*Polish Journal of Veterinary Sciences Vol. 14, No. 3 (2011), 467-472*

DOI 10.2478/v10181-011-0069-9

*Original article*

# Antimicrobial resistance patterns to beta-lactams of gram-positive cocci isolated from bovine mastitis in Lithuania

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## Abstract

The aim of the study was to isolate gram-positive cocci from cows with mastitis and to determine their resistance to beta-lactamic antibiotics. Eight hundred and nine strains were isolated and identified as staphylococci (n=516), streptococci (n=199) and enterococci (n=94) from sub-clinical and clinical cases of bovine mastitis in Lithuania. The most common causative agents of udder disease included: *S. epidermidis* (n=176), *S. aureus* (n=176), *S. agalactiae* (n=134), *S. hyicus* (136) and *E. hirae* (n=68). Isolates were analysed for antimicrobial resistance to penicillin, ampicillin, amoxicillin, cephalothin, cephalexin, amoxicillin + clavulanate. The susceptibility patterns were analysed using the agar disk diffusion method. *S. aureus* showed the highest level of resistance to amoxicillin (81.3%), penicillin (76.7%) and ampicillin (78.4%). The corresponding values for CNS strains were 59.7%, 59.7% and 50.6% against penicillin, ampicillin and amoxicillin respectively. *Streptococci* were the most frequently resistant to amoxicillin (29.3%), and enterococci to penicillin (27%), amoxicillin (27.5%) and amoxicillin/clavulanic acid (23.8%). The resistance of all tested mastitis pathogens to aminopenicillins and penicillin highly correlated (r=0.83). Compared with other antibiotics, amoxicillin and clavulanic acid combination tended to be more effective ($p<0.05$) against all tested bacteria *in vitro*. However, *S. aureus*, in 38.1% of cases, was resistant to this combination of antimicrobials.

This study demonstrates that *S. epidermidis, S. aureus, S. hyicus, S. agalactiae* and *E. hirae* remain the most frequent mastitis causative agents on Lithuanian cattle farms. The highest resistance *in vitro* to penicillins was demonstrated by *S. aureus*, *S. hyicus* and *S. intermedius*. Resistance to cephalosporins remains low, irrespective of bacterial species of gram-positive cocci.

**Key words**: antimicrobial agent, cows, cocci, resistance, mastitis

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## Introduction

Bovine mastitis is a multifactorial disease and is one of the most difficult to control. It can be caused by many different bacterial species, the most common of which are *Staphylococcus* and *Streptococcus* genera. The prevalence of different species varies geographically, temporally and also due to control measures adopted in herds. In addition, different pathogens are typical of different types of mastitis (clinical, subclinical or heifer mastitis). Bovine mastitis is the single most common cause for antimicrobial use in lactating cattle worldwide. There is a variety of antimicrobials that are used for the prevention and treatment of mastitis. Therefore, resistance to antimicrobials is expected. Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Bradley 2007, Malinowski et al. 2008). Tenhagen et al. (2006) in a recent survey suggest that penicillin, half synthetic penicillin G and cephalosporins are the antibiotics of choice in dairy herds. In the Nordic countries penicillin is used as the first-line antibiotic treatment for bovine mastitis because of a low resistance rate and narrow spectrum. According to Tenhagen et al. (2006) and Roesch et al. (2006) penicillin was used on 31 – 66% of the farms.

According to studies in the United States and Finland the existence of antibiotic resistance of udder pathogens is worldwide, with certain regional differences. In Switzerland there has been no increase in antibiotic resistance of mastitis pathogens during the last 20 years. In other countries, for example Norway and Sweden, levels of resistance have remained fairly constant at around 10-15%. It is clear that the rate of penicillin resistance varies per country and also over time within the countries (Rossitto et al. 2002, Pitkälä et al. 2004). Makovec and Ruegg (2003) indicate that antimicrobial resistance was identified in 24.5% of the analyzed bacterial isolates. According to Vintov et al. (2003) considering the studies of European herds, penicillin resistance is more heterogeneous, ranging from only 2% (Norway) to more than 70% (Ireland), with an overall average of ca. 32%. It should be noted that resistance of microorganisms directly depends not only on the use of antimicrobial drugs in medical practice but also on their use in farming, animal treatment, prophylaxis and growth stimulation. Despite considerable use and sometimes misuse, many antimicrobials remain effective today.

