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Udder pathogens isolated from milk of cows before drying off and their antibiotic sensitivity*)

WOJCIECH BARAŃSKI, MAŁGORZATA RAŚ, TOMASZ JANOWSKI, SŁAWOMIR ZDUŃCZYK, JEROEN DEWULF*, AART DE KRUIF*, SARNE DE VLIEGHER*, GEERT OPSOMER*

Zespół Rozrodu Zwierząt Katedry Nauk Klinicznych Wydziału Medycyny Weterynaryjnej UWM, ul. Oczapowskiego 14, 10-950 Olsztyn
*Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Gent University, Gent, Belgium

Barański W., Raś M., Janowski T., Zduńczyk S., Dewulf J., De Kruif A., De Vliegher S., Opsomer G.
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Summary

The aim of this study was to describe the incidence of bacteria and fungi in the milk of dairy cows at the moment of drying off in 8 herds in North-East Poland. Additionally, tests for antibiotic sensitivity of the isolated bacteria were performed. In total, milk samples of 1145 quarters of 288 cows were examined. In 41 cows coagulase negative staphylococci (CNS) were isolated, while Strep. agalactiae was isolated in 22 cows, Strep. uberis in 18 cows, Staph. aureus in 16 cows, E. coli in 10 cows, Strep. dysgalactiae in 3 cows, and Arcanobacter pyogenes in 1 cow. Fungal infections were found in 28 cows. The lowest level of antibiotic resistance among the isolated CNS strains was found for cefquinome, Nafpenzal® and cephalexin while the highest number of resistant strains was found when novobiocin and streptomycin were tested. For Strep. agalactiae the lowest resistance level could be found when cefquinome and cephoperazone were tested, while the highest level was found for streptomycin and kanamycin. When testing Strep. uberis – the less resistance was found for cefquinome, cephalexin and Nafpenzal® and the highest for streptomycin and kanamycin. Staph. aureus strains were mostly sensitive for amoxicillin with clavulanic acid and Nafpenzal®, while streptomycin and neomycin were found to have the highest resistance level. The results of the present study show that bacteria can be isolated in the milk of one third of the cows at the moment of drying off and that resistance levels among the isolated bacteria were lowest for cephalosporins.

Keywords: dairy cows, udder, bacteria, antibiotic sensitivity

Despite considerable research efforts, mastitis and udder health problems in general remain the most costly disease found in modern dairy herds (10, 13, 19). The incidence of intramammary infections (IMI) increases at the end of lactation, but does not necessarily result in an increased number of clinical mastitis cases (16). Bacteria present inside of the mammary gland at the end of lactation may further develop during the dry period and result in an increased number of clinical cases (9, 22) after parturition (3). The incidence of mastitis during lactation is assumed to be a growing problem in Poland because of increased milk production and enlargement of herds. Many studies have investigated the causes of clinical mastitis and antimicrobial resistance of isolated pathogens during lactation (11, 14, 16, 17), but there are relatively few studies available concerning the prevalence of bacteria present in the udders just before drying off or during the dry period (12, 15, 21).

Administrating long acting antibiotics to cows during the drying off period is a widely accepted method of mastitis prophylaxis. The aim of this treatment is to kill bacteria which are present in the udder at the moment of drying off, and to prevent the udder from new infections during the dry period. On the other hand, antibiotics available for drying off are relatively limited and differ widely from country to country. Therefore, it is of crucial importance to evaluate the efficacy of the available products and to monitor the emergence of resistance towards antibiotics used to treat udder health problems. This risk of increased resistance may be due to an increased use of antibiotics (18). New compounds are quite seldom introduced and the efficacy of antibiotics already on the market to treat udder infections has changed significantly over recent years. Therefore, knowledge of the bacteria present in the udder in the drying off period and their sensitivity to antibiotics may significantly contribute to improve udder health and prevent clinical mastitis in the following lactation.

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The aim of the study was to evaluate the prevalence of bacteria in the milk of cows during the drying off period in herds in North-East Poland as well as to assess the level of antibiotic sensitivity of the isolated bacteria.

