ABSTRACT

The objective of this study was to describe the in vitro resistance of *Staphylococcus (S.) aureus* from bovine quarter milk samples obtained by the udder health laboratory of the Bavarian Animal Health Services between 2012 and 2022. All *S. aureus* samples were tested for β-lactamase production and only forwarded to further microbroth susceptibility testing either if the β-lactamase result was positive or upon explicit request by the submitter.

The growth of most *S. aureus* isolates was inhibited at the lowest evaluated minimum inhibitory concentration (MIC) of tested antimicrobials, with the MIC$_{50}$ and MIC$_{90}$ mostly beneath the respective breakpoint. On average, about a quarter (24%, n = 5,718) of tested isolates was resistant to erythromycin. However, the prevalence of resistant isolates dropped from 53% (n = 1,018) in 2012 to 8% (n = 113) in 2022. The second highest prevalence of in vitro resistance was to penicillin (17%, of all isolates tested for β-lactamase production, n = 28,069). Less than 14% of isolates were resistant to the remaining assessed antimicrobial agents (cefoperazone, pirlimycin, kanamycin-cefalexin, marbofloxacin, amoxicillin-clavulanate, ceftinome, or cefazolin, respectively). Over the years, 4% (n = 959) of the *S. aureus* isolates selected for microbroth susceptibility testing (and 0.8% (n = 1,392) of all submitted *S. aureus* isolates) were methicillin-resistant *S. aureus* (MRSA), and 5% (n = 1,162) of *S. aureus* isolates were multidrug-resistant. However, there was an overall trend toward fewer resistant isolates. These findings are consistent with those of several European monitoring programs that reported a slight decrease of AMR of bovine *S. aureus* in countries where antibiotic use in veterinary medicine was reduced.

Notably, isolates of clinical mastitis cases were consistently less likely to express in vitro resistance than isolates obtained from milk of healthy cows or subclinical mastitis cases.

In conclusion, antimicrobial resistance (AMR) of *S. aureus* was decreasing and penicillin should remain the first-choice antimicrobial in the attempt of treating *S. aureus* intramammary infections in Bavaria.

Key words: *Staphylococcus aureus*, mastitis, dairy cattle, antimicrobial resistance

INTRODUCTION

Bovine mastitis is the most prevalent and expensive disease in the dairy industry worldwide (Ruegg, 2017). It is defined as an inflammation of the mammary gland and is mostly caused by bacteria (Radostits et al., 2007). Therefore, mastitis therapy accounts for the majority of antimicrobial treatments of dairy cows (Nobrega et al., 2017). The use of antimicrobials with potentially resulting emergence of resistant bacteria has become a concern to dairy farmers, consumers, and public health authorities. One of the most frequently isolated contagious pathogens from bovine mastitis in Germany is *Staphylococcus (S.) aureus* (Kadlec et al., 2019; Tenhagen et al., 2006). Extensive fibrosis and micro abscess formation by *S. aureus* (Zadoks et al., 2011) allow the pathogen to avoid both the immune response of the host as well as antimicrobial agents (Barkema et al., 2011). In addition, the successful treatment of this infection might be hampered by antimicrobial resistance (AMR).

To monitor AMR trends over time, various international (World Organization for Animal Health, European Medicines Agency etc.) and national organizations in Germany, e.g., BVL (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit), have implemented monitoring programs for AMR as well as antimicrobial use on farms (Toutain et al., 2017). However, while the total veterinary antimicrobial sales observed between 2011 and 2020 in Germany decreased by 65% (BVL, 2022), *S. aureus* isolates from mastitis
cases were associated with an increasing prevalence of resistance. For example, the reported percentage of isolates resistant to penicillin increased from 14% to 24% between 2011 and 2017 (BVL, 2019). Although these programs provide overview data on conditions throughout Germany, the studies are limited by small sample sizes and missing information about regional differences. Consequently, supporting research is needed to better understand the temporal development of AMR of \textit{S. aureus} and to identify current therapeutic options for \textit{S. aureus} mastitis. Therefore, the objective of this study was to describe the in vitro resistance of \textit{S. aureus} isolates from quarter milk samples obtained through the udder health laboratory of the Bavarian Animal Health Services (TGD) between 2012 and 2022.

