Occurrence of *Prototheca* spp. in cow milk samples

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SUMMARY

Protothecosis is a potential zoonotic disease associated with bovine mastitis which can be transmitted to humans through contaminated milk. Considering the increasing prevalence of bovine mastitis due to *Prototheca* species, individual cow milk samples were analyzed using microbiological examination and biomolecular assay. Aspects related to health requirements for milk production, clinical and histological bovine mastitis were also described. The results showed 24/257 (9.3%) culture-positive samples and 42/257 (16.3%) PCR-positive samples. Moreover in 5 cows with somatic cell count over 10^6/mL presented histological features of mastitis. This study reveals that the presence of *Prototheca* species in dairy herds was related to the hygienic conditions of the milking equipment, showing an emerging public health issue.

KEY WORDS: Prototheca, Bovine mastitis, Milk, Italy.

Received December 12, 2014 *Accepted* July 3, 2014

INTRODUCTION

The algae of the genus *Prototheca* (*P.*) is closely related to the green algae *Chlorella*, although it lacks chlorophyll (Jagielski *et al*., 2007). Some of these heterotrophic algae are of phylogenetic, medical, and biomedical interest (Huss *et al.*, 1990). The *Prototheca* genus includes five species: *P. blaschkeae*, *P. stagnora*, *P. ulmea*, *P. wickerhamii*, and *P. zopfii* (Marques *et al*., 2008). *P. zopfii* has been differentiated into three biotypes and *P. zopfii* genotype II is the predominant pathogen generally associated with bovine mastitis (Moller *et al*., 2007; Osumi *et al*., 2008). Recent phylogenetic investigations, based on 18S rDNA, revealed discriminating molecular characteristics among the three different *P. zopfii* biotypes. Therefore, the previous biotype 3 of *P. zopfii* was re-classified as a new species (*P. blaschkeae* sp. nov.), while *P. zopfii* biotypes 1 and 2 were re-classified as *P. zopfii* genotypes I and II (Roesler *et al*., 2006).

In humans, *P. wickerhamii* is usually associated with cutaneous or subcutaneous lesions as well as generalized infections (Zaitz *et al*., 2006; Lass-Florl and Mayr 2007; Narita *et al*., 2007). In 1952, *P. zopfii* was identified for the first time as bovine mastitis pathogen associated with reduced milk production and characterized by a thin watery secretion with white flakes (Osumi *et al*., 2008). *P. blaschkeae* was first isolated and described from human onychomycosis (Roesler *et al*., 2006). *P. zopfii* and *P. wickerhamii* are also etiological agents of human protothecosis (Buzzini *et al*., 2004).

Many reports (Segal *et al*., 1976; Camargo *et al*., 1978; McDonald *et al*., 1984; Cheville *et al*., 1984; Hodges *et al*., 1985; Kirk 1991; Costa *et al*., 1996; Anderson and Walker 1998) showed the endemic incidence of bovine mastitis due to *P.* spp., in many regions of the world. As observed in several studies worldwide, there are many reports describing *P.* spp. in association

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with bovine mastitis in Italy (Ricchi *et al*., 2010 a, b; Ricchi *et al*., 2013; Cremonesi *et al*., 2012), although these data were mostly collected in Northern Italy, where the climate and hydrogeological conditions are markedly different from those observed in the southern regions.
The aim of the study was to investigate the presence of *P.* spp. in individual cow milk samples from Apulia region dairy farms (Italy) using microbiological examination and bimolecular assay. In particular PCR assay was targeted to the 18S rDNA gene. Aspects related to health requirements for milk production and histological bovine mastitis were also described.

MATERIALS AND METHODS

Sampling
Two hundred and fifty-seven individual milk samples were collected between January and July 2012 from 5 dairy herds in the Apulia Region (Italy), marked with the letters A (70 samples collected), B (52 samples), C (35 samples), D (40 samples), and E (60 samples). Before milk sample collection, the teats were washed, dried and disinfected (chlorhexidine 5%). Milk samples from all 257 animals were aseptically collected immediately before milking and stored at 0-4°C.

