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1 **Antimicrobial susceptibility of udder pathogens from cases of**  
2 **acute clinical mastitis in dairy cows**

3 **Running title: Antimicrobial susceptibility of udder pathogens from acute clinical mastitis**

4  
5 Björn Bengtsson<sup>1\*</sup>, Helle Ericsson Unnerstad<sup>1</sup>, Torkel Ekman<sup>2</sup>, Karin Artursson<sup>1</sup>, Maria Nilsson-Öst<sup>1</sup> and Karin  
6 Persson Waller<sup>1</sup>

7  
8 1) National Veterinary Institute, SE 751 89 Uppsala, Sweden.

9 2) Swedish Dairy Association, Uppsala, Sweden

10

11 \*Corresponding author: Björn Bengtsson, National Veterinary Institute, SE 751 89 Uppsala, Sweden. Tel +46

12 (0)18 674000, Fax: +46 (0)18 309162, E-mail: Bjorn.Bengtsson@sva.se

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16 **Abstract**

17 To investigate occurrence of acquired antimicrobial resistance in udder pathogens MICs in *Staphylococcus*  
18 *aureus* (n=211), coagulase-negative staphylococci (CNS) (n=56), *Streptococcus uberis* (n=113), *Streptococcus*  
19 *dysgalactiae* (n=152), *Streptococcus agalactiae* (n=6), *Escherichia coli* (n=163), and *Klebsiella* spp. (n=42)  
20 were determined using microdilution. Isolates were from a nation wide survey employing strict inclusion criteria.  
21 Presence of acquired resistance was evaluated by species-specific epidemiological cut-off values issued by  
22 EUCAST. Penicillin or methicillin resistance in staphylococci were however evaluated by  $\beta$ -lactamase  
23 production or presence of the *mecA* gene, respectively.

24  
25 Staphylococci were mostly susceptible to antimicrobials tested but 7.1% of *S. aureus* and 12.5% of CNS were  
26 resistant to penicillin by  $\beta$ -lactamase production. Methicillin resistance was not found in *S. aureus*. All  
27 *Streptococcus dysgalactiae* and *S. agalactiae* were susceptible to penicillin. Bimodal MIC distributions for  
28 tetracycline in *S. dysgalactiae* and *S. uberis* indicate acquired resistance in some isolates. Among *E. coli* 12.3 %  
29 of isolates were resistant to one or more antimicrobials. Resistance to streptomycin (11.0%), sulphamethoxazole  
30 (8.6%), ampicillin (7.4%), or tetracycline (4.9%) were the most common traits. *Klebsiella* spp. were resistant to  
31 ampicillin and some isolates also to tetracycline (7.1%) or sulphonamide (9.5%).

32  
33 The study shows that in Sweden bacteria associated with acute clinical mastitis for the most part are susceptible  
34 to antimicrobials used in therapy but resistance to penicillin in *S. aureus* is not uncommon. Penicillin is  
35 recommended for treatment of mastitis caused by gram-positive pathogens and regular monitoring of  $\beta$ -  
36 lactamase production in *S. aureus* is therefore recommended in herds with udder health problems.

37  
38 **Keywords:**  
39 Mastitis; Antimicrobial resistance; Cattle; *Staphylococcus aureus*; Coagulase negative staphylococci;  
40 *Escherichia coli*; *Klebsiella* spp.; *Streptococcus uberis*; *Streptococcus agalactiae*; *Streptococcus dysgalactiae*.

41  
42

## 43 **1 Introduction**

44 Acquired antimicrobial resistance in bacteria is an increasing threat in human as well as in veterinary medicine.  
45 Hence, monitoring antimicrobial susceptibility in pathogenic as well as in commensal bacteria in animals is  
46 recommended by OIE (Acar and Rostel, 2001). Such monitoring generates data of importance for therapeutic  
47 decisions and provides information on trends in resistance that might be cause for interventions regarding  
48 antimicrobial use.