The aim of this study was to isolate gram-positive bacteria which usually cause mastitis in Lithuanian bovine farms and to determine their resistance to beta-lactamic antibiotics.

## Materials and Methods

A survey of quarter milk cultures obtained in 1340 samplings at the cow level from 34 Lithuanian dairy herds was conducted between January, 2008, and November, 2010. The medium age of cows in this study was 6.7 years (range 2.2 to 10.4). The diagnosis of mastitis was made on the basis of anamnesis and clinical examination of the udder, macroscopic evaluation of secretion and California Mastitis Test (CMT). A quarter of milk (inflamed secretion) samples from cows with subclinical or clinical forms of mastitis was collected aseptically. The closing streams were 2-4 ml of the secretion and were collected into sterile tubes. The samples were cooled and immediately transported to the laboratory.

Bacteriological testing was based on the National Mastitis Council (NMC) recommendations for bovine milk cultures, with some impovements. The bacteria were identified based on colony morphology; Gram's staining; and tests as required, for catalase, coagulase, oxidase production, and finally, using API Staph and API 20 Strep identification strips ("Biomerieux"). Antimicrobial resistance of *Streptococcus* spp., *Staphylococcus* spp. and *Enterococcus* spp. was tested using the disc diffusion method and performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The following discs with antibiotics (Oxoid) were used: penicillin (P; 10 UI), amoxicillin (25 μg), amoxicillin with clavulanic acid (30 μg), ampicillin (10 μg), cephalothin (30 μg ) and cephalexin (30 μg).

The statistical analysis was performed by 'Epi Info' (1996) Centers for Disease Control & Prevention (CDC), U. S. A., Version 6.04). Arithmetical means (X), standard deviation (SD) and coefficients of variation (Cv) were set by minimal and maximal meanings. The Student multiple comparison method was applied to determine the criterion of significance concerning the differences among groups (p). The difference was considered statistically significant at $p<0.05$.

## Results

During this study 809 strains of gram-positive cocci were isolated from cows with mastitis (Table 1). The most frequently isolated genera was *Staphylococcus* spp. – in total 516 isolates (63.8%) were obtained ($p<0.05$). One hundred and ninety nine isolates of *Streptococcus* spp. and 94 strains of *Enterococcus* spp. were isolated as remaining causative agents. Analysis of staphylococci distribution within the genera indicated that 3 species of

Table 1. Distribution of gram-positive cocci in cases of bovine mastitis.

| Species | Year of isolation and number of isolates (n) | | | | Mean ± SD | % |
|---|---|---|---|---|---|---|
| | 2008 | 2009 | 2010 | Total | | |
| *S. dysgalactiae* | 11 | 7 | 12 | 30 | 10.0 ± 2.6 | 3.7 |
| *S. uberis* | 8 | 9 | 8 | 25 | 8.3 ± 0.6 | 3.1 |
| *S. equinus* | 6 | 0 | 4 | 10 | 3.3 ± 3.0 | 1.2 |
| *S. agalactiae* | 52 | 42 | 40 | 134 | 44.7 ± 6.4 | 16.6 |
| *E. hirae* | 14 | 22 | 32 | 68 | 22.7 ± 9.0 | 8.4 |
| *E. faecium* | 4 | 4 | 5 | 13 | 4.3 ± 0.6 | 1.6 |
| *E. faecalis* | 3 | 5 | 5 | 13 | 4.3 ± 1.2 | 1.6 |
| *S. intermedius* | 6 | 9 | 13 | 28 | 9.3 ± 3.5 | 3.5 |
| *S. hyicus* | 73 | 44 | 19 | 136 | 45.3 ± 27.0 | 16.8 |
| *S. aureus* | 91 | 35 | 50 | 176 | 58.7 ± 28.9 | 21.8 |
| *S. epidermidis* | 65 | 54 | 57 | 176 | 58.7 ± 5.7 | 21.8 |
| Tested strains | 333 | 231 | 245 | 809 | | 100 |

Table 2. Antimicrobial resistance of gram-positive cocci to penicillins.