Material and methods

The animals (n = 288) used in the study belonged to 8 dairy herds containing 36 to 153 milking cows. The average annual milk production in these herds ranged from 5200 to 8200 l of milk per cow. None of the cows suffered from clinical mastitis at the moment of sampling. Single milk samples were collected between October 2003 and September 2005 from 1145 quarters of 288 cows a week before drying off. Before sampling, the teats were cleaned with dry paper towels and, if necessary, washed with water and dried thereafter to remove any visible soil. The first three strippings of milk were discarded and subsequently the teats were disinfected with cotton balls moistened with 70% ethyl alcohol. Samples (10 ml) were collected from each quarter in sterile tubes, transported under cooled conditions (approximately 4°C) and delivered to the laboratory within 4 hours, and then tested for the presence of bacteria and fungi.

For bacteriology, 10 µl of milk was used both for aerobic and anaerobic bacteria using following media: Blood Agar Base (Bio Merieux), Mc Conkey (BTL), Muller--Hilton (BTL) and Edwards (Oxoid). Plates were incubated at 37°C and read 24 and 48 hours later. Bacteria were identified by their gross colony morphology and by Gram staining. Detailed identification of isolated bacteria was performed using API tests (API 20 Staph., API 20 Strep., API 20 NE, API 20 E, API 20 A). They allow a further isolation at strain level.

Sabouraud medium was used for fungal examination. Incubation took 5 days and 1ml of milk was used. Fungi were identified only on the basis of colony morphology.

All isolated bacteria were checked for antibiotic sensitivity. Most of the tested antibiotics were pure antimicrobial agents used in different medicines designed for udder treatment of lactating and dry cows. The only exception was Nafpenzal® which is a ready-to-use medicine. A total of 17 different commercially available discs containing a known amount of a specific antibiotic were used (ampicillin – 25 µg – Oxoid, amoxicillin and clavulanic acid – 30 µg – Oxoid, bacitracin - 10 units – Oxoid, cefoperazone – 30 µg – Mast Diagnostics, cefquinome – 30 µg – Rosco, cephalexin – 30 µg – Mast Diagnostics, cloxacillin – 5 µg – Oxoid, erythromycin – 15 µg – Oxoid, kanamycin – 30 µg – Oxoid, lincomycin – 15 µg – Oxoid, Nafpenzal® – 35 µg – Oxoid, neomycin – 30 µg – Oxoid, novobiocin – 5 µg – Rosco, penicillin G – 10 units – Oxoid, rifaximin – 40 µg – Mast Diagnostics, streptomycin – 25 µg – Oxoid and tetracycline – 30 µg – Oxoid). After incubation at 37°C for 24 h the zone of growth inhibition was measured and according to the instructions received from the producer, results were categorized as susceptible, intermediate or resistant.

Results and discussion

In 145 samples obtained from 111 cows, bacteria and fungi were found. Species of micro organisms and frequency of IMI are presented in tab. 1. Coagulase--negative staphylococci (CNS) were the most frequently isolated. The most frequently found CNS was *Staph. haemoliticus*, *epidermidis* and *chromogenes* (tab. 2).

Fungal infections were found in 28 cows – *Candida sp.* in 5 cows, *Mucor sp.* in 8 animals, *Penicillium sp.* in 11 cows, and *Aspergillius sp.* in 4 animals.

Antibiotic resistance was examined for all bacteria isolated from the udders. Results are presented in tab. 3 and 4. For CNS the antibiotics with the lowest level of resistance were cefquinome, Nafpenzal® and cephalexin, whereas the highest level of resistance was observed for novobiocin and streptomycin (tab. 3). Isolated *Staph. aureus* strains were most sensitive for amoxicillin with clavulanic acid and Nafpenzal®. Many of those *Staphylococci* were also susceptible for cephalosporin, while they were resistant for streptomycin and neomycin in most of the cases (tab. 3).

