

Proposal of Epidemiological Cutoff Values for Apramycin 15 µg and Florfenicol 30 µg Disks Applicable to *Staphylococcus aureus*

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Apramycin and florfenicol are two antimicrobial agents exclusively used in veterinary medicine. Resistance determinants to these antimicrobial agents have been described in several staphylococci, yet no inhibition zone-based epidemiological cutoff (ECOFF) values are available to detect populations harboring resistance mechanisms. In this study, we propose disk diffusion inhibition zone ECOFF values of *Staphylococcus aureus* for apramycin and florfenicol. The susceptibility to apramycin and florfenicol was evaluated by disk diffusion of five *S. aureus* collections, comprising 352 isolates of animal ($n = 265$) and human ($n = 87$) origin. The aggregated distributions of inhibition zone diameters were analyzed by the normalized resistance interpretation method to obtain normalized wild-type (WT) population distributions and corresponding ECOFF values. The putative WT populations of *S. aureus* were characterized by an inhibition zone ≥ 15 mm (ECOFF = 15 mm) for apramycin and ≥ 21 mm for florfenicol (ECOFF = 21 mm). Five nonwild-type (NWT) isolates were detected for apramycin, all without inhibition zone and harboring the *apmA* gene, whereas five NWT isolates were identified for florfenicol, all carrying the *fexA* gene. The proposed ECOFF values for apramycin and florfenicol may be a valuable tool in future antimicrobial resistance monitoring and surveillance studies to identify *S. aureus* NWT populations toward these antimicrobial agents.

Keywords: *Staphylococcus aureus*, epidemiological cutoff value, apramycin, florfenicol

Introduction

STAPHYLOCOCCUS AUREUS is an important pathogen in veterinary medicine, mainly associated with skin and soft-tissue infections in companion animals, mastitis in cattle and systemic infections in poultry.^{1,2} The past decades have witnessed an increasing prevalence of antimicrobial-resistant *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA) strains, in various animal species.³ As such, the occurrence of MRSA in animals has been identified as a microbiological hazard for human health.⁴

Apramycin is an aminocyclitol antimicrobial agent produced by *Streptomyces* spp. approved only for use in veter-

inary medicine for clinical purposes in Member States of the European Union.⁵ It is administered in oral formulations in feed or drinking water for the treatment of enteric infections caused by, for example, *Salmonella enterica* or *Escherichia coli* in pigs, calves, lambs, poultry, and rabbits.^{5,6} Florfenicol is a fluorinated thiamphenicol derivative. Similarly to apramycin, its use is restricted to veterinary medicine and it is administered parenterally for the treatment of respiratory bacterial infections in pigs, cattle, and ovine as well as in oral formulations in feed and drinking water for the treatment of bacterial infections in pigs, poultry, and fish.^{6,7} Florfenicol is also available, in combination with an antifungal and a steroid, for the topical treatment of otitis externa in dogs caused

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by *Staphylococcus pseudintermedius* and occasionally *S. aureus*.⁶ Importantly, both antimicrobial agents belong to classes that are considered as veterinary critically important antimicrobials,⁸ as well as critically or highly important antimicrobials in human medicine.⁹

Although apramycin is not used for the treatment of infections caused by staphylococci in animals, the presence of a resistance determinant to this antibiotic, *apmA*, was identified in MRSA isolates from cattle and pigs belonging to the clonal complex CC398.¹⁰ Since then, *apmA* has been described in MRSA isolates collected from a broiler,¹¹ from a nasal swab of a human working on a broiler farm and the environment of his residence¹¹ and from the environmental dust of a pig farm.¹² This gene was also identified in a *Staphylococcus lentus* (recently reclassified as *Mammaliococcus lentus*)¹³ isolate from a veal calf¹⁴ and in a *Staphylococcus sciuri* (recently reclassified as *Mammaliococcus sciuri*)¹³ isolate from an environmental sample of a pig farm.¹⁵ The *apmA* gene, which is usually located on small plasmids or large multiresistance plasmids,¹⁶ encodes an acetyltransferase and is associated with elevated minimum inhibitory concentrations (MICs) of apramycin (32 to ≥ 128 mg/L) and gentamicin (8 mg/L).^{10,12,16}

