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# Estimation of epidemiological cut-off values for eight antibiotics used for treatment of bovine mastitis caused by *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae*

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ABSTRACT

Interpretive criteria for antimicrobial susceptibility testing are lacking for most antimicrobials used for bovine streptococcal mastitis. The objectives of this study were to determine (tentative) epidemiological cut-off ((T)ECOFF) values for clinically relevant antibiotics used for treatment of bovine mastitis, and to estimate the proportion of acquired resistance (non-wild-types) in *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus uberis*. A total of 255 *S. uberis* and 231 *S. dysgalactiae* subsp. *dysgalactiae* isolates were obtained in Denmark and Norway from bovine mastitis. The isolates were tested for susceptibility to 10 antibiotics using broth microdilution. In accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard operating procedure, additional published MIC distributions were included for the estimation of ECOFFs for cloxacillin, cephapirin, lincomycin and tylosin, and TECOFFs for amoxicillin, benzylpenicillin, cephapirin and oxytetracycline. The proportion of non-wild-type (NWT) isolates for the beta-lactams was significantly higher in the Danish *S. uberis* (45–55%) compared to the Norwegian isolates (10–13%). For oxytetracycline, the proportion of NWT was significantly higher in the Danish isolates, both for *S. uberis* (28% vs. 3%) and *S. dysgalactiae* (22% vs. 0%). A bridging study testing in parallel MICs in a subset of isolates (n = 83) with the CLSI-specified and the EUCAST-specified broths showed excellent correlation between the MICs obtained with the two methods. The new ECOFFs and TECOFFs proposed in this study can be used for surveillance of antimicrobial resistance, and - for antimicrobials licensed for streptococcal bovine mastitis - as surrogate clinical breakpoints for predicting their clinical efficacy for this indication.

## 1. Introduction

Bovine mastitis accounts for most antimicrobial treatments in adult dairy cattle (Barlow, 2011; Ruegg, 2017; DANMAP 2022). Diagnosis of mastitis is typically based on bacterial culture and in certain cases followed by antimicrobial susceptibility testing (AST) of suspected pathogens. Correct interpretation of AST for bovine udder pathogens is, however, hampered by a shortage of host- and infection-specific clinical breakpoints (CBPs) for most of the antimicrobials available for bovine mastitis. Consequently, AST results for most antimicrobials are interpreted with alternative breakpoints specific for other infections, other animal species or for humans. Adding to this, some of the antimicrobials indicated for bovine mastitis are not used in other species. In those cases, the only option is to use AST for other antimicrobials belonging to the

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same class as surrogates. The use of non-specific breakpoints to guide antimicrobial therapy could lead to misleading interpretation of AST results, which may have severe consequences such as treatment failure and selection of antimicrobial resistance.

Development of CBPs is costly and time-consuming, involving production of pharmacokinetic (PK) and pharmacodynamic (PD) data as well as subsequent PK/PD modelling (Toutain et al., 2017). A simpler alternative that does not take into consideration drug PK properties, could be to use epidemiological cut-off values (ECOFFs) (Kahlmeter and Turnidge, 2022) for interpretation of Minimum Inhibitory Concentration (MIC) data. An ECOFF is defined as the highest MIC for wild-type organisms devoid of phenotypically detectable, acquired resistance mechanisms. ECOFFs can be established according to criteria provided by the European Committee for Antimicrobial Susceptibility Testing (EUCAST). One of these criteria is that an ECOFF should be based on MIC distributions deriving from at least five laboratories, and a tentative ECOFF (TECOFF) from three or four laboratories (EUCAST SOP 10.2, 2021). Whereas ECOFFs are used to predict acquired resistance, CBPs should always be preferred to predict clinical efficacy. Nevertheless, until CBPs become available, ECOFFs may be considered as surrogates to estimate clinical efficacy provided that corresponding medicinal products are known to be clinically effective against the wild-type of target pathogens.

*Streptococcus uberis* and *Streptococcus dysgalactiae* subspecies *dysgalactiae* (abbreviated to *S. dysgalactiae* in this paper) are among the most frequently isolated *Streptococcus* spp. from bovine mastitis (Riekerink et al., 2008; Bradley et al., 2015; Heikkilä et al., 2018; Astrup et al., 2022). EUCAST ECOFFs for these bovine streptococci have been defined for trimethoprim-sulfamethoxazole, but not for any of the other antimicrobials available for mastitis treatment in Europe. A complicating factor in producing additional ECOFFs is that MIC data for veterinary streptococci are typically produced using CLSI-specified broth medium, which differs slightly from that recommended by EUCAST.

