Veterinary Microbiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis

Monika Entorf[a,b], Andrea T. Feßler[a,*], Heike Kaspar[c], Kristina Kadlec[a], 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

ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 30 November 2015
Accepted 9 December 2015

Keywords:
Macrolides
Disk diffusion
Broth microdilution
Inducible resistance
Resistance genes

ABSTRACT

Tylosin, a 16-membered macrolide, is – besides other indications – used for the treatment of bovine mastitis. So far, there is only limited information available on the tylosin susceptibility of streptococci isolated from mastitis. The aim of the present study was to comparatively investigate 303 streptococci from bovine mastitis, including 101 *Streptococcus agalactiae*, 100 *Streptococcus dysgalactiae* and 102 *Streptococcus uberis*, for their tylosin and erythromycin susceptibility by broth microdilution and agar disk diffusion. Both tests followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI). For erythromycin, the results were interpreted using the CLSI-approved clinical breakpoints. Moreover, erythromycin-resistant isolates were tested for the presence of macrolide resistance genes and for inducible macrolide resistance. In general, both testing methods showed a good correlation for the three streptococcal species, although for the erythromycin susceptibility testing 11 *S. uberis* isolates fell into the very major error category. All but one of the erythromycin-resistant isolates harbored at least one macrolide resistance gene, with the *erm*(B) gene being most common. Moreover, single isolates of *S. agalactiae* and *S. dysgalactiae* proved to be inducibly macrolide-resistant. Since inducible macrolide resistance can easily switch to constitutive resistance, tylosin should not be used for the treatment of infections caused by inducibly resistant streptococci.

1. Introduction

Bovine mastitis is an economically important disease among dairy cattle. According to Jones and Bailey (2009), bovine mastitis costs the U.S. dairy industry about 1.7–2 billion US dollars annually, which corresponds to 11% of the total U.S. milk production. *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* are considered as major pathogens in bovine mastitis (Denamiel et al., 2005; Persson et al., 2011; Pillar et al., 2014). *Escherichia coli* is intrinsically resistant to macrolides (CLSI, 2015b), which are therefore not a therapeutic option for *E. coli* infections. However, macrolides are used for the treatment of bovine mastitis caused by staphylococci and streptococci/enterococci. Tylosin, a 16-membered macrolide, is licensed for bovine mastitis therapy (http://www.vetidata.de; Entorf et al., 2014). Before or in parallel to the treatment, antimicrobial susceptibility testing (AST) of the causative isolates should be performed (http://www.fve.org/news/publications/pdf/antibioen.pdf), especially since considerable percentages of macrolide-resistant isolates have been detected among staphylococci and streptococci from bovine mastitis (Lüthje and Schwarz, 2006; Feßler et al., 2010; Lindeman et al., 2013; Pinto et al., 2013). In routine diagnostics, broth microdilution and agar disk diffusion are the most commonly used AST methods (Jorgensen et al., 1999; Schwarz et al., 2003).

As an internal control for AST, it is indispensable to test defined quality control (QC) strains side-by-side with the test isolates. Only if the QC strains are in the acceptable range for the antimicrobial agents tested, the test results can be regarded as valid. Acceptable ranges of the QC strains *Staphylococcus aureus* ATCC® 25923 and *Streptococcus pneumoniae* ATCC® 49519, *S. aureus* ATCC® 29213 and/or *Enterococcus faecalis* ATCC® 29212, approved by the Clinical and Laboratory Standards Institute (CLSI), have been available for many years for erythromycin and tylosin broth microdilution tests as well as for 15 μg erythromycin disks to be used in agar disk diffusion tests. In contrast, acceptable QC ranges for *S. aureus* ATCC® 25923 and 30 μg tylosin disks have recently

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

http://dx.doi.org/10.1016/j.vetmic.2015.12.003
0378-1135/

been determined (Buß et al., 2014), approved by CLSI and included into the CLSI document VET01-S (CLSI, 2015a). The availability of acceptable QC ranges for *S. aureus* ATCC® 25923 allows the lege artis performance of disk diffusion tests using 30 μg tylosin disks. Despite the availability of QC parameters for tylosin, there are still no approved clinical breakpoints for tylosin on the basis of which bacteria may be classified as susceptible, intermediate or resistant. The development of such clinical breakpoints requires besides susceptibility testing data, also information on clinical efficacy, dosing, route of administration of the antimicrobial agents, as well as species-specific pharmacokinetic and pharmacodynamic data (Schwarz et al., 2010).

Macrolide resistance among streptococci and staphylococci is commonly mediated by *erm* genes, whose gene products confer combined resistance to macrolides, lincosamides and streptogramin B antibiotics by methylation of the ribosomal binding site of these drugs. The *erm* genes can be expressed either inducibly or constitutively. While 14- and 15-membered macrolides can act as inducers, 16-membered macrolides – such as tylosin –, lincosamides, ketolides or streptogramins are not able to induce *erm* gene expression (Weisblum, 1995; Schmitz et al., 2002; Entorf et al., 2014). The *msr* genes confer resistance to macrolides and streptogramin B antibiotics and the *mef* genes confer resistance to macrolides via efflux while *mph* genes confer macrolide resistance by enzymatic inactivation (Lüthje and Schwarz, 2007).

The aim of this study was to comparatively investigate streptococci from bovine mastitis for their erythromycin and tylosin susceptibility by agar disk diffusion and broth microdilution and to investigate the resistant isolates for the resistance genes present.

