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Intensity of slime production by yeast strains isolated from bovine mastitis cases and their susceptibility to polyenes

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Summary

The purpose of this study was to determine the intensity of slime production by yeast-like fungi isolated from cases of bovine mastitis. Yeast isolates were recovered from 105 quarters of 87 cows with clinical and subclinical mastitis from 30 dairy herds in Poland over the period of October 2004 – February 2010. Slime production was detected using a modified Christensen macrotube method established for coagulase-negative staphylococci. The adherent slime layer was scored as negative (–), weak positive (1+), moderate positive (2+) or strong positive (3+). Out of 105 strains of yeast causing udder inflammation in cows, 22 (about 21%) were able to produce slime. 10.47% of yeast-like fungi isolated from bovine mastitis cases were able to form slime at the intensity level of 1+, 9.52% at the intensity level of 2+, while one strain (0.95%) produced slime at the intensity level defined as strong positive (3+). It was demonstrated that 22.34% of Candida genus strains were slime positive, while 40% of Geotrichum genus strains showed this activity. Yeast-like fungi of the genus Trichosporon did not produce slime. In an in vitro amphotericin B susceptibility assay only 9.63% of the 83 non-slime-producing (NSP) and 4.54% of the 22 slime-producing (SP) isolates were susceptible, 34.93% NSP and 13.63% SP isolates were moderately susceptible, whereas 55.42% non-slime-producing and 81.81% slime-producing strains were resistant to amphotericin B ($p \leq 0.05$). 20 (24.09%) non-slime-producing and 1 (4.54%) slime-producing strains of yeast were susceptible to nystatin ($p \leq 0.05$), 49 NSP (59.03%) and 17 SP (77.27%) fungi were moderately (adequately) susceptible, 14 NSP (16.87%) and 4 SP (18.18%) isolates were resistant to nystatin. Our studies indicate that slime-producing yeast strains isolated from the bovine mammary gland are more resistant to amphotericin B and less susceptible to nystatin in comparison with non-slime-producing strains.

Keywords: yeast, slime production, mastitis, susceptibility, polyenes, cows

Mastitis can occur in both clinical and subclinical forms. Clinical mastitis is characterised by changes in the udder and in milk that are directly observable, whereas the subclinical form is characterised by an increased number of somatic cells in milk and the absence of clinical signs. Both forms of mastitis cause a substantial loss of milk production potential and reduce milk quality (7, 21). A wide variety of microorganisms (bacteria, fungi and algae) are involved as causative agents in bovine mastitis (1, 4, 5, 10-12, 18, 29, 39), which proves to be the most common disease affecting dairy cows, and the importance of yeast infections in the etiology of this condition is recognized (34). Among over 72 000 described species of fungi that are ubiquitous in nature (soil, plants, water, air), more than 300 are now considered as real or potential pathogens responsible for mycoses in humans and animals. Yeast and fungi are normal flora of the soil that may colonize udder skin in small numbers (17, 34). They are known to be opportunists and produce disease when natural defense mechanisms are compromised (34). The prevalence of mycotic mastitis is usually very low (1-12% of all mastitis cases) in dairy herds, but in tropical countries the percentage can be more elevated (10, 34). Fungal infections of the mammary gland are predominantly caused by yeasts of the *Candida* genus (10, 13, 15, 20, 22, 24, 26, 28, 35). Strains of yeast-like fungi possess a number of virulence factors that enable the organism to cause hematogenously disseminated infections in susceptible hosts.

One of these factors, which favour the colonization of the host tissue and the persistence of the infection (2, 6, 23, 32), is the production of slime. Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections. Fungal biofilm formation is a complex and diverse phenomenon (32). *Candida albicans* biofilm formation, though, has been studied more extensively than biofilms of other *Candida* species (23). Evidence suggests that biofilms demonstrate a significantly lower susceptibility to antifungal drugs (27). The aim of this study of yeast-like fungi isolated from cases of bovine mastitis was to determine the intensity of their slime production and their susceptibility to polyenes.

Material and methods

Yeast isolates were recovered from 105 quarters of 87 cows with clinical and subclinical mastitis from 30 dairy herds in Poland over the period of October 2004–February 2010. Milk samples were collected aseptically into sterile glass vials, then cooled and immediately transported to the laboratory. 0.01 ml of milk was plated as soon as possible on sheep (5%) blood agar, Mac Conkey agar, aesculin--talium acetate crystal violet agar and Sabouraud agar with chloramphenicol (bioMerieux Poland). When fungal growth was noted on blood and Sabouraud agars, yeasts were identified in terms of their gross morphological, microscopic, and physiologic characteristics. The genera and species of yeast were determined by the API 20 C AUX system and the computer program API WEB (bioMerieux).