| Species | Number of resistant isolates (%) | | | | Mean | Std.dev |
|---|---|---|---|---|---|---|
| | Penicillin | Ampicillin | Amoxicillin | Amoxicillin and clavulanic acid | | |
| *S. dysgalactiae* | 23.3 | 26.6 | 26.6 | 6.7 | 20.80 | 9.53 |
| *S. uberis* | 12.0 | 12.0 | 36.0 | 8.0 | 17.0 | 12.81 |
| *S. equinus* | 20.0 | 20.0 | 30.0 | 10.0 | 20.0 | 8.17 |
| *S. agalactiae* | 28.3 | 46.3 | 24.6 | 16.4 | 28.9 | 12.62 |
| *E. hirae* | 11.8 | 7.4 | 13.2 | 17.6 | 12.5* | 4.20 |
| *E. faecalis* | 7.7 | 7.7 | 30.8 | 15.4 | 15.4 | 10.89 |
| *E. faecium* | 61.5 | 23.1 | 38.5 | 38.5 | 40.4 | 15.83 |
| *S. epidermidis* | 59.7 | 59.7 | 50.6 | 7.4 | 44.4 | 25.00 |
| *S. aureus* | 76.7 | 78.4 | 81.3 | 38.1 | 68.6* | 20.44 |
| *S. intermedius* | 53.6 | 71.2 | 46.4 | 17.9 | 47.3 | 22.18 |
| *S. hyicus* | 57.4 | 71.3 | 55.1 | 16.9 | 50.2* | 23.31 |
| Mean | 37.5 | 38.5 | 39.4 | 17.5* | | |
| SD | 24.60 | 27.54 | 18.49 | 11.12 | | |

* $P<0.05$

*Staphylococcus* were divided quite equally in the tested samples: *S. epidermidis* (21.8%), *S. aureus* (21.8%) and *S. hyicus* (16.8%). *S. intermedius* ($p<0.05$) was the rarest and constituted only 3.5 percent of all staphylococci. *S. agalactiae* ($p<0.05$) was the leading species among streptococci. *E. hirae* dominated among enterococci.

76.7% of *S. aureus* isolates were resistant to penicillin (Table 2). Other species demonstrated low resistance to penicillin: *E. faecalis* (7.7%), *S. uberis* (12.0%), *E. hirae* (11.8%). Resistance to ampicillin varied according to bacterial species. The most frequent resistance was observed among *S. intermedius* (71.2%) and *S. aureus* (78.4%) and less frequent resistance was demonstrated by *S. agalactiae* (46.3%), *E. faecium* (23.1%) and *S. equinus* (20.0%). The susceptibility of the tested mastitis pathogens to ampicillin, amoxacillin and penicillin correlates $r=0.83$. The majority of strains resistant to amoxicillin were determined among *S. aureus* (81.3%), *S. hyicus* (55.1%) and *S. epidermidis* (50.6%). Other species of bacteria were more frequently susceptible to this

Table 3. Antimicrobial resistance of gram-positive cocci to cephalosporins.

| Species | Number of resistant isolates (%) | | Mean | Std.dev |
|---|---|---|---|---|
| | Cephalothin | Cephalexin | | |
| *S. dysgalactiae* | 6.7 | 10.0 | 8.4 | 2.33 |
| *S. uberis* | 16.0 | 12.0 | 14.0 | 2.82 |
| *S. equinus* | 10.0 | 10.0 | 10.0 | 0.0 |
| *S. agalactiae* | 8.2 | 12.0 | 10.0 | 2.82 |
| *S. epidermidis* | 10.8 | 8.0 | 9.4 | 1.98 |
| *S. aureus* | 4.5 | 6.8 | 5.6 | 1.62 |
| *S. intermedius* | 14.3 | 10.7 | 12.5 | 2.54 |
| *S. hyicus* | 13.2 | 15.4 | 14.3 | 1.56 |
| Mean | 10,5 | 10,6 | | |
| SD | 3,93 | 2,64 | | |

antibiotic. Compared with other antibiotics, a combination of amoxicillin and clavulanic acid was more effective *in vitro* ($p<0.05$) against all tested gram-positive cocci. However, *S. aureus* in 38.1% of cases was resistant to this combination.