The primary objective of this study was to generate data to propose (T)ECOFFs for clinically relevant antimicrobials used for treatment of bovine mastitis caused by *S. uberis* and *S. dysgalactiae*. A bridging study was performed for a subset of the isolates for comparison of results generated by the EUCAST and CLSI methodology. A secondary objective was to report the prevalence of acquired resistance in Norwegian and Danish bovine mastitis isolates representing these two bacterial species.

## 2. Materials and methods

### 2.1. Bacterial isolates

The project included 255 *S. uberis* and 231 *S. dysgalactiae* isolates obtained in Denmark and Norway from cases of clinical or subclinical bovine mastitis.

The Danish isolates (81 *S. uberis* and 78 *S. dysgalactiae*) were obtained from samples from large-scale randomized research studies, submitted during 2018–2021 to the Center for Diagnostics, Technical University of Denmark. If a bacterial species was isolated more than once from a farm, only the isolate from the first sample was included. Each isolate was identified by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). In both countries, *S. dysgalactiae* subsp. *dysgalactiae* was differentiated from other subspecies by observation of α-haemolysis.

The Norwegian isolates (174 *S. uberis* and 153 *S. dysgalactiae*) were retrieved from milk samples submitted to the routine mastitis diagnostic service at TINE Mastitis Laboratory. The laboratory performs all bacteriological analyses of milk samples in Norway from all over the country. The *S. uberis* isolates were obtained from 157 herds in 2021, while the *S. dysgalactiae* isolates were obtained from 143 herds in 2020. All isolates were identified at species level by MALDI-TOF (Bruker Daltonics). The Norwegian isolates were sent to the Norwegian Veterinary Institute for further susceptibility testing.

### 2.2. Antimicrobial susceptibility testing

All isolates were subjected to antimicrobial susceptibility testing using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, 2023). The CLSI methodology was chosen to enable inclusion of data from previous studies in the estimation of (T)ECOFFs. A custom-made Sensititre panel from ThermoFisher Scientific (Waltham, Massachusetts, USA) was used for MIC testing. The panel contains two-fold dilution ranges of antimicrobials available in Denmark and Norway for treatment of Gram-positive udder infections in cattle: amoxicillin (0.004–4 mg/L), benzylpenicillin (0.002–2 mg/L), cloxacillin (0.015–2 mg/L), cephapirin (0.004–8 mg/L), cefalexin (0.03–16 mg/L), tylosin (0.03–8 mg/L), lincomycin (0.03–8 mg/L), streptomycin (0.5–64 mg/L), trimethoprim-sulfamethoxazole (0.008/0.15–1/19 mg/L), and oxytetracycline (0.063–8 mg/L). This project also included MIC data from the EJP IMPART study (Veldman, 2019), where different Sensititre panels were applied.

Similar cultivation methods were used in this study and in the IMPART study: For each batch of Sensititre panels and media, batch control was performed at least weekly with the reference strain *Streptococcus pneumoniae* ATCC 49619, during the trial period. After thawing, each bacterial isolate was cultivated on a 5% calf blood agar, incubated at 35–37 °C overnight (18–20 h) in ambient air supplemented with 5% $CO_2$. Ready-made cation-adjusted Mueller Hinton Broth with lysed horse blood (Ca-MHB-LHB) from ThermoFisher Scientific, with between 2.5–5% lysed horse blood was used for inoculation of all the IMPART isolates, the Norwegian isolates, and the Danish *S. dysgalactiae*. For the Danish *S. uberis*, cation-adjusted Mueller Hinton Broth (Ca-MHB) mixed with 3.5% lysed horse blood (both from ThermoFisher Scientific) was applied. For each isolate, a McFarland 0.5 adjusted suspension in sterile saline was prepared. To obtain the required bacterial concentration of 5 $\times 10^5$ cfu/ml (2 $\times 10^5$ – 7 $\times 10^5$ cfu/ml), a volume, A (see below), of the suspension was mixed with 11 ml of Ca-MHB-LHB. The Sensititre panel was inoculated with 100 µl inoculum per well, and the plates were sealed. All isolates were incubated at 35–37 °C for 20–24 h in ambient air. Initially, A= 100 µl was applied for five isolates of each species followed by quantification of the concentration of the positive control well by serial dilution and plate counting on blood agar. For each laboratory, the volume A was then adjusted to ensure that the concentration was within the required range (2 $\times 10^5$ – 7 $\times 10^5$ cfu/ml). In the Danish and Norwegian laboratories, the transfer volume A was 80 µl (DK) or 100 µl (N) for *S. uberis* and 100 µl (DK, N) for *S. dysgalactiae*. Within the IMPART project, a transfer volume varied between 55 µl – 100 µl between the different laboratories for both species. For each species, the bacterial concentration of the inoculum was controlled by colony counts once a week during the trial period. Purity control was performed for all panels by streaking 10 µl of the inoculated Ca-MHB-LHB onto a blood agar plate, with 18–24 h incubation at 37 °C in ambient air.