The florfenicol resistance gene *fexA* was first identified in a bovine *S. lentus* isolate.¹⁷ To date, this determinant has been described in various *S. aureus* and other staphylococcal isolates (including *S. pseudintermedius* and *Staphylococcus simulans*) collected from pigs,¹⁸ cattle,^{14,19} poultry,²⁰ cats,²¹ a dog,²² a horse,²³ and a marmot²⁴ as well as from soil surrounding pig farms.²⁵ The *fexA* gene has also been reported in MRSA isolates from nasal swabs of pig farmers.²⁶ This gene is located in the Tn558 transposon or its variants, which can be found on small or large plasmids,^{3,27} or even integrated in the staphylococcal chromosomal DNA.²³ It encodes the efflux pump FexA, a 14 transmembrane segment transporter of the Major Facilitator Superfamily and is associated with florfenicol MIC values ranging from 16 to 64 mg/L.^{17,23,28} Other determinants can convey resistance to florfenicol, namely, the *cfr*, *optrA*, and the *poxA* genes. The *cfr* gene encodes an rRNA methylase and was first identified in a bovine *S. sciuri* isolate,²⁹ but its presence is now reported in several staphylococci, including *S. aureus*, from various animal origins.³ It is usually located on plasmids but can also be found in the staphylococcal chromosome.³ The *optrA* gene codes for an ABC-F protein that has a ribosomal protection effect and has been described in *S. sciuri* isolates from cats, dogs, and pigs.³ Recently, it has also been reported in MRSA isolates collected from pigs and poultry.²⁷ The *poxA* gene codes also for an ABC-F protein, which, however, is only distantly related to *OptrA*.³⁰ Besides resistance to florfenicol, all three genes also mediate resistance to oxazolidinones. The gene *cfr* confers additional resistance to lincosamides, pleuromutilins, and streptogramin A.³ The *poxA* gene also confers elevated MICs to tetracycline.³⁰

Despite the occurrence of *S. aureus* strains carrying apramycin and florfenicol resistance genes, there are no inhibition zone-based epidemiological cutoff (ECOFF) values established to identify such strains in a *S. aureus* population during antimicrobial resistance surveillance studies. Hence, this study focused on proposing ECOFFs of *S. aureus* for apramycin and florfenicol based on inhibition zones that may allow a rapid identification of isolates with resistance mechanisms toward these two antimicrobial agents.

Materials and Methods

Bacterial isolates

The collection studied included 352 *S. aureus* isolates, 87 of human origin (from 2006 to 2007 and 2014), and 265 of animal origin (from 2001 to 2020). The isolates of human origin were collected from several infection sites of hospitalized patients ($n=53$)^{31,32} and ambulatory patients ($n=34$) in the Lisbon area. The *S. aureus* of animal origin were collected from cattle ($n=83$), dogs ($n=46$), cats ($n=44$), pigs ($n=41$), horses ($n=22$), rabbits ($n=16$), poultry ($n=6$), dolphins ($n=4$), and a bird ($n=1$). The animal host species was not known for two isolates.

These isolates were analyzed at five independent laboratories; two located in Portugal (cities of Lisbon [Lab1] and Oeiras [Lab2]) and three located in Germany (two in Berlin [Lab3, Lab4], another in Wunstorf [Lab5]). In total, the collection comprised 178 MRSA isolates and 174 methicillin-susceptible *S. aureus* isolates.

This study involved only bacterial strains that were already isolated and thus, no ethics approval was necessary.

Antimicrobial susceptibility testing

Inhibition zone diameters were determined for apramycin (15 μ g) and florfenicol (30 μ g) disks by the Kirby–Bauer method according to EUCAST (Lab1, for human isolates) or CLSI standards.^{33,34} Antibiotic disks were acquired from MAST Group Ltd. (Liverpool, United Kingdom) or Thermo Scientific™ Oxoid™ (Basingstoke, United Kingdom or Wesel, Germany). In brief, fresh overnight cultures were obtained for each isolate on Tryptic Soy Agar (Thermo Scientific Oxoid) or blood agar plates (Thermo Scientific Oxoid), from which isolated colonies were transferred to 0.85% (p/v) NaCl to obtain a cellular suspension with turbidity equivalent to 0.5 McFarland. The cellular suspension was swabbed onto Mueller–Hinton agar (Thermo Scientific Oxoid) plates and the antibiotic disks were placed on the inoculated media within 15 minutes. After 5 minutes, plates were inverted and placed in an incubator at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After incubation for 18–20 hours, inhibition zone diameters were measured in millimeters. In agreement with the CLSI and EUCAST recommendations, *S. aureus* ATCC®25923™ and *S. aureus* ATCC®29213™ were used as quality control strains in this study.^{33,34}

