A retrospective analysis of the changes in antimicrobial resistance of *Staphylococcus aureus* and MRSA in bovine quarter milk samples from Southern Germany between 2012 and 2022

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ABBREVIATIONS

AMR	Antimicrobial resistance
blaZ	β-lactamase gene
BMD	Broth microdilution
BTM	Bulk tank milk
BVL	Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit)
CA	Community associated
CC	Clonal complex
CFU	Colony forming unit
CM	Clinical mastitis
CNS	Coagulase negative staphylococci
Coa	Coagulase
DNA	Desoxyribonucleic acid
E. coli	Escherichia coli
E.g.	Exempli gratia
EFSA	European Food Safety Authority
EMA	European Medicines Agency
Etc.	Et cetera
EU	European Union
HA	Hospital associated
i.e.	Id est
IFN	Interferon
IL	Interleukin
IMI	Intramammary infection
LA	Livestock associated
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry
mec	Methicillin resistance gene
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MLST	Multi locus sequence typing
MRSA	Methicillin resistant *Staphylococcus aureus*
Nuc	Thermonuclease
OIE	World Organization for Animal Health
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PMN	Polymorphonuclear neutrophils
QMS	Quarter milk sample
RNA	Ribonucleic acid
S.	*Staphylococcus*
SCC	Somatic cell count
SCC mec	Staphylococcal cassette chromosome mec
SCV	Small colony variant
Spa	Staphylococcal protein A
ST	Sequence type
Staph.	*Staphylococcus*
Strep.	*Streptococcus*
TÄHAV	Veterinary Drug Prescription Legislation (Tierärztliche Hausapothekenverordnung)
TGD	Animal Health Service (Tiergesundheitsdienst)
WHO	World Health Organization
z.B.	Zum Beispiel

I. INTRODUCTION

Staphylococcus aureus is a major pathogen causing bovine mastitis, a highly contagious intramammary infection in cattle that results in significant economic losses for dairy farmers. It is among the most frequently isolated mastitis pathogens worldwide, leading to mainly subclinical, persistent infections of the bovine mammary gland. The infection often responds poorly to antimicrobial treatment and necessitates the culling of many affected animals (Radostits et al., 2007). After transmission during the milking process, *S. aureus* often persists in the mammary gland, generating decline in milk quantity and quality and therefore establishing staphylococcal mastitis as one of the most expensive bovine diseases in dairy industry (Heikkilä et al., 2018; Sørensen et al., 2010). Its significance remains exceptional despite decades of developing control programs, including vaccines, hygiene standards, and therapy plans; it culminates in the public health threat from these potentially zoonotic bacteria and their emerging antimicrobial resistance (Brahma et al., 2022; van Loo et al., 2007). In case of infection, a microbial culture commonly identifies the causative agent. An additional susceptibility test is needed to select a suitable antimicrobial therapy. Penicillins and cephalosporins remain the most used antimicrobials in sensitive *S. aureus* (Tenhagen et al., 2006). However, increasing use of antibiotic treatment over the last 70 years may have fostered the development of resistant strains and even multidrug-resistant MRSA. This added to the common failure of treatment and unsatisfactory control, imposing a serious challenge on dairy farmers, veterinarians, and public health (Ruegg, 2017). As the general awareness of resistant bacteria increased drastically in the recent years, national as well as international organizations like OIE (World Organization for Animal Health), EMA (European Medicines Agency) and BVL (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit) implemented surveillance programs and control guidelines for prudent use of antimicrobials in veterinary medicine (Toutain et al., 2017). In order to support these efforts for successful *S. aureus* mastitis prevention and control, further monitoring about isolate distribution and trends of resistance patterns are required. Therefore, the objective of this thesis was to determine the prevalence and antimicrobial resistance of mastitis causing *S. aureus* and MRSA in cattle in Southern Germany over the course of eleven years

between 2012 and 2022.

II. LITERATURE REVIEW

1. *Staphylococcus aureus*

1.1. History and characteristics

Staphylococcal infections were first described in the late 19th century by the Scottish surgeon Sir Alexander Ogston, who published studies about these infectious microorganisms as causative agents for abscesses. He named them after the Greek *staphyle* (bunch of grapes) and *kokkos* (grain), because of their clustered formation visible under the microscope (Ogston, 1880, 1882). A few years later, the German surgeon Friedrich J. Rosenbach designed a taxonomy of *Staphylococcus (S.) pyogenes aureus*, which he differentiated from *S. pyogenes albus* by colony pigmentation (Rosenbach, 1884). The first staphylococcal mastitis cases in sheep and cattle were reported by Nocard (1887) and Guillebeau (1890), respectively, establishing the pathogenic role of *S. aureus* in animals (Jonsson & Wadstorm, 1993).

The morphology reveals *S. aureus* as a gram-positive bacterium often grouping into pairs, tetrads or irregular grape-like clusters (RKI, 2000). Colonies incubated on blood agar for 24 hours appear as smooth, raised and translucent, showing golden pigmentation along with a wide zone of strong haemolysis (PHE & NHS, 2020). *S. aureus* is a catalase-positive, mostly coagulase-positive, non-motile, and non-sporulating facultative anaerobic organism that is capable of mannitol and trehalose fermentation.

The genus *Staphylococcus* currently contains more than 70 validated species, with further distribution in subspecies and subtypes (Parte et al., 2020). Therefore, this genus presents a great variability in manifestation, with only the *S. aureus* subtype *aureus* splitting up in some strains that are animal and human skin commensals, and some that can be pathogenic and cause soft tissue infections, bone infections, and toxin-mediated diseases like Staphylococcal Scalded Skin Syndrome (SSSS) and Toxic Shock Syndrome (Linde & Lehn, 2002). The different outcome in manifestation depends on the strain-specific set of virulence factors like surface binding proteins and toxins, biofilm formation and antimicrobial resistance (Magro et al., 2017).

Many *S. aureus* strains are also known for causing food poisonings, because of their production of enterotoxins, which are heat tolerant and therefore resistant to pasteurisation (Jørgensen et al., 2005). Furthermore, the zoonotic potential of *Staphylococcus aureus* has led to reports about transmissions from animals to humans and vice versa (Heaton et al., 2020). Still, the major importance of this pathogen lies in its usually host specific infections.

2. Bovine *S. aureus* mastitis

2.1. Bovine mastitis

Mastitis is the most prevalent infectious disease in bovine dairy production, being the subject of steadily rising counts of research projects over the past decades worldwide (Ruegg, 2017). It is specified as an inflammation of the mammary gland mostly caused by bacteria invading the udder (Radostits et al., 2007). The manifestation varies from acute to chronic as well as subclinical to clinical cases, depending on multiple factors like species of causative bacteria, age and lactation stage of the cow along with environmental impacts like housing system and hygiene (Lundberg, 2015). As to the mastitis causing bacteria, the most common representatives are staphylococci, streptococci and coliforms, which can be further differentiated based on their reservoirs and transmission patterns between environmental and contagious pathogens (Blowey & Edmondson, 2010). The first group survives in the barn environment, causing infections in cows between the milkings and often leading to plain clinical symptoms (e.g., *Streptococcus (Strep.) uberis* and coliforms). The contagious organisms persist in the mammary gland and are transmitted from cow to cow during the milking process, rather inducing subclinical intramammary infections (IMIs) with raised cell counts in the bulk tank milk (e.g., *S. aureus*, *Strep. agalactiae* etc.). Over the recent years, environmental mastitis causing pathogens like *Streptococcus uberis* and *Escherichia coli* have been increasingly isolated of mastitis milk samples (Oliveira et al., 2013; Ruegg, 2017). However, previous research accounts this rather to the reduction of contagious mastitis cases by steadily improving prevention and control programs like milking hygiene, antibiotic treatment, and culling of infected animals (PHE & NHS, 2020; Ruegg, 2017).