### 2.3. Epidemiological cut-off values

ECOFF and TECOFF values were estimated following the methodology described in EUCAST SOP 10.2 (EUCAST, 2021). In brief, each individual MIC distribution is run through the 'ECOFFinder' algorithm (Turnidge et al., 2006) to estimate 99.9% ECOFF of each contributing distribution (log2 value). The means and standard deviations of contributing 99.9% ECOFFs are calculated (using their log2 values). The (T)ECOFF is the log2 mean after conversion to its arithmetic value and rounding up to the next two-dilution. The log2 standard deviation is used to determine the 95% confidence interval (on the log2 scale), after which the lower and upper values of the interval are converted to their numerical vales and rounded down (lower value) and up (upper value) to the next two-fold dilution.

The MIC distributions determined in the Danish and Norwegian laboratories were supplemented with MIC distributions (mostly deriving from IMPART) published on the EUCAST website (https://mic.eucast.org/, accessed May 2023). Additional relevant MIC distributions were identified in PubMed (www.ncbi.nlm.nih.gov/) by using all combinations of bacterial species and each antimicrobial agent (one combination at a time) in the search string. In accordance with EUCAST SOP 10.2, MIC distributions were only used for the setting of (T)ECOFFs if they fulfilled the following criteria:

- the assumed wild-type distributions should visually approximate log-normal distributions,
- the dilution ranges should include at least two dilutions below the wild-type mode,
- the modes of the wild-type distributions from the different laboratories should be identical or deviate no more than one two-fold dilution,
- the distributions should have at least 15 isolates in the wild-type distribution.

### 2.4. Proportion of non-wild-type isolates

The estimated (T)ECOFF values were used to estimate the proportion of acquired resistance (non-wild-type – NWT) in the Norwegian (2020–2021) and the Danish isolates (2018–2021), because these isolate selections were considered representative for the respective populations. For this comparison, only one isolate per herd was included. The proportion of resistance in the Danish and Norwegian isolates was compared using the Yates $\chi^2$ test (two tailed, with a significance level at 0.05). When the expected number within one category was $< 5$, the Fishers Exact test was used for comparing two proportions.

### 2.5. Bridging study

MIC data resulting from studies using CLSI methodology may be used for setting ECOFFs only if the resulting distributions are similar to distributions from studies using EUCAST methodology. EUCAST determines whether such data are applicable. In order to compare MICs obtained using the CLSI and the EUCAST methodology for streptococci, a bridging study with a subset of the Danish isolates (41 *S. uberis* and 42 *S. dysgalactiae*) was performed in the Danish laboratory. MICs were determined twice for each isolate with the only difference between replicates being the use of EUCAST-specified MH-F broth (from the microbiology laboratory at Herlev Hospital[1]) vs. the use of CLSI-specified CA-MHB-LHB. The testing was performed in parallel, i.e., using the same bacterial suspension (transfer suspension), with incubation at the same time, in the same incubator. Comparison of MIC distributions was undertaken according to the International organization for Standardization (ISO) (Anonymous, 2021).

