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Short communication: Epidemiology and genotyping of *Candida rugosa* strains responsible for persistent intramammary infections in dairy cows

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ABSTRACT

The present study was undertaken during an outbreak of clinical and subclinical mastitis in 14 dairy cows caused by *Candida rugosa*, in which high somatic cell counts were seen and cases did not respond to antibiotic treatment. Intramammary infection cured spontaneously in 10 cows, whereas 4 cows were culled as a result of persistent infections. Repeated sampling of these cows and biomolecular analysis of the isolates showed that the infections were caused by the same genotype, even over a period of 2 lactations. Random amplification of the genome of *C. rugosa* milk isolates gave 3 different DNA banding patterns (genotypes G1, G2, and G3). Viable cells of *C. rugosa* were also isolated from various environmental sources and were present in high concentrations in total mixed ration samples, which could be considered the primary source of diffusion of viable yeast cells in the environment, as demonstrated by genotyping. The proven capacity of these microorganisms to survive in the environment of the cow, such as the total mixed ration, bedding, water, and cow skin, and to cause persistent intramammary infections highlights the importance of mycotic spread in dairy herds.
Key words: *Candida rugosa*, environmental source, persistent intramammary infection, dairy cow

Short Communication

Bovine mastitis has been defined as an inflammation of the mammary gland usually caused by microbial infection. A variety of microorganisms has been implicated as causative agents of bovine mastitis, which is the most important disease affecting dairy cows (Watts, 1988). Most cases are caused by bacteria, but yeasts and fungi also contribute to the importance of the problem (Seker, 2010). Yeasts are single-celled organisms that are ubiquitous in the environment and they are considered opportunistic pathogens of the mammary gland that produce disease when natural defense mechanisms are lowered (de Casia dos Santos and Marin, 2005). Sources of infection include the skin of the udder, udder secretion, milkers' hands, milking machines, treatment instruments, floor, straw, feed, dust, soil, drug mixtures, and sanitizing solutions (Richard et al., 1980; Elad et al., 1995). Yeast-like fungi have been identified in milk samples since as early as 1901 (Pengov, 2002), and potentially pathogenic species of yeasts have been isolated from 7% of routine milk samples in central and northern Europe (Aalbaek et al., 1994). These cases of mastitis regressed spontaneously within 2 to 4 wk (Farnsworth and Sorensen, 1972; Richard et al., 1980). The organisms most frequently implicated are yeasts belonging to the genus *Candida*, several species of which have been recovered from infected glands (de Casia dos Santos and Marin, 2005). Among them, *Candida rugosa* has been described as being responsible for IMI after intramammary antibiotic treatments (Crawshaw et al., 2005).

An unusual increase in the number of severe cases of subclinical and clinical mastitis in a dairy herd in northern Italy occurred from March 2009 to December 2010, and samples were submitted to the Section of Infectious Diseases, Veterinary College of Milan, for bacteriological culture. The herd, a 200-cow Holstein-Friesian herd in freestall facilities bedded with sawdust and straw, had an average daily milk production of 34.08 kg, a 305-d mature-equivalent (i.e., age-, season-, and fat-corrected milk production) yield of 12,503 kg, and bulk milk SCC between 113,000 and 281,000 cells/mL. Quarter milk samples (n = 597) from 152 cows were collected routinely after calving for a *Staphylococcus aureus* control program, and 13 quarters with clinical mastitis were submitted to the laboratory for bacteriological culture.

Milk bacteriological procedures were performed according to the recommendations of the National Mastitis Council (NMC, 1999). These procedures revealed an IMI caused by *C. rugosa* in 17 of 134 (12.69%) culture-positive samples, corresponding to 14 dairy cows. These culture-positive samples had pure cultures (mean 3.5 ×

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10^3 cfu/mL) and high SCC (mean $2{,}033 \times 10^3$ cells/mL). Culture-positive samples revealed *Streptococcus* spp. in 56 samples (41.79%), CNS in 32 samples (23.88%), *Staph. aureus* in 17 samples (12.69%), gram-negative species in 6 samples (4.48%), and other microorganisms in 6 samples (4.48%).