**MATERIALS AND METHODS**

**Sample Population**

This retrospective study included all \textit{S. aureus} positive bovine quarter milk samples that were submitted to the udder health laboratory of the TGD between 2012 and 2022. The submissions consisted of quarter milk samples from whole herd screenings by TGD technicians as well as samples of individual cows submitted by farmers or their veterinarians. All quarter milk samples had simultaneously been tested with the California Mastitis Test (CMT) and categorized according to its score into “negative” or “subclinical mastitis.” If samples showed abnormal milk or the cow had other signs of clinical mastitis (e.g., swollen udder) they were classified as “clinical mastitis” cases either by the technicians on the farm or by visual examination of the milk in the laboratory.

**Laboratory Analysis**

\textbf{a) Bacteriology} The laboratory methods were based on the respective valid guidelines for diagnosis of mastitis of the DVG (German veterinary medical society; DVG, 2009; DVG, 2018). Since all quarter milk samples used in this study were collected as part of routine mastitis diagnostics at the TGD, the laboratory methods were designed to detect various mastitis-causing pathogens, not only \textit{S. aureus}. Briefly, all quarter milk samples were inoculated using calibrated loops, with an inoculum size of 0.01 mL for samples of whole herd screenings and 0.05 mL for samples of clinical mastitis cases. The inocula were placed on Esculin blood agar plates (Oxoid) supplemented with 5% sheep blood and incubated aerobically at 36 ± 1°C. Evaluation was performed after 18–24 h and 48 h incubation. The phenotypic identification of \textit{S. aureus} was based on colony morphology and hemolysis. Clumping factor and coagulase were determined only in isolates that did not show a clear zone of β-hemolysis (DVG, 2018). After 2013, \textit{S. aureus} was additionally identified by MALDI TOF if necessary (microflex MALDI Biotyper, reference database V.3.3.1.0., Bruker Daltonik GmbH).

\textbf{b) In vitro antimicrobial resistance} All visually identified staphylococcal isolates were tested for β-lactamase (penicillinase) production by the iodometric method as recommended by Rosselet et al. (1977) and Gedek (1978), using an iodine/iodine-potassium stock solution with phosphate buffer, aqua dest., and penicillin G. The β-lactamase-positive samples were either directly forwarded to microbroth susceptibility testing or transferred to Brilliance-MRSA 2 agar (Oxoid) to clarify whether they belonged to the group of methicillin-resistant \textit{Staphylococcus aureus} (MRSA). In case of MRSA-like growth, MIC determination was conducted subsequently on all individual samples obtained from cases of subclinical and clinical mastitis as well as randomly on stock samples to clarify the diagnosis. The breakpoint ≥4 mg/l for oxacillin confirmed the presence of MRSA (Becker, 2004). If a quarter milk sample was positively tested for more than one \textit{S. aureus} strain, the more pathogenic strain (e.g., β-lactamase-positive strain or MRSA) was used for further analysis.

