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Isolation of yeasts from bovine milk in Belgium

P.E. Lagneau[1], K. Lebtahi[2] & Danielle Swinne[2]
[1] *Fédération du Hainaut de Lutte contre les Maladies du Bétail (Dir. Dr. J. Durez) Drève du Prophète 2, 7000 Mons;* [2] *Lab. for Mycology (Prof. Ch. De Vroey) Institute of Tropical Medicine Nationalestraat 155, 2000 Antwerpen, Belgium*

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Abstract

A total of 436 milk samples from non-infected and 80 from infected quarters were investigated: 24.5% of the samples collected from non-infected and 55% of those collected from infected quarters were positive. Normal milk yielded not less than 16 different species and among them many potentially pathogenic yeast species such as *C. parapsilosis*, *C. guilliermondii*, *C. tropicalis*, *C. glabrata* and *T. asahii*, all five able to grow at 40 °C. In contrast, the yeasts isolated from infected quarters were from 3 species : *C. kefyr*, *C. catenulata* and *C. lambica*, which were also among the yeasts species recovered from normal milk. Among the three species, only one i.e. *Candida kefyr* is able to grow above 40 °C and from there can be considered as potentially pathogenic, even if bacterial association is necessary to cause mastitis.

Key words: cattle, milk, yeasts

Introduction

If fungal mastitis existed in cattle before the arrival of antibiotics, there has been since then an ever increasing number of cases reported, almost invariabily associated with prior antibiotic treatment of suspected or proven bacterial mastitis.

An overview of literature leads Chermette and Bussiéras [1] to conclude that, in our countries, more or less 6% of the mastitis are due to fungal organisms which are almost always yeasts.

In the United States, Kirk and Bartlett [2] mention a prevalence of fungal mastitis between 2 and 7%.In tropical countries, the percentage can be more elevated i.e. 12% in Brazil [3]. The most frequent organisms implicated are yeasts and belong to the genera Cryptococcus, Trichosporon and Candida with among them, *Cryptococcus neoformans*, which causes meningeal cryptococcosis in the mammals and can be responsible for acute mastitis in cattle and even now and again in goats [4].

Nevertheless, mastitis due to *Cr. neoformans* seems to be more frequently observed in tropical [5,6] than in temperate climates [7] and has never been seen in Belgium as far as we know [8,9,10]. However, in the present survey, special attention was given to the isolation of this yeast species.

The first report of bovine mastitis caused by *Prototheca* sp., a genus of achlorophyllous algae, was published by Lerch in 1952 [11]. Later on, his observations were confirmed by others [12,13]. Among the different potentially pathogenic species of Prototheca, *P. zopfii* seems especially to be linked with bovine intramammary infections [14,15]. Cases of mastitis due to *P. zopfii* were recently identified for the first time in Belgium [16,17] so that we decided to look for it during this survey.

Material and methods

516 milk samples[1] collected from quarters of 183 cows of 9 dairy herds were examined. Three hundred and fifty-six were from herds without mastitis cases and 160 were from herds with mastitis cases but only 80 out of the latter were from diseased animals. In total,

[1] The samples were supplied by the "Fédération du Hainaut de Lutte contre les Maladies du Bétail".

436 milk samples were from non-infected quarters and 80 from infected ones.

The milk samples were always aseptically collected, kept at a temperature of 0–4 °C and plated at the latest 24h after sampling.

Five ml milk were centrifuged at 4000 rpm and 3.5 ml of the supernatant eliminated: 0.5 ml of the sediment was inoculated on blood agar complemented with 0.2‰ chloramphenicol, incubated at 37 °C for the isolation of *Prototheca* sp., 0.5 ml on *Guizotia abyssinica* medium,[2] incubated at 25 °C for the isolation and the identification of *Cr. neoformans* which gives brown colonies on this medium and 0.5 ml on Sabouraud agar medium complemented with 0.2‰ of chloramphenicol incubated at 37 °C for the isolation of all the other potentially pathogenic yeasts. Incubation was 7 days.

The identification of the yeasts and of the *Prototheca* sp. occurred respectively according to Barnett et al. [19] and Camargo et al. [20]. Chi square test was used for the statistical analysis of the results.

Results

Nor Prototheca sp. neither *Cr. neoformans* were isolated. One hundred and fifty yeast isolates grew on Sabouraud agar complemented with chloramphenicol. Among them, 106 were recovered from the 436 normal milk samples (24.5%) and 44 from 80 milk samples from infected quarters (55%).

The frequency and degree of contamination by yeast species of milk samples from normal and infected quarters are mentioned in Table 1.

The frequency of yeast contamination of normal milk collected in dairy herds without mastitis cases was compared with that of normal milk samples collected in one dairy herd with mastitis cases. The percentages of contamination were respectively 20% (71/356) and 43.7% (35/80). The difference was statistically significant ($p < 0.001$).

Comparing the species isolated in a same dairy herd from 8 infected and from 80 non-infected quarters, we observed that 3 out of the 8 infected quarters were contaminated by yeasts and yealded only *C. kefyr* whereas the 15 out of the 80 non-infected quarters also contaminated by yeast, yealded *Cr. laurentii, Cr. curvatus, P. membranaefaciens* and *Cr. albidus*.