Determination of ECOFF values

The inhibition zone-based ECOFF values were estimated using the normalized resistance interpretation (NRI) method.^{35,36} This method uses the distributions of inhibition zone diameters to make a least-square regression analysis to determine the putative wild-type (WT) population, the mean inhibition zone diameter and the associated standard deviation (SD) for each species-antimicrobial agent combination. The ECOFF corresponds to the smallest inhibition zone diameter presented by the putative WT population and is calculated at $2.5 \times$ the SD above the mean value and rounded up to the lowest absolute value.^{35,36} Thus, the ECOFF allows the distinction between putative WT populations (devoid of phenotypically detectable acquired resistance mechanisms) and nonwild-type (NWT) populations (with phenotypically detectable acquired resistance mechanisms).³⁷ The ECOFF

estimated by the NRI method will include 99.4% of the WT population.³⁶ The NRI method was used with permission from the patent holder, Bioscand AB, TÄBY, Sweden (European Patent No. 1383913, U.S. Patent No. 7,465,559). The automatic and manual excel programs were made available through courtesy by P. Smith, W. Finnegan, and G. Kronvall at www.bioscand.se/nri/

The ECOFF values generated in this study are based on five data sets from five independent laboratories located in two countries, which provided data for 142 (Lab 1), 33 (Lab 2), 108 (Lab 3), 32 (Lab 4), and 37 (Lab 5) *S. aureus* isolates.

Results

The five data sets generated in this study were analyzed individually before aggregation (Supplementary Table S1). Each distribution was validated, abiding the minimum number required of WT isolates (at least $n = 15$)³⁷ and an SD below the acceptable SD upper limit of 3.38 mm, recommended by the NRI method.

The aggregated distributions of inhibition zone diameters of apramycin and florfenicol for the 352 *S. aureus* isolates included in this study are shown in Fig. 1. Both aggregated distributions were bimodal, with inhibition zone diameters ranging from 6 (=growth until the disk) to 30 mm for apramycin and from 9 to 38 mm for florfenicol. The aggregated distributions of the putative WT populations and associated ECOFF values of both antimicrobial agents were calculated using the NRI method (Table 1). Both normalized distributions of the WT populations were validated as they included >100 observations in the putative WT distribution³⁷ and the SDs were below the acceptable SD upper limit of 3.38 mm (Table 1).

For apramycin, the NRI analysis characterized a WT population ≥ 15 mm with an associated SD of 1.55 mm (Table 1). Applying the estimated ECOFF=15 mm, an NWT population was identified comprising five isolates (1.4%) collected from cattle ($n=2$) and pigs ($n=3$). All these isolates were MRSA, showed no inhibition zone, and carried the *apmA* gene for apramycin resistance.

For florfenicol, a WT population with inhibition zone diameters ≥ 21 mm was estimated with an associated SD of 2.30 mm (Table 1). The application of the calculated

ECOFF=21 mm identified an NWT population of 1.4%, corresponding to five MRSA isolates collected from cattle ($n=2$), pigs ($n=2$), and a dog ($n=1$). These isolates presented inhibition zone diameters ranging between 9 and 11 mm and all carried the *fexA* gene mediating resistance to florfenicol in staphylococci.¹¹

Discussion

The aggregated distribution analyzed in this study revealed an NWT population (1.4%) toward apramycin. This low frequency of NWT isolates is not surprising since dissemination of apramycin resistance determinants is still rare in *S. aureus* and is mainly associated with food-producing animals and humans with professional contact to them.^{3,11} In fact, all the five NWT isolates for apramycin in this study were collected from food-producing animals, either cattle or pigs. Apramycin is not affected by most aminoglycoside-modifying enzymes and in staphylococci, a single acetyl-transferase encoded by the *apmA* gene has been identified as mediating resistance to this antimicrobial agent.³ In this study, the five isolates carrying *apmA* showed no inhibition zone for apramycin. All isolates of human origin were categorized as WT for apramycin, in agreement with Truelson *et al.*, who analyzed the distribution of apramycin MICs for a collection of >100 *S. aureus* (mainly of human origin), proposing an MIC-based cutoff value of 32 mg/L and also finding no NWT population among human *S. aureus*.³⁸

For florfenicol, the application of the ECOFF value proposed in this study detected the presence of an NWT population (1.4%), comprising five isolates collected from cattle, pigs, and a dog, all harboring *fexA*. The NWT MRSA isolate of canine origin belongs to the clonal lineage ST398 and presents a multidrug resistance phenotype, showing additional resistance to fluoroquinolones and tetracyclines.³⁹ The ECOFF estimated in this study may complement the MIC-based ECOFF established by EUCAST for the combination florfenicol—*S. aureus*, ECOFF_{EUCAST}=8 mg/L.