For microbroth susceptibility testing by breakpoint analysis, a selection of \textit{S. aureus} isolates was included according to the following routine guidelines: In herd screenings, up to 3 β-lactamase positive isolates were selected. Furthermore, all quarter milk samples were included if they originated from a cow showing subclinical or clinical mastitis signs, after treatment was conducted, or at the specific request of the client. The same selection standards were implemented for individual sample submissions. Antimicrobial susceptibility testing was performed with broth microdilution using the breakpoint method (mastitis 3 plate, Merlin Diagnostika GmbH). This commercial system complied with the CLSI guidelines (CLSI, 2015), with quality control testing (\textit{S. aureus} ACTT 29 213) performed weekly and within the established ranges, in accordance with the guidelines from the accreditation authority. Here, the most common antimicrobial agents for intramammary therapy were tested: β-lactams (penicillin, ampicillin, amoxicillin-clavulanate and oxacillin, the last as representative of penicillinase-stable isoxazolyl penicillins), along with cephalosporins of the first, third and fourth generation (cefazolin, cefoperazone and cefquinome, respectively), aminoglycosides (kanamycin-cefalexin), macrolides (erythromycin), quinolones (marbofloxacin) and lincosamides (pirlimycin). The respective breakpoints were analyzed using the program MCN 6 (version MCN 6.00–08.01.2018 Rel. 89; Demo Computer...
GmbH and Merlin Diagnostica GmbH). The program used the official breakpoints from the standards in effect at the time (e.g., NCCLS M31-A3, CLSI Vet01, CLSI M100). If the formerly valid breakpoints were identical to the currently valid ones, the current standards were cited as the source in the respective tables. If no official breakpoint for the indication S. aureus mastitis cattle existed, the program would either use former breakpoints (e.g., NCCLS M31-A3), human breakpoints (CLSI, 2023a), manufacturers’ information (e.g., marbofloxacin; HPRA, 2011; e.g., cefquinome; BASG, 2012), or values from publications (e.g., kanamycin-cefalexin; Pillar et al., 2009). In February 2023, the newest CLSI Vet01S Standard provided the first official breakpoint for kanamycin-cefalexin for S. aureus mastitis in cattle (CLSI, 2023b). Thus, both values were considered in this study. Results for ampicillin, gentamicin and tetracyclin were discarded due to incomplete susceptibility testing and/or missing MIC values. Therefore, susceptibility testing for a total of 10 antimicrobial agents was included in this study. Multidrug resistance was defined as resistance to 3 or more antimicrobial classes, according to Magiorakos et al. (2012) and Sweeney et al. (2018). The antimicrobial classes included in this study were β-lactams, amino-glycosides, macrolides, quinolones and lincosamides. Intermediate results were categorized as resistant and only acquired resistance was included.

Statistical Analysis

The statistical software SAS 9.4 (SAS Institute Inc.) was used for data analysis. Descriptive statistics were applied for MIC observations by year and isolate (PROC FREQ). Fisher’s exact test was used for comparing the association between mastitis status and isolates evaluated with MIC as well as mastitis status and resistant isolates. The MIC50 and MIC90 were the MIC where 50 and 90% of isolates were inhibited by the tested antibiotics, respectively. Multi-resistance was determined by the number of antimicrobial classes that the isolates were tested resistant to in vitro. All graphics were created in Microsoft Excel 2010. Missing data were ignored and α was set at 0.05.

RESULTS

Sample Population Descriptions

Table 1 provides an overview of the samples included in this study. From all quarter milk samples submitted to the TGD between 2012 and 2022, a total of 167,651 isolates were identified as S. aureus. They originated from 94,058 cows of 12,052 herds and were mostly (~80%) collected during whole herd screenings. Overall, 17% (n = 27,998) of all S. aureus isolates were β-lactamase positive, and the prevalence of methicillin-resistant S. aureus (MRSA) was 0.8% (n = 1,392).

Based on the selection criteria mentioned above, a total of 23,446 S. aureus isolates were further analyzed with broth microdilution. Six isolates were β-lactamase negative but penicillin resistant according to their breakpoint. They were included in the further analysis as penicillin resistant. Among all isolates analyzed with broth microdilution, S. aureus isolates originating from cases of subclinical (66%, n = 15,568) or clinical mastitis (27%, n = 6,213) were more likely to be evaluated for their MIC than those from healthy quarters (7%, n = 1,664, Table 1; P < 0.001). Furthermore, isolates from clinical mastitis quarters were less likely to exhibit resistance to at least one of the antimicrobials tested (16%, n = 2,064) than those from healthy quarters or with subclinical mastitis (Table 1; P < 0.001). The prevalence of methicillin-resistant S. aureus (MRSA) among S. aureus isolates analyzed with broth microdilution was 4% (n = 959).