Yeast colonies were identified phenotypically (Crawshaw et al., 2005) and by biochemical profiles using 2 colorimetric sugar utilization tests, the API ID32C and API 20CAUX Diagnostic kits (BioMerieux, Marcy l'Etoile, France).

One hundred twenty-one samples were collected from various environmental sources: well water, watering trough water, water used during milking, TMR, unused sawdust, unused straw, teat cup liners premilking, teat cup liners during milking, treatment syringes, milkers' hands during milking, premilking teat dip, postmilking teat dip, and bulk tank milk samples (Table 1). Samples were also collected from animals randomly selected from *C. rugosa*-infected cows and healthy cows: teat skin swabs collected before milking and fecal samples collected directly from the rectum (Table 1). *Candida rugosa* cells from swabs were directly isolated by streaking on potato dextrose agar (Microbiol Diagnostics, Milan, Italy) amended with 0.1% tetracycline. Viable counts were performed by serial dilution and plating on the same medium. *Candida rugosa* cells were isolated from water samples by membrane filtration (pore size of 0.22 μm) and growth on potato dextrose agar, whereas isolation from unused bedding materials, feed, and feces was done through a dilution procedure in buffered peptone water (Crawshaw et al., 2005) and subsequent streaking on potato dextrose agar.

No *C. rugosa* viable cells were recovered from well water, water used during milking, unused bedding materials, treatment syringes, milkers' hands, premilking teat cup liners, or teat dip samples (Table 1). *Candida rugosa* was isolated from teat skin swabs, feces, watering trough water, teat cup liner swabs during milking, and TMR samples (Table 1). Positive samples from animal feces and teat skin were found in both healthy and infected cows. The bulk tank milk sample also revealed the presence of *C. rugosa* cells (Table 1).

Biomolecular analysis was carried out to study the epidemiology of *C. rugosa* strains from the environmental and animal samples. Genomic DNA extraction was based on the *micro*LYSIS-PLUS protocol (*micro*LYSIS, Microzone Ltd., Haywards Heath, West Sussex, UK). For genotyping of *C. rugosa* isolates, primer M13 (5′-GAGGGTGGCGGTTCT-3′) was used in a random amplification of polymorphic DNA (RAPD)-PCR protocol (Fadda et al., 2010) to evaluate DNA banding patterns. Random amplification of the genome of *C. rugosa* milk isolates gave 3 different DNA banding patterns, designated genotype 1 (**G1**), genotype 2 (**G2**), and genotype 3 (**G3**; Figure 1). In particular, 7 cows were infected by G1 (9 positive quarters), 6 cows were infected by G3 (7 positive quarters), and only 1 cow was infected by G2 (1 positive quarter).

Milk samples from dairy cows infected with *C. rugosa* were collected after 2 wk. Dairy cows were considered persistently infected when the milk sample was positive (White et al., 2010). Intramammary infection self-cured spontaneously in 10 cows, demonstrated by culture-negative samples with low SCC, as often reported in the literature (Farnsworth and Sorensen, 1972; Richard et al., 1980), whereas 4 cows were slaughtered as a result of persistent infections (Table 2). The latter were repeatedly sampled, including during slaughter, with intracanalicular mammary swabs and parenchymal samples being collected. Persistence of IMI was demonstrated by culture-positive samples with high SCC during the follow-up period (Table 2), that consequently led to the culling of these cows. Samples of the quarters

Table 1. Prevalence and concentration of *Candida rugosa* in 121 environmental samples