49  
50 Mastitis is one of the most costly diseases for the dairy industry (Kossaibati and Esslemont, 1997) and  
51 antimicrobials are important parts of therapy of the disease, although not the solution for poor udder health. For  
52 the last twenty years, the Swedish recommended antimicrobial treatment for acute clinical mastitis caused by  
53 gram-positive bacteria is penicillin administered systemically for 3-6 days contingently in combination with  
54 intramammaries (Ekman et al., 1994). Intramammary treatment alone, using penicillin, is recommended at  
55 drying off in cases of subclinical mastitis. Annually about one fifth of Swedish dairy cows are treated for  
56 mastitis during lactation (Valde et al., 2004) and thus mastitis is by far the disease in Swedish animal production  
57 where the largest amounts of antimicrobials are used (SVARM 2003). In Sweden, use of antimicrobials is on  
58 prescription only and a recent study revealed that most veterinarians comply with the therapeutic  
59 recommendations and that penicillin administered systemically is used in over 80% of treatments for mastitis  
60 (Landin, 2006). The selective pressure enforced by this use might cause an increase in the prevalence of  
61 antimicrobial resistance and regular monitoring of susceptibility of udder pathogens is therefore warranted.

62  
63 Antimicrobial susceptibility of udder pathogens has been investigated on several occasions over the past fifty  
64 years in Sweden (Wallmark and Thörne, 1958; Holmberg, 1975; Franklin and Horn af Rantzien, 1983;  
65 Robertsson and Franklin, 1987). The most recent nation-wide survey was performed in 1995 (Nilsson et al.,  
66 1997) and an update was therefore urgent. The objective of this study was to determine antimicrobial  
67 susceptibility of udder pathogens from Swedish dairy cows with acute clinical mastitis.

## 68 **2 Materials and methods**

### 69 **2.1 Sampling**

70 Isolates tested are from a study on prevalence of udder pathogens in acute clinical mastitis. In the study, 51  
71 veterinary practices distributed thorough out Sweden collected milk samples from a specified number of cases,  
72 i.e. cows with acute clinical mastitis, during each of six consecutive two-month periods from May 1st 2002 to

73 April 31st 2003. Each practice sampled cases meeting the inclusion criteria (see below) consecutively as  
74 encountered in practice. The number of cases assigned to each practice was proportional to the number of dairy  
75 cows in the county where the practice operated and based on the number of participating practices in the county.  
76 The objective was to obtain milk samples from 1000 cases distributed geographically in proportion to the  
77 number of dairy cows in different regions of the country.

78  
79 Only lactating cows with macroscopically altered milk in one or more quarters and with no previous episode of  
80 clinical mastitis in the current lactation were sampled. Moreover, the previous routine cow composite milk  
81 somatic cell count (CSCC) of the cow should have been <200 000 cells/mL and the cow should not have been  
82 treated with antimicrobials in the preceding 30 days. When a case was identified, the responsible veterinarian  
83 collected individual milk-samples aseptically from all quarters with macroscopic signs of mastitis.

## 84 **2.2 Bacteriological culture**

85 Milk samples were cultured at the respective veterinary practice according to their routine procedures, usually  
86 employing blood-, mannitol-salt- and McConkey agar plates incubated at 37 °C for 16-24 h. After evaluation at  
87 the practice, plates were sent to the National Veterinary Institute (SVA) irrespective of culture results. Most  
88 plates arrived within one week after sampling and mostly within four days (>80%). At SVA, growth on the  
89 plates was evaluated and additional laboratory tests performed in accordance with the routines at the Mastitis  
90 laboratory. Briefly, *Staphylococcus aureus* was identified by means of typical colony morphology,  $\alpha$ - and  $\beta$ -  
91 haemolysis and positive coagulase reaction. Staphylococci other than *S. aureus* were identified by typical colony  
92 morphology and negative coagulase reaction but were not determined to species level. Streptococci were typed  
93 to species level employing the CAMP reaction and 12 biochemical reactions (hippurate, aesculin, salicine,  
94 sorbitol, mannitol, raffinose, lactose, saccharose, inuline, trehalose, starch, and glycerine). For isolates not  
95 identified with these methods, Lancefield grouping (Streptex, Murex Biotech limited, Dartford, UK) was used.  
96 Gram-negative bacteria with typical colony morphology and positive for p-Nitrophenyl- $\beta$ -D-  
97 glucopyranosiduronic acid (PGUA) and indole were considered *Escherichia coli*. For other Gram-negative  
98 bacteria, oxidase reaction and API 20 E or API 20 NE (Bio-Mérieux, France) was used. A milk sample was  
99 classified as positive if at least one colony-forming unit (CFU) of *S. aureus* or *S. agalactiae* was isolated. For  
100 other agents, the presence of at least three CFU was needed for positive classification. Samples were classified as  
101 contaminated and excluded from the study if three or more bacterial types were isolated from one milk sample  
102 and growth of a major udder pathogen was not identified.