Minimum Inhibitory Concentrations and Resistance

Figure 1 shows the changes in resistance for the different antimicrobials in S. aureus isolates between 2012 and 2022, and Table 2 the respective trends of MIC50 and MIC90. On average, 24% (n = 5,718) of the isolates showed in vitro resistance to erythromycin. This was the highest proportion of resistant isolates in this study. The MIC50 and MIC90 steadily decreased over the 11 years (P < 0.001, Figure 2). Similarly, the resistance prevalence to erythromycin dropped from 53% (n = 1,018) in 2012 to 8% (n = 113) in 2022 (P < 0.001, Figure 1). When only clinical mastitis cases were assessed (n = 6,211), the proportion of resistant isolates decreased over the same time from 46% (n = 132) to 5% (n = 29), with an average resistance prevalence of 16% (n = 1,002).

The second most frequent resistance was against penicillin. Here, 17% (n = 28,069) of all S. aureus isolates (submitted to the TGD, n = 167,651) were in vitro resistant. In the study period, the percent of in vitro resistant isolates decreased from 23% (n = 4,482) to 14% (n = 1,287; Figure 1). The MIC50 and MIC90 did not change over the 11 years, with the MIC50 constantly at the lowest and the MIC90 at the second highest MIC tested (Table 2).

For the remaining antimicrobials tested, the in vitro AMR remained below 14% for each. An average of 6% (n = 1,490) of isolates were resistant to pirlimycin, even if only clinical cases were included (6%, n = 369). Both MIC50 and MIC90, as well as resistance prevalence,
remained at a consistently low level during the study period (Table 2 and Figure 1, respectively).

Three percent of all isolates (n = 676) were in vitro resistant against marbofloxacin and this share decreased from 3% (n = 56) in 2012 to 1% (n = 16) in 2022 (P < 0.001, Figure 1). If only clinical cases (n = 6,211) were considered, the overall prevalence of resistant isolates was 2% (n = 128). The MIC$_{50}$ and MIC$_{90}$ consistently remained at the lowest and second lowest evaluated concentrations, respectively (Table 2).

Similarly, the MIC$_{50}$ and MIC$_{90}$ for Oxacillin remained at the lowest evaluated concentration during the study period (Table 2). Among all S. aureus isolates analyzed with broth microdilution, the prevalence of methicillin-resistant S. aureus (MRSA) was 4% (n = 959). However, this prevalence increased from 2% (n = 43) in 2012 to 5% (n = 71) in 2022 (P < 0.001, Figure 1).

On average, 3% (n = 692) of all S. aureus isolates was resistant in vitro to cefquinome. The resistance prevalence increased from 2% (n = 31) in 2012 to 3% (n = 47) in 2022 (P < 0.01, Figure 1).

On average, 3% (n = 692) of all S. aureus isolates was resistant in vitro to cefquinome. The resistance prevalence increased from 2% (n = 31) in 2012 to 3% (n = 47) in 2022 (P < 0.01, Figure 1).

Similar to this, 2% (n = 391) of the S. aureus isolates showed resistance to cefazolin, and this percentage increased from 1% (n = 11) in 2012 to 2% (n = 26) in 2022 (P < 0.01, Figure 1).

Overall, 2% (n = 348) of all isolates were resistant in vitro to amoxicillin-clavulanate. If only clinical mastitis samples (n = 6,210) were considered, this prevalence was only 1% (n = 68). The resistance prevalence increased from 1% (n = 18) to 2% (n = 25) in the study period (P < 0.05, Figure 1). For each of these 3 antimicrobials (cefquinome, cefazolin and amoxicillin-clavulanate), the MIC$_{50/90}$ was at the lowest concentration tested (Table 2).

In vitro resistance to multiple antimicrobials

Figure 3 shows the number of antimicrobial substances that S. aureus isolates were in vitro resistant to between 2012 and 2022. In the first 3 years, more than two thirds (72%; n = 4,314) of the isolates showed resistance to at least one of the 10 antimicrobials tested. Starting 2015, the share of in vitro resistant isolates constantly decreased, with a general downward trend from 58% (n = 1,480) in 2015 to 40% (n = 1,391) in 2022. The only exception to this trend was in 2018, where the percentage of resistant isolates increased briefly to 41% (n = 989). Over all isolates, 5% (n = 1,162) of all MIC tested S. aureus were considered multidrug-resistant.