Cefquinome and cefoperazone seemed to be the best choice to treat *Str. agalactiae* infections, while a high level of resistance was found for streptomycin and kanamycin (tab. 4). To successfully treat *Str. uberis* infections, the lowest level of antimicrobial resistance was found for cefquinome, cephalexin and Nafpenzal® while an insufficient number of these bacteria were sensitive for streptomycin and kanamycin (tab. 4).

Tab. 1. Species isolated from mammary glands at the moment of drying off

Pathogen	No of infected cows	%	No of infected quarters	%
Coagulase-negative staphylococci (CNS)	41	14.20	58	5.00
Strep. agalactiae	22	7.60	28	2.40
Strep. uberis	18	6.25	21	1.80
Strep. dysgalactiae	3	1.00	4	0.30
Staph. aureus	16	5.50	22	1.90
E. coli	10	3.50	11	1.00
Arcanobacter pyogenes	1	0.30	1	0.09
Fungi	28	9.70	48	4.20

Tab. 2. Species of CNS isolated from udders and number of IMI in quarters and cows

Staph. epidermidis		Staph. chromogenes		Staph. haemoliticus	
cows	*quarters*	*cows*	*quarters*	*cows*	*quarters*
15	20	15	19	15	20
Staph. hyicus		**Staph. warneri**		**Staph. saprophiticus**	
cows	*quarters*	*cows*	*quarters*	*cows*	*quarters*
2	3	1	2	1	3

Tab. 3. Resistance of CNS and *Staph. aureus* to antibiotic

Antibiotic	CNS (n = 41)			*Staph. aureus* (n = 16)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Amoxicillin + clavulanic acid	7.3	17.1	75.6	0	0	100
Ampicillin	4.9	14.6	80.5	0	31.2	68.8
Bacytracin	58.5	36.6	4.9	50	37.5	12.5
Cephalexin	0	9.8	90.2	0	6.2	93.8
Cefoperazon	0	12.5	87.5	0	6.2	93.8
Cloxacilin	24.4	43.9	31.7	6.2	43.8	50.0
Cefquinome	0	0	100	6.2	6.2	87.6
Erythromycin	29.3	26.8	43.9	12.5	56.3	31.2
Kanamycin	29.3	51.2	19.5	87.6	12.4	0
Lincomycin	43.9	26.8	29.3	18.7	81.3	0
Nafpenzal®	0	7.3	92.7	0	0	100
Neomycin	29.3	39.0	31.7	87.6	6.2	6.2
Novobiocin	75.6	14.6	9.8	68.8	25.0	6.2
Penicillin	26.8	29.3	43.9	0	18.7	81.3
Rifaksymin	26.8	22.0	51.2	12.5	25.0	62.5
Streptomycin	63.4	31.7	4.9	93.8	6.2	0
Tetracycline	24.4	53.7	21.9	68.8	25.0	6.2

Explanations: R – Resistant; I – Intermediate; S – Susceptible

Tab. 4. Sensitivity of *Str. agalactiae* and *Str. uberis* to antibiotic

Antibiotic	*Str. agalactiae* (n = 22)			*Str. uberis* (n = 18)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Amoxicillin +clavulanic acid	4.6	13.6	81.8		11.1	88.9
Ampicillin		13.6	86.4		16.7	83.3
Bacytracin	31.8	63.6	4.6	72.2	27.8	
Cephalexin	4.6	4.6	90.8		5.6	94.5
Cefoperazon	4.6		95.4		11.1	88.9
Cloxacilin	27.2	36.4	36.4	61.1	16.7	22.2
Cefquinome		4.6	95.4			100
Erythromycin	22.7	31.8	45.5	27.8	33.4	27.8
Kanamycin	86.4	13.6		100		
Linkomycin	45.5	40.9	13.6	44.4	16.7	38.9
Nafpenzal®		4.6	95.4		5.6	94.4
Neomycin	72.8	13.6	13.6	77.8	16.7	5.5
Novobiocin	81.8	13.6	4.6	83.3	16.7	
Penicillin	18.2	31.8	50.0	16.7	66.6	16.7
Rifaksymin	36.4	18.2	45.4	50.0	27.8	22.2
Streptomycin	86.4	13.8		94.4	5.6	
Tetracycline	22.7	50.0	27.3	66.7	27.8	5.5

