 and *P. blaschkeae* SAG 2064, both obtained from Sammlung von Algenkulturen der Universität Göttingen, Göttingen, Germany (Roesler et al., 2006), and *P. zopfii* 219509/2, attributed to genotype 2 after auxonographical, biochemical, and sequencing analysis (our unpublished data), were used as control strains. No discrepant results were observed in repeated experiments.

Statistical Evaluation of Data

Correlation between the percentage of milk and environmental samples positive for *Prototheca* spp. was calculated by using the nonparametric Spearman rank correlation test (Myers and Well, 2003).

RESULTS AND DISCUSSION

Two-hundred thirty-nine and 69 *Prototheca* spp. strains were isolated from 3,208 individual milk samples and 411 environmental samples, respectively. Among them, a representative subset of strains (108 and 53 strains, respectively) was analyzed by genotype-specific PCR procedure. The percentage of milk and environmental samples positive for *Prototheca* spp., together with the results of genotype-specific PCR analysis on *Prototheca* spp. isolates, are reported (herd by herd) in Table 2.

When primers Proto 18-4f and Proto 18-4r were used, the 450-bp fragment of the internal amplification control, specific for *Prototheca* genus, was detected in all investigated strains (Figure 1). On the contrary, the presence of the *P. zopfii* genotype-1–specific amplicon (150 bp; Figure 1) was detected in only one strain isolated from drinking water (Table 2).

The *P. zopfii* genotype-2 pattern (165 bp; Figure 1) was observed in 97.2% of milk samples and in 84.9% of environmental samples analyzed (Table 2). When prim-

Table 2. Prevalence of cows infected by *Prototheca* spp. in all sampled herds, number of environmental samples positive for *Prototheca* spp., and results of genotype-specific PCR analysis on *Prototheca* spp. isolates

Herd code	Lactating cows (n)	Positive milk samples (n)	Prevalence (%)	Typed strains (milk) (n)	Typing results by genotype-specific PCR	Total Environmental samples (n)	Positive environmental samples (n)	Positive samples (%)	Positive typed strains (environment) (n)	Typing results by genotype-specific PCR
1	69	21	30.4	5	*P. zopfii* genotype 2	29	7	24.1	5	*P. zopfii* genotype 2
2	232	29	12.5	22	*P. zopfii* genotype 2 (n = 19) *P. blaschkeae* (n = 3)	38	2	5.3	1	*P. zopfii* genotype 2
3	362	11	3.0	7	*P. zopfii* genotype 2	20	10	50.0	2	*P. zopfii* genotype 2
4	325	13	4.0	8	*P. zopfii* genotype 2	8	2	25.0	1	*P. zopfii* genotype 2
5	118	22	18.6	2	*P. zopfii* genotype 2	20	5	25.0	4	*P. zopfii* genotype 2
6	340	69	20.2	3	*P. zopfii* genotype 2	37	6	16.2	4	*P. zopfii* genotype 2
7	140	2	1.4	2	*P. zopfii* genotype 2	16	3	18.8	2	*P. zopfii* genotype 2
8	167	9	5.3	2	*P. zopfii* genotype 2	10	0	0	0	0
9	33	2	6.1	2	*P. zopfii* genotype 2	5	1	20.0	1	*P. zopfii* genotype 2
10	42	2	4.8	2	*P. zopfii* genotype 2	6	1	16.7	1	*P. zopfii* genotype 2
11	86	5	5.8	5	*P. zopfii* genotype 2	5	1	20.0	1	*P. zopfii* genotype 2
12	54	3	5.5	3	*P. zopfii* genotype 2	6	0	0	0	0
13	65	5	7.7	5	*P. zopfii* genotype 2	12	0	0	0	0
14	95	12	12.6	12	*P. zopfii* genotype 2	4	1	25.0	1	*P. zopfii* genotype 2
15	490	14	2.9	9	*P. zopfii* genotype 2	85	12	14.1	12	*P. zopfii* genotype 1 (n = 1) *P. zopfii* genotype 2 (n = 9) *P. blaschkeae* (n = 2)
16	275	12	4.4	11	*P. zopfii* genotype 2	51	10	19.6	10	*P. zopfii* genotype 2 (n = 7) *P. blaschkeae* (n = 3)
17	41	3	7.3	3	*P. zopfii* genotype 2	15	2	13.3	2	*P. zopfii* genotype 2
18	55	5	9.1	5	*P. zopfii* genotype 2	14	6	42.9	6	*P. zopfii* genotype 2 (n = 4) *P. blaschkeae* (n = 2)
19	45	0	0	0	0	4	0	0	0	0
20	33	0	0	0	0	2	0	0	0	0
21	38	0	0	0	0	6	0	0	0	0
22	24	0	0	0	0	2	0	0	0	0
23	51	0	0	0	0	12	0	0	0	0
24	28	0	0	0	0	4	0	0	0	0