Table 1. Overview of Staphylococcus aureus quarter milk samples in vitro tested for β-lactamase activity and the subset of isolates further analyzed with broth microdilution (BMD) between 2012 and 2022

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Isolates (n)</th>
<th>Cows (n)</th>
<th>Herds (n)</th>
<th>CMT Negative n (%)</th>
<th>Subclinical Mastitis n (%)</th>
<th>Clinical Mastitis n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All S. aureus</td>
<td>167,651</td>
<td>94,058</td>
<td>12,052</td>
<td>62,702</td>
<td>95,729</td>
<td>9,221</td>
</tr>
<tr>
<td>β-lactamase +</td>
<td>27,998</td>
<td>15,919</td>
<td>2,765</td>
<td>14,867</td>
<td>12,739</td>
<td>392</td>
</tr>
<tr>
<td>β-lactamase -</td>
<td>139,653</td>
<td>81,923</td>
<td>11,888</td>
<td>47,901</td>
<td>82,954</td>
<td>8,798</td>
</tr>
<tr>
<td>BMD Analysis³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All S. aureus</td>
<td>23,446</td>
<td>21,721</td>
<td>7,925</td>
<td>1,665</td>
<td>15,568</td>
<td>6,213</td>
</tr>
<tr>
<td>Resistant⁴</td>
<td>12,582</td>
<td>11,586</td>
<td>5,159</td>
<td>1,195</td>
<td>9,336</td>
<td>2,063</td>
</tr>
<tr>
<td>Susceptible</td>
<td>10,864</td>
<td>10,519</td>
<td>5,121</td>
<td>478</td>
<td>6,236</td>
<td>4,14</td>
</tr>
</tbody>
</table>

¹California Mastitis Test.
²Isolates tested positive (+) or negative (-) for β-lactamase activity.
³Subset of isolates forwarded to BMD analysis.
⁴Resistant to at least one antimicrobial tested. Includes resistant and intermediate results.
DISCUSSION

Previously published studies mostly focused on a few isolates, and only a few publications have analyzed regional trends. In contrast, the strength of this study was, that it included a large number of samples and farms with a California Mastitis Test result available for each sample. Furthermore, this study included AMR data from a single laboratory over more than a decade, which allowed for trend evaluation within a region.

Table 2. MIC50 and MIC90 of the respective antimicrobials in Staphylococcus aureus isolates between 2012 and 2022, based on breakpoint method. Areas highlighted in green indicate values below the respective breakpoint.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC in µg/ml</th>
<th>2012</th>
<th>2014</th>
<th>2016</th>
<th>2018</th>
<th>2020</th>
<th>2022</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>MIC50</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Penicillin</td>
<td>MIC50</td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>≤0.125</td>
<td>≤0.125</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Pirlimycin</td>
<td>MIC50</td>
<td>≤2</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>MIC50</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>≤0.25</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>MIC50</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>MIC50</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
<td>≤2</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Kanamycin-cefalexin</td>
<td>MIC50</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
<td>≤4/0.4</td>
</tr>
<tr>
<td>Cefquinome</td>
<td>MIC50</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>MIC50</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
</tr>
<tr>
<td></td>
<td>MIC90</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
<td>≤4</td>
</tr>
</tbody>
</table>

Figure 1. Percentage of resistant Staphylococcus aureus isolates by year and antimicrobial substance based on breakpoint method. For kanamycin-cefalexin the breakpoint of CLSI Vet01S 2023 was applied (CLSI, 2023b). ERY = erythromycin, PEN = penicillin, CEP = cefoperazone, PIR = pirlimycin, KAN/CEF = kanamycin-cefalexin, OXA = oxacillin, MAR = marbofloxacin, CEQ = cefquinome, CEZ = cefazolin, AMX/CLV = amoxicillin-clavulanate.