2.2. Prevalence and significance

Despite the aforementioned declining trends of mastitis cases caused by contagious pathogens, *S. aureus* continues to be one of the most frequently isolated pathogens from bovine IMI worldwide (Gianneechini et al., 2002; Heikkilä et al., 2018; Østerås & Sølverød, 2009; Pascu et al., 2022; Tenhagen et al., 2006). To name a few examples from the past three decades, the prevalence of *S. aureus* among dairy herds was 70% in Bavaria (Groh et al., 2022), 93% in Great Britain (Wilson & Richards, 1980), 43% in USA (Lombard et al., 2008), and 77% in China (Li et al., 2009). Using bulk tank milk (BTM) for prevalence testing, 70% of the herds in Hungary (Peles et al., 2007), 30% in Mexico (Miranda-Morales et al., 2009), and 74% in Canada (Riekerink et al., 2010) were positive. Furthermore, quarter milk samples positive for *S. aureus* accounted for 27% in Korea (Moon et al., 2007) and 21% in Sweden (Ericsson Unnerstad et al., 2009).

In Germany, previous studies revealed a prevalence of *S. aureus* in quarter milk samples of 5,7% in 2006 (Tenhagen et al., 2006), 5% in 2010 (Schwarz et al., 2010), 2,5% in 2019 (Kadlec et al., 2019), and 2,9% in 2023 (Groh et al., 2023).

The greatest significance of mastitis lies in the economic losses for the dairy farmers, with especially *S. aureus* causing greater expenses than the average mastitis case (Gröhn et al., 2004; Heikkilä et al., 2018; Kreausukon, 2011; Swinkels et al., 2005). Similar to the large spread of prevalence, the international comparison of economic calculations reveals great differences from country to country. This is due to the variations in study design, regional prevention and treatment methodology, and time-dependent factors like regulations for producers (Halasa et al., 2007).

Basically, the economic losses due to mastitis can be separated in direct and indirect costs: Expenses for discarded milk (because of lowered quality and antibiotic residue), veterinary consult, and drug treatment as well as increased workload for the dairy farmer count as direct costs. Whereas indirect costs include decreased milk production, widespread failure of treatment (due to resistance and evasion by abscess formation), premature culling and replacement along with the loss of genetic potential (Berry et al., 2004; Erskine et al., 2003; Keefe, 2012;

McDougall et al., 2009; Murphy, 1956; Seegers et al., 2003).

According to an article published by the University of Glasgow in 2016, the global mastitis costs are estimated to be €16 – 26 billion per year. In the US, mastitis causes annual losses up to $2 billion, with production losses accounting for most of it (iGEM, 2016). Per mastitis case, the estimated losses accumulate to €146 in Denmark (Østergaard et al., 2005) or €182 in Netherlands (Huijps et al., 2008). The costs vary depending on clinical or subclinical manifestation, as shown in studies from Sweden (€278 for a clinical case, €60 for a subclinical case) (Nielsen et al., 2010) and the Netherlands (up to €235 and €120 for clinical and subclinical IMI, respectively) (Huijps et al., 2008). Furthermore, Rollin et al. (2015) documented the economic loss of $444 per average clinical mastitis case in the US. Studies of pathogen specific mastitis costs are very rare, but a stochastic model from Denmark estimated expenses ranging between €149 and €570 per mastitis case. In this project, *S. aureus* mastitis was the costliest mastitis type, exceeding costs of other pathogens like CNS, *E. coli*, *Streptococcus dysgalactiae* and *Strep. uberis* with €570 per case (Sørensen et al., 2010).

2.3. Reservoirs, transmission and risk factors

Staphylococcus aureus is ubiquitous in the barn environment, such as bedding material, dust, equipment and feed (Matos et al., 1991; Radostits et al., 2007). Also, non-bovine animals, insects as well as milking personnel are reservoirs - even though their role as source of infection is less important (Roberson et al., 1998). Healthy cows are persistently colonized with *S. aureus* on various body sites, especially the skin and mucosa. Hence, it is likely for calves to have first contact with the contagious bacteria at birth and becoming a reservoir for other cows and themselves. Nevertheless, the infected mammary gland of lactating cows is considered as the main source of *S. aureus* IMI. The infection is easily transmitted during the milking process, as previous researchers confirmed by finding similar *S. aureus* strains in both milking machinery and infected milk (Davidson, 1961; Roberson et al., 1994; Svennesen et al., 2019; Zadoks et al., 2002).

There are many risk factors facilitating the transmission, including animal, pathogen and environmental risk factors. On cow level, teat skin lesions (e.g.,

caused by malfunctioning milking machinery or other cows) are paving the way for staphylococcal infections, as the epithelial barrier no longer exists (Myllys et al., 1994). There is also a higher risk for mastitis with increasing parity of the cow, due to prolonged exposure to pathogens, decreased capability of the immune system and structural changes of the teat canal (Radostits et al., 2007; Sinha et al., 2021; Verbeke et al., 2014). With other reproductive system diseases present (like dystocia, retained placenta and ketosis) the local immune defence is weakened and therefore an IMI more likely (Radostits et al., 2007). Pathogen risk factors which are positively correlated with mastitis are the forementioned strain specific risk factors (virulence factors), as well as the antimicrobial resistance, leading to more persistent cases and therefore higher risk of transmission in the herd (Zaatout et al., 2020). At last, several environmental and managemental risk factors influence the spread of *S. aureus*, like the hygiene of udder and milking machinery, disinfection and teat dipping before and after every milking plus dry bedding facilities (Sato et al., 2008; Schnitt & Tenhagen, 2020).

2.4. Pathogenesis and interaction with host

To cause intramammary infection, already quantities as small as 10 colony forming units (CFUs) of *S. aureus* can be sufficient, as shown in a previous experimental study (Reiter et al., 1970). The bacteria must overcome the physical barrier of the teat canal, which is normally closed between the milkings. Therefore, any form of injury on the teat increases the risk of colonization, as mentioned before. The pathogenesis of staphylococcal IMIs can generally be divided into three steps: first, the entry and adhesion to cells of the bovine mammary gland epithelium, facilitated by various virulence factors. This step can only be completed, if the bacteria can resist the pulling effect of milk flushing out of the udder. Therefore, *S. aureus* adheres to fat globules, thus further distributing into the upper mammary gland (Frost et al., 1977; Sandholm et al., 1989). *S. aureus* adheres especially well to these upper bovine mammary epithelial cells, and the presence of milk further enhances it (Mamo & Fröman, 1994). A different form of attachment is the formation of biofilms, where a three-dimensional complex of bacteria binds to living or non-living surfaces. The second step consists of interaction and later evasion of the host immune system. The defense mechanisms can be divided into innate or unspecific immunity and acquired or

specific immunity. During the entry of pathogens, nonspecific somatic cells like neutrophils, macrophages, and natural killer cells influx from the blood stream, rapidly eliminating any bacteria before the acquired immune system is activated. Additionally, there are nonspecific bacteriostatic proteins and enzymes present in the milk: lactoferrin, a protein binding free iron molecules needed by bacteria for growth, lactoperoxidase, producing a reactive bacteriostatic metabolite, lysozyme, a bactericidal protein damaging bacterial cell walls, and complement proteins, capable of opsonization (antigen presentation) and cell lysis. Only if this first response fails, for instance due to immune evasion techniques of the pathogen, the specific defense is triggered. Therefore, antigen presentation mediated by macrophages, dendritic cells and B lymphocytes neutralizing pathogenic material through phagocytosis and binding it to their major histocompatibility complex (MHC) II activates cytotoxic T cells. These T lymphocytes not only produce cytokines like interleukin (IL)-2 and interferon (IFN)-γ for further immune response, but also induce proliferation and differentiation of B lymphocytes into plasma cells (secreting more antibodies for bacterial opsonization) or memory cells (for specific pathogen recognition) (Rainard et al., 2003; Sordillo & Streicher, 2002).

The accumulation of these immune cells around vital *S. aureus* bacteria leads to the formation of abscesses, as great amounts of polymorphonuclear neutrophils (PMN) eliminate pathogenic material through phagocytosis and in consequence to that go into necrotic cell lysis, building a wall of cell detritus around living bacteria. As the abscess matures, a fibrous capsule forms in the periphery of the abscess due to fibroblastic proliferation to shield healthy tissue from the pathogen (Kobayashi et al., 2015).