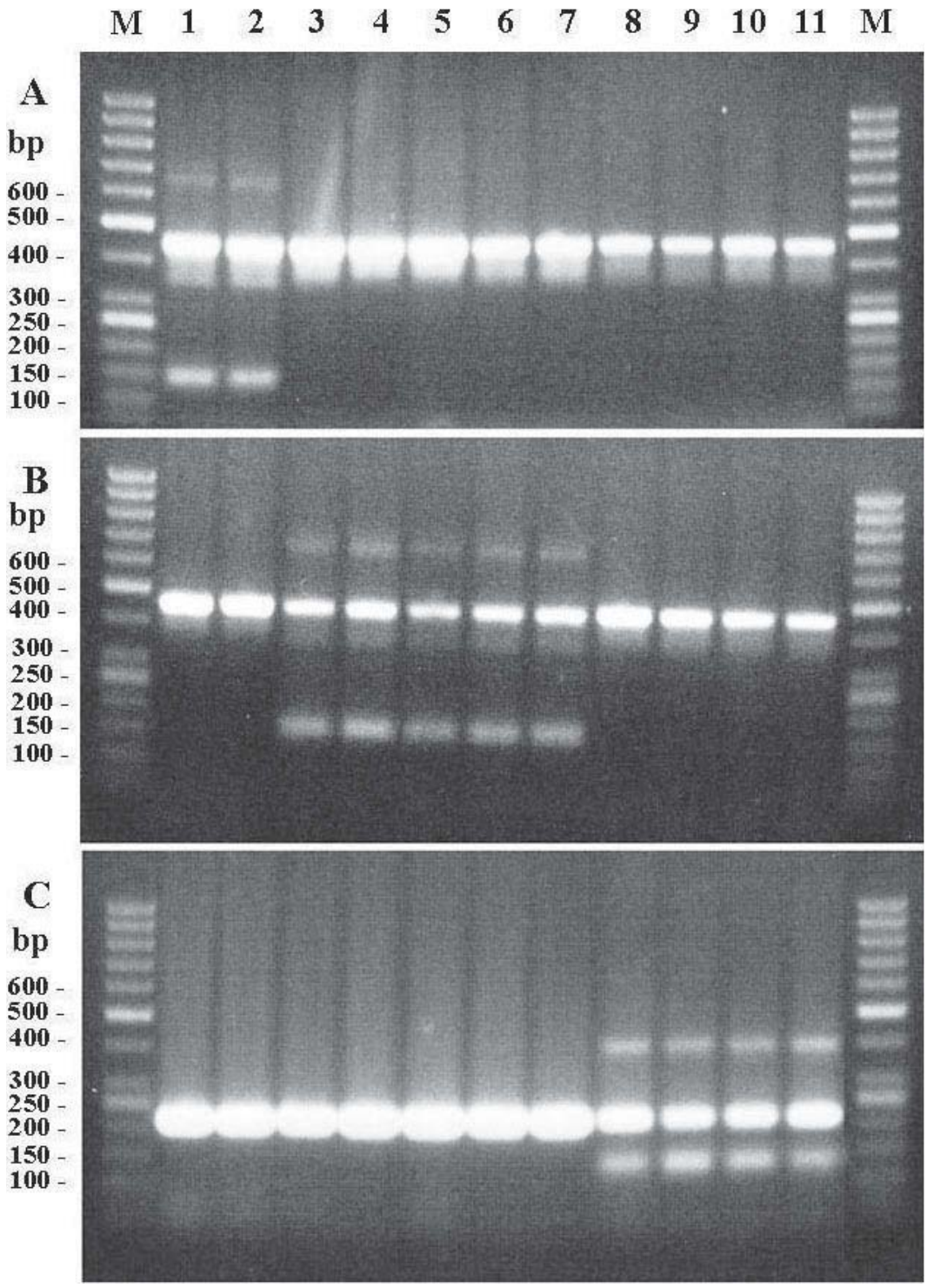

Figure 1. Results of genotype-specific PCR analysis for *Prototheca* spp. isolates. A) Analysis specific for *Prototheca zopfii* genotype 1; B) analysis specific for *P. zopfii* genotype 2; C) analysis specific for *Prototheca blaschkeae*. M = molecular size marker; lane 1 = *P. zopfii* genotype 1 SAG 2063 (control strain); lane 2 = *P. zopfii* genotype 1 strain isolated in the present study; lane 3 = *P. zopfii* genotype 2 219509/2 (control strain); lanes 4–7 = *P. zopfii* genotype 2 strains isolated in the present study; lane 8 = *P. blaschkeae* SAG 2064 (control strain); lanes 9–11 = *P. blaschkeae* strains isolated in the present study.

ers PZGT 3-IK/f and PZGT 3-IK/R were used, the 213-bp fragment of the internal amplification control was detected in all investigated strains (Figure 1). The presence of *P. blaschkeae*-specific pattern (126 bp) was found in 3 of 108 (2.8%) milk samples and in 7 of 53 (13.2%) environmental samples (in particular, in bedding, feces, and drinking water; Table 2).

No significant ($P < 0.01$) correlation was observed ($\rho = 0.06$) between the percentage of milk and environmental samples positive for *Prototheca* spp.

The present study represents the first large-scale screening survey aimed at characterizing the diffusion of both *P. zopfii* genotypes and *P. blaschkeae* from bovine mastitis milk and cow barn surroundings of Italian dairy herds.

The data reported herein confirmed the role of *P. zopfii* genotype 2 as a major mastitis agent, whereas *P. blaschkeae* was sporadically isolated. *Prototheca zopfii* genotype 2 strains were isolated from individual milk sampled from cows exhibiting clinical (milk alterations