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Tylosin susceptibility of staphylococci from bovine mastitis

Monika Entorf [a,b], Andrea T. Feßler [a,*], Kristina Kadlec [a], Heike Kaspar [c], Joachim Mankertz [c], Thomas Peters [b], Stefan Schwarz [a]

[a] Institute of Farm Animal Genetics, Friedrich-Loeffler-Institut (FLI), Neustadt-Mariensee, Germany
[b] Milchtierherden-Betreuungs- und Forschungsgesellschaft mbH (MBFG), Wunstorf, Germany
[c] Federal Office of Consumer Protection and Food Safety (BVL), Berlin, Germany

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ABSTRACT

Although the 16-membered macrolide tylosin is commonly used for the treatment of bovine mastitis, little information is currently available about the susceptibility of mastitis pathogens to tylosin. In the present study, 112 *Staphylococcus aureus* and 110 coagulase-negative *Staphylococcus* (CoNS) spp. isolates from cases of bovine mastitis were tested by broth microdilution and agar disk diffusion with 30 μg tylosin disks. Susceptibility to erythromycin was tested by broth microdilution and disk diffusion using 15 μg disks. Both test populations showed bimodal distributions of minimal inhibitory concentrations (MICs) and zone diameters with eleven *S. aureus* and eight CoNS isolates showing tylosin MICs of ≥256 μg/ml and no zones of growth inhibition around the tylosin 30 μg disks. All 19 isolates with tylosin MICs of ≥256 μg/ml were also resistant to erythromycin. For six additional erythromycin-resistant isolates, tylosin MICs of 1–8 μg/ml were observed. One *S. aureus* and two CoNS isolates showed inducible macrolide resistance. PCR analysis of the 25 erythromycin-resistant staphylococcal isolates identified the resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(T), *mph*(C) and *msr*(A) alone or in different combinations. An excellent correlation between the results of the different tylosin susceptibility tests (broth microdilution versus disk diffusion) was seen for *S. aureus* and CoNS isolates. Since tylosin does not induce the expression of the aforementioned *erm* genes, isolates with an inducible resistance phenotype may – if only tylosin is tested – be falsely classified as tylosin-susceptible. Thus, erythromycin should be tested in parallel and tylosin should only be used for the treatment of infections caused by erythromycin-susceptible staphylococci.

1. Introduction

The 16-membered macrolide tylosin is commonly used for the treatment of bovine mastitis (Ziv and Sulman, 1973; El-Sayed et al., 1986; McDougall et al., 2007). Many prudent use guidelines request that the causative pathogen should be identified and tested for its susceptibility against antimicrobial agents before starting an antimicrobial therapy. In mastitis diagnostic laboratories, routine susceptibility testing is conducted mainly by using either broth dilution assays or agar disk diffusion (Watts and Lindeman, 2005). Tylosin susceptibility testing by broth microdilution is not problematic as (i) the antimicrobial agent is commercially available for the production of microtitre plates for broth microdilution or for use in broth macrodilution assays and (ii) quality control (QC) ranges for reference strains have been approved by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013b). For disk diffusion, most disk producers have only the tylosin 30 μg disk in their regular range of products and produce the tylosin 15 μg and 60 μg disks only upon request. In contrast, CLSI-approved QC ranges have been published exclusively for the tylosin 60 μg disk (CLSI, 2013b). To validate the results obtained with tylosin disks other than

* Corresponding author. Tel.: +49 5034 871 254; fax: +49 5034 871 143.
E-mail address: andrea.fessler@fli.bund.de (A.T. Feßler).

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the 60 μg disk (Schwarz et al., 2010), QC ranges for the reference strain *Staphylococcus aureus* ATCC® 25923 and the tylosin 30 μg and 15 μg disks have been determined recently in an interlaboratory trial (Buß et al., 2014). The Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee of CLSI has approved the QC ranges for the 30 μg disk in June 2013 and decided that the 30 μg disk should be the preferred disk for tylosin susceptibility testing by disk diffusion.

In the present study, *S. aureus* and coagulase-negative staphylococci (CoNS) from cases of bovine mastitis were comparatively investigated for their susceptibility to tylosin and erythromycin by broth microdilution and disk diffusion using the tylosin 30 μg disk. Moreover, emphasis was put on the detection of macrolide resistance genes and the analysis of inducibly resistant isolates since tylosin is a non-inducer and inducibly resistant isolates may be falsely recognized as susceptible.

2. Material and methods

2.1. Tylosin susceptibility testing

In total, 112 *S. aureus* isolates and 110 CoNS, all from cases of bovine mastitis during 2007–2010 from various diagnostic laboratories all over Germany, were included in this study. These isolates were from the strain collection of the German National Resistance Monitoring program GE*RM*-Vet and from diagnostic submissions to the Milchtierherden-Betreuungs- und Forschungsgesellschaft mbH (MBFG), Wunstorf, Germany. Most of the *S. aureus* isolates originated from cases of clinical mastitis while the majority of the CoNS was from cases of subclinical mastitis. Only one isolate per dairy farm was included to ensure that the isolates were not epidemiologically related. Most of the *S. aureus* isolates and CoNS have previously been used in studies on cefoperazone susceptibility (Feßler et al., 2012) and/or oxacillin susceptibility (Feßler et al., 2010a).