Health requirements for milk production and SCC (Somatic Cell Count) value
During individual milk sampling a detailed report on the hygienic conditions of the herds and the milking equipment was compiled. This analysis was followed by the examination of individual SCC values.

Histological analysis
As mastitis caused by *P. zopfii* is most often recognized as a chronic symptom-free process with a very high somatic cell count (SCC) (over 10^6/mL) (Jánosi *et al.* 2001), a preliminary histological examination was performed exclusively on cows diagnosed with bovine mastitis with SCC values >10^6/mL. Biopsies were collected by fine needle aspiration and fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4 µm and stained by haematoxylin eosin (HE).

Microbiological analysis
The individual milk samples were cultured on blood agar and incubated at 37 °C for 48 h. The resulting colonies were picked and stained using the methylene blue (MB) method.

Biomolecular analysis
Reference materials. Laboratory collection *P. blaschkeae*, *P. stagnora*, *P. ulmea*, *P. wickerhamii*, and *P. zopfii* genotype I-II strains, supplied by IZS LER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna), were used as positive and negative controls.
DNA extraction and purification. Aliquots of 257 individual milk samples from five different Italian dairy herds were subjected to DNA extraction and purification using a DNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions.
Oligonucleotide Primers. The oligonucleotide primers described by Roesler *et al*. (2006) and synthesized by PRIMM Srl (Milan, Italy) were used in this study (Table 1).
PCR assay. The PCR reactions were performed in a final volume of 25 µl, using 12.5 µl of HotStarTaq Master Mix 2X (QIAGEN, Hilden, Germany) containing 2.5 units of HotStarTaq DNA Polymerase, 1.5 mM of $MgCl_2$ and 200 µl of each dNTP.
Then, 1 µM of each oligonucleotide primer and 1 µl of DNA were added. The amplification profile involved an initial denaturation step at 95°C for 15 min. This was followed by 30 cycles at 94°C for 30 s, 58°C for *P. zopfii* and 63°C for *P. blaschkeae* for 30 s, 72°C for 30 s. The positive and negative controls for the extraction and PCR were included. The PCR reactions were processed in a MasterCycler Personal (Eppendorf, Milan, Italy). All reactions were performed in duplicate.
Detection of amplified products. PCR amplified products were analyzed by electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in a 1X TBE buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with ethidium bromide. A Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania) was used as the molecular weight marker. Image acquisition was performed using UVITEC (Eppendorf).

TABLE 1 - *Oligonucleotide primers.*

Primer	*Target*	*Sequence (5'-3')*
Proto18-4f	*P. zopfii* genotype I and II	GACATGGCGAGGATTGACAGA
PZGT 1/r	*P. zopfii* genotype I	GCCAAGGCCCCCCGAAG
PZGT 2/r	*P. zopfii* genotype II	GTCGGCGGGGCAAAAGC
PZGT 3-IK/f	*P. blaschkeae*	CAGGGTTCGATTCCGGAGAG
PZGT 3/r	*P. blaschkeae*	GTTGGCCCGGCATCGCT

RESULTS

Health requirements for milk production

Hygiene inspection of the milking equipment identified non-compliances in herds A, B, C and E:

1) utensils, containers, tanks for milk collection and transport were not clean and so, for the purposes of this study, these were disinfected;
2) the teats and adjacent parts of the cow were not cleaned before milking started;
3) faecal contamination of the feed was evident. By contrast, dairy herd D complied with all of the requirements imposed by current legislation (Commission Regulation (EC) No 853/2004).

In particular, in dairy herd D

1) milking was carried out hygienically and, immediately after milking, the milk was stored in a clean place designed and equipped to avoid contamination;
2) milking equipment and premises where milk was stored, handled or cooled were located and constructed so as to limit the risk of milk contamination;
3) milk storage premises were protected against vermin, and properly separated from the premises where animals were housed;
4) surfaces of equipment that came into contact with the milk were easy to clean and disinfect;
5) hot and/or cold potable water and animal feed was adequate;
6) high degree of personal cleanliness was observed.