103

104 Isolates considered primary udder pathogens were kept frozen (-70°C) pending further analysis. From these  
105 isolates, *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus uberis*, *Streptococcus*  
106 *dysgalactiae*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella* spp. were selected for susceptibility  
107 testing. When the same bacterial species was isolated from more than one udder quarter in the same cow, only  
108 one isolate was included to avoid a clustering effect. Isolates of different bacterial species from different udder  
109 quarters in the same cow, or different species isolated from the same udder quarter, were included however.

## 110 **2.2 Susceptibility testing**

111 Isolates were tested for antimicrobial susceptibility by determination of minimum inhibitory concentration  
112 (MIC) using a microdilution method. Testing was performed according to CLSI (formerly NCCLS)  
113 recommendations (NCCLS, 2002) using VetMIC™ panels (National Veterinary Institute, Uppsala, Sweden) and  
114 cation adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, USA). Antimicrobials and range of  
115 concentrations tested are given in Tables 1-3. For testing of oxacillin susceptibility in staphylococci, 2% NaCl  
116 was added to the broth. Quality control strains, *S. aureus* ATCC 29213, *S. aureus* ATCC 25923 and *E. coli*  
117 ATCC 25922, tested in parallel with each batch of isolates, were on all occasions within acceptable ranges. All  
118 isolates of staphylococci were in addition examined for  $\beta$ -lactamase production by the "clover-leaf" method as  
119 described by Bryan and Godfrey (1991). Staphylococci with MIC for oxacillin >2 mg/L were retested using the  
120 same VetMIC panel but at a lower temperature, 30°C. Isolates with MIC >2 mg/L on retesting were examined  
121 for presence of the *mecA*-gene by PCR according to Smyth and others (2001).

122

123 Isolates were classified as susceptible or resistant based on species-specific epidemiological cut-off values issued  
124 by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (<http://www.eucast.org>). For  
125 staphylococci, the EUCAST cut-off value for clindamycin (>0.25 mg/L) could not be used since it is outside the  
126 range of tested concentrations. Moreover, in *S. aureus* the EUCAST cut-off value for neomycin (>1 mg/L)  
127 would have split the distribution of MICs an inappropriate way and a higher value (>2 mg/L) was therefore used.  
128 For the same reason, a higher cut-off value (>4 mg/L) for gentamicin than recommended by EUCAST (>2 mg/L)  
129 was used in *E. coli*. Cut off-values used are given in Table 1-3. Classification of staphylococci as resistant to  
130 penicillin or oxacillin was based on production of  $\beta$ -lactamase and presence of *mecA* gene respectively. Isolates  
131 were not classified as susceptible or resistant when cut-off value from EUCAST were not available.

132

133 **3 Results**

134 In all, 987 udder quarters from 829 cows were sampled. The number of cases sampled per 1000 cows in each of  
135 24 counties ranged between 0.3 and 3.5. In the majority, 19 counties, 1.4 to 2.5 cases per 1000 cows were  
136 sampled. In each of the six sampling periods, 126–152 cases were sampled. After exclusion of duplicate isolates  
137 of the same bacterial species within cows, 743 isolates from 694 udder quarters in 669 cows were tested for  
138 antimicrobial susceptibility: *Staphylococcus aureus* (n=211), CNS (n=56), *Streptococcus uberis* (n=113),  
139 *Streptococcus dysgalactiae* (n=152), *Streptococcus agalactiae* (n=6), *Escherichia coli* (n=163), and *Klebsiella*  
140 spp. (n=42). The results are given as distributions of MICs and, when appropriate cut-off values from EUCAST  
141 are available, as percent resistant isolates in Tables 1-3.

142  
143 Twenty-four (11.3%) of 211 isolates of *S. aureus*, were resistant to one or more antimicrobial. Penicillin  
144 resistance, i.e.  $\beta$ -lactamase production, occurred in 15 isolates (7.1%) and was the most common trait (Table 1).  
145 All these isolates had MICs for penicillin  $>0.12$  mg/L. Four isolates (1.9%) were resistant to more than one  
146 antimicrobial: one isolate to penicillin and erythromycin, two isolates to neomycin and streptomycin and one  
147 isolate to penicillin, erythromycin, chloramphenicol, neomycin and streptomycin. The latter isolate also had high  
148 MICs to spiramycin ( $>32$  mg/L) and clindamycin ( $>8$  mg/L).