## 3. Results

Additional comprehensive MIC distributions were published and available from the VetPath project (Thomas et al., 2015; de Jong et al., 2018; El Garch et al., 2020). For oxytetracycline, the available MIC distributions from McDougall and associates (2014) were included, while no other relevant distributions were available. Additionally, for *S. dysgalactiae*, a MIC distribution for cloxacillin was obtained from McDougall et al. (2014).

[1] www.herlevhospital.dk/english/Sider/default.aspxhttps://research.regionh.dk/en/organisations/klinisk-mikrobiologi

### 3.1. Streptococcus uberis

All the included MIC distributions for *S. uberis* isolates are available in Supplementary Table S1. The corresponding aggregated MIC distributions are shown in Supplementary Figs. S1-S9.

The mode for the assumed wild-type distribution was the same for the Danish and Norwegian distributions, except for a one dilution step difference for oxytetracycline (Table 1 and S1).

MIC distributions from EUCAST, VetPath (Thomas et al., 2015; de Jong et al., 2018; El Garch et al., 2020) and McDougall et al. (2014) were also included to achieve data from five or at least three laboratories for each (T)ECOFF estimation.

Table 1 shows the estimated (T)ECOFFs based on the analysis of all collected distributions. For *S. uberis*, the MIC distributions (Supplementary Figs. S1-S9) were clearly bimodal for most of the antimicrobials. Only for cefalexin and trimethoprim-sulfamethoxazole, the distributions were unimodal, while for lincomycin an apparent

**Table 1**
Proposed ECOFFs and TECOFFs[a] for *Streptococcus uberis*.

| Antimicrobial agent (no. of distributions) | Mode range[b] (mg/L) | (T) ECOFF (mg/L)[c] | 95% Confidence Interval for (T)ECOFF (mg/L) | Additional data[a] |
|---|---|---|---|---|
| Oxytetracycline (3) | 0.25–0.5 | (1) | 0.5–1 | McDougall et al. (2014) |
| Amoxicillin (3) | 0.03–0.06 | (0.125) | 0.06–0.25 | El Garch et al. (2020) |
| Benzylpenicillin (4) | 0.03 | (0.125)[d] | 0.03–0.25 | Thomas et al. (2015), deJong et al. 2018, El Garch et al. (2020). |
| Cloxacillin (9) | 0.25–0.5 | 1 | 0.25–2 | Thomas et al. (2015), deJong et al. 2018, El Garch et al. (2020), EUCAST |
| Cephapirin (3) | 0.06 | (0.125) | 0.06–0.25 | Thomas et al. (2015), deJong et al. 2018. |
| Cephalexin (5) | 0.25–0.5 | 1 | 0.5–2 | Thomas et al. (2015), deJong et al. 2018, El Garch et al. (2020), EUCAST |
| Trimethoprim-sulfamethoxazole (1:19) (8) | 0.125–0.25 | 0.5 | 0.25–1 | EUCAST |
| Tylosin (5) | 0.5–1 | 2 | 0.5–8 | El Garch et al. (2020), EUCAST |
| Lincomycin (9) | 0.06–0.25 | 0.25[e] | 0.06–0.5 | El Garch et al. (2020), EUCAST |
| Streptomycin (2) | 64–128 | Na[f] | Na[e] | |

[a] (T)ECOFFs were estimated from weighted aggregated data from the Norwegian Veterinary Institute and the Center for Diagnostics (DK) and supplemented with published data from at least one additional laboratory.
[b] The range of mode values from the included MIC distributions.
[c] TECOFFs are displaying in brackets.
[d] Due to the overlap of the WT and NWT distributions, a TECOFF at 0.06 mg/L may be more appropriate.
[e] Visual inspection indicates that a TECOFF at 0.5 is more accurate (supplemental Table S9)
[f] Not analyzed since only two distributions were available.

multimodal distribution was observed.

The estimated (T)ECOFFs were applied to evaluate occurrence of NWT isolates in the individual distributions from Norway and Denmark, respectively (Table 2). Very few NWT isolates were observed among the Norwegian isolates. A significantly higher proportion of NWT was observed for oxytetracycline, amoxicillin, benzylpenicillin, cloxacillin, cephapirin, and lincomycin (Table 2) among the Danish isolates, compared to the Norwegian isolates.