This is already part of the third step of pathogenesis, the survival and tissue invasion. Although abscess formation is a host defense mechanism in purpose of eliminating the pathogen, it simultaneously can lead to failure of antimicrobial treatment and unhindered bacterial replication (Cheng et al., 2011). Furthermore, as soon as the encapsuled bacteria is in the need for more energy supplies, it secretes the enzyme staphylokinase, breaking the abscess capsule and therefore allowing further systemic spreading of *S. aureus* (Kwiecinski et al., 2013). Another survival technique of *S. aureus* is the formation of small colony variants (SCVs), which can evade the host immune defence by intracellular infestation.

Their modified metabolism allows intracellular replication even when facing nutritional stress, making these subpopulations a potent cause of persistent infections with high rates of antimicrobial resistance (Proctor et al., 2014; Sendi & Proctor, 2009). And finally, *S. aureus* can evade the host immune system by its ability of biofilm formation. This bacterial population, coated by a self-created polymeric matrix, can escape phagocytosis by macrophages and additionally becomes resistant to antimicrobial treatment (Monzón et al., 2002; Prakash et al., 2003).

2.5. Clinical manifestation, treatment and control

S. aureus mastitis mostly presents itself as a chronic, subclinical infection of the mammary gland. Many cases do not show typical symptoms but lead to periodic clinical flare-ups. The condition may persist over several months. This leads to increased milk SCCs and great reduction in milk yield (Radostits et al., 2007). In rare occasions, staphylococcal mastitis can manifest as acute or even peracute, with plain clinical symptoms and high fatal counts despite aggressive treatment methods. In acute cases, the milk texture appears watery with clots and flakes, and the mammary gland shows severe swelling plus induration, as the affected tissue turns extensively fibrotic and dysfunctional. Peracute *S. aureus* mastitis mostly occurs in the narrow postpartum phase, additionally leading to systemic reactions like fever, anorexia, and the inability to stand. Moreover, gangrenous forms have been described, showing blueish discoloration, subcutaneous emphysema up to wide necrotic areas of the udder, often leading to early death (Nickerson, 2011). As mentioned above, infection severity is depending on risk factors of the host, antigen and environment, hence the great variability of clinical outcome and treatment success (Barkema et al., 2006).

As to the therapy of *S. aureus* mastitis, it can be administered either while lactation or as dry cow therapy. In case of a subclinical mastitis, usually treatment occurs at early dry-off, because of the higher cure rates (20 – 70% in comparison to 10 – 30% during lactation) and the economic losses due to discarded milk in the withholding period (Nickerson, 2011; Radostits et al., 2007). Treatment success of clinical cases can be maximized by combining intramammary antimicrobial therapy with parenteral injections of antibiotics, plus anti-

inflammatory drugs such as meloxicam and corticoids and maybe fluid therapy (Radostits et al., 2007). The choice of antibiotic is depending on many factors, such as antimicrobial susceptibility, ability to penetrate the udder, bacteriostatic or bactericidal effects, costs and withdrawal period (Gruet et al., 2001). Different recommendations exist with regards to bovine mastitis treatment based on susceptibility testing. The antimicrobial treatment of clinical mastitis cases commonly precedes the outcome of susceptibility testing and may be adjusted afterwards accordingly (Krömker & Leimbach, 2017). A different treatment approach is the selective dry-off on quarter level, continuing the cows' production without the expenses for antibiotic treatment, moreover decreasing the risk of elevated bulk tank SCCs and transmission of infection (Swinkels et al., 2021). Still, the success rate of *S. aureus* mastitis therapy is rather low in comparison to other mastitis pathogens (Radostits et al., 2007). Especially factors like the duration of infection, age of the cow, number of infected quarters, and high SCC worsen the prognosis of cure (Blowey & Edmondson, 2010; Radostits et al., 2007). Hence, the importance of further research on prevalence and resistance of mastitis-causing *S. aureus*, as the development of long-term satisfactory control and treatment methods is still in progress. Newer research about alternative treatment methods has delivered interesting results for bacteriophage (Mohammadian et al., 2022; Titze et al., 2020) or nanoparticle (Algharib et al., 2020; Kour et al., 2023) therapy. However, the current status of research on these alternative treatment options would mostly allow their use as adjunct therapy, until further research is conducted (Tomanić et al., 2023).

In order to control mastitis, a 5-point-plan was implemented in the 1960s and later extended to the 10-point-plan by the National Mastitis Council, attempting to cover effective udder health management practices (Neave et al., 1969; NMC, 2001). These publications determined the five most important control measures as 1) treatment and recording of clinical cases, 2) post-milking teat disinfection, 3) dry cow therapy, 4) culling of chronic cases and 5) proper milk machine maintenance. The high standards of hygiene must also be accompanied with the right milking order, where first-lactation cows are being milked before older animals, uninfected cows next, and finally known infected animals last. This is because infected animals can contaminate the milking gear and therefore the next six to eight cows. With these steps applied, many studies have shown their

effectiveness in the fight against contagious as well as environmental mastitis pathogens (Blowey & Edmondson, 2010; Hillerton & Booth, 2018). Still, this method shows far better results in the control of *S. agalactiae*, probably due to the rareness of chronic cases as well as antimicrobial resistance (Nickerson, 2011). Furthermore, mastitis vaccines have been produced with the intention of decreasing IMI with certain mastitis pathogens and upholding the economic profits of the industry, however only very few studies have obtained acceptable results for *S. aureus* IMI (Kour et al., 2023).

2.6. Antimicrobial resistance

Antimicrobial resistance (AMR) refers to the ability of microorganisms, such as bacteria, to withstand the effects of antimicrobial drugs, leading to treatment failure, elevated mortality rates and increased treatment expenses (Prestinaci et al., 2015). The world health organization (WHO) declared that AMR is one of the top ten global public health threats facing humanity. As a One-Health problem it requires actions across human, agricultural, and environmental sectors (Walsh et al., 2023). In modern mastitis control programs, antimicrobial therapy against *S. aureus* is one of the most important aspects up to this day. Resistance to various antimicrobial agents is frequent (Brahma et al., 2022; Pascu et al., 2022).

The history of AMR is just as long as that of antimicrobial therapy itself. Since its discovery in the 1940s, penicillin remains the first choice antimicrobial used in veterinary medicine (Prescott John, 2017). However, reports on bacterial enzymes inhibiting the effects of penicillin on a *S. aureus* agar plate existed as early as one year before the antimicrobial was introduced for therapeutic use (Abraham & Chain, 1940). Penicillins disrupt the synthesis of bacterial cell walls by binding the penicillin-binding proteins (PBPs) of the microorganisms, which are required for the cross-linking of peptidoglycans (Tipper & Strominger, 1965). Penicillin resistant *S. aureus* strains express the enzyme β-lactamase, causing hydrolysis of the β-lactam ring of penicillin. It is encoded by the gene blaZ, which can either be located in a plasmid or integrated within the chromosome (Jensen & Lyon, 2009).

2.7. Methicillin-resistant *S. aureus* (MRSA)

Since the prevalence of *S. aureus,* that are resistant to β-lactamase-labile penicillins, increased over time, the development of β-lactamase-stable drugs (e.g. methicillin and cloxacillin) as well as β-lactamase inhibitors were available since 1959. Less than one year after the introduction of methicillin, the first human infection caused by methicillin-resistant *Staphylococcus aureus* (MRSA) was observed (Harkins et al., 2017). MRSA emerged with the acquisition of the *mec*A (or *mec*C) gene. This causes the formation of an alternative PBP2a, which binds beta-lactams with lower affinity (Miragaia, 2018). The *mec*A gene is located on a mobile genetic island called the staphylococcal cassette chromosome *mec* (SCC*mec*). This cassette can be transferred between different *S. aureus* strains and carries different genes of antimicrobial resistance, serving as a mechanism for multi-drug resistance (Matthews et al., 1987).