149  
150 Twelve (21.4%) of the 56 CNS isolates tested were resistant to one or more antimicrobial. Seven (12.5 %)   
151 isolates were resistant to penicillin through  $\beta$ -lactamase production, all of these had MICs for penicillin  $>0.12$   
152 mg/L (Table 1). Five isolates (8.5%) were resistant to more than one antimicrobial: three isolates to penicillin  
153 and in addition tetracycline, gentamicin or erythromycin, one isolate to erythromycin and tetracycline and one  
154 isolate to penicillin, oxacillin and tetracycline. The last also had high MICs to cephalothin ( $>1$  mg/L) and  
155 streptomycin (64 mg/L).

156  
157 One isolate of *S. aureus* and four of CNS had MIC  $>2$  mg/L for oxacillin on testing at 37 °C. Two of the CNS-  
158 isolates still had MICs  $>2$  mg/L on retesting at 30 °C and in one of these the *mecA* gene was confirmed by PCR.  
159 This isolate was phenotypically resistant to the  $\beta$ -lactam antimicrobials penicillin and oxacillin and had a high  
160 MIC to cephalothin ( $>1$  mg/L). In addition the isolate was resistant to tetracycline and had a high MIC to  
161 streptomycin (64 mg/L). The isolate was confirmed as *S. epidermidis* on typing using the Staph-Zym® test  
162 (Rosco Diagnostics, Taastrup, Denmark).

163

164 The results for streptococci are difficult to evaluate since EUCAST cut-off values are lacking for the species  
165 tested. Of the six *S. agalactiae* isolates, two were resistant to tetracycline (Table 2). One of these isolates had  
166 high MICs to clindamycin (>8 mg/L), erythromycin (1 mg/L) and spiramycin (>64 mg/L) indicating acquired  
167 resistance also to these antimicrobials. For both *S. dysgalactiae* and *S. uberis* there are no cut off values for  
168 tetracycline but distributions of MICs for this antimicrobial were bimodal indicating acquired resistance in some  
169 isolates (Table 2). One isolate of *S. uberis* had a deviating high MIC to trimethoprim sulphonamide (2 mg/L).  
170 MICs to penicillin were below the epidemiological cut-off values in *S. agalactiae* and *S. dysgalactiae*. For *S*  
171 *uberis* there is no cut-off value for penicillin but seven isolates had deviating high MICs (0.12-0.25 mg/L) (Table  
172 2).

173

174 The results for *Klebsiella* spp. are also difficult to interpret since cut-off values are lacking. However most  
175 isolates were resistant to ampicillin and a few also to tetracycline (7.1%) or sulphonamide (9.5%) (Table 3).  
176 Bimodal distributions of MICs indicate acquired resistance in some isolates to streptomycin or trimethoprim.

177

178 Among *E. coli*, 20 isolates (12.3%) were resistant to one or more antimicrobial. Sixteen isolates (9.8%) were  
179 resistant to at least two, and 12 (7.4%) to three or more antimicrobials (Table 4). The most common traits were  
180 resistance to streptomycin (11.0%), sulphonamides (8.6%) or ampicillin (7.4%) (Table 3). Ten isolates (6.1%)  
181 were resistant to all these antimicrobials.

#### 182 4 Discussion

183 Udder pathogens were isolated from milk samples collected under strict inclusion criteria by systematic  
184 sampling from all parts of Sweden. Data presented should therefore provide a good estimate of antimicrobial  
185 susceptibility of udder pathogens encountered in acute clinical mastitis in the field. Susceptibility data was  
186 interpreted according to species-specific epidemiological cut-off values separating the wild type population from  
187 the subpopulation that has acquired reduced susceptibility, here classified as resistance. To identify acquired  
188 reduced susceptibility is most relevant for monitoring purposes and recommended by the European Food Safety  
189 Authority (EFSA, 2008) but it should be understood that reduced susceptibility not always implies clinical  
190 resistance. Susceptibility data presented as distributions of MICs, as in the present paper, can easily be  
191 reevaluated using other cut-off values and is thereby available for other interpretations.