### 3.2. *Streptococcus dysgalactiae subsp. dysgalactiae*

All the included MIC distributions for *S. dysgalactiae* isolates are available in Supplementary Table S2. The corresponding aggregated distributions are shown in Supplementary Figs. S10-S18.

The mode for the assumed wild-type distribution was the same for the Danish and Norwegian distributions, except for amoxicillin, for which the mode was one dilution step higher (0.03 mg/L) in the Danish distribution (Table 3). The modes found in the additional MIC distributions from VetPath (El Garch et al., 2020; de Jong et al., 2018) and McDougall et al. (2014) were either identical or deviating no more than one two-fold dilution from the modes of the Norwegian and Danish MIC distributions (Supplementary Table S2).

Table 3 shows the estimated (T)ECOFFs based on the analysis of all collected distributions. The aggregated MIC distributions (Supplementary Tables S10-S18) were unimodal for most of the antimicrobials. One exception was oxytetracycline for which we detected a wild-type mode at MIC= 2 mg/L and a second peak at MIC= 8 mg/L.

The estimated (T)ECOFFs were used to evaluate the occurrence of NWT isolates in the individual distributions from Norway and Denmark, respectively (Table 4). The occurrence of NWT isolates was at a very low level in both Danish and Norwegian isolates, except for a significantly higher proportion of oxytetracycline NWT among Danish isolates.

### 3.3. *Bridging study*

Correlation between broth microdilution testing using CLSI- and EUCAST-specified media is shown in Table 5. There were high rates of essential agreement (>90%) for all correlations, and all wild-type modes were within one two-fold dilution of each other. This is within the acceptance criteria for inclusion in (T)ECOFF analysis according to EUCAST SOP 10.2 (EUCAST, 2021).

**Table 2**
Occurrence[a] of non-wild-type (NWT) *Streptococcus uberis* from Denmark and Norway.

| | Percent non-wild-type[b] | | $X^2$-value | p-value[c] |
|---|---|---|---|---|
| | Norway | Denmark | | |
| Oxytetracycline | 3.2 | 28 | 30.3 | < 0.001 |
| Amoxicillin | 10 | 53 | 50.4 | < 0.001 |
| Benzylpenicillin | 6.4 | 51 | 59.5 | < 0.001 |
| Cloxacillin | 10 | 56 | 55.3 | < 0.001 |
| Cephapirin | 10 | 52 | 48.1 | < 0.001 |
| Cephalexin | 1.3 | 4.9 | – | 0.047 |
| Trimethoprim-sulfamethoxazole (1:19) | 3.8 | 0 | – | 0.098 |
| Tylosin | 4.5 | 3.7 | – | 1.0 |
| Lincomycin | 10 | 27 | 10.2 | 0.001 |
| No. isolates tested | 157 | 81 | – | – |

[a] Only one isolate per herd was included.
[b] (T)ECOFF's estimates (Table 1) were applied.
[c] Fishers exact test (2-sided) was applied for cefalexin, trimethoprim-sulfamethoxazole and tylosin.

**Table 3**
Proposed ECOFFs and TECOFFs[a] for *Streptococcus dysgalactiae* subsp. *dysgalactiae*.

| Antimicrobial agent (no. of distributions) | Mode range[b] (mg/L) | (T) ECOFF (mg/L)[c] | 95% Confidence Interval (mg/L) | Additional data[a] |
|---|---|---|---|---|
| Oxytetracycline (3) | 2 | (8) | 4–16 | McDougall et al. (2014) |
| Amoxicillin (3) | 0.016–0.03 | (0.03)[d] | 0.008–0.125 | El Garch et al. (2020) |
| Benzylpenicillin (4) | 0.008 | (0.016) | 0.004–0.016 | deJong et al. 2018, El Garch et al. (2020) |
| Cloxacillin (8) | 0.125 | 0.25 | 0.125–0.25 | McDougall et al. (2014), EUCAST |
| Cephapirin (3) | 0.06 | (0.06)[e] | 0.03–0.125 | deJong et al. 2018 |
| Cephalexin (5) | 0.25–0.5 | 1 | 0.25–1 | deJong et al. 2018, El Garch et al. (2020), EUCAST |
| Trimethoprim-sulfamethoxazole (1:19) (7) | 0.06–0.125 | 0.25 | 0.06–0.25 | EUCAST |
| Tylosin (5) | 0.25 | 0.5 | 0.125–1 | deJong et al. 2018, El Garch et al. (2020), EUCAST |
| Lincomycin (9) | 0.125–0.25 | 0.5 | 0.125–0.5 | El Garch et al. (2020), EUCAST |
| Streptomycin (2) | 16 | Na[f] | Na[f] | |