The first cases of bovine mastitis caused by MRSA were reported in 1962 (Devriese et al., 1972); its worldwide prevalence has been growing since (Zaatout & Hezil, 2021). In Germany, the proportion of MRSA among *S. aureus* isolates in bovine mastitis from 2009 to 2019 ranged between 2% and 14% (BVL, 2017, 2019). Eradication of MRSA from dairy herds is challenging and treatment failure often leads to culling as a last option, causing great economical losses for dairy farmers (Spohr et al., 2011). Transmission of bovine MRSA to humans has been documented. Consequently, people working or living in close contact with cows are at increased risk of becoming infected with MRSA (Schmidt et al., 2017; van Loo et al., 2007). The majority of the human livestock-associated (LA-) MRSA cases in Germany and Europe are attributed to clonal complex CC398. However, these LA-MRSA have the smallest influence on human *S. aureus* infections (3% of all nosocomial MRSA infections in Germany), compared to hospital-acquired (HA) or community-associated (CA) MRSA (Cuny et al., 2015). MRSA strains and their transmission to consumers have also been described for different food products, including cheese and raw milk (Normanno et al., 2007). Although pigs are considered the most important reservoir of LA-MRSA (Golob et al., 2022), the zoonotic potential of MRSA from dairy cows and raw milk cheeses provides an additional reason for ongoing research about bovine MRSA mastitis.

2.8. Monitoring programs

Because the transmission of bacteria and associated AMR from livestock to

humans has become a public concern, various international (World Organization for Animal Health, European Medicines Agency etc.) and national organizations have implemented monitoring programs for zoonotic and indicator bacteria, in line with preceding EU legislation (Directive 2003/99/EC; EU Decision 2013/652/EU) (Schrijver et al., 2018). Monitoring programs of several European nations (e.g., Sweden, France, United Kingdom, Denmark) provide data about *S. aureus* and MRSA from bovine mastitis cases, describing prevalence, AMR trends, and control methods since several years (Korsgaard et al., 2020; Swedres-Svarm, 2014; UK-VARSS, 2021). The German national monitoring program includes prevalence and AMR data of bovine *S. aureus* since 2009, as well as the prevalence of MRSA since 2009 and AMR of MRSA since 2019 (BVL, 2021). Although the approaches and laboratory techniques vary widely, these monitoring programs deliver important data on AMR trends and the effectiveness of control measures in the respective country.

3. Laboratory analysis of *S. aureus* in bovine mastitis

3.1. Identification of *S. aureus*

Numerous methods have been established for the identification of *S. aureus* isolates. The conventional phenotypic identification method for *S. aureus* in mastitis diagnostics is a microbial culture from milk samples. Using a sheep blood agar which is incubated for 24 hours at 37°C, species-specific parameters like colony morphology and haemolysis type can be identified, enabling further examinations like Gram stain morphology, catalase, and coagulase reaction. The large, smooth and mostly golden colonies can show different types of haemolysis in bovine *S. aureus* mastitis samples, which can be complete (alpha-haemolysin), incomplete (beta- or delta-haemolysin), a mixed form of both (double haemolysis), or non-haemolytic (gamma-haemolysin) (NMC, 2017; Wang et al., 2020; Younis et al., 2000). These cultures are often the basis for further phenotypical or biochemical identification methods, such as performing the Gram stain, where *S. aureus* appears as gram-positive cocci in pairs or clusters. Coagulase can be detected either in free form with the tube coagulase test or in bound form with the slide test. In both tests, a staphylococcal culture is mixed with rabbit plasma to generate enzymatic conversion from fibrinogen to fibrin and therefore clotting of the plasma (Samanta & Bandyopadhyay, 2020). Latex

agglutination tests for the simultaneous detection of clumping factor, Protein A, and capsule polysaccharides are unreliable for bovine staphylococcal mastitis, with many isolates showing latex agglutination-negative phenotypes (NMC, 2017).

There are many selective and differential media to identify *S. aureus*, like mannitol salt agar (MSA), lipovitellin salt mannitol agar (LSM), Vogel-Johnson agar (VJ), Baird Parker agar, and potassium thiocyanate-actidione-sodium azide-egg yolk-pyruvate agar (KRANEP). Because of the halotolerance of *S. aureus*, the selective media have a proportion of 5%-10% sodium chloride to inhibit the growth of other bacteria (MSA, LSM). Other selective agents are potassium tellurite and lithium chloride (in Vogel-Johnson and Baird Parker agar) as well as the pH indicator phenol red, which will change the colour of the medium yellow due to the ability of *S. aureus* to ferment mannitol. While showing a wide range of pH tolerance, its optimum is at 7,4. Furthermore the bacteria can be enriched in broths before inoculated on agar plates, and an increased CO_2 also stimulates growth (Samanta & Bandyopadhyay, 2020).

Molecular techniques are complementing phenotypic methods in *S. aureus* identification, as they guarantee sensitive and specific detection of microorganisms and therefore timely decisions on appropriate antimicrobial therapy. In veterinary medicine, polymerase chain reaction (PCR) is the most common method to identify different *S. aureus* genotypes (Taponen et al., 2009a). Current studies emphasize the role of MALDI-TOF MS (matrix-assisted laser desorption ionisation, time of flight mass spectrometry), defining it as equally effective in *S. aureus* identification as PCR typing (Kour et al., 2023; Ngassam Tchamba et al., 2019).

Genotypic identification of *S. aureus* using PCR first requires the isolation of DNA from a bacterial culture. PCRs are usually developed with oligonucleotide primers for the detection of *Staphylococcus*-specific 16S ribosomal RNA or specific genes like nuc, coa (thermonuclease and coagulase) as well as enterotoxin genes sea to sej and blaZ (penicillin resistance) (Graber et al., 2009; Taponen et al., 2009a). This fast and affordable typing method was proven to exhibit specificity of 100% and sensitivity close to this value, therefore it is frequently used in clinical veterinary diagnostic laboratories (Saraiva et al., 2017).

The proteomic identification by MALDI-TOF MS is comparable to modern PCR analysis in accuracy, efficiency and cost-effectiveness, and has been used for more than a decade in routine clinical microbiology laboratories (Nonnemann et al., 2019). Either staphylococcal colonies or their extracted proteins are applied as a thin film onto a 24-spot steel plate, where they are covered with a matrix and subsequently dried. The mass spectrometer then generates the protein spectral profile of an *S. aureus* isolate and compares it to a reference database for identification (Alatoom Adnan et al., 2011). This method allows the identification of *S. aureus* at serotype or strain level, as well as antimicrobial resistance profiling within minutes, while costing only up to a third of conventional phenotypic methods (Seng et al., 2009). Some clinical veterinary diagnostic laboratories may not yet use MALDI-TOF MS as standard identification method due to high purchase costs and insufficient reference spectra included in the commercial database. But as expansions of the database can easily be performed, the use in larger laboratories increased considerably in the recent years, and development of cheaper models by the diagnostics industry will only further augment its significance (da Motta et al., 2014; Seng et al., 2009; Wanecka et al., 2019).

All these identification methods are used for rapid and reliable detection of mastitis-causing bacteria at species level, in order to find suitable treatment options for afflicted dairy cows. For the distinction of different strains or isolates in the scope of epidemiological and evolutionary investigations, a plethora of *S. aureus* typing methods is available (Dendani Chadi et al., 2022).

3.2. Antimicrobial susceptibility testing

3.2.1. β-lactamase testing

As mentioned before, some *S. aureus* strains express the enzyme β-lactamase and are therefore resistant to β-lactam antimicrobials. The β-lactamase enzyme (induced by *bla*Z gene) production may be constitutive or inducible by exposure to certain antimicrobials (de Oliveira et al., 2000). The detection of β-lactamase by chromogenic, iodometric, and acidometric tests delivers results earlier than regular susceptibility tests (e.g., broth microdilution or disk diffusion, see below) and can be used for a first overview of the resistance patterns in *S. aureus*-positive samples. Alternatively, a PCR assay of the *bla*Z gene can be performed. However, this test can provide false negative results if the β-lactamase activity of an isolate

is based on the expression of other genes, or if *blaZ* is detectable but not actively expressed. (Robles et al., 2014).