with watery appearance, chronic subacute mastitis with fibrosis), subclinical (absence of milk alterations but increase of SCC) and, rarely, latent mastitis (neither milk alterations nor increase of SCC). On the other hand, the 3 strains of *P. blaschkeae* were isolated from milk produced by one cow with clinical mastitis and from 2 cows with subclinical mastitis (exhibiting only an increase of SCC).

Our data contradict previous hypothesis about the supposed exclusive role of *P. zopfii* genotype 2 as the causative agent of protothecal mastitis (Möller et al., 2007; Osumi et al., 2008) and, in contrast, confirm the study of Marques et al. (2008), supporting the hypothesis that such pathology could be caused by *P. blaschkeae*, although less frequently. Moreover, we confirmed the status of *P. zopfii* genotype 1 as an environment-related organism, with no involvement in mastitis etiology. However, compared with other reported data (Osumi et al., 2008), its occurrence on Italian dairy herds was surprisingly low.

The isolation of *P. zopfii* genotype 2 and *P. blaschkeae* strains (about 85 and 13% of strains, respectively) from the environment surrounding the cow barn suggests that the potential exposure of cow mammary glands to these yeast-like microalgae through contaminated bedding, feces, and water is considerable. No protothecal contamination of environmental samples was observed in the 6 uninfected herds. However, in view of the absence of any significant ($P < 0.01$) correlation between the prevalence of *Prototheca* spp. in milk and in environmental samples, the existence of a relationship between udder infection and environmental contamination by *Prototheca* spp. remains questionable.