192

193 In Sweden systemic administration of penicillin is used in over 80 % of cows treated for clinical mastitis  
194 (Landin, 2006). Therefore penicillin resistance in gram-positive udder pathogens is of greatest concern from a  
195 clinical perspective. In the present study 7.1% of *S. aureus* were resistant penicillin. This agrees with Swedish  
196 studies from 1982 (10.4%) and 1985 (10.0%) (Franklin and Horn af Rantzien, 1983; Robertsson and Franklin,  
197 1987) in which  $\beta$ -lactamase production was used to define resistance, as in the present study. In fact similar  
198 frequencies (10-13%) were found already in the 1950s, -60s and -70s using disc diffusion (Holmberg, 1975) or  
199 agar-cup methods (Wallmark and Thörne, 1958). Comparisons between studies should be made with caution and  
200 in the light of possible differences in methodology and selection of isolates but the prevalence of penicillin  
201 resistance among *S. aureus* seems to have changed very little over the last 25 years. In fact the prevalence in the  
202 present study (7.1%) is about the same (6.1%) as among 181 isolates from acute clinical mastitis tested in 1995  
203 using the same methodology as in the present study (Nilsson et al., 1997). In CNS occurrence of penicillin  
204 resistance was numerically higher in 1995 (25.7%) than in the present study (12.5%) but the limited number of  
205 isolates tested precludes conclusions on trends.

206  
207 Despite a substantial usage of penicillin the situation regarding resistance in *S. aureus* is favourable and appears  
208 stable. Current practice in Sweden includes bacteriological diagnosis of most cases of mastitis and examination  
209 of udder health before trade of animals. Cows with mastitis caused by *S. aureus*, and especially penicillin  
210 resistant strains, are to be milked separately and culled if bacteriological cure is not attained. Treatment of  
211 chronic cases is not recommended and trade of infected animals discouraged. These measures prevent spread of  
212 *S. aureus* within and between herds and thereby in combination with prudent use of antimicrobials probably also  
213 counteract an increase in prevalence of penicillin resistance. Most likely prevalence of penicillin resistance in *S.*  
214 *aureus* is largely influenced by spread of resistant clones as suggested by Aarestrup and Jensen (1998). Use of  
215 penicillin likely imposes a selection pressure by leaving animals with unresolved infections as reservoirs of  
216 resistant *S. aureus*. Resistance arising *de novo* is probably less important since horizontal transmission of the  
217 *blaZ* gene is an extremely rare event in staphylococci causing mastitis (Olsen et al., 2006). This agrees with the  
218 predominance of a limited number of clones/types of *S. aureus* within a dairy herd or geographical region  
219 (Vintov et al., 2003; Anderson et al., 2006) and the apparent association between resistance phenotype and strain  
220 type (Waage et al., 2002; Vintov et al., 2003; Anderson et al., 2006).

221

222 All *S. dysgalactiae* and the majority of *S. uberis* had low MICs to penicillin indicating clinical susceptibility but  
223 seven isolates of the latter species had MICs 0.12-0.25 mg/L. Due to the range of concentrations tested it cannot  
224 be determined if the MIC distribution is bimodal but in other studies *S. uberis* with MICs for penicillin > 0.12  
225 mg/L in deviate from the majority of isolates (Guérin-Faubleé et al., 2002, Rossitto et al., 2002; MARAN-2005).  
226 Such bimodal MIC distributions indicate the presence of acquired resistance in a bacterial population (Turnidge  
227 et al., 2006) but it is unclear if MICs  $\geq 0.12$ mg/L in *S. uberis* indicate reduced susceptibility of clinical  
228 importance.

229  
230 Susceptibility data for tetracycline in *S. uberis* and *S. dysgalactiae* are good examples of bimodal MIC  
231 distributions where the majority of isolates had MICs  $\leq 8$  mg/L, but occasional isolates had MICs >32 mg/L.  
232 Similar bimodal distributions are reported elsewhere but with a substantially larger proportion (24-68%) of  
233 isolates with MICs >16 mg/L (MARAN-2005; Guérin-Faubleé et al., 2002; Rossitto et al., 2002).

234  
235 Methicillin resistance was not found in *S. aureus* but was confirmed in one isolate of CNS identified as *S.*  
236 *epidermidis*. Reports on methicillin resistance in CNS associated with mastitis are rare but it has been  
237 demonstrated in *S. epidermidis* (Kaszanyitzky et al., 2004) and *S. sciuri* (Devriese et al., 2002; Guérin-Faubleé et  
238 al., 2003). Likewise, there are only a few reports on methicillin resistant *S. aureus* (MRSA) associated with  
239 mastitis (Devriese and Hommez, 1975; Kaszanyitzky et al., 2004; Lee, 2006). In contrast, MRSA are  
240 increasingly reported in companion animals and horses (for a review see Leonard and Markey, 2008) and the  
241 common occurrence in livestock such as pigs and veal calves in some countries could be an important reservoir  
242 for human infections (Wulf and Voss, 2008). In human healthcare, MRSA is considered a great threat and  
243 animal reservoirs of such bacteria are of concern from a zoonotic point of view. Infections in animals could also  
244 be of concern from a clinical perspective since all  $\beta$ -lactams, including cephalosporins, would be lost from the  
245 therapeutic arsenal. Detection and containment in dairy herds of MRSA associated with mastitis is therefore  
246 essential.