The finding of NWT populations for these antimicrobial agents confirms the presence of apramycin and florfenicol resistance determinants in *S. aureus* isolates from food-producing and companion animals.

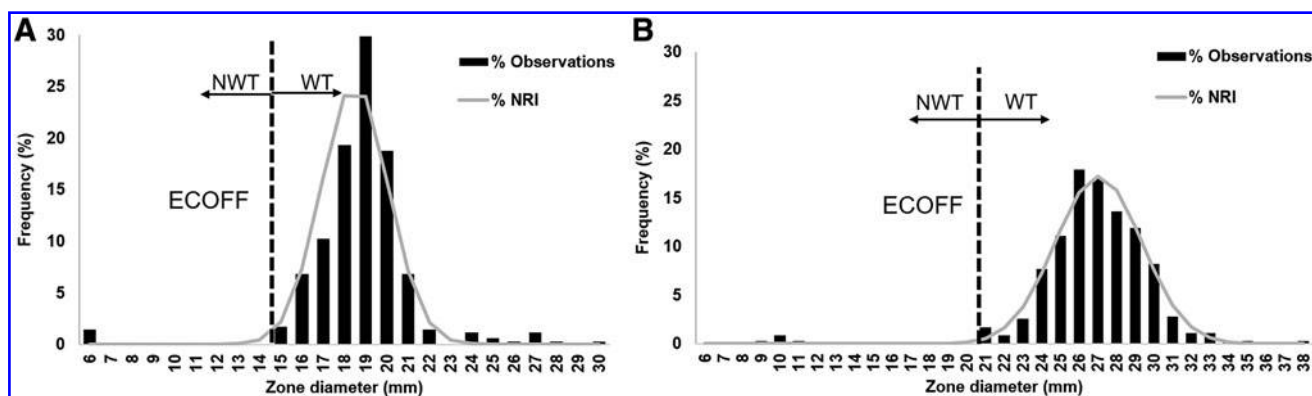


FIG. 1. Aggregated distributions of inhibition zone diameters of apramycin (A) and florfenicol (B) for the 352 *Staphylococcus aureus* isolates and respective estimated ECOFF values by the NRI method. Black columns represent the inhibition zone distributions, whereas the gray line indicates the NRI-generated normalized distribution of the WT populations. The estimated ECOFF values are shown by dashed vertical lines, whereas the WT and NWT populations are indicated by arrows. ECOFF, epidemiological cutoff; NRI, normalized resistance interpretation; NWT, nonwild-type; WT, wild-type.

TABLE 1. EPIDEMIOLOGICAL CUTOFF VALUES OF *STAPHYLOCOCCUS AUREUS* FOR APRAMYCIN AND FLORFENICOL ESTIMATED USING THE NORMALIZED RESISTANCE INTERPRETATION METHOD

	ECOFF (mm)	SD (mm)	WT population (mm)	NWT population (mm)
Apramycin	15	1.55	≥15	<15
Florfenicol	21	2.30	≥21	<21

The ECOFF values were determined based on the aggregated distributions of inhibition zone diameters for 352 *S. aureus* isolates.

ECOFF, epidemiological cutoff; NWT, nonwild-type; SD, standard deviation; WT, wild-type.

In this study, we propose ECOFF values for two antimicrobial agents used exclusively in veterinary medicine, apramycin and florfenicol, based on the inhibition zone diameter distributions obtained for a collection of 352 *S. aureus* independent isolates of animal and human origin from different geographic regions. The application of the proposed ECOFF values to other collections by other laboratories will be valuable in antimicrobial resistance monitoring and surveillance studies to identify *S. aureus* NWT populations toward these antimicrobial agents, in a One Health context.

Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Table S1

References

- Peton, V., and Y. Le Loir. 2014. *Staphylococcus aureus* in veterinary medicine. *Infect. Genet. Evol.* 21:602–615.
- Weese J.S. 2005. Methicillin-resistant *Staphylococcus aureus*: an emerging pathogen in small animals. *J. Am. Anim. Hosp. Assoc.* 41:150–157.
- Schwarz, S., A.T. Feßler, I. Loncaric, *et al.* 2018. Antimicrobial resistance among staphylococci of animal origin. *Microbiol. Spectr.* 6. DOI: 10.1128/microbiolspec.ARBA-0010-2017.