In the framework of managing protothecal mastitis outbreaks, the importance of a molecular characterization of *Prototheca* spp. strains isolated from both individual milk and environmental samples must be stressed. This information is critical to clarify the route of infection for bovine udder in order to set up an effective prophylactic program. Moreover, worldwide, large-scale investigations on the occurrence of different *Prototheca* species and genotypes may represent the first step to explore the distribution of these etiological agents in different regions and countries.

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REFERENCES

Buzzini, P., S. Menichetti, C. Pagliuca, C. Viglianisi, E. Branda, and B. Turchetti. 2008a. Antimycotic activity of 4-thioisosteres of flavonoids towards yeast and yeast-like microorganisms. Bioorg. Med. Chem. Lett. 18:3731–3733.

Buzzini, P., B. Turchetti, E. Branda, M. Goretti, M. Amici, P. E. Lagneau, L. Scaccabarozzi, V. Bronzo, and P. Moroni. 2008b. Large-scale screening of the *in vitro* susceptibility of *Prototheca zopfii* towards polyene antibiotics. Med. Mycol. 46:511–514.

Buzzini, P., B. Turchetti, R. Facelli, R. Baudino, F. Cavarero, L. Mattalia, P. Mosso, and A. Martini. 2004. First large-scale isolation of *Prototheca zopfii* from milk produced by dairy herds in Italy. Mycopathologia 158:427–430.

Casal, M., and J. Gutierrez. 1983. In vitro activity of ribostamycin against *Prototheca* spp. Mycopathologia 83:21–23.

Castagna de Vargas, A., A. Lazzari, J. M. Santurio, S. H. Alves, G. Ferreira, and L. C. Kreutz. 1998. Isolation of *Prototheca zopfii* from a case of bovine mastitis in Brazil. Mycopathologia 142:135–137.

Cunha, L. T., S. M. Pugine, M. R. Silva, E. J. Costa, and M. P. De Melo. 2010. Microbicidal action of indole-3-acetic acid combined with horseradish peroxidase on *Prototheca zopfii* from bovine mastitis. Mycopathologia 169:99–105.

Furuoka, H., A. Anri, Y. Arita, N. Tuzuki, H. Satoh, and C. Itakura. 1989. Protothecal mastitis in a cow. Jpn. J. Vet. Sci. 51:197–199.

Janosi, S., F. Ratz, G. Szigeti, M. Kulcsar, J. Kerenyi, T. Lauko, F. Katona, and G. Huszenicza. 2001. Review of the microbiological, pathological and clinical aspects of bovine mastitis caused by the alga *Prototheca zopfii*. Vet. Q. 23:58–61.

Kawai, K., K. Shimazaki, H. Higuchi, and H. Nagahata. 2007. Antibacterial activity of bovine lactoferrin hydrolysate against mastitis pathogens and its effect on superoxide production of bovine neutrophils. Zoonoses Public Health 54:160–164.

Lass-Flörl, C., and A. Mayr. 2007. Human protothecosis. Clin. Microbiol. Rev. 20:230–242.

Lagneau, P. E. 1996. First isolation of *Prototheca zopfii* in bovine mastitis in Belgium. J. Mycol. Med. 6:145–148.

Marques, S., E. Silva, J. Carvalheira, and G. Thompson. 2006. In vitro antimicrobial susceptibility of *Prototheca wickerhamii* and *Prototheca zopfii* isolated from bovine mastitis. J. Dairy Sci. 89:4202–4204.

Marques, S., E. Silva, C. Kraft, J. Carvalheira, A. Videira, A. R. Volker, and G. Thompson. 2008. Bovine mastitis associated with *Prototheca blaschkeae*. J. Clin. Microbiol. 46:1941–1945.

McDonald, J. S., J. L. Richard, and J. Cheville. 1984. Natural and experimental bovine intramammary infections with *Prototheca zopfii.* Am. J. Vet. Res. 45:592–595.

Möller, A., U. Truyen, and U. Roesler. 2007. *Prototheca zopfii* genotype 2. the causative agent of protothecal mastitis? Vet. Microbiol. 120:370–374.