The SSC values from individual milk samples from the 5 dairy herds examined were:

1) in dairy herd A, mean SCC value was 342,000/mL while five of these animals showed a value >1,000,000/mL;
2) in dairy herd B, mean SCC value was 312,000/mL;
3) in dairy herd C, mean SCC value was 273,000/mL;
4) in dairy herd D, mean SCC value was 128,000/mL;
5) in dairy herd E, mean SCC value was 273,000/mL.

Histological analysis

Histological examination performed on five samples showed mammary gland tissues with chronic interstitial mastitis associated with dilated mammary acini and severe damage to the epithelium. In the lumen of the damaged mammary alveoli, *P.* organisms were present in dark clusters (Figure 1).

Microbiological analysis

Microbiological analysis on blood agar and morphological examination by the MB meth-

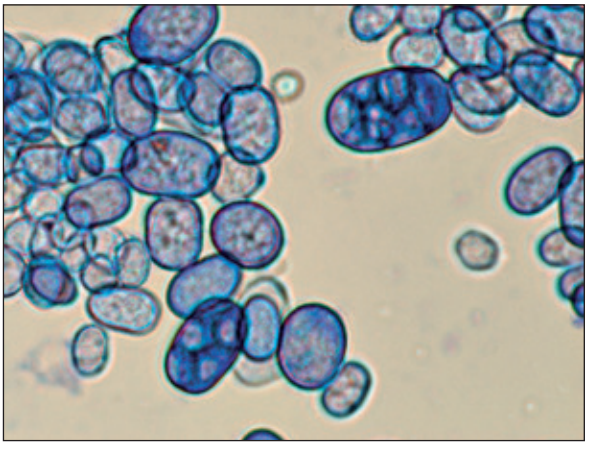

FIGURE 1 - *P. spp. isolated from milk samples. Characteristic microscopic morula appearance of P. spp. using light microscopy (MB 40x).*

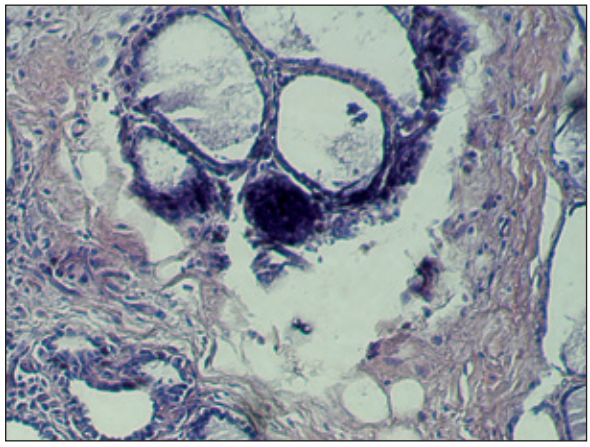

FIGURE 2 - *Mammary gland: damaged mammary alveoli with P. organisms in dark clusters (HE 20x).*

od, performed on each individual milk sample, were positive for *P.* spp. in 24/257 (9.3%). Microscopic examination showed globose-ovoid cells, ranging from 1.5 to 25 µm and the endospores formed their own walls while still enclosed within the mother cell (Figure 2). The production of endospores inside the mother cell is characteristic of the genus of *P.* The positivity rates were thus differentiated by sample origin: 15, 5 and 4 positive individual milk samples came from A, B and C respectively. There were no positive results in samples from herds D and E (Figure 2).

Biomolecular analysis

PCRs performed on each individual milk samples gave positive results for *P.* spp. in 42/257 (16.3 %) samples (Table 2). In particular, *P. zopfii* was highlighted in 42 individual milk samples, with 13 assigned to *P. zopfii* genotype I and 26 to genotype II. *P. blaschkeae* was observed in only 3 samples, all from the same herd. Molecular analysis revealed that the milk from only 1 herd did not contain *P.* spp. Also, three individual milk samples were positive both *P. zopfii* and *P. blaschkeae* (Table 2). All PCR assays targeting the 18S rDNA gene produced specific amplicons with expected sizes. The specificity of PCR products was confirmed by sequence analysis. The results refer to 3 experiments and did not highlight any significant variability.