Slime production was detected using a modified Christensen macrotube method established for coagulase--negative staphylococci (9) according to Ozkan et al. (32). A loopful of organisms from the surface of a Sabouraud agar plate was inoculated into a tube containing 10 ml of Sabouraud liquid medium supplemented with glucose (8%). The tubes were incubated at 35°C for 24 h, after which the broth was aspirated and the walls of the tube were stained with safranin. The adherent slime layer was scored as negative (–), weak positive (1+), moderate positive (2+) or strong positive (3+). Each tube was scored independently by two observers.

The study was conducted on the basis of control strains from the collection of the Department of Microbiology, Center of Oncology in Bydgoszcz. Strain CTR 78 produced slime (positive control, 3+), while strain CAL 998 did not (negative control, –).

Yeast isolates for the polyene susceptibility assay were cultured on Sabouraud agar for 24 h at 37°C. A portion of the colony was suspended in 5 ml of sterile saline. The suspension was adjusted to contain approximately 5×10^5 cells per 1 ml, using the densitometer (bioMerieux Poland). This suspension was streaked onto a Petri dish containing Yeast Nitrogen Base (YNB) agar (Dom Nauki, Krakow, Poland). The discs (Dom Nauki, Krakow, Poland) containing nystatin (100 U) and amphotericin B (10 µg) were applied equidistant on the surface of plates with YNB. The plates were incubated at 37°C for 24-48 h; the zone inhibition of growth was measured (in mm) and recorded. The following interpretations of susceptibility were applied: for nystatin: susceptible > 18 mm, moderately susceptible 14-18 mm, resistant < 14, for amphotericin B: susceptible > 16 mm, moderately susceptible 12-16 mm, resistant < 12. Statistical analysis was performed using the proportion test.

Results and discussion

Research results for the intensity of slime production are presented in table 1. Out of 105 strains of yeast causing udder inflammation in cows, 22 (20.95%) were able to produce slime. Importantly, 10.47% of fungi isolated from bovine mastitis cases were able to form slime at the intensity level of 1+, 9.52% at the intensity of 2+, while one strain (0.95%) produced slime at the intensity level defined as strong positive (3+). These were the following fungi: *Candida albicans* (1 strain, moderate positive), *Candida lusitaniae* (1 strain, weak positive), *Candida sp.* (2 strains were scored as weak positive and 1 strain as moderate positive), *Candida tropicalis* (1 strain was scored as weak positive and 1 strain as moderate positive), *Candida kefyr* (1 strain – weak positive), *Candida inconspicua* (1 strain – weak positive and 1 strain moderate positive), *Candida krusei/inconspicua* (4 strain were scored as weak positive and 6 strains as moderate positive) and *Geotrichum sp.* (1 strain weak positive and 1 strain strong positive). Yeast-like fungi of the genus *Trichosporon* and other species of the genus *Candida* were shown not to produce slime.

Tab. 1. Slime production by yeast-like fungi isolated from a case of bovine mastitis

Genera and species	Number of isolates	Slime production			
		–	1+	2+	3+
Candida sp.	16	13	2	1	0
Candida rugosa	14	14	0	0	0
Candida humicola	9	9	0	0	0
Candida kefyr	10	9	1	0	0
Candida inconspicua	4	2	1	1	0
Candida tropicalis	11	9	1	1	0
Candida krusei/inconspicua	12	2	4	6	0
Candida lusitaniae	11	10	1	0	0
Candida lambica	1	1	0	0	0
Candida glabrata	1	1	0	0	0
Candida albicans	2	1	0	1	0
Candida guiliermondii	2	2	0	0	0
Candida pseudotropicalis	1	1	0	0	0
Trichosporon cutaneum	3	3	0	0	0
Trichosporon capitatum	1	1	0	0	0
Trichosporon asahii	1	1	0	0	0
Trichosporon mucoides	1	1	0	0	0
Geotrichum sp.	5	3	1	0	1
Total	105	83	11	10	1

Tab. 2. In vitro susceptibility of yeast isolated from mastitic milk of cows to polyenes

Genera and species (n)	Nystatin						Amphotericin B					
	NSP			SP			NSP			SP		
	S	I	R	S	I	R	S	I	R	S	I	R
Candida humicola (9)	2	5	2	0	0	0	2	2	5	0	0	0
Candida lusitaniae (11)	4	4	2	0	1	0	0	5	5	0	0	1
Candida sp. (16)	5	7	1	0	2	1	1	1	11	0	1	2
Candida rugosa (14)	0	12	2	0	0	0	0	8	6	0	0	0
Candida tropicalis (11)	1	7	1	0	2	0	2	4	3	1	1	0
Candida kefyr (10)	2	6	1	0	1	0	0	0	9	0	0	1
Candida inconspicua (4)	1	1	0	0	2	0	0	0	2	0	0	2
C. krusei/inconspicua (12)	1	0	1	0	8	2	1	0	1	0	0	10
C. lambica (1)	0	1	0	0	0	0	0	1	0	0	0	0
C. glabrata (1)	0	0	1	0	0	0	0	1	0	0	0	0
C. guiliermondii (2)	0	0	2	0	0	0	0	1	1	0	0	0
C. pseudotropicalis (1)	1	0	0	0	0	0	0	0	1	0	0	0
C. albicans (2)	0	0	1	1	0	0	0	1	0	0	1	0
Trichoporon cutaneum (3)	2	1	0	0	0	0	0	2	1	0	0	0
Trichosporon mucoides (1)	0	1	0	0	0	0	0	0	1	0	0	0
T. capitatum (1)	0	1	0	0	0	0	0	1	0	0	0	0
T. asahii (1)	0	1	0	0	0	0	1	0	0	0	0	0
Geotrichum sp. (5)	1	2	0	0	1	1	1	2	0	0	0	2
Total (105)	20	49	14	1	17	4	8	29	46	1	3	18
%	24.09[a]	59.03	16.87	4.54[a]	77.27	18.18	1.09.1963	34.93	55.42[b]	1.04.1954	13.63	81.81[b]