- Pomba, C., M. Rantala, C. Greko, *et al.* 2017. Public health risk of antimicrobial resistance transfer from companion animals. *J. Antimicrob. Chemother.* 72:957–968.
- European Medicines Agency (EMA). 2018. Reflection paper on use of aminoglycosides in animals in the European Union: development of resistance and impact on human and animal health. EMA/CVMP/AWP/721118/2014. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-use-aminoglycosides-animals-european-union-development-resistance-impact-human_en.pdf (accessed January 11, 2021).
- Direção Geral de Alimentação e Veterinária (DGAV). Medvet—Database of medicines and biocides of veterinary use. Lisbon, Portugal. Available at: <http://medvet.dgav.pt/> (Accessed February 20, 2020).
- European Medicines Agency (EMA). 2002. Committee for Veterinary Medicinal Products: summary report for florfenicol (extension to all food producing species). EMEA/MRL/822/02-Final. Available at: https://www.ema.europa.eu/en/documents/mrl-report/florfenicol-extension-all-food-producing-species-summary-report-6-committee-veterinary-medicinal_en.pdf (accessed January 11, 2021).
- World Organisation for Animal Health (OIE). 2019. OIE list of antimicrobial agents of veterinary importance. Available at: https://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/AMR/A_OIE_List_antimicrobials_July_2019.pdf (accessed January 11, 2021).
- World Health Organisation (WHO). 2019. Critically important antimicrobials for human medicine, 6th revision. Geneva: WHO. Available at: <https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1> (accessed January 11, 2021).
- Feßler, A.T., K. Kadlec, and S. Schwarz. 2011. Novel apramycin resistance gene *apmA* in bovine and porcine methicillin-resistant *Staphylococcus aureus* ST398 isolates. *Antimicrob. Agents Chemother.* 55:373–375.
- Wendlandt, S., K. Kadlec, A.T. Feßler, *et al.* 2013. Transmission of methicillin-resistant *Staphylococcus aureus* isolates on broiler farms. *Vet. Microbiol.* 167:632–637.
- Kadlec, K., A.T. Feßler, N. Couto, C. F. Pomba, and S. Schwarz. 2012. Unusual small plasmids carrying the novel resistance genes *dfrK* or *apmA* isolated from methicillin-resistant or -susceptible staphylococci. *J. Antimicrob. Chemother.* 67:2342–2345.
- Madhaiyan, M., J. S. Wirth, and V. S. Saravanan. 2020. Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promotion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliococcus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. *Int. J. Syst. Evol. Microbiol.* 70:5926–5936.
- Argudín, M.A., W. Vanderhaeghen, and P. Butaye. 2015. Diversity of antimicrobial resistance and virulence genes in methicillin-resistant non-*Staphylococcus aureus* staphylococci from veal calves. *Rev. Vet. Sci.* 99:10–16.
- Schoenfelder, S.M.K., Y. Dong, A.T. Feßler, S. *et al.* 2017. Antibiotic resistance profiles of coagulase-negative staphylococci in livestock environments. *Vet. Microbiol.* 200:79–87.
- Feßler, A.T., K. Kadlec, Y. Wang, *et al.* 2018. Small antimicrobial resistance plasmids in livestock-associated methicillin-resistant *Staphylococcus aureus* ST398. *Front. Microbiol.* 9:2063.