The chromogenic nitrocefin test relies on a chromogenic cephalosporin (nitrocefin), that indicates the presence of β-lactamase producing *S. aureus* by changing disk colour from yellow to red (positive test result). If the test is negative, no colour change can be observed (Chaudhary et al., 2021).

In the acidimetric method, the reaction of penicillin-phenol red substrate with the β-lactamase enzyme results in the production of penicilloic acid (by penicillin hydrolyzation), leading to a colour change from red to yellow (positive test result). No change in colour again indicates a negative test (Livermore & Brown, 2001).

The iodometric method relies again on penicillin hydrolyzation. The product penicilloic acid reduces iodine, therefore preventing its complexion with starch. If the blue coloured iodine starch complex is hindered by β-lactamase positive *S. aureus*, the disc shows discoloration (Chaudhary et al., 2021).

3.2.2. Susceptibility testing and identification of MRSA

For the identification of antimicrobial resistance in *S. aureus*, many susceptibility testing techniques have been developed over time; they can again be separated into phenotypic and genotypic methods. Phenotypic resistance is assessed by growing *S. aureus* in the presence of specific antimicrobial concentrations. This allows the subsequent classification of the *S. aureus* isolate as susceptible, intermediate, or resistant to the antimicrobial agent. The broth microdilution method (BMD) and disk diffusion method are currently considered as gold standards in phenotypic antimicrobial susceptibility testing (AST) (Sanchini, 2022).

In Broth Microdilution (BMD), the Minimum Inhibitory Concentration (MIC) - defined as the lowest concentration of an antimicrobial that prevents visible growth of the bacteria – is used to interpret the antimicrobial susceptibility. A bacterial culture is inoculated in liquid media (e.g., cation-adjusted Mueller-Hinton broth (CAMHB)), usually in a commercially prepared 96-multiwell plate with 50 µl/well. These wells contain standardized concentration gradients of frozen or freeze-dried antibiotics, which are then inoculated with an emulsion of the bacterial culture. This inoculum must reach a certain standardised turbidity

(0,5 McFarland), in order to correctly indicate bacterial growth despite a specific concentration of the antimicrobial. It is incubated at 35°C +/- 2°C and MICs can be interpreted after 16-24 hours (CLSI, 2023b). BMD is a cost-effective, but time-consuming method.

The interpretation of MICs usually depends on specific breakpoints for the respective antimicrobial, bacteria, and host species and target tissue (e.g., mastitic udder). MIC values lower than the breakpoint indicate susceptibility of the bacteria, MICs higher than the breakpoint indicate resistance. Veterinary specific interpretive criteria for MICs are provided by either the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, their approaches vary notably and for certain animal species, antimicrobials and indications, official veterinary breakpoints are not yet established. Especially for the indication *S. aureus* mastitis in cattle, these institutions often have to refer to human breakpoints (CLSI, 2023a), manufacturers' information (Vetoquinol UK Ltd, 2011), or values from publications (Pillar et al., 2009).

For disk diffusion test, or standard Kirby-Bauer test, a bacterial culture in the concentration of 1–2 × 10^8 colony-forming unit (CFU)/mL (equivalent to the 0.5 McFarland turbidity unit) is inoculated in Mueller-Hinton agar (MHA) plates. Paper disks with specific antimicrobial concentrations are stamped on top. After incubation (35°C +/- 2°C, 16-24 h), the diameter of the growth inhibition zone of each disk is measured and interpreted (CLSI, 2023b). This method allows the classification as susceptible, intermediate or resistant, but not MIC values.

A traditional phenotypic method of MRSA identification is the inoculation on chromogenic media. These selective media contain oxacillin or cefoxitin in order to inhibit the growth of competitor organisms and allow for the detection of MRSA within 18-26 hours after incubation. For example, chromogenic media based on phosphatase activity use a chromogen which yields a blue colour as a result of phosphatase activity, which is the case for all MRSA (Xu et al., 2016). This method only provides categorization into susceptible or resistant.

Similar to *S. aureus* identification, genotypic methods have surpassed phenotypic methods in accuracy and efficiency of MRSA identification. However, they do not determine MICs, but detect known DNA sequences or resistance genes (e.g.,

*mec*A). This limits the value of these methods for the case of new, unknown resistance genes, or MRSA isolates that possess the resistance gene without the allele being expressed, which makes the isolate susceptible to the antimicrobial (Pu et al., 2014).

The *mec*A-PCR is considered a gold-standard molecular method to detect MRSA. However, this method would not detect MRSA that obtained resistance through the *mec*C gene (Schlotter et al., 2014). Therefore, PCR assays have been developed that detect both mecA and mecC genes (Becker et al., 2016).

4. Objectives

Based on the available literature on *S. aureus* in bovine mastitis, it can be concluded that ongoing research about current isolate distribution and regional resistance trends is required to support efforts for successful *S. aureus* and MRSA prevention and control in Bavarian dairy cattle farming. The aim of this work was therefore to determine the prevalence and antimicrobial resistance of mastitis-causing *S. aureus* and MRSA in cattle in Southern Germany over an eleven-year period between 2012 and 2022.

III. PUBLICATIONS

1. Publication I

Cumulative doctoral achievement: Publication

Changes in antimicrobial resistance of *Staphylococcus aureus* in bovine quarter milk samples from Southern Germany between 2012 and 2022

Karell, J.; Petzl, W.; Gangl, A.; Huber-Schlenstedt, R.; Sorge, U.S.

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Changes in antimicrobial resistance of *Staphylococcus aureus* in bovine quarter milk samples from southern Germany between 2012 and 2022

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ABSTRACT

The objective of this study was to describe the in vitro resistance of *Staphylococcus 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} (the MIC where 50% and 90% of isolates were inhibited by the tested antibiotics, respectively) mostly beneath the respective breakpoint. On average, about one-fourth (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, cefquinome, 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*, 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 antimicrobial resistance (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, 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 aureus* (Tenhagen et al., 2006; Kadlec et al., 2019). 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., 2006). 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, and so on) 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, although 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,

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

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 *S. aureus* and to identify current therapeutic options for *S. aureus* mastitis. Therefore, the objective of this study was to describe the in vitro resistance of *S. aureus* isolates from quarter milk samples obtained by 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 *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 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

Bacteriology. The laboratory methods were based on the respective valid guidelines for diagnosis of mastitis of the DVG (German veterinary medical society; DVG, 2009, 2018). Because 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 *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 to 24 h and 48 h incubation. The phenotypic identification of *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, *S. aureus* was additionally identified by MALDI TOF if necessary (microflex MALDI Biotyper, reference database V.3.3.1.0., Bruker Daltonik GmbH).

In Vitro AMR. 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 methicillin-resistant *Staphylococcus aureus* (**MRSA**) 2 agar (Oxoid) to clarify whether they belonged to the group of 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 *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 *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 Clinical & Laboratory Standards Institute (**CLSI**) guidelines (CLSI, 2015), with quality control testing (*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* masti-

tis 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 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, aminoglycosides, 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 $\mathbf{MIC_{50}}$ and $\mathbf{MIC_{90}}$ were the MIC where 50% and 90% of isolates were inhibited by the tested antibiotics, respectively. Multiresistance 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 (Microsoft Corp.). 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 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 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 MIC_{50} and MIC_{90}. On average, 24% (n = 5,718) of the isolates

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

				CMT[1] score and clinical outcome		
Item	Isolate (n)	Cow (n)	Herd (n)	CMT negative, n (%)	Subclinical mastitis, n (%)	Clinical mastitis, n (%)
Pathogen						
All *S. aureus*	167,651	94,058	12,052	62,702 (37.4)	95,729 (57.1)	9,221 (5.5)
β-lactamase +[2]	27,998	15,919	2,765	14,867 (53.1)	12,739 (45.5)	392 (1.4)
β-lactamase −[2]	139,653	81,923	11,888	47,901 (34.3)	82,954 (59.4)	8,798 (6.3)
BMD analysis[3]						
All *S. aureus*	23,446	21,721	7,925	1,665 (7.1)	15,568 (66.4)	6,213 (26.5)
Resistant[4]	12,582	11,586	5,159	1,195 (9.5)	9,336 (74.2)	2,063 (16.4)
Susceptible	10,864	10,519	5,121	478 (4.4)	6,236 (57.4)	4,14 (38.2)