2. Materials and methods

2.1. Streptococcal isolates from bovine mastitis

In total, 303 streptococcal isolates (101 *S. agalactiae*, 100 *S. dysgalactiae* and 102 *S. uberis*) from cases of clinical and subclinical bovine mastitis collected from farms all over Germany in the period of 2007–2010 were included in this study. These isolates originated from the strain collection of the German National Resistance Monitoring program GE*RM*-Vet and from diagnostic submissions to the mastitis diagnostic laboratory Milchtierherden-Betreuungs- und Forschungsgesellschaft mbH (MBFG = Dairy Herd Care and Research Company), Wunstorf, Germany. Since the testing of epidemiologically related isolates should be avoided, only one isolate per dairy farm was included. Most of the streptococcal isolates have previously been used in a study on cefoperazone susceptibility testing (Feßler et al., 2012).

2.2. Antimicrobial susceptibility testing

The susceptibility testing for the streptococcal isolates was performed in parallel by broth microdilution and agar disk diffusion according to the recommendations given in the CLSI document VET01-A4 (CLSI, 2013). *S. aureus* ATCC® 29213 and *S. aureus* ATCC® 25923 served as quality control strains for broth microdilution and agar disk diffusion, respectively. Custom-made microtitre plates (MCS Diagnostics, Swalmen, The Netherlands), which contained erythromycin (0.015–32 μg/ml) and tylosin (0.06–128 μg/ml) in 2-fold serial dilutions, were used for broth microdilution. Disk diffusion tests with erythromycin 15 μg disks (Oxoid, Wesel, Germany) and tylosin 30 μg (Biolab, Budapest, Hungary) also followed the aforementioned CLSI standard (CLSI, 2013). AST of the streptococci included the following specifications. The inoculum has been adjusted to McFarland standard 0.5. For agar disk diffusion, Mueller-Hinton agar plates supplemented with 5% lysed sheep blood were used. Zone diameters were read after incubation for 20–24 h at 35 °C ±2 °C. For broth microdilution, the inoculum was diluted to a final concentration of 5×10^5 cfu/ml in cation-adjusted Mueller-Hinton broth supplemented with 2.5–5% lysed horse blood. MIC results were read after incubation for 20–24 h at 35 °C ±2 °C (CLSI, 2015a). The following erythromycin breakpoints for agar disk diffusion and broth microdilution, respectively, were used to classify streptococci as susceptible (≥21 mm, ≤0.25 μg/ml), intermediate (16–20 mm, 0.5 μg/ml) or resistant (≤15 mm, ≥1 μg/ml) (CLSI, 2015a). Since there were no CLSI-approved clinical breakpoints for tylosin available, a classification of the isolates as susceptible, intermediate or resistant was not possible.

Inducible resistance to tylosin was assumed for isolates that were classified as erythromycin-resistant by at least one method, but had distinctly lower tylosin MICs and larger zone diameters around the tylosin disk. For such isolates, broth microdilution and agar disk diffusion were repeated after pre-incubation of the isolates in the presence of a subinhibitory concentration of 0.25 μg/ml erythromycin to see whether there was a change in the tylosin MIC values and/or zone diameters. Moreover, the CLSI-recommended screening test for inducible clindamycin resistance was conducted by placing a clindamycin 2 μg disk 15 mm away from the edge of the erythromycin 15 μg disk (CLSI, 2015a,b).

Table 1a
Characteristics of the 31 isolates, classified as erythromycin-resistant.

Number of isolates	Species	Category	Erythromycin		Tylosin		Macrolide resistance genes[a]			
			MIC (μg/ml)	Zone diameter (mm)	MIC (μg/ml)	Zone diameter (mm)	*erm*(B)	*erm*(C)	*mef*(A)	*msr*(D)
7	*S. agalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	–	+
3	*S. agalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	–	–
1	*S. agalactiae*	Resistant	≥64	≤6	≥256	≤6	+	+	–	–
1	*S. agalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	+	+
1	*S. agalactiae*	Resistant	8	15	128	≤6	–	–	–	+
1[b]	*S. agalactiae*	Resistant	4	12	4	19	+	–	–	+
1	*S. agalactiae*	Resistant	4	15	128	≤6	+	–	+	+
4	*S. dysgalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	–	–
2	*S. dysgalactiae*	Resistant	≥64	≤6	≥256	≤6	+	+	–	–
1	*S. dysgalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	+	+
1	*S. dysgalactiae*	Resistant	≥64	≤6	≥256	≤6	+	–	+	–
1	*S. dysgalactiae*	Resistant	≥64	≤6	64	16	–	–	–	–
1[b]	*S. dysgalactiae*	Resistant	≥64	≤6	4	18	+	–	–	–
5	*S. uberis*	Resistant	≥64	≤6	≥256	≤6	+	–	–	–
1[b]	*S. uberis*	Resistant	8	13	0.5	20	–	–	+	+

[a] The resistance genes *erm*(A), *erm*(T) and *msr*(A) were negative for all isolates tested and the results are therefore not displayed in the table.
[b] Isolates tested for inducible macrolide resistance.

Please cite this article in press as: M. Entorf, et al., Comparative erythromycin and tylosin susceptibility testing of streptococci from bovine mastitis, Vet. Microbiol. (2015), http://dx.doi.org/10.1016/j.vetmic.2015.12.003