Myers, J. L., and A. D. Well. 2003. Research Design and Statistical Analysis. 2nd ed. Lawrence Erlbaum Associates, Mahwah, NJ.

National Mastitis Council. 1999. Laboratory Handbook on Bovine Mastitis. Revised. National Mastitis Council Inc., Madison, WI.

Osumi, T., Y. Kishimoto, R. Kano, H. Maruyama, M. Onozaki, K. Makimura, T. Ito, K. Matsubara, and A. Hasegawa. 2008. *Prototheca zopfii* genotypes isolated from cow barns and bovine mastitis in Japan. Vet. Microbiol. 131:419–423.

Pore, R. S. 1973. Selective medium for the isolation of *Prototheca.* Appl. Microbiol. 26:648–649.

Pore, R. S. 1998. *Prototheca* Krüger. Pages 883–887 in The Yeasts: A Taxonomic Study. C. P. Kurtzman and J. W. Fell, ed. Elsevier, New York, NY.

Ribeiro, M. G., M. Rodrigues de Farias, U. Roesler, K. Roth, S. M. Rodigheri, M. A. Ostrowsky, T. Salerno, A. K. Siqueira, and M. C. Fernandes. 2009. Phenotypic and genotypic characterization of *Prototheca zopfii* in a dog with enteric signs. Res. Vet. Sci. 87:479–481.

Roesler, U., A. Möller, A. Hensel, D. Baumann, and U. Truyen. 2006. Diversity with the current algal species *Prototheca zopfii*: A proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. Int. J. Syst. Evol. Microbiol. 56:1419–1425.

Roesler, U., H. Scholz, and A. Hensel. 2003. Emended phenotypic characterization of *Prototheca zopfii*: A proposal for three biotypes and standards for their identification. Int. J. Syst. Evol. Microbiol. 53:1195–1199.

Salerno, T., M. G. Ribeiro, H. Langoni, A. K. Siqueira, E. O. Costa, P. A. Melville, V. F. Bueno, A. A. Yamamura, U. Roesler, and A. V. Silva. 2010. In vitro algaecide effect of sodium hypochlorite and iodine based antiseptics on *Prototheca zopfii* strains isolated from bovine milk. Res. Vet. Sci. 88:211–213.

Satoh, K., K. Ooe, H. Nagayama, and K. Makimura. 2010. *Prototheca cutis* sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin. Int. J. Syst. Evol. Microbiol. 60:1236–1240.

Scaccabarozzi, L., B. Turchetti, P. Buzzini, G. Pisoni, L. Bertocchi, N. Arrigoni, P. Boettcher, V. Bronzo, and P. Moroni. 2008. Isolation of *Prototheca* spp. strains from environmental sources of dairy herds. J. Dairy Sci. 91:3474–3477.

Segal, E., A. A. Padhye, and L. Ajello. 1976. Susceptibility of *Prototheca* species to antifungal agents. Antimicrob. Agents Chemother. 10:75–79.

Shahan, T. A., and R. S. Pore. 1991. In vitro susceptibility of *Prototheca* spp. to gentamicin. Antimicrob. Agents Chemother. 35:2434–2435.

Tortorano, A. M., A. Prigitano, G. Dho, R. Piccinini, V. Daprà, and M. A. Viviani. 2008. In vitro activity of conventional antifungal drugs and natural essences against the yeast-like alga *Prototheca.* J. Antimicrob. Chemother. 61:1312–1314.

Turchetti, B., P. Buzzini, M. Goretti, E. Branda, G. Diolaiuti, C. D'Agata, C. Smiraglia, and A. Vaughan-Martini. 2008. Psychrophilic yeasts in glacial environments of Alpine glaciers. FEMS Microbiol. Ecol. 63:73–83.

Ueno, R., N. Urano, and M. Suzuki. 2003. Phylogeny of the nonphotosynthetic green micro-algal genus *Prototheca* (Trebouxiophyceae, Chlorophyta) and related taxa inferred from SSU and LSU ribosomal DNA partial sequence data. FEMS Microbiol. Lett. 223:275–280.