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

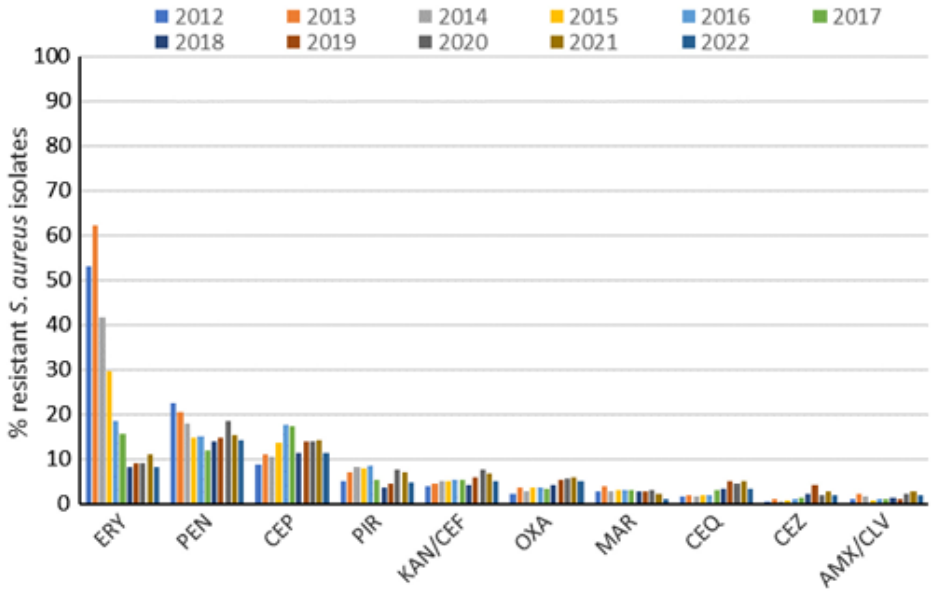

Figure 1. Percentage of resistant *Staphylococcus aureus* isolates by year and antimicrobial substance based on breakpoint method. For kanamycin-cefalexin the breakpoint of Clinical & Laboratory Standards Institute Vet01S 2023 was applied (CLSI, 2023b). ERY = erythromycin, PEN = penicillin, CEP = cefoperazone,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

showed in vitro resistance to erythromycin. This was the highest proportion of resistant isolates in this study. The MIC_{50} and MIC_{90} 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).

Table 2. Minimum inhibitory concentration where 50% and 90% of isolates were inhibited by the tested antibiotics (MIC_{50} and MIC_{90}, respectively) of the respective antimicrobials in *Staphylococcus aureus* isolates between 2012 and 2022, based on breakpoint method[1]

Antimicrobial	MIC (µg/mL)	2012	2014	2016	2018	2020	2022
Erythromycin	MIC_{50}	1	**0.5**	**0.5**	**0.25**	**0.25**	**0.25**
	MIC_{90}	2	4	1	**0.5**	**0.5**	**0.5**
Penicillin	MIC_{50}	**≤0.125**	**≤0.125**	**≤0.125**	**≤0.125**	**≤0.125**	**≤0.125**
	MIC_{90}	8	8	8	4	8	8
Pirlimycin	MIC_{50}	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**
	MIC_{90}	**2**	**2**	**2**	**2**	**2**	**2**
Marbofloxacin	MIC_{50}	**≤0.25**	**≤0.25**	**≤0.25**	**≤0.25**	**≤0.25**	**≤0.25**
	MIC_{90}	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**	**0.5**
Oxacillin	MIC_{50}	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**
	MIC_{90}	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**
Cefoperazone	MIC_{50}	**≤2**	**≤2**	**≤2**	**≤2**	**≤2**	**≤2**
	MIC_{90}	**≤2**	4	4	4	4	4
Kanamycin-cefalexin	MIC_{50}	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**
	MIC_{90}	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**	**≤4/0.4**
Cefquinome	MIC_{50}	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**
	MIC_{90}	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**	**≤1**
Cefazolin	MIC_{50}	**≤4**	**≤4**	**≤4**	**≤4**	**≤4**	**≤4**
	MIC_{90}	**≤4**	**≤4**	**≤4**	**≤4**	**≤4**	**≤4**
Amoxicillin or clavulanate	MIC_{50}	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**
	MIC_{90}	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**	**≤4/2**

[1]Bold numbers indicate values below the respective breakpoint.

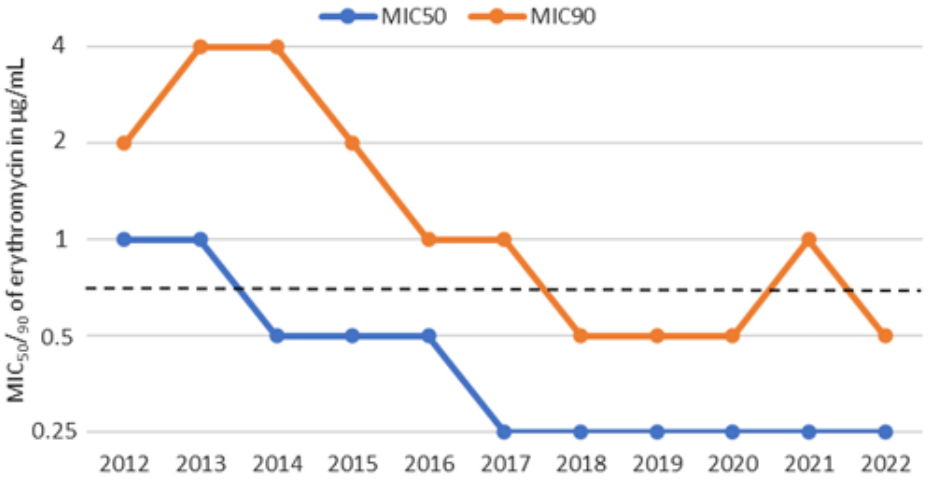

Figure 2. Minimum inhibitory concentration where 50% and 90% of isolates were inhibited by the tested antibiotic (MIC_{50} and MIC_{90}) of erythromycin for *Staphylococcus aureus* isolates per year based on breakpoint method. The dashed line indicates the breakpoint.

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 MIC_{50} and MIC_{90} did not change over the 11 years, with the MIC_{50} constantly at the lowest and the MIC_{90} 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 MIC_{50} and MIC_{90}, 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 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).

Overall, 13% (n = 3,136) of the *S. aureus* isolates were resistant in vitro to cefoperazone and this percentage increased from 9% (n = 167) in 2012 to 11% (n = 159) in 2022 ($P < 0.01$, Figure 1). The MIC_{50} and MIC_{90} were consistently at the lowest and second lowest assessed MIC, respectively (Table 2).

For kanamycin-cefalexin, the resistance prevalence was 5% (n = 1,222) and did not change significantly in the study period. Over the 11 years, both the MIC_{50} and MIC_{90} were constantly at the lowest assessed MIC (Table 2). With the former breakpoint applied, the MIC_{50} and MIC_{90} did not change and the overall resistance prevalence was at 2% (n = 729).