Source	Frequency of samples		Mean concentration, cfu/mL
	No.	No. positive	
Well water samples	2	0	—
Watering trough water	7	2	250
Water used during milking	2	0	—
TMR	2	1	26,000
Unused sawdust	2	0	—
Unused straw	2	0	—
Teat cup liners premilking (swabs)	24	0	—
Teat cup liners during milking (swabs)	32	7	157
Treatment syringes (swabs)	16	0	—
Milkers' hands during milking (swabs)	4	0	—
Premilking teat dip (swabs)	2	0	—
Postmilking teat dip (swabs)	2	0	—
Bulk tank milk	2	1	60
Teat skin before milking (swabs)	11	5	920
Rectal feces	11	2	1,650

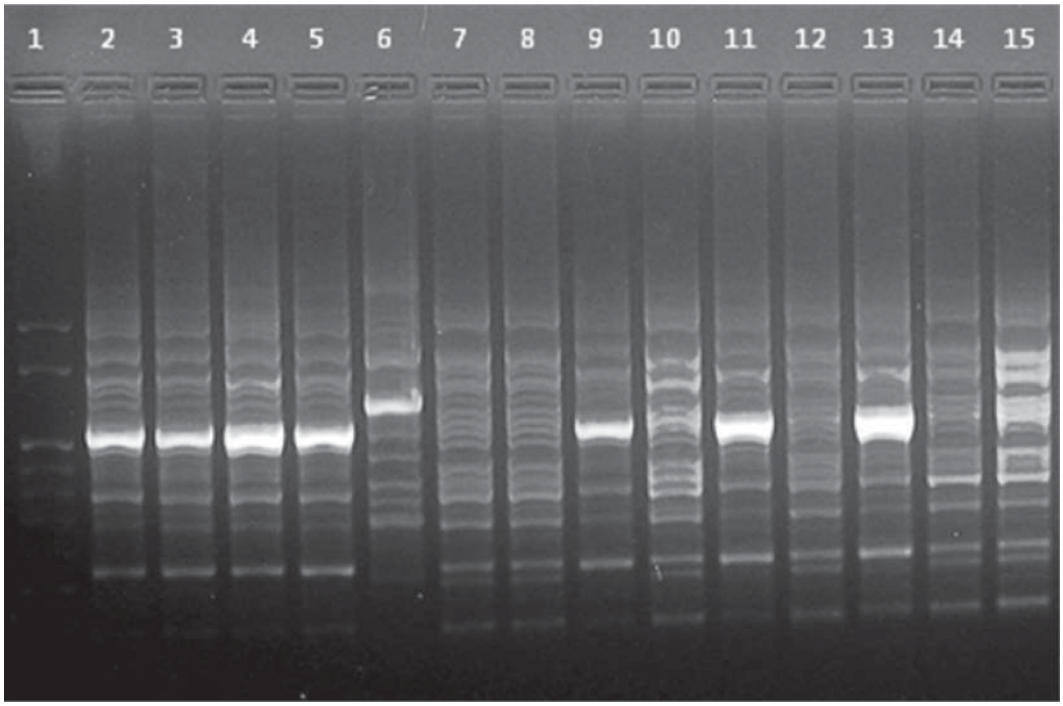

Figure 1. *Candida rugosa* DNA banding patterns from milk isolates, representing all 14 infected cows; each lane corresponds to a specific affected cow. Lane 1 = DNA ladder; lanes 2, 3, 4, 5, 9, 11, and 13 = G1 strain; lane 6 = G2 strain; lanes 7, 8, 10, 12, 14, and 15 = G3 strain.

collected at slaughterhouse were fixed in 10% (vol/vol) buffered formalin and processed routinely for histopathology. Four-micrometer sections were stained with hematoxylin-eosin and periodic acid–Schiff stains. The swabs revealed the presence of viable *C. rugosa* cells in the infected quarters. Nevertheless, histopathological examination of those quarters revealed only multifocal, mild to moderate, subacute mastitis with no evidence of yeast cells, even with periodic acid–Schiff stain. Bacteriological and genotypic analysis of milk samples from infected quarters at different intervals revealed that the infection was caused by the same strain (Table 2). The G1 and G3 strains seemed to be responsible for persistent infections. In particular, one cow (cow number 4) showed a persistence of infection during 2 lactations with survival of viable yeast cells in the mammary gland during the dry period and for about 9 mo afterward (Table 2).