Table 3 illustrates the first-generation cephalosporin class potency to all bacterial species, (except *Enterococcus* spp.) from this study. From this table it can be seen that cephalothin and cephalexin would be effective in the treatment of mastitis caused by streptococci and staphylococci Overall, cephalosporins were more frequently effective against staphylococci compared with the group of penicillins.

## Discussion

Our study demonstrated the importance of gram-positive cocci as aethiological agents in udder infections of cows. Different sources indicate that *S. aureus,* coagulase negative staphylococci (CNS), *S. agalactiae* and environmental streptococci are predominant aetiological agents in both subclinical and clinical forms of mastitis (Olde Riekerink et al. 2006, Ferguson et al. 2007). There are indications that environmental mastitis pathogens are now emerging as the most frequent cause of mastitis in many, particularly well-managed, herds (Ebrahimi 2008). *Staphylococcus* was a predominant genus in our study, with a rate of 63.8% among all cocci ($p<0.05$). The prevalence of two important pathogenic species – *S. epidermidis* and *S. aureus* among the genera was 21.8% however, the majority of the intramammary infections (IMI) (65.8%) were caused by non-*aureus* staphylococci. The distribution of pathogens isolated from mastitis samples differ considerably among countries and even among studies within a country. *Staphylococcus spp*. also had the highest prevalence in reports from the United States and Norway (Makovec and Ruegg 2003, Reksen et al. 2006). Although the control of *S. aureus* mastitis is often deemed to be difficult, many herds have successfully achieved this through implementation of the standard mastitis prevention program. As a result the prevalence of *S. aureus* IMI has decreased in several European countries (Pitkälä et al. 2004, Bradley et al. 2007). Previous studies in Lithuania demonstrated that *S. aureus* was one of the most frequent pathogens (Klimienė et al. 2005).

According to Ebrahimi et al. (2008), Olde Riekerink et al. (2008), Gianneechini et al. (2002) *S. agalactiae, S. dysgalactiae* (16-42.4%), *S. uberis* (18-39.9%), *S. bovi*s as well as *Enterococcus* spp. were the main causative agents of bovine mastitis in California, Norway, Uruguay, Sweden and New Zealand. Authors indicate that *S. agalactiae* was found in 1-13.1% of the positive samples in Sweden (Ebrahimi et al. 2008) and in 7.7% of the positive samples in Wisconsin (Makovec and Ruegg 2003). Makovec and Ruegg (2003), Olde Riekerink et al. (2006), Piepers et al. (2007) described a constant decline of the prevalence of *S. agalactiae* in their diagnostic material. The reports also suggest the re-emergence of *S. agalactiae* in some European countries e.g., Finland and Denmark (Pyörälä 2008). Several other mastitis pathogens are thought to have the potential to spread in a contagious manner. This is particularly true for *S. dysgalactiae* and to a lesser extent for *S. uberis* (Tikofsky and Zadoks 2005). In this study *S. agalactiae* was found more frequently than other streptococci (16.6% of all pathogens), while *S. dysgalactiae* (3.7%), *S. uberis* (3.1%) and *S. equinus* (1.2%) contained non-significant part among other pathogens of mastitis. Klimienė et al. (2009) indicate that *S. agalactiae* was the causative agent of mastitis, detected in 18.04% of cases.

Little information is available on enterococci as pathogens isolated from milk samples, and most stu-

dies focus on the two major species – *E. faecalis* and *E. faecium*. While data are sparse, the incidence of enterococci as aetiological agents of bovine mastitis according to different sources varies from 0 to 21.2% (Pitkälä et al. 2004, Bradley et al. 2007).

The resistance of the identified bacteria to beta-lactams depends on the species of bacteria and particular antibiotic. *Staphylococcus spp.*, particularly *S. aureus* demonstrated frequent resistance to aminopenicillins. Significant resistance of *S. aureus* and other staphylococci to penicillin and ampicillin was reported in Poland, Finland (Pitkälä 2004, Malinowski et al. 2008). According to these authors ampicillin and penicillin are consistently the antimicrobial drugs to which *S. aureus* are most commonly resistant. Other species of staphylococci were much more susceptible than *S. aureus*, though they often (approximately half of all strains) were resistant to penicillin but not to a combination of amoxicillin and clavulanic acid. The high resistance rate of staphylococci to amoxicillin and cluvalanic acid combination was described previously by other authors (Malinowski 2008).