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} and MIC_{90} were 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

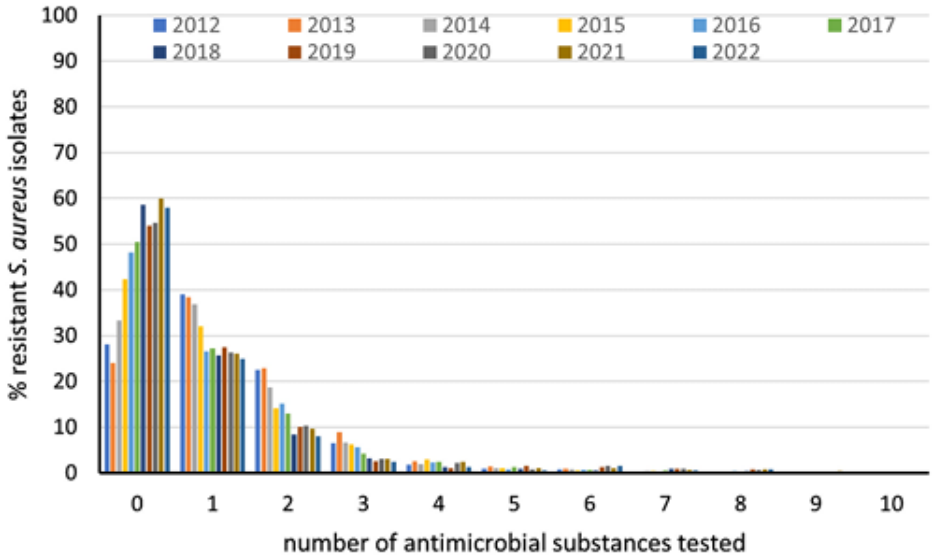

Figure 3. Number of antimicrobial substances that *Staphylococcus aureus* isolates tested in vitro resistant to by year. For kanamycin-cefalexin the breakpoint of Clinical & Laboratory Standards Institute Vet01S 2023 standard was applied (CLSI, 2023b).

(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. However, the percentage declined from 6% (n = 198) in 2012 to 3% (n = 176) in 2022.

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.

Although 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, because 1 to 2 isolates already 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, although 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). Although 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 = 5,717) of isolates resistant to erythromycin, only 18% (n = 1,004) also showed resistance to pirlimycin. Because resistance to erythromycin is usually mediated by *erm* genes that also cause pirlimycin resistance (when the *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 *erm* genes that are inducibly expressed. The latter could be identified by a D test (Shrestha 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 *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 *S. aureus* isolates from bovine mastitis. The 6% in vitro resistance to pirlimycin (for all isolates [n = 1,490] and for clinical mastitis cases only [n = 369]) obtained in this study were 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 = 1,004) were also resistant to erythromycin ($P < 0.001$). The remaining 33% (n = 486) of isolates resistant to pirlimycin probably have other resistance genes than *erm* (e.g., *lnu*/*lin*, *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. (2019) in Austria. 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). Because the distribution of MIC 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) *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 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-stabile penicillins (amoxicillin-clavulanate).

Although the recommendation to report all MRSA strains as resistant to β-lactams was initially developed from severe cases of human septicemia, 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 MIC_{50} and MIC_{90} of both cefazolin and cefquinome were 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. Because 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 multidrug resistant isolates.

Although 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 (TÄHAV). 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 and Huber-Schlenstedt, 2021). 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.

Although 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 t) between 2011 and 2021 (Sander et al., 2022). Furthermore, penicillin sales decreased sharply (−55%, 293 t) 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. Although 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 (Kasabova et al., 2021; Sander et al., 2022)—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; Korsgaard et al., 2020; VMD and APHA, 2021), 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). Because 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.

NOTES

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Abbreviations used: AMR = antimicrobial resistance; AMX/CLV = amoxicillin-clavulanate; BMD = broth microdilution; CEP = cefoperazone; CEQ = cefquinome; CEZ = cefazolin; CLSI = Clinical & Laboratory Standards Institute; ERY = erythromycin; KAN/CEF = kanamycin-cefalexin; MAR = marbofloxacin; MIC_{50} = MIC for 50% of isolates inhibited by tested antibiotics; MIC_{90} = MIC for 90% of isolates inhibited by tested antibiotics; MRSA = methicillin-resistant *Staphylococcus aureus*; OXA = oxacillin; PEN = penicillin; PIR = pirlimycin; TGD = the udder health laboratory of the Bavarian Animal Health Services.

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2. Publication II

Cumulative doctoral achievement: Publication

Prevalence and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine quarter milk samples from Southern Germany (2013 – 2022)

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Prevalence and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine quarter milk samples from Southern Germany (2013 – 2022)

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ABSTRACT

The objective of this study was to determine the prevalence and antimicrobial resistance (AMR) pattern of methicillin-resistant *Staphylococcus aureus* (MRSA) from bovine quarter milk samples isolated by the Bavarian Animal Health Services (TGD) between 2013 and 2022. All *Staphylococcus (S.) aureus* submissions were tested for β-lactamase production. Only those isolates positive for β-lactamase or upon request by the client were forwarded to susceptibility testing. Identification of MRSA was either based upon oxacillin resistance determined by broth microdilution or alternatively growth on Brilliance MRSA 2 Agar.

Almost all MRSA isolates (n=910) were *in vitro* resistant to penicillin (99%, n=905) and cefoperazone (98%, n=894). More than half of the isolates showed resistance to cefquinome (65%, n=592) and cefazolin (53%, n=486). Furthermore, nearly half (46%, n=417) of the MRSA isolates were *in vitro* resistant to erythromycin, with a remarkable drop from 92% (n=78) to 39% (n=28) between 2013 and 2022. The overall resistance prevalence for kanamycin-cefalexin, pirlimycin, marbofloxacin, and amoxicillin-clavulanate remained below 35% for each. In the study period, the percentage of *in vitro* resistant MRSA isolates from bovine quarter milk samples slightly decreased, but mostly due to less resistance against erythromycin.

In conclusion, the prevalence of MRSA among quarter milk samples containing *S. aureus* was overall low and increased only slightly, whereas the percentage of

antimicrobial resistance of MRSA (especially against β-lactams) was high but moderately decreased. Consequently, successful therapy of MRSA infections in Bavarian dairy cows remains unlikely.

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

INTRODUCTION

Mastitis in dairy cattle is the most common and costly disease in this industry worldwide [1]. It is defined as an inflammation of the mammary gland usually caused by bacteria and therefore treated mainly with antimicrobial agents [2]. Bacteria resistant to antimicrobials have become a public concern, and although methicillin-resistant *Staphylococcus aureus* (MRSA) in bovine mastitis is rarely found, it has been increasingly reported [3]. MRSA show resistance to most broad-spectrum β-lactam antimicrobials [4]. This limits treatment options, because they constitute the majority of antimicrobials approved for bovine mastitis therapy and every antimicrobial approved for dry cow therapy in Germany [5]. In addition, MRSA evade both the host immune response and antimicrobial agents through extensive fibrosis, micro abscess formation and other pathogen-specific immune-evasion strategies [6]. Therefore, the eradication of MRSA from dairy herds is challenging [7, 8]. In addition to the aspect of animal health, the zoonotic potential of MRSA poses an infection risk to dairy farmers and veterinarians [9, 10]. Contamination of raw milk products with MRSA and transmission to consumers have been described [11]. Although the likelihood of the latter two events is low, they contribute to providing a rationale for research on MRSA in dairy herds. For this reason, the Bavarian Animal Health Services (TGD) perform MRSA diagnostics on all *S. aureus* isolates from quarter milk samples since 2013 [12, 13].

In Germany, MRSA are included in the national monitoring program for antimicrobial resistance (GermVet) as representatives of potentially zoonotic bacteria in veterinary medicine. Of all bovine mastitis cases caused by *S. aureus* between 2011 and 2019, the prevalence of MRSA was only 6% [14]. But while these records provide an overview of MRSA occurrence, they do not include data on the resistance profile as is the case with other pathogens. This would be necessary to evaluate the success of antimicrobial stewardship programs and their direct impact on MRSA. In particular, the efficacy of

non-β-lactam antimicrobials to treat MRSA infections in dairy cows needs to be investigated, as culling infected animals alone is not sufficient to eradicate MRSA from dairy farms [4]. Therefore, the aim of this study was to describe the *in vitro* resistance of MRSA isolates from quarter milk samples obtained by the udder health laboratory of the Bavarian Animal Health Services between 2013 and 2022.