[a] (T)ECOFF were estimated from weighted aggregated data from the Norwegian Veterinary Institute and Center for Diagnostics (DK) and supplemented with data from at least one additional laboratory.
[b] The range of mode values from the included MIC distributions.
[c] TECOFFs are displaying in brackets.
[d] Visual inspection indicates that a TECOFF at 0.06 mg/L may be more appropriate.
[e] Visual inspection indicates that a TECOFF at 0.125 mg/L may be more appropriate.
[f] Not analyzed since only two distributions were available.

**Table 4**
Occurrence[a] of non-wild-type (NWT) *Streptococcus dysgalactiae* subsp. *dysgalactiae* from Denmark and Norway.

| | Percent NWT[b] | | $X^2$-value | p-value[c] |
|---|---|---|---|---|
| | Norway | Denmark | | |
| Oxytetracycline | 0 | 22 | 30.4 | < 0.001 |
| Amoxicillin | 0.7 | 2 | – | 0.30 |
| Benzylpenicillin | 0.7 | 0 | – | 1.0 |
| Cloxacillin | 1.4 | 1.2 | – | 1.0 |
| Cephapirin | 1.4 | 6 | – | 0.01 |
| Cephalexin | 0.7 | 0 | – | 1.0 |
| Trimethoprim-sulfamethoxazole (1:19) | 0.7 | 0 | – | 1.0 |
| Tylosin | 3.5 | 6.2 | – | 0.50 |
| Lincomycin | 0.7 | 2.5 | – | 0.30 |
| No. isolates tested | 143 | 81[d] | | |

[a] Only one isolate per herd was included.
[b] For trimethoprim-sulfamethoxazole, the EUCAST ECOFFs were applied. For all other antimicrobials, the estimated (T)ECOFF (Table 3) were applied.
[c] Fishers exact test (two-sided) has been applied for all but oxytetracycline.
[d] For oxytetracycline, two of the Danish isolates were not tested.

**Table 5**
Agreement analysis on CLSI versus EUCAST-specified broths for determination of the minimum inhibitory concentration for *Streptococcus* spp.

| Antimicrobial agent | *S. uberis* | | *S. dysgalactiae* subsp. *dysgalactiae* | |
|---|---|---|---|---|
| | Essential Agreement % | Bias % | Essential Agreement % | Bias % |
| Oxytetracycline | 100 | -29.3[a] | 97.5 | -47.1 |
| Amoxicillin | 90.2 | + 73.4 | 100 | + 69.0 |
| Benzylpenicillin | 97.5 | + 50.0 | 100 | + 14.2[a] |
| Cloxacillin | 100 | + 43.1 | 100 | + 9.5[a] |
| Cephapirin | 97.6 | + 4.9[a] | 100 | + 2.4[a] |
| Cefalexin | 100 | 26.8 | 97.6 | -47.6 |
| Trimethoprim-sulfamethoxazole | 92.7 | + 58.5 | 100 | 0[a] |
| Tylosin | 97.6 | + 7.7[a] | 97.6 | + 30.1 |
| Lincomycin | 92.7 | -1.3[a] | 100 | + 60.1 |
| Streptomycin | 100 | + 4.0[a] | 100 | + 8.0[a] |

The analysis followed the methods described in ISO 20776–2 (2021).
[a] Bias within ISO 20776–2 acceptance criteria.

## 4. Discussion

In this study, ECOFFs for cephapirin, cloxacillin, lincomycin and tylosin, and TECOFFs for amoxicillin, benzylpenicillin and cephapirin and oxytetracycline were proposed for *S. uberis* and *S. dysgalactiae*. Furthermore, the results for trimethoprim-sulfamethoxazole corroborated the established EUCAST ECOFFs for this combination. These antimicrobials represent most of the veterinary antimicrobial products registered for mastitis treatment in Scandinavia and other European countries. The (T)ECOFFs are most relevant for surveillance of antimicrobial resistance, whereas CBPs should be preferred to predict clinical outcomes. (T)ECOFFs can only be used to distinguish wild-type isolates from isolates with acquired resistance and cannot by themselves predict clinical outcome. Nevertheless, when an antimicrobial product is licensed for treatment of bovine mastitis caused by these specific streptococci, it is likely that therapeutic exposures effective against the wildtype can be achieved with the approved dosage. In those cases, ECOFFs may be used as surrogate CBPs to guide mastitis treatment.