Furthermore, the 5 individual milk samples collected from the cows on which histological examinations were performed were positive for *P. zopfii* genotype II.

TABLE 2 - *Biomolecular results.*

Herd	*Positive samples*	*P. zopfii*	*P. blaschkeae*
A	21/70	*P. zopfii* genotype I (7) / II (14)	-
B	11/52	*P. zopfii* genotype I (3) / II (5)	3
C	8/35	*P. zopfii* genotype I (3) / II (5)	-
D	0	-	-
E	2/60	*P. zopfii* genotype I	-

DISCUSSION

This study represents the first investigation into the occurrence of the microalga *P.* spp. in milk samples from family dairy herds in the Apulia region (Italy).

The occurrence of *P.* spp. was evident in 42/257 of the samples and in 4/5 of the herds evaluated. In only 1 herd were milk samples negative for *P.* spp., while mean SSC value was 128,000/mL. These data, in agreement with previous studies, (Baumgartner *et al.*, 1997; Malinowski *et al.*, 2002) showed that the presence of *P.* spp. in dairy herds seems to correlate with the hygienic conditions of the milking equipment and proper management of animal feeding and potable water.

Highly contaminated spoiled feed may also be the source of large numbers of *P.* spp. in the farm environment, and may be responsible for mastitis (Baumgartner *et al.*, 1997). Therefore, monitoring contaminations in animal feeds is necessary to guarantee animal heath. Additionally, in order to effectively prevent contamination, all the controls in food chain must be combined with proper implementation of the HACCP (Hazard Analysis Critical Control Point) system on the farms, also assessing the occurrence of emerging foodborne pathogens as *P.* spp.

To find an effective approach to control the spread of these microalgae its analogies with several aspects for environmental bacteria contamination may be used: a number of Critical

Control Points (CCPs) can thus be identified:
1) maintain excellent hygiene both of the litter and during milking procedures through proper use of water on dirty teats and udders;
2) prohibit the feeding of milk unsuitable for human consumption to heifers that may in turn become intermittent shedders of the microalga following bowel contamination;
3) separate infected animals from healthy ones both during lactation and just before delivery;
4) given the lack of treatment options dispose of infected animals safety.

Biomolecular analysis showed that *P. zopfii,* genotype II, was present more often than *P. zopfii,* genotype I, and *P. blaschkeae*. Genotype II occurred in 26/42 of the samples; genotype I occurred in 13/42 samples; while *P. blaschkeae* occurred in only 3 of 42 samples.

The study showed that coupling the conventional culture methods based on plating and isolating *P.* spp. and DNA-based methods may easy differentiate of *P.* spp., directly from the milk sample, with greater sensitivity and specificity.

The histological examinations by fine needle aspiration, in agreement with the study by Hanaa *et al*. (2010), revealed chronic interstitial mastitis associated to dilated mammary acini, damage to the epithelium, and the presence of *P.* organisms in dark clusters.

Protothecosis is a potential zoonotic disease which can be transmitted to humans through contaminated milk. It causes intestinal infections and enteritis because of its resistance to pasteurization (Melville *et al*., 1999). Indeed, *P.* spp. not only affects milk quality due to its destruction of mammary gland tissues, but also poses a potential threat to human health still not entirely known.

CONCLUSION

In conclusion, by coupling cultural and molecular assays, *P.* spp. was detected in as many as 16.3% of the animals tested. Not surprisingly, the PCR assays were more sensitive than the microbiological methods. Also, 100% of the animals with high SCC (over 10^6/mL) tested positive for *P.* spp. and *P.* organisms were clearly visible upon histological examinations in the lumen of the mammary glands. The findings of these investigations seem to confirm that *P.* spp. can impact on milk production. More extensive structured studies are necessary to evaluate the magnitude of this problem in more depth in dairy herds in the local and global livestock industry.

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