2.2. Antimicrobial susceptibility testing and detection of resistance genes

All isolates were tested in parallel by broth microdilution and agar disk diffusion according to the CLSI document VET01-A4 (CLSI, 2013a). For broth microdilution, custom-made microtitre plates (MCS Diagnostics, Swalmen, The Netherlands) were used which contained tylosin (0.06–128 μg/ml) and erythromycin (0.015–32 μg/ml) in 2-fold serial dilutions. Disk diffusion tests with tylosin 30 μg (Biolab, Budapest, Hungary) and erythromycin 15 μg disks (Oxoid, Wesel, Germany) also followed the aforementioned CLSI standard (CLSI, 2013a). *S. aureus* ATCC® 29213 and *S. aureus* ATCC® 25923 served as quality control strains. Scattergrams were constructed by plotting tylosin 30 μg zone diameters against tylosin MICs (Feßler et al., 2012). The same approach was conducted for the erythromycin 15 μg zone diameters and the corresponding erythromycin MICs.

Inducible resistance to non-inducers, such as 16-membered macrolides (tylosin, spiramycin) and lincosamides (pirlimycin, lincomycin, clindamycin), was assumed for isolates that showed high erythromycin MICs and small to no zone diameters around the erythromycin disk, but distinctly lower tylosin MICs and larger zone diameters around the tylosin disk. For such isolates, broth microdilution and disk diffusion were repeated after preincubation of the isolates in the presence of a subinhibitory concentration of 0.25 μg/ml erythromycin. Moreover, the CLSI-recommended screening test for inducible lincosamide resistance was conducted by placing a clindamycin 2 μg disk 15 mm away from the edge of the erythromycin 15 μg disk (CLSI, 2013b).

Isolates with elevated erythromycin MICs were tested by PCR for the presence of the macrolide resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(T), *mph*(C) and *msr*(A). The respective PCR primers and assay conditions have been described previously (Lüthje and Schwarz, 2006, 2007a; Feßler et al., 2010b).

3. Results and discussion

3.1. Tylosin susceptibility testing

The 112 *S. aureus* isolates showed a bimodal MIC distribution with tylosin MICs of 1 μg/ml ($n = 46$), 2 μg/ml ($n = 52$), 4 μg/ml ($n = 3$) and ≥256 μg/ml ($n = 11$). While the *S. aureus* isolates with MICs of ≥256 μg/ml did not show a zone of growth inhibition, the remaining *S. aureus* isolates had zone diameters of 20–29 mm around the 30 μg tylosin disks (Fig. 1a). Twelve *S. aureus* isolates were classified as erythromycin-resistant by their erythromycin MIC of ≥64 μg/ml and no zone diameter ($n = 11$) or 8 μg/ml and a zone diameter of 11 mm ($n = 1$) (CLSI, 2013b). The remaining isolates were classified as erythromycin-susceptible with MICs of 0.5 μg/ml ($n = 83$) or 0.25 μg/ml ($n = 17$) and zone diameters of 24–32 mm around the 15 μg erythromycin disks (Fig. 1b).

The 110 CoNS isolates showed a bimodal MIC distribution with tylosin MICs of 0.5 μg/ml ($n = 1$), 1 μg/ml ($n = 22$), 2 μg/ml ($n = 68$), 4 μg/ml ($n = 7$), 8 μg/ml ($n = 4$) and ≥256 μg/ml ($n = 8$). As seen for the *S. aureus* isolates, the CoNS with MICs of ≥256 μg/ml also did not display an inhibition zone while the remaining CoNS revealed zone diameters of 18–29 mm around the 30 μg tylosin disks (Fig. 2a). Among the CoNS, 13 isolates were classified as erythromycin-resistant by their erythromycin MIC of ≥64 μg/ml and no zone diameter ($n = 11$) or 32 μg/ml and zone diameters of 10 or 11 mm ($n = 2$) (CLSI, 2013b). Seven isolates were classified as intermediate by their erythromycin MICs of 2 μg/ml or 1 μg/ml, but classified as erythromycin-susceptible by their zone diameters of 24–34 mm (CLSI, 2013b). The remaining isolates with erythromycin MICs of 0.5 μg/ml ($n = 78$) or 0.25 μg/ml ($n = 12$) and zone diameters of 24–36 mm proved to be susceptible to erythromycin by either method (Fig. 2b).

3.2. Resistance genes

Eleven high-level erythromycin-resistant *S. aureus* isolates, which also showed tylosin MICs of ≥256 μg/ml were detected. None of these isolates harbored the macrolide ABC transporter gene *msr*(A) or the macrolide