All MIC distributions included in the (T)ECOFF analysis were generated using the CLSI-specified medium. EUCAST-specified medium for streptococci is very similar to that of CLSI, except that it is supplemented with 20 mg/L β-NAD. It was considered unlikely that this difference would result in meaningful differences in distributions, but in order investigate this, we performed a bridging study on a subset of the Danish isolates for each species. This comparison was favourable, meaning that the estimated (T)ECOFFs are equally useful for MIC data generated by the EUCAST-specified MH-F broth.

Visual inspection of MIC distributions is an important second step in (T)ECOFF estimation, as described in EUCAST SOP 10.2 (EUCAST, 2021). Visual evaluation of the distributions displayed in Supplemental Figs. S1-S19 generally supported the estimated (T)ECOFFs. However, some of the wild-type distributions were only 2–3 dilutions wide. Consequently, for some of the antimicrobials, the estimated (T)ECOFF may be too low in an international (trans-laboratory) perspective, especially when the estimates are based on data from only three laboratories and do not represent a potentially larger variation across laboratories. For *S. uberis*, particularly the lincomycin estimated ECOFF of 0.25 mg/L may be too low, as by visual inspection, a cut-off at 0.5 mg/L seems more appropriate (Fig. S9). Similarly, for *S. dysgalactiae*, the estimated TECOFFs for amoxicillin and cephapirin were determined by ECOFFinder to be 0.03 mg/L and 0.06 mg/L, respectively, but visually, two-fold higher TECOFFs seem more accurate (Figs. S11 and S15). This underscores the importance of including data from multiple laboratories for determining cut-off values. Visual inspection also reveals that for some antimicrobials, the NWT and the WT distributions are in very close proximity or even overlap, and in some cases the proposed TECOFF may need adjusting to detect the NWT more confidently. For example, for *S. uberis*, a TECOFF for benzylpenicillin at 0.06 mg/L appears more appropriate than the calculated value of 0.125 mg/L (Fig. S3).

The proportion of NWT isolates was generally higher among the Danish compared to the Norwegian *S. uberis* isolates (Table 2). We discuss here the beta-lactams, as these – benzylpenicillin in particular – constitute by far the most widely used antimicrobial agents for bovine mastitis in Denmark and Norway (DANMAP 2022; NORM/NORM-VET 2021). Regarding the penicillins (benzylpenicillin, amoxicillin and cloxacillin) and cephapirin, approximately half of the Danish isolates were NWT, which was significantly higher than the 6–10% observed among the Norwegian isolates. Although streptococci are often considered "intrinsically susceptible" to penicillins (Anonymous, 2015), penicillin-resistant *S. uberis* isolates have been described in several countries within the recent years (Zhang et al., 2022). The high proportion of benzylpenicillin NWT in the Danish isolates is similar to the 44% NWT found among 61 isolates in Switzerland in 2019. Interestingly, and for unknown reasons, the proportion of benzylpenicillin NWT among Swiss *S. uberis* isolates declined to 31% and 14% in 2020 and 2021, respectively (Anon, 2022). A study by de Jong et al. (2018) reported no resistance to benzylpenicillin among 188 *S. uberis* bovine mastitis isolates from across Europe, based on a human CBP (R>2 mg/L). By re-calculating their data according to the proposed TECOFF (0.06 mg/L), 62% of isolates were NWT. This example illustrates that extreme caution should be taken when comparing susceptibility data from different studies. It is yet unknown whether the high proportion of penicillin NWT in Danish isolates has any clinical implications, but the MIC levels and proportion of NWT should be closely monitored ahead for potentially increasing trends. This emphasizes the importance of continuing surveillance and initiating research trying to relate the MIC to clinical outcome in cattle, and to establish CBPs.