The data for streptococci resistance to antimicrobial drugs are not as extensive as for *S. aureus*. Variability in drug resistance is more pronounced among individual streptococcal species than among studies of staphylococcus. Ebrahimi et al. (2008) identified that *S. agalactiae* showed high resistance rates to amoxicillin (76.92%), penicillin (69.23%), and ampicillin (61.53%). Berghash et al. (1983) reported that minimal inhibitory concentration levels for *S. agalactiae* isolates were higher for herds that reported dry cow treating of all cows, as opposed to herds that did not dry cow treat or only selectively treated cows. In our study *S. agalactiae* and *S. dysgalactiae* demonstrated resistance to beta-lactams, though they were more frequently susceptible to amoxicillin and clavulanic acid combination rather than tested against separate antimicrobial agents. It may be presumed that the resistance of the above mentioned bacteria may be associated with the production of beta-lactamase. It is known that mastitis caused by *S. dysgalactiae* and *S. agalactiae* is much more easily cured than mastitis caused by *S. aureus* (Rositto et al. 2002).

According to Ebrahimi et al. (2008) a high resistance rate was observed among *S. uberis* isolates, in particular, against penicillin (100%) and ampicillin (88.88%). In our experiment, only about 12% of *S. uberis* were resistant both to penicillin and ampicillin. An earlier study, prepared in the USA, investigated the change of susceptibility of mastitis agents in a period of 7 years and showed that the proportions of resistant and susceptible isolates of *S. uberis* changed by increasing the quantity of resistant isolates (Erskine et al. 2002).

*S. equinus* is a rare species of bacteria and there is not much information regarding the diseases caused by it. While estimating the resistance of this species to penicillin we determined that its resistance was not in significant contrast to other species of streptococci. Three isolates demonstrated susceptibility to amoxicillin (out of 10). One strain was resistant to a combination of amoxicillin and clavulanic acid**.**

*Enterococcus* spp. are used as indicator bacteria for the development of antimicrobial resistance (DANMAP 2008) and provide accurate information on previous antibiotic treatment of animals. As bovine mastitis is the single most common reason for antimicrobial use in dairy farms, knowledge of resistance profiles of enterococci isolated from milk samples are useful for assessment and monitoring of changes in resistance rates of dairy enterococci isolates. Analysis of the literature shows that *E. faecalis* as well as *E. faecium* may produce beta-lactamases. *Enteroccocus* may be resistant to penicillin and ampicillin since is it also able to produce little afinitet conjuncting protein (PBP). The agar disk diffusion method may help to isolate microorganisms where PBP are modified, but it can not be reliably to discover beta-lactamases producing strains. As Piepers (2007) reports, the *Enterococcus spp*. were even more resistant than streptococci. This corresponds to our data. *E. faecium* was very often resistant to penicillin during our study (23.1-61.5%, depending on the particular antibiotic). This is similar to the data of previous years (Ružauskas et al. 2008).

Other studies (Ebrahimi 2008, Malinowski et al. 2008) indicate that resistance to beta-lactams is increasing and such data is in agreement with the data obtained in the present study. The percentage of penicillin-susceptible strains (62.5%) remained low, and penicillin is one of the most frequently used antimicrobial agents for the treatment of udder infections. In recent years, CNS has emerged as an important minor mastitis pathogen and causes substantial economic losses. CNS tends to be more resistant than *S. aureus* and easily develops multiresistance. The current study demonstrates that the resistance of CNS to cephalosporins and the combination of amoxicillin-clavulanic acid remains low.

The present study indicates that *S. epidermidis, S. aureus, S. hyicus, S. agalactiae* and *E. hirae* remain frequent mastitis causative agents on Lithuanian cattle farms. The highest resistance *in vitro* to penicillins was demonstrated by *S. aureus*, *S. hyicus* and *S. intermedius*. Resistance to cephalosporins remain low irrespective of bacterial species of gram-positive cocci.

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