[2] *Guizotia abyssinica* medium (Staib's medium modified) 50 g *Guizotia abyssinica* (niger seed) ground, boiled in 1000 ml distilled water for 30 min and filtered. Filtrate q.s. 1000 ml with dist.water + 1 g glucose, 1 g creatinine, 1 g KH_2PO_4, 1 g chloramphenicol, 15 g agar, 1 g diphenyle 50 ml ethanol

Discussion

Cryptococcus neoformans was not isolated. It confirms that this yeast species doesn't seem to be an organism commonly found as an agent of mastitis in Belgium even if it is regularly isolated from pigeon droppings which is probably the most common source of mastitis due to *Cr. neoformans* [20].

Prototheca zopfii was also not isolated. We conclude that this organism remains an uncommon infectious agent even if it was recently isolated in Belgium from mastitis cases [16]. Moreover its non-isolation from normal milk leads to think that it isn't a usual contaminant of the gland and from there it would be interesting to look for its natural biotope in the environment, which remains unknown.

The quantification of the yeasts showed that there were no differences between the degree of contamination of milk from infected or non-infected glands, the difference between the two being only qualitative since only 3 different *Candida sp.* were recovered from infected glands whereas 16 different species of Candida, Cryptococcus, Pichia, Debaryomyces and Trichosporon could be isolated from normal milk.

Candida kefyr (syn./older name: *Candida pseudotropicalis*), *Candida catenulata* and *Candida lambica* were isolated from infected quarters. In a recent article on mycotic mastitis observed in Denmark, Aalbaek et al. [15] also isolated *C. kefyr* and *C. catenulata* from mastitis milk. *C. kefyr* is a well known pathogenic yeast as demonstrated experimentally by the same authors [19], whereas they denied any pathogenicity to *C. catenulata.* Even if, according to Bodey [22], this species has been isolated from skin lesions, from onychomycosis in human beings, it can't grow at 40 °C which is one of the conditions that is required to infect the mammary gland, according to Kirk and Bartlett [2]. In contrast, *C. kefyr* is able to grow till 42 °C.

The association of *C. kefyr* with mastitis cases was yet reinforced by its exclusive presence in milk sampled from diseased animals in a herd where only *Cr. laurentii, Cr. curvatus, P. membranaefaciens* and *Cr. albidus* were recovered from normal milk.

The third species *C. lambica*, also unable to grow at 40 °C, has never, as far as we know, been mentioned to be occurring in milk. Notice that those 3 species are

Table 1. Frequency and degree of contamination by yeast species of milk samples from normal and infected quarters.

Samples from	Species	No of isolates	Frequency (%)	N° of CFU/ml*		
				<11	12–50	>50
Normal quarters (n = 436)	*Candida kefyr*	20	4.5%	4	16	0
	Candida catenulata	17	3.8%	13	3	1
	Candida lambica	16	3.6%	9	3	4
	Candida inconspicua	2	0.4%	0	1	1
	Candida parapsilosis	1	0.2%	0	1	0
	Candida guilliermondii	1	0.2%	1	0	0
	Candida diversa	1	0.2%	0	0	1
	Candida tropicalis	1	0.2%	0	1	0
	Candida glabrata	1	0.2%	0	0	1
	Cryptococcus laurentii	26	5.9%	19	5	2
	Cryptococcus curvatus	10	2.2%	6	3	1
	Cryptococcus luteolus	1	0.2%	1	0	0
	Cryptococcus albidus	1	0.2%	1	0	0
	Pichia membranaefaciens	4	1.1%	3	0	1
	Debaryomyces hansenii	3	0.8%	0	1	2
	Trichosporon asahii	1	0.2%	1	0	0
Total		106				
Infected quarters (n = 80)	*Candia kefyr*	18	22.5%	7	9	2
	Candida catenulata	15	18.7%	10	5	0
	Candida lambica	11	13.7%	9	2	0
Total		44				

*CFU = colony forming unit.

also present in normal milk, among other well known potentially pathogenic yeasts such as *C. parapsilosis*, *C. guilliermondii*, *C. tropicalis*, *C. glabrata* and *T. asahii*, all five able to grow at 40 °C [23].

An interesting information was given by the comparison of the percentages of positivity of normal milk from dairy herds without mastitis cases (20%) and that of normal milk collected in a dairy herd with some mastitis cases (43.7%).

This difference statistically significant leads us to believe that even the healthy animals in a dairy herd where mastitis cases are observed, offer favourable conditions for the multiplication of yeasts in milk. This could be the result of a serial treatment with antibacterial antibiotics given blindly to all the animals, which constitutes a well known predisposing factor [2,3].

In conclusion, it seems that the yeast species associated with mastitis in cows are present in normal milk among many other different species present sometimes in great quantities. As all the isolation mediums were complemented with chloramphenicol, we were unable to say whether the isolated yeasts were or were not associated with bacteria in the mammary gland. This is in fact very important since from there, we cannot possibly evaluate the actual role of the yeast in mastitis. However, if, at least for some yeast species, the association with bacteria is necessary to provoque mastitis, it is high time to pay special attention to the ever increasing implications of yeasts in the bovine mammary pathology.

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Address for correspondence: Danielle Swinne, Associate Professor, Department of Microbiology, ITM, Nationalestraat 155–2000 Antwerpen, Belgium
Phone: 32 3 2476666; Fax: 32 3 2161431