The higher proportion of beta-lactam NWT for Danish *S. uberis* isolates suggests differences in antimicrobial usage patterns between the two countries. In Denmark, the 1st generation cephalosporins cephapirin and cephalexin have for more than a decade been frequently used for dry-cow treatment, accounting for approximately one fifth of such treatments in recent years (DANMAP 2022). On the contrary, these antimicrobials are not used for treatment of cattle in Norway according to the annual NORM/NORM-VET reports. In these reports, antimicrobial prescription statistics are only partially divided into different species, therefore the use of beta-lactams for cattle in the two countries cannot be compared. There are important structural differences between the dairy production in Denmark and Norway, which may explain differences in antimicrobial use and resistance between the two countries (Rajala-Schultz et al., 2021). For example, the Danish herds are generally larger, and are dominated by Holstein-Friesian cattle, whereas in Norway the Norwegian Red cattle predominate. Due to this species difference, the milk-yield is considerably higher in the Danish dairy cattle and the somatic cell-counts are also higher (Rajala-Schultz et al., 2021).

For *S. dysgalactiae*, the proportion of NWT was low for most antibiotics in both countries (Table 4). The only exception was oxytetracycline for which the proportion of NWT was 22% among Danish isolates. This is significantly higher than the 0% in Norwegian isolates, but lower than the 43% we calculated with our proposed TECOFF of 8 mg/L (applied on tetracycline data) in 227 bovine isolates from across Europe (El Garch et al., 2020; de Jong et al., 2018). In Denmark, tetracyclines are rarely used for treating mastitis but have for decades been one of the two most frequently used antimicrobials for respiratory infections in calves (Fertner et al., 2016), hence it is possible that usage at an early age has selected for the resistance observed in the adult cattle population. In contrast, the absence of tetracycline NWT in isolates from Norway could be because tetracyclines have constituted less than 5% of prescriptions (in kg active compound) for terrestrial food producing animals since the

1990's according to the Norwegian surveillance on antimicrobial usage (NORM/NORM-VET 2021).

## 5. Conclusion

(T)ECOFFs for *S. uberis* and *S. dysgalactiae* were proposed for most of the antimicrobials licensed for treatment of bovine mastitis in Europe. These (T)ECOFFs can be considered as the second-best option for interpretation of antimicrobial susceptibility testing until CBPs for bovine mastitis become available, to guide treatment with antimicrobial products licensed for streptococcal bovine mastitis. The bridging study successfully showed concordance between the CLSI and EUCAST method for MIC testing of *S. uberis* and *S. dysgalactiae*. In order to facilitate future comparison of MIC data from different sources, it would be advisable if the two breakpoint-setting organisations strive to further harmonize their methods, similar to the effort of the CLSI-EUCAST joint disk diffusion working group. While low levels of NWT were detected in *S. dysgalactiae*, high proportions of NWT isolates were found - particularly for beta-lactams - in *S. uberis* isolates from Danish herds. The clinical relevance of these susceptibility results remains to be determined, but results illustrate the importance of monitoring susceptibility trends over time and of relating pathogen susceptibility to clinical outcome.

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## CRediT authorship contribution statement

**Jensen Vibeke Frøkjær:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Damborg Peter:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualisation. **Norström Madelaine:** Writing – original draft, Validation, Software, Formal analysis, Data curation. **Nonneman Bettina:** Writing – original draft, Data curation, methodology, Investigation. **Slettemås Jannice Schau:** Writing – original draft, Project administration, Investigation, Methodology, Data curation. **Smistad Marit:** Writing – original draft, Investigation, Methodology. **Sølverød Liv:** Writing – original draft, Investigation, Methodology. **Turnidge John:** Writing - original draft, validation, Supervision, Software, Methodology, Formal analysis, Data curation. **Urdahl Anne Margrete:** Writing – original draft, Project administration, Investigation, Funding acquisition. **Kees Veldman:** Writing – original draft, Project administration, Investigation, Methodology, Data curation. **Van Essen-Zangenberg Alieda:** Investigation, Data Curation. **Astrup Lærke Boye:** Writing – original draft, Data curation, methodology, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors of the manuscript entitled "Estimation of epidemiological cut-off values for eight antibiotics used for treatment of bovine mastitis caused by *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae*" declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2024.109994.

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