Infections were stated in 38.5% of the examined cows and in 12.6% of the quarters. Only one quarter was infected in 54 cows while in 57 animals bacteria were presented in two or more quarters. Green et al. (12) found bacteria in 45.8% of the examined cows that were sampled during the whole dry period. The most often isolated bacteria were major pathogens (63% of infected cows) mainly *Staph. aureus* and *Strep. agalactiae*. In 37% of the infected cows, minor pathogens (CNS) were isolated. The prevalence of *Staph. aureus* at quarter level (1.92%) was comparable with the data of Green et al. (12) (1.52%). In the above mentioned study (12), no *Str. agalactiae* were isolated during the dry period, while in the present study this germ seemed to be quite common (2.44% of the infected quarters) and may have been responsible for an elevated somatic cell count at the end of lactation. This may be related to the fact that these bacteria are sensitive to many antibiotics. Total dry cow therapy (concerning all cows in a herd) has been used in Poland for a relatively short time but is becoming increasingly popular. This way of treatment has long been in use in Western countries where the administration of long-acting antibiotics over an extended period has decreased the prevalence of bacteria. Thus it is possible that within a few years the number of mammary infections caused by this germ will also decrease in Poland.

Blowey and Edmondson (2) claimed that there is an increase in the number of *Str. uberis* infections in barns that use straw as bedding material. As a conclusion they suggest sawdust, shavings or sand as better alternatives. While straw was the most popular bedding material for cows in our region the prevalence of *Str. uberis* before drying off was not very high. To verify this theory a comparison of the present data with information about bacteria isolated in clinical mastitis cases in our region would be necessary. Bradley and Green (3) also investigated the prevalence of enterobacterial infections during the dry period. The main bacterium present at and during drying-off in that study was *E. coli* (2.14% and 5.18% of quarters infected, respectively). This is higher in comparison with our data (only 0.96%). In contrast with the study of Bradley and Green (3), no other *Enterobacteriaceae* were isolated in our study. This could be caused by the fact that samples were collected only from healthy animals or that the number of examined cows was too small to find other *Enterobacteriaceae.*

To our knowledge, there are currently no data available that describe the different types of CNS isolated from samples collected at drying off time. However, isolation and strain typing of CNS has been done by some authors in clinical mastitis cases (1, 6, 11, 20). The group of CNS consists of at least 40 different species and subspecies (8). In the present trial only six species were isolated with special attention to *Staph. chromogenes*, *Staph. epidermidis*, and *Staph. haemoliticus* (tab. 2). Rajala-Shultz et al. (20) found *Staph. chromogenes* being the most prevalent CNS species (105/158) in cows with clinical mastitis. This finding agrees partially with our results in which *Staph. chromogenes* was one of the most frequently isolated bacterium from that group (5.2%). The prevalence of *Staph. chromogenes*, *Staph. epidermidis* and *haemoliticus* were very similar in our study. Other authors isolated only *Staph. epidermidis* (1) or did not notice them at all (20). On the contrary, in the presented study they were found in 5.2% of examined cows.

In Finland the largest group of pathogens responsible for *mastitis* was *Staphylococci* (17). Moreover, this study mentioned that the number of infections caused by *Staph. aureus* decreased, whereas the number of infections caused by CNS increased between 1988-1995. In the study, *Staph. aureus* was only isolated in 1.92% of the investigated quarters. It is possible that this is an underestimation of the real prevalence since our data are based on only one sampling (just before drying off). It has been noted that for a reliable diagnosis of *Staph. aureus* 3 milk samples are recommended (www.nmconline.org/docs/NMC10steps.pdf.). A quarter is defined as being infected if bacteria have been found in it twice or three times.

The results of this study clearly show that there is a much bigger problem with contagious, rather than environmental organisms, within the examined herds. This is related to the fact that cows with clinical mastitis were excluded from that trial. If we had also sampled those animals, environmental bacteria would have been found much more often. Other explanations for this situation may be the fact that the owners are, due to financial constrains, reluctant to cull cows with persistent mastitis. Obviously these cows are a persistent source of infection for the other cows in the herd.