17. Kehrenberg, C., and S. Schwarz. 2004. *fexA*, a novel *Staphylococcus lentus* gene encoding resistance to florfenicol and chloramphenicol. *Antimicrob. Agents Chemother.* 48: 615–618.
18. Kadlec, K., R. Ehricht, S. Monecke, U. *et al.* 2009. Diversity of antimicrobial resistance pheno- and genotypes of methicillin-resistant *Staphylococcus aureus* ST398 from diseased swine. *J. Antimicrob. Chemother.* 64:1156–1164.
19. Feßler, A., C. Scott, K. Kadlec, R. Ehricht, S. Monecke, and S. Schwarz. 2010. Characterization of methicillin-resistant *Staphylococcus aureus* ST398 from cases of bovine mastitis. *J. Antimicrob. Chemother.* 65:619–625.
20. He, T., Y. Wang, S. Schwarz, Q. Zhao, J. Shen, and C. Wu. 2014. Genetic environment of the multi-resistance gene *cfr* in methicillin-resistant coagulase-negative staphylococci from chickens, ducks, and pigs in China. *Int. J. Med. Microbiol.* 304:257–261.
21. Haenni, M., P. Châtre, C. Dupieux-Chabert, *et al.* 2017. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in horses, cats, and dogs over a 5-year period in France. *Front. Microbiol.* 8:2493.
22. Couto, N., A. Belas, C. Rodrigues, S. Schwarz, and C. Pomba. 2016. Acquisition of the *fexA* and *cfr* genes in *Staphylococcus pseudintermedius* during florfenicol treatment of canine pyoderma. *J. Glob. Antimicrob. Resist.* 7: 126–127.
23. Kehrenberg, C., and S. Schwarz. 2006. Distribution of florfenicol resistance genes *fexA* and *cfr* among chloramphenicol-resistant *Staphylococcus* isolates. *Antimicrob. Agents Chemother.* 50:1156–1163.
24. Monecke, S., D. Gavier-Widén, H. Hotzel, *et al.* 2016. Diversity of *Staphylococcus aureus* isolates in European wildlife. *PLoS One.* 11:e0168433.
25. Zhao, Q., Y. Wang, S. Wang, *et al.* 2016. Prevalence and abundance of florfenicol and linezolid resistance genes in soils adjacent to swine feedlots. *Sci. Rep.* 6:32192.
26. Brennan, G.I., Y. Abbott, A. Burns, *et al.* 2016. The emergence and spread of multiple livestock-associated clonal complex 398 methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains among animals and humans in the Republic of Ireland, 2010–2014. *PLoS One.* 11:e0149396.
27. Li, S.M., Y. F. Zhou, L. Li, *et al.* 2018. Characterization of the multi-drug resistance gene *cfr* in methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from animals and humans in China. *Front. Microbiol.* 9:2925.
28. Couto, N., A. Belas, M. Centeno, E. van Duijkeren, and C. Pomba. 2014. First description of *fexA*-positive methicillin-resistant *Staphylococcus aureus* ST398 from calves in Portugal. *J. Glob. Antimicrob. Resist.* 2:342–343.
29. Schwarz, S., C. Werckenthin, and C. Kehrenberg C. 2000. Identification of a plasmid-borne chloramphenicol-florfenicol resistance gene in *Staphylococcus sciuri*. *Antimicrob. Agents Chemother.* 44:2530–2533.
30. Antonelli, A., M.M. D’Andrea, A. Brenciani, *et al.* 2018. Characterization of *poxTA*, a novel phenicol-oxazolidinone-tetracycline resistance gene from an MRSA of clinical origin. *J. Antimicrob. Chemother.* 3:1763–1769.
31. Costa, S.S., C. Falcão, M. Viveiros, *et al.* 2011. Exploring the contribution of efflux on the resistance to fluorquinolones in clinical isolates of *Staphylococcus aureus*. *BMC Microbiol.* 11:241.
32. Costa, S.S., C. Palma, K. Kadlec, *et al.* 2016. Plasmid-borne antimicrobial resistance of *Staphylococcus aureus* isolated in a Hospital in Lisbon, Portugal. *Microb. Drug Resist.* 22:617–626.
33. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2020. EUCAST disk diffusion method v 8.0. Available at: www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2020_manuals/Manual_v_8.0_EUCAST_Disk_Test_2020.pdf (accessed December 17, 2020).
34. Clinical Laboratory and Standards Institute (CLSI). 2020. VET01S ED5:2020—Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 5th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
35. Kronvall, G., G. Kahlmeter, E. Myhre, and M.F. Galas. 2003. A new method for normalized interpretation of antimicrobial resistance from disk test results for comparative purposes. *Clin. Microbiol. Infect.* 9:120–132.
36. Kronvall, G., and P. Smith. 2016. Normalized resistance interpretation, the NRI method: review of NRI disc test applications and guide to calculations. *APMIS.* 124:1023–1030.
37. The European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2019. MIC distributions and epidemiological cut-off values (ECOFF) setting. EUCAST SOP 10.1. European Committee on Antimicrobial Susceptibility Testing, Växjö, Sweden.
38. Truelson, K.A., T. Brennan-Krohn, K.P. Smith, and J.E. Kirby. 2018. Evaluation of apramycin activity against methicillin-resistant, methicillin-sensitive, and vancomycin-intermediate *Staphylococcus aureus* clinical isolates. *Diagn. Microbiol. Infect. Dis.* 92:168–171.
39. Couto, N., C. Monchique, A. Belas, C. Marques, L.T. Gama, and C. Pomba. 2016. Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period. *J. Antimicrob. Chemother.* 71:1479–1487.

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