247  
248 Only a few isolates of *E. coli* and *Klebsiella* spp. were resistant enrofloxacin or the combination  
249 trimethoprim/sulphonamide i.e. antimicrobials in Sweden used for therapy of mastitis caused by gram-negative  
250 bacteria. Three isolates (1.8%) of *E. coli* were resistant to enrofloxacin. Enrofloxacin resistance in *E. coli*  
251 associated with mastitis is reported elsewhere (Kaspar, 2006) but was not observed in the Swedish study from

252 1995 (Nilsson et al., 1997). This could be because a higher breakpoint for resistance (>0.5 mg/L) than in the  
253 present study was used but probably reflects that enrofloxacin was introduced on the Swedish market in the early  
254 1990s, i.e. shortly before the study in 1995.

255  
256 Resistance to more than one antimicrobial was rare among staphylococci and streptococci, which is consistent  
257 with other studies on isolates from bovine mastitis (Sabour et al., 2004; Anderson et al., 2006). In contrast, *E.*  
258 *coli* were often resistant to more than one antimicrobial. Half of the 20 resistant *E. coli* isolates had streptomycin,  
259 sulphonamide and ampicillin resistance in their phenotype and six also tetracycline resistance. All these  
260 resistance traits, often in combination, are common in *E. coli* from the gastrointestinal tract of young cattle in  
261 Sweden (SVARM 2003) but rare in *E. coli* from the gastrointestinal tract of healthy dairy cows (SVARM 2006).  
262 Since ampicillin and tetracycline are rarely used for treatment of mastitis in Sweden, selection of resistant *E. coli*  
263 clones and/or resistance determinants probably occurs outside the udder by use of antimicrobials for treatment of  
264 other diseases than mastitis and possibly in other animal categories than adult dairy cows. This is consistent with  
265 a high genetic diversity of *E. coli* associated with mastitis, as reported by Srinivasan et al. (2007).

266

## 267 **Conclusions**

268 This study shows that in Sweden bacteria associated with acute clinical mastitis mostly are susceptible to  
269 antimicrobials used in therapy. Most important, the majority of streptococci and staphylococci were sensitive to  
270 penicillin, the drug recommended for treatment of mastitis caused by these bacteria. Accordingly, antimicrobial  
271 resistance is probably not a major cause for therapeutic failures in treatment of acute clinical mastitis in Swedish  
272 dairy cows. However, 7.1% of *S. aureus* were resistant to penicillin. Therefore, to guide therapy and measures to  
273 counteract spread of resistant clones, isolates of this bacterium should be tested for  $\beta$ -lactamase production on a  
274 regular basis in herds with udder health problems.

275

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278

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**Table 1.** Resistance (percent, 95% CI in brackets) and distribution (percent) of MIC for *Staphylococcus aureus* (n=211) and coagulase negative staphylococci (CNS)(n=56) from acute clinical mastitis in dairy cows. White fields denote range of dilutions tested for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Bold vertical lines indicate EUCAST epidemiological cut-off values. When no cut-off value is available isolates are not classified as susceptible or resistant.