Explanation: NSP – non slime producing, SP – slime producing, S – susceptible, I – intermediate, R – resistant, a,b – $p \leq 0.05$

Results of the assays of two antifungals against cultures of yeast are presented in table 2. In an *in vitro* amphotericin B susceptibility assay only 9.63% of the 83 non-slime-producing (NSP) and 4.54% of the 22 slime-producing (SP) isolates were susceptible, 34.93% NSP and 13.63% SP isolates were moderately susceptible, whereas 55.42% non-slime-producing and 81.81% slime-producing strains were resistant to amphotericin B ($p \leq 0.05$). 20 (24.09%) non-slime-producing and 1 (4.54%) slime-producing strains of yeast were susceptible to nystatin ($p \leq 0.05$), 49 (59.03%) and 17 (77.27%) of the fungi were moderately (adequately) susceptible, 14 NSP (16.87%) and 4 SP (18.18%) isolates were resistant to nystatin.

A number of research papers addressed the problem of slime production by yeast of the *Candida* genus isolated from humans (8, 19, 33, 38) and by *Staphylococcus aureus* isolated from mastitic cows (3, 14, 16, 30, 36). In our previous work (25) we demonstrated that *Candida* causing mastitis was capable of producing slime but the intensity of the process had not been established. Therefore, our present study included the genera *Trichosporon* and *Geotrichum* and focused on the intensity of slime production by these three yeast genera.

Out of 105 strains under investigation, 22 strains (20.95%) were shown to produce slime. It was demonstrated that 22.34% of the *Candida* genus strains were slime positive, while 40% of the *Geotrichum* genus strains showed this activity. Yeast-like fungi of the genus *Trichosporon* did not produce slime. Out of the genus *Candida*, the following 6 species of fungi belonging to *Candida non-albicans* (CNA) were found to form slime: *Candida lusitaniae*, *Candida sp.*, *Candida tropicalis*, *Candida kefyr*, *Candida inconspicua*, and *Candida krusei/inconspicua.* Other CNA species (*Candida humicola*, *Candida rugosa*, *Candida lambica*, *Candida guilliermondii*, *Candida pseudotropicalis*, *Candida glabrata*) did not form slime. One out of two isolates of *Candida albicans* (CA) was able to produce slime. This species of yeast is rarely isolated from mastitic bovine milk, because it mainly belongs to CNA (10, 12, 13, 15, 20, 22, 24, 26, 28, 35). Other authors (38) also report that CNA yeasts recovered from humans produced slime more frequently than CA.

Slime production was weak (1+) in cases of 11 strains (10.47%) and moderate (2+) in cases of 10 strains (9.52%). Among 105 strains, only one strain (*Geotrichum sp.*) exhibited the slime formation ability scored as strong positive (3+). The present research high-

lights the fact that as many as 83.33% of the fungi of the species *Candida krusei/inconspicua* were capable of slime formation.

In vitro antifungal resistance is frequently divided into two categories: primary and secondary resistance. Primary resistance is characteristic of microorganisms that are innately resistant to antifungal drugs. Secondary resistance is only developed during or after exposure to antifungals (37).

Results of studies presented by Melchior et al. (30) and Cucarella et al. (14) indicate that *S. aureus* recovered from mastitis cases is highly resistant to antimicrobial agents when it grows in a biofilm. *S. aureus* strains isolated from mastitic ruminants were used in experimental challenge by Baselga et al. (3). The SP (slime-producing) variant showed a significantly higher colonization capacity compared to the NSP (non--slime-producing) variant of the same strain ($p \leq 0.001$). However, the NSP variant was characterized by higher virulence than the SP one ($p \leq 0.001$).

Our studies indicate that slime-producing yeasts isolated from the bovine mammary gland are more resistant to amphotericin B and less susceptible to nystatin in comparison with non-slime-producing strains. A characteristic feature of yeast-like fungi cells present in a biofilm environment is their resistance to antifungal drugs, most probably related to drug-exempt penetration (23, 33). This problem requires further research on the role of a yeast biofilm in the pathogenesis of mastitis in cows.

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