Many studies have already been carried out to evaluate the effect of antibiotic treatment at the moment of drying off (1, 4, 5, 15). Practitioners often send milk samples to labs to discover to which antibiotics the isolated bacteria are sensitive. The results (obtained *in vitro*) are then transferred to an *in vivo* situation and treatment. Unfortunately it has been noted that despite the wide use of antibiograms treatment fails in many cases. The latter may be caused by a wrong sampling procedure (contamination of the sample with bacteria from the skin or air), or a misidentification of the germ in the lab. Another explanation simply is that the *in vitro* situation on the plate is too different from the *in vivo* situation in the udder. Treatment failure may furthermore be caused by an insufficient distribution of the antibiotics in the udder caused by the presence of milk, pus, micro abscesses, or the presence of scar tissue adhesions. However, there is so far no better way to know the sensitivity of isolated germs towards antibiotics, though the imperfections of this procedure should always be kept in mind.

It has been found that amoxicillin with clavulanic acid, Nafpenzal® and cephalosporins are the most efficient in inhibiting the growth of *Staph. aureus* (tab. 3). Other authors (7) reported that 75% of the tested *Staph. aureus* strains were resistant to penicillin, whereas this study found no resistance to penicillin at all. As far as tetracycline is concerned we could show that only 6.2% of the isolated strains were susceptible, while the above cited researchers mentioned 58.33% of susceptibility.

There is a shortage of data describing which kind of bacteria may be isolated from the udders of dairy cows before drying off in Poland. Additionally, the level of resistance towards antibiotic treatment among the isolated bacteria is generally not known. Malinowski et al. (15) performed a trial to compare the clinical effectiveness of different combinations of antibiotics used for treatment of clinical *mastitis*. Unfortunately they did not describe bacteria that had been isolated before drying off, so a comparison with the present study is not possible.

In this study, CNS was the most susceptible for cefquinome, Nafpenzal® and other cephalosporins. The highest frequency of resistance among these bacteria was for novobiocin and streptomycin. The latter is in agreement with data of other authors (1) who also reported that cephalotin and methicillin were the most active beta-lactam antibiotics against *Staph. epidermidis*. Rajala-Schultz et al. (20) found the highest resistance of CNS against penicillin (31.7%), followed by resistance against ampicillin (12.2%) and tetracycline (11.5%). Resistance for penicillin was found in 28.6%, which is in agreement with the literature (11), while 4.9% and 24.4% of isolates were resistant for ampicillin and tetracycline, respectively.

Isolated *Str. agalactiae* were susceptible for cephalosporins and amoxicillin with clavulanic acid. However, cephalosporin C may be the least effective for those micro organisms (1). Blowey and Edmondson (2) demonstrated that almost all antibiotics are effective against *Str. agalactiae* but we could not confirm this because 9 out of 17 examined antibiotics presented susceptibility lower or equal to 50%.

Many strains of isolated bacteria were susceptible to cephalosporins and amoxicillin with clavulanic acid. These antimicrobial agents are quite new in Poland which explains their high level of susceptibility. Products containing penicillin, streptomycin and tetracycline have mostly been used in the past. It has also been very popular to use medicines with 3-5 agents in one tube, but this, in turn, has led to none of the commercially available medicines being useful after some time.

To summarize, it can be stated that more than 30% of clinically healthy cows were infected at the moment of drying off – mostly with CNS, *Staph. aureus*, *Strep. agalactiae* and *Str. uberis*. Infections caused by fungi were also relatively high. Most isolated bacteria were susceptible to different antimicrobial agents, particularly cephalosporins. Thus, based on the results of the present study, the use of long acting antibiotics at the moment of drying off should be encouraged in Poland as there are a high number of cows suffering from bacteriological udder infections. Further studies are needed, however to gauge the results of these treatments under practical circumstances.

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Adres autora: dr Wojciech Barański, Ruszajny 18J, 11-010 Barczewo; e-mail: wojbar@uwm.edu.pl