Substance	Species	Resistance (%)	Distribution (%) of MICs (mg/L)														
			≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256	
Cephalothin	<i>S. aureus</i>	0 (0.0-1.7)		19.9	72.0	8.1											
	CNS	-		8.9	66.1	23.2		1.8									
Chloramphenicol	<i>S. aureus</i>	0.9 (0.1-3.4)							10.4	87.7	0.9		0.9				
	CNS	0 (0.0-6.4)						8.9	50.0	41.1							
Clindamycin	<i>S. aureus</i>	-					99.1	0.5				0.5					
	CNS	-					94.6	5.4									
Erythromycin	<i>S. aureus</i>	1.9 (0.5-4.8)				62.1	36.0	1.4		0.5							
	CNS	3.6 (0.4-12.3)				73.2	23.2			3.6							
Gentamicin	<i>S. aureus</i>	0.5 (0.0-2.6)			13.7	53.6	27.5	4.7	0.5								
	CNS	1.8 (0.0-9.6)			73.2	25.0	1.8										
Neomycin	<i>S. aureus</i>	2.4 (0.8-5.4)					84.8	12.8	2.4								
	CNS	-					98.2	1.8									
Oxacillin <sup>a</sup>	<i>S. aureus</i>	0 (0.0-1.7)				20.9	52.6	26.1	0.5 <sup>d</sup>								
	CNS	1.8 (0.0-9.6)				42.9	35.7	14.3	3.6 <sup>d</sup>	3.6 <sup>e</sup>							
Penicillin <sup>b</sup>	<i>S. aureus</i>	7.1 (4.0-11.4)	81.5	11.4		0.5		0.5	1.9	0.5	3.8						
	CNS	12.5 (5.2-24.1)	76.8	10.7	1.8	1.8	3.6			1.8	3.6						
Spiramycin	<i>S. aureus</i>	-							1.9	5.2	77.7	14.7	0.5				
	CNS	-							10.7	21.4	64.3	3.6					
Streptomycin	<i>S. aureus</i>	1.9 (0.5-4.8)						3.8	41.2	41.2	11.8	1.4		0.5			
	CNS	-						48.2	37.5	10.7			1.8	1.8			
Tetracycline	<i>S. aureus</i>	0 (0.0-1.7)				80.6	19.4										
	CNS	5.4 (1.1-14.9)				82.1	12.5						1.8	3.6			
Trim/sulpha <sup>c</sup>	<i>S. aureus</i>	0 (0.0-1.7)			98.6	1.4											
	CNS	7.2 (2.0-17.3)			69.1	23.6	3.6		1.8		1.8						

<sup>a</sup> No cut-off value given, classification according to presence of the *mecA*-gene.

<sup>b</sup> No cut-off value given, classification according to beta-lactamase production.

<sup>c</sup> Concentration of trimethoprim given, tested in concentration ratio 1/20 (trimethoprim/sulphamethoxazole).

<sup>d</sup> these isolates (1 *S. aureus* and 2 CNS) had MICs for oxacillin 0.5-1 mg/L on retesting at a lower temperature (30 °C).

<sup>e</sup> these two isolates had MIC for oxacillin >4 mg/L also on retesting at a lower temperature (30 °C), one of the isolates carried the *mecA*-gene.

**Table 2.** Resistance (percent, 95% CI in brackets) and distribution (percent) of MIC for *Streptococcus agalactiae* (n=6), *Streptococcus dysgalactiae* (n=152) and *Streptococcus uberis* (n=113) from acute clinical mastitis in dairy cows. White fields denote range of dilutions tested for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Bold vertical lines indicate EUCAST epidemiological cut-off values. When no cut-off value is available isolates are not classified as susceptible or resistant.

Substance	Species	Resistance (%)	Distribution (%) of MICs ( $\mu\text{g/L}$ )											
			$\leq 0.06$	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Cephalothin	<i>S. agal.</i>	0 (0.0-45.9)		83.3	16.7									
	<i>S. dysg.</i>	-		75.0	24.3	0.7								
	<i>S. uberis</i>	-		54.9	41.6	3.5								
Chloramphenicol	<i>S. agal.</i>	-							66.7	33.3				
	<i>S. dysg.</i>	-							21.1	76.3	2.6			
	<i>S. uberis</i>	-							15.9	84.1				
Clindamycin	<i>S. agal.</i>	-					83.3					16.7		
	<i>S. dysg.</i>	-					99.3	0.7						
	<i>S. uberis</i>	-					100.0							
Erythromycin	<i>S. agal.</i>	-				83.3	16.7							
	<i>S. dysg.</i>	-				100.0								
	<i>S. uberis</i>	-				100.0								
Penicillin	<i>S. agal.</i>	0 (0.0-45.9)	83.3	16.7										
	<i>S. dysg.</i>	0 (0.0-2.4)	100.0											
	<i>S. uberis</i>	-	93.8	5.3	0.9									
Spiramycin	<i>S. agal.</i>	-							83.3			16.7		
	<i>S. dysg.</i>	-							99.3	0.7				
	<i>S. uberis</i>	-							100.0					
Tetracycline	<i>S. agal.</i>	33.3 (4.3-77.7)				66.7				16.7	16.7			
	<i>S. dysg.</i>	-				2.6	19.7	44.7	21.7	8.6		0.7	2.0	
	<i>S. uberis</i>	-				92.9	4.4	0.9				1.8		
Trim/sulpha <sup>a</sup>	<i>S. agal.</i>	-			100.0									
	<i>S. dysg.</i>	-			97.4	2.6								
	<i>S. uberis</i>	-			93.8	5.3	0.9							

<sup>a</sup> Concentration of trimethoprim given, tested in concentration ratio 1/20 (trimethoprim/sulphamethoxazole).