Random amplification of the genome of *C. rugosa* isolated from TMR, feces, watering trough, and teat skin indicated identity with the milk G1 strain. The high level of *C. rugosa* measured in TMR (at least 10^2 cfu/mL higher than the other environmental samples) led us to assume that it could be the main source of

Table 2. Persistent intramammary infections with *Candida rugosa*

Cow	Milk sample date	SCC, $\times 10^3$cells/mL	cfu/mL	Genotype[1]
1	Sep. 22, 2010	3,748	1,000	1
	+14 d	1,283	2,300	1
2	Dec. 1, 2009	2,386	1,400	3
	+15 d	1,919	150	3
3	May 11, 2010	2,024	5,000	3
	+7 d	1,602	2,600	3
	+16 d	1,913	3,000	3
4	Sep. 22, 2009	69	1,000	3
	+231 d	263	100	3
	+238 d	933	400	3
	+245 d	2,614	2,500	3
	+258 d	4,601	7,900	3

[1]Random amplification of the genome of *C. rugosa* milk isolates gave 3 different DNA banding patterns, designated genotypes 1, 2, and 3.

viable yeast cells in the environment. Consequently, we hypothesized that contamination of the teat skin with *C. rugosa* occurred via cows' feces, where the yeast was then introduced into the teat canal. The spread could also have occurred during milking, following contamination of teat cup liners.

Genotyping evaluation confirmed the hypothesis that contamination of the environment with G1 strains probably started from the TMR. Because feed had the highest concentration of organisms, the spread of viable G1 cells would occur via contamination of watering trough water by cow feces and subsequent exposure to the teat canal; another important potential contamination of drinking water could be oral contamination by cows after eating. Additionally, teat skin, teat cup liners, and bulk tank milk isolates revealed both G1 and G3 strains, indicating the possibility of intramammary contamination during milking for both genotypes. Positive results for teat cup liners could result from teat skin or milk from infected quarters. This introduces the possibility that new IMI could occur not only via environmental contamination, but also during milking procedures.

The affected animals had been treated intramammarily for clinical mastitis in the previous months or lactations so a control on treatment syringes and protocol was done to exclude them as a potential source of infection.

These assumptions agree with the view of *Candida* spp. as opportunistic yeast pathogens. Yeast has evolved to become a successful commensal organism in healthy hosts and to express variant traits critical for existence on mucosal and cutaneous surfaces. In the weakened host, the same traits become virulence characteristics, accounting for invasive abilities as the delicate balance between *Candida* and the host shifts in favor of the yeast (Cutler, 1991). The different fate of infection in the affected cows and the possibility of persistence of *C. rugosa* inside the mammary gland could be due to host health status and its immune defense activity. *Candida rugosa* has been demonstrated to persist during the dry period and through to the subsequent lactation along with the mammary pathogens such as *Escherichia coli* and *Staph. aureus* (Osterås et al., 1999; Green et al., 2002). Strain G2 was not found among environmental isolates and was found in only one cow. We hypothesize that the diffusion of this strain was less than that of the other strains, probably because of a lower environmental persistence and a weak capacity to colonize the udder.

Yeast mastitis is an increasing problem in some countries (Krukowski et al., 2006; Seker, 2010), as seen by the manifestation and progression of mycotic IMI. Mycotic IMI affecting the mammary gland for long durations are difficult to control due to poor understanding of the pathobiology of the infections and lack of therapeutic protocols; subsequently, culling of the infected animals is sometimes the only solution. Thus, to reduce the development of this type of mastitis, it is essential to manage environmental contamination associated with hygiene during milking.

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