However, the percentage declined from 6% (n = 198) in 2012 to 3% (n = 176) in 2022.
While more sensitive laboratory techniques such as nitrocefin for β-lactamase testing and mec PCR for MRSA detection have become available over time, the methods employed in this study have remained consistent within the TGD for several decades. These methods were chosen because they provided comparable diagnostic outcomes to newer methods and were convenient in routine diagnostics of large sample sizes. Additionally, the continued use of these methods ensured the consistency necessary for trend analyses.

The high prevalence of β-lactamase positive isolates in our study will have introduced some bias when comparing our results with other studies on resistance profiles in bovine *S. aureus*. A similar risk applies for the selection of up to 3 *S. aureus* isolates per herd, since already 1–2 isolates would cover 80% of the infections caused by *S. aureus* (Woudstra et al., 2023). However, it is important to note that the AMR patterns from our study largely matched those reported in other publications from Germany and Europe, as demonstrated below. Furthermore, this initial selection of isolates did not affect the overall resistance trends we observed, as we found the same trends when analyzing *S. aureus* isolates without β-lactamase. In summary, while our study population may not be entirely representative for herd-level observations, it nevertheless offers reliable insights into resistance trends as the same selection criteria were used over time.

A noteworthy observation of this study was that isolates from clinical mastitis quarters were less likely to be in vitro resistant than isolates from healthy quarters or those diagnosed with subclinical mastitis. This had also been previously reported by (Ender et al., 2004) for *S. aureus* and by Sorge et al. (2021) for other mastitis pathogens. Ender et al. (2004) provided a potential explanation, as they observed a much slower growth of methicillin-resistant *S. aureus* and thus a significant loss of fitness in vitro. As a certain threshold concentration of bacteria in the udder tissue is necessary to trigger an inflammatory reaction, this could consequently explain the higher prevalence of resistant, potentially slower growing, *S. aureus* in subclinical or healthy cases (Rainard et al., 2018). While we were unable to analyze the association of AMR with cfu/ml in this study, the clinical status of the quarters (i.e., the sample population) will likely influence the results of different studies. This needs to be considered when comparing their observations to our study.

In this study, the most common resistance was against erythromycin as 24% (n = 5,718) of isolates were resistant to it. This was higher than the 3% (n = 40) and 4% (n = 9) reported by the German monitoring program between 2011 and 2019 (BVL, 2021) and the European monitoring program between 2015 and 2016 (El Garch et al., 2020), respectively. However, our prevalence was below the 41% (n = 41) reported for Austria (Wald et al., 2019). The reason for this discrepancy is unclear, because this effect remained, when we focused only on clinical isolates (16% resistant, n = 1,002). However, there was a strong downward trend in resistant isolates during the study period (2022: only 8% (n = 113) were in vitro resistant) so that the average period percentage might not adequately represent the current status (Figure 1). From the 24% (n =

![Figure 2. MIC\textsubscript{50} and MIC\textsubscript{90} of erythromycin for *Staphylococcus aureus* isolates per year based on breakpoint method. Dashed line indicates breakpoint.](image)
of isolates resistant to erythromycin, only 18% (n = 1004) also showed resistance to pirlimycin. Since resistance to erythromycin is usually mediated by \textit{erm} genes that also cause pirlimycin resistance (when the \textit{erm} gene is constitutively expressed; EMA, 2011), the 82% (n = 4,713) of isolates showing resistance to erythromycin but sensitivity to pirlimycin could rely on \textit{erm} genes that are inducibly expressed. The latter could be identified by a D test (Shreshta and Rana, 2014). Unfortunately, genotypic distinctions or D tests were not available for our study population.

In contrast to the observations for erythromycin, the prevalence of resistant isolates against penicillin (17%, n = 28,069) was comparable to the average 18% (n = 227) reported by the German national survey between 2011 and 2019 (BVL, 2021), and below the 26% (n = 63) found by the European monitoring program VetPath (El Garch et al., 2020). Our results also align with the findings of Naranjo-Lucena and Slowey (2023) regarding penicillin resistance prevalence in several European countries, which could confirm the effectiveness of German \textit{S. aureus} control programs when compared on an international scale.