**Table 3** Resistance (percent, 95% CI in brackets) and distribution (percent) of MIC for *Escherichia coli* (n=163) and *Klebsiella* spp. (n=42) isolated from cases of acute clinical mastitis in dairy cows. White fields denote range of dilutions tested for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. Bold vertical lines indicate EUCAST epidemiological cut-off values. When no cut-off value is available isolates are not classified as susceptible or resistant.

Substance	Species	Resistance (%)	Distribution (%) of MICs (mg/L)															
			≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512
Ampicillin	<i>E. coli</i>	7.4 (3.9–12.5)					0.6	12.3	62.0	17.2	0.6	0.6		6.7				
	<i>Klebsiella</i>	97.6 (87.4–99.9)								2.4	2.4	16.7	78.6					
Ceftiofur	<i>E. coli</i>	0 (0.0–2.2)				21.5	74.8	3.7										
	<i>Klebsiella</i>	-				19.0	71.4	9.5										
Chloramphenicol	<i>E. coli</i>	1.8 (0.4–5.3)							3.1	84.0	11.0	1.8						
	<i>Klebsiella</i>	-							26.2	59.5	14.3							
Enrofloxacin	<i>E. coli</i>	3.1 (1.0–7.0)	11.0	77.9	8.0	1.2		0.6			1.2							
	<i>Klebsiella</i>	-	2.4	45.2	42.9	9.5												
Florfenicol	<i>E. coli</i>	0 (0.0–2.2)							1.8	65.6	31.9	0.6						
	<i>Klebsiella</i>	-							16.7	64.3	19.0							
Gentamicin	<i>E. coli</i>	0 (0.0–2.2)					1.2	48.5	42.3	8.0								
	<i>Klebsiella</i>	0 (0.0–8.4)					57.1	40.5	2.4									
Nalidixic acid	<i>E. coli</i>	3.1 (1.0–7.0)							1.2	28.2	65.6	1.2	0.6	0.6	0.6		1.8	
	<i>Klebsiella</i>	-							14.3	71.4	11.9	2.4						
Neomycin	<i>E. coli</i>	1.2 (0.1–4.4)							1.2	44.2	45.4	8.0			0.6	0.6		
	<i>Klebsiella</i>	-							47.6	47.6	4.8							
Streptomycin	<i>E. coli</i>	11.0 (6.7–16.9)								3.7	39.9	45.4	1.2	0.6	1.2	0.6	7.4	
	<i>Klebsiella</i>	-								64.3	19.0		2.4	4.8	4.8		4.8	
Sulphamethoxazole	<i>E. coli</i>	8.6 (4.8–14.0)												79.8	11.7			8.6
	<i>Klebsiella</i>	9.5 (2.7–22.6)												50.0	40.5			9.5
Tetracycline	<i>E. coli</i>	4.9 (2.1–9.4)						11.0	68.1	15.3	0.6						4.9	
	<i>Klebsiella</i>	7.1 (1.5–19.5)						16.7	52.4	23.8							7.1	
Trimethoprim	<i>E. coli</i>	3.1 (1.0–7.0)		4.9	28.8	53.4	9.2	0.6						3.1				
	<i>Klebsiella</i>	-		2.4		33.3	54.8	7.1						2.4				

**Table 4.** Resistance phenotypes of *Escherichia coli*

(n=20) resistant to at least one antimicrobial. “R”

indicates resistance.

Number of isolates	Resistance phenotype <sup>a</sup>									
	Sm	Su	Am	Tc	Nal	Ef	Tm	Cm	Nm	
1	R	R	R	R	R	R	R	R	R	
1	R	R	R	R	R	R	R	R		
1	R	R	R	R	R	R				
1	R	R	R	R				R		
1	R	R	R	R						
1	R	R	R	R			R		R	
1	R	R	R				R		R	
1	R	R	R				R			
2	R	R	R							
3	R	R								
4	R									
1	R		R	R	R	R			R	
1			R	R						
1		R			R	R				

<sup>a</sup> Sm: streptomycin; Su: sulphamethoxazole; Am: ampicillin; Tc: tetracycline; Nal: nalidixic acid; Ef: enrofloxacin; Tm: trimethoprim; Cm: chloramphenicol; Nm: neomycin.