Among all antimicrobials tested, pirlimycin, cefoperazone, and kanamycin-cefalexin were the only ones with official clinical breakpoints for \textit{S. aureus} isolates from bovine mastitis. The 6% in vitro resistance to pirlimycin (both overall (n = 1,490) and for clinical cases only (n = 369)) was comparable to the findings of the monitoring programs, with - , on average 4% (n = 51) in Germany (BVL, 2021) and 3% (n = 8) in Europe (El Garch et al., 2020). From our 6% (n = 1,490) of isolates resistant to pirlimycin, a total of 67% (n = 1004) were also resistant to erythromycin ($P < 0.001$). The remaining 33% (n = 486) of isolates resistant to pirlimycin probably have other resistance genes than \textit{erm} (e.g., \textit{tnu/lin}, \textit{lsa}; EMA, 2011).

Furthermore, the prevalence of cefoperazone resistance (here 13%, n = 3,136) was similar to the 10% (n = 10) observed by Wald et al. in Austria (2019). As the new breakpoint for kanamycin-cefalexin (CLSI, 2023b) was only published recently, we can compare the 5% (n = 1,222) in vitro resistance found here only to the 0% (n = 0) evaluated in northern Germany (Bolte et al., 2020). Since the distribution of MICs did not show trends during our study period, this difference may be due to the regional rather than temporal differences. However, this hypothesis could only be verified if more comparable studies included this antibiotic in their susceptibility testing, which would certainly be encouraged by a specific official breakpoint.

Unlike the other antimicrobials, the classification of marbofloxacin depended solely on the breakpoints provided in the manufacturer’s information. Kroemer et al. (2012) worked with the same breakpoint and found 0.7% (n = 4) \textit{S. aureus} isolates of clinical mastitis cases resistant to marbofloxacin. This was lower than the 3% (n = 676, overall) or 2% (n = 128, clinical cases only) of our study, which could be due to the origin of the isolates, as they collected quarter milk samples from

**Figure 3.** Number of antimicrobial substances that \textit{Staphylococcus aureus} isolates tested in vitro resistant to by year. For kanamycin-cefalexin the breakpoint of CLSI Vet01S 2023 was applied (CLSI, 2023b).
several European countries without further regional differentiation.

To evaluate the results for the remaining 3 antimicrobial agents, the CLSI recommendations regarding susceptibility testing for *S. aureus* had to be considered (Dien Bard et al., 2014). They suggest that β-lactam antibiotics other than penicillin and oxacillin should not be included in susceptibility testing for *S. aureus*. Instead, all *S. aureus* isolates showing resistance to penicillin should be considered resistant to all penicillinase-labile penicillins (ampicillin, cefazolin, cefquinome, cefoperazone), and resistance to oxacillin (MRSA) should be applied to the other penicillinase-stable penicillins (amoxicillin-clavulanate).

Although the recommendation to report all MRSA strains as resistant to β-lactams was initially developed from severe cases of human sepsis, it could be justified in the context of bovine mastitis, due to the ineffective immune response of the mammary gland during *S. aureus* infections (Egyedy and Ametaj, 2022) and the often subtherapeutic concentrations of antimicrobials in udder tissue (de Jong et al., 2023). Nevertheless, we have included amoxicillin-clavulanate, cefquinome, and cefazolin in our discussion, but must point out that the respective in vitro results may be insufficiently predictive of therapeutic outcome.

Cefquinome or cefazolin resistances were not included in any of the monitoring programs. But our in vitro resistance to cefquinome (3%, *n* = 692) and cefazolin (2%, *n* = 391) were slightly below the 6% (*n* = 1) found by Monistero et al. (2020) in Germany. Our MIC50 and MIC90 of both cefazolin and cefquinome at the lowest MIC tested (≤4 µg/ml for cefazolin and ≤ 1 µg/ml for cefquinome), which was identical to the results from Käppeli et al. (2019). For amoxicillin-clavulanate, the resistance prevalence of 2% (*n* = 348) found in this study was slightly higher than in the European (1%, *n* = 1) monitoring program (2009–2012; De Jong et al., 2015), but identical if we only included clinical cases.

Looking at the AMR prevalence of the individual antimicrobials combined, 5% (*n* = 1,162) of *S. aureus* were multidrug-resistant in this study. This result is lower than the 19% (*n* = 7) found in Brazil (Gonçalves et al., 2023) and considerably lower than reported by other studies (65% (*n* = 73) from Elemo et al., 2017; 100% (*n* = 48) from Salauddin et al., 2020). It must be noted that other publications worked with more antimicrobial classes (e.g., folate pathway inhibitors, glycopeptides, tetracyclines), which could explain the different results. Since all MRSA are considered to be multidrug-resistant (Magiorakos et al., 2012), we can assume that our MRSA prevalence of 4% (*n* = 959; among *S. aureus* isolates tested with broth microdilution) represented a large proportion of our multi-drug resistant isolates.

While the prevalence of AMR in *S. aureus* dropped over the study period, there was a particularly large drop in AMR prevalence in 2018. This coincided with a legislative change in veterinary drug prescriptions in Germany (TAHVA). This change aimed to minimize the use of critically important antimicrobials (third- and fourth-generation cephalosporins, macrolides, fluoroquinolones) by introducing mandatory susceptibility testing before their use, and to decrease the extended use or changes of antimicrobial agents during therapy of individual cases. As a result, more quarter milk samples of clinical cases were submitted to the Bavarian Animal health services (Sorge et al., 2021b). However, we were unable to find a significantly higher prevalence of clinical mastitis quarters for *S. aureus* in 2018 that could have explained the drop in AMR prevalence.

While we are aware that associations on population level with individual risk factors might be subject to ecological fallacy (Dohoo et al., 2009), it was noteworthy that the trends in AMR in our study were indeed correlated with the results of the national programs about antimicrobial sales (BMG et al., 2011) and antimicrobial use in German veterinary medicine (Kasabova et al., 2021). For instance, the greatest reduction of resistance prevalence in our study was measured for erythromycin (Figure 1). The same antimicrobial class also had one of the biggest declines in sales (−73%, 127 tons) between 2011 and 2021 (Sander et al., 2022). Furthermore, penicillin sales decreased sharply (−55%, 293 tons) during the same time, which could explain the drop in resistant isolates that we observed (Figure 1). However, cefoperazone and cefquinome did not follow this pattern. While resistance to them stagnated or marginally increased (Table 1), sales and use of third- and fourth-generation cephalosporins for veterinary medicine had dropped by more than 50% between 2013 and 2020 (Sander et al., 2022; Kasabova et al., 2021) – particularly for 2018 (Sander et al., 2022). Without data about cow individual treatment history and AMR changes, the cause for this discrepancy remains elusive.

When comparing the AMR trends from our study with the results from national monitoring programs of Germany (BVL, 2021) and other European countries (Swedres-Svarm, 2014; UK-VARSS, 2021; Korsgaard et al., 2020), a slight reduction of AMR prevalence in bovine *S. aureus* isolates can be observed in most of them. All these countries developed their AMR monitoring and reduction programs based on the corresponding preceding EU regulations (Naranjo-Lucena and Slowey, 2023). It is an encouraging observation that, even though approaches varied widely, in most countries that reduced antibiotic use, AMR of *S. aureus*
also decreased. However, the decreasing trend in AMR prevalence among S. aureus isolates observed in our study was probably not only due to AMR reduction programs, but also a result of successfully implemented mastitis control programs (e.g., the 5-point mastitis control plan; Hillerton and Booth, 2018). Since they aim to prevent new infections while eliminating existing infections, they consequently contribute to reduce the spread of AMR.

CONCLUSIONS

The overall percentage of in vitro resistant S. aureus isolates from bovine mastitis cases was low and decreased over the study period. This included the prevalence of resistance against critically important antimicrobials and penicillin. Therefore, penicillin should remain the first-choice antibiotic in the attempt of S. aureus mastitis therapy in Bavaria. Our findings agree with the observations of several European monitoring programs, which described that in countries where veterinary antibiotic consumption was reduced, AMR of bovine S. aureus also decreased.

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