MATERIALS AND METHODS

Sample Population

All bovine quarter milk samples that were submitted to the udder health laboratory of the TGD between 2013 and 2022 and that contained MRSA were included in this retrospective case series. Most samples were from whole herd screenings by TGD technicians, the rest consisted of individual cow submissions by farmers or their veterinarians. The California Mastitis Test (CMT) was used to categorize all samples as either negative (healthy quarter) or positive in the case of subclinical mastitis. Samples that stemmed from a cow with signs of clinical mastitis (abnormal milk, swollen udder, fever etc.) were classified as clinical mastitis cases either by the technicians on the farm or based on examination of the milk in the laboratory.

Laboratory Analysis

a) Bacteriology

The laboratory methods were based on the DVG (German veterinary medical society) guidelines for diagnosis of mastitis valid at the time [15, 16]. Since the quarter milk samples for this study were obtained as part of routine mastitis diagnostics at the TGD, the laboratory methods were designed to identify various mastitis-causing pathogens, not only MRSA. Accordingly, inoculum sizes of 0,01 mL for samples of whole herd screenings and 0,05 mL for samples of clinical mastitis cases were used. The inocula were transferred to Aesculin blood agar plates (Oxoid) supplemented with 5% sheep blood and incubated aerobically at 36 ± 1°C. They were evaluated after 18-24 hours and 48 hours of incubation. The phenotypic identification of *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 [16]. MALDI TOF was

used for differentiation of strains when results were unclear (microflex MALDI Biotyper, reference database V.3.3.1.0., Bruker Daltonik GmbH).

b) In-vitro antimicrobial resistance and MRSA classification

Following the recommendations of Rosselet et al. [17] and Gedek [18], all *S. aureus* isolates were tested for the synthesis of β-lactamase by the iodometric method, using an iodine/iodine-potassium stock solution with phosphate buffer, aqua dist. and penicillin G. To determine if the β-lactamase-positive samples belonged to the group of methicillin-resistant *Staphylococcus aureus* (MRSA), they were either directly analyzed by microbroth susceptibility testing or transferred to Brilliance-MRSA 2 agar (Oxoid). To confirm the diagnosis in case of MRSA-like growth, MIC determination was then carried out on each individual sample (with positive CMT or changes in secretion) as well as random stock samples. The breakpoint ≥ 4 mg/l for oxacillin indicated the presence of MRSA [19].

According to routine guidelines, a sample selection of all isolates was forwarded to further antimicrobial susceptibility testing. At herd screenings, up to 3 β-lactamase positive *S. aureus* 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 if the dairy farmer or veterinarian specifically requested it. The same selection standards were implemented for individual sample submissions. Susceptibility testing was performed with broth microdilution using the breakpoint method (mastitis 3 plate, Merlin Diagnostica GmbH). This commercial system complied with the CLSI guidelines [20], with quality control testing (*S. aureus* ACTT 29 213) performed weekly and within the established ranges, in accordance with the guidelines from the accreditation authority. Here, the following common antimicrobials applied in mastitis therapy were tested: β-lactams (penicillin, ampicillin, amoxicillin-clavulanate and oxacillin, cephalosporins of the first, third and fourth generation (cefazolin, cefoperazone and cefquinome, respectively)), macrolides (erythromycin), quinolones (marbofloxacin), lincosamides (pirlimycin), and an aminoglycoside-cephalosporin combination (kanamycin-cefalexin). The program MCN 6 (version MCN 6.00–08.01.2018 Rel. 89; Demo Computer GmbH and Merlin Diagnostica GmbH) was used for breakpoint analysis, applying official breakpoints from standards in effect at the time (e.g., NCCLS M31-A3, CLSI Vet01, CLSI M100).

In lack of official breakpoints for the indication *S. aureus* mastitis in cattle, the program would either use former breakpoints [21], human breakpoints [22], manufacturers' information [23, 24], or values from publications [25]. The first official breakpoint for kanamycin-cefalexin for *S. aureus* mastitis in cattle was only published recently [26], which is why both breakpoints were considered in this study. All MRSA isolates were assumed to be multidrug resistant as defined by Magiorakos et al., based on resistance of MRSA to most β-lactam antimicrobials [27, 28]. Results for ampicillin, gentamicin and tetracyclin were discarded due to incomplete susceptibility testing and/or missing MIC values. Therefore, results for a total of ten antimicrobial agents were included in this study. Intermediate results were categorized as resistant and only acquired resistance was included.

Statistical Analysis

The statistical analysis was done in SAS 9.4 (SAS Institute Inc., Cary, NY, USA). Descriptive statistics were applied for MIC observations by year and isolate (PROC FREQ), as well as Fisher's exact test comparing the association between mastitis status and MRSA. Prevalence trends were evaluated with the Wilcoxon rank-sum test. The MIC_{50} and MIC_{90} were the MIC where 50 and 90% of isolates were inhibited by the tested antibiotics, respectively. All graphics were created in Microsoft Excel 2010. Missing data were ignored and α was set at 0.05.

RESULTS

Sample Population and Prevalence

Table 1 provides an overview of the samples included in this study. From all quarter milk samples submitted to the TGD between 2013 and 2022, a total of 147,718 isolates were identified as *S. aureus*. They were mostly (~80%) collected during whole herd screenings. Overall, 0.9% (n=1,341) of the *S. aureus* isolates were MRSA, originating from 976 cows of 389 herds.

A total of 910 MRSA isolates from quarter milk samples were forwarded to MIC determination (with broth microdilution). Here, the proportion of diagnostic samples of herd screenings was 56% (n=510), due to the selection criteria for susceptibility testing (e.g., clients request).

During the study period, we noted a slight overall increase in MRSA prevalence in our study population (Table 1). Among all herds positive for *S. aureus*, there were more herds positive for MRSA, with a prevalence of 2.2% in 2013 increasing to 2.3% in 2022 ($P < 0.05$). Similarly, the prevalence of quarters testing positive for MRSA among quarters with *S. aureus* isolates increased from 0.6% in 2013 to 1.2% in 2022 ($P < 0.001$). However, the percentage of cows positive for MRSA in herds positive for MRSA did not change significantly.

Minimum Inhibitory Concentrations (MIC) and *in vitro* resistance

Figure 1 shows the trend of resistance for the different antimicrobials in MRSA isolates between 2013 and 2022, and Table 2 the respective trends of MIC50 and MIC90. Since oxacillin resistance was used for the identification of MRSA, 100% (n=910) of the MRSA isolates were *in vitro* resistant (Figure 1).

Almost all MRSA isolates were *in vitro* resistant to penicillin, with an average resistance prevalence of 99% (n=905; Figure 1). In the study period, both the MIC_{50} and MIC_{90} were constantly at the highest MIC tested (Table 2).

Similar results were obtained for cefoperazone, as 98% (n=894) of the MRSA isolates were resistant to this antimicrobial (Figure 1). The MIC_{50} and MIC_{90} remained above the breakpoint over all 10 years (Table 2).

On average, 65% (n=592) of isolates were resistant to cefquinome. There was a noteworthy peak of resistance in 2019, with 89% (n=110) of the MRSA isolates ($P<0.001$; Figure 1). However, the resistance prevalence (as well as the MICs) remained relatively stable over the rest of the study period.

Comparably, 53% (n=486) of all MRSA isolates were in vitro resistant to cefazolin, with a peak of 87% resistance prevalence *in vitro* in 2019 ($P<0.001$; Table 1).

For erythromycin, on average 46% (n=417) of the isolates were *in vitro* resistant, with a remarkable drop from 92% (n=78) in 2013 to 39% (n=28) in 2022 ($P < 0.001$; Figure 1). The MIC results showed a similar trend, as the MIC_{50} decreased at a constant rate over the study period ($P < 0.001$).

For the other antimicrobials tested, the average *in vitro* resistance remained below 35% for each and with no remarkable MIC changes in the study period (Table 2). For kanamycin-cefalexin, the prevalence of *in vitro* resistance was 34% (n=311) and increased from 32% (n=27) in 2013 to 48% (n=34) in 2022 ($P < 0.05$; if the breakpoint from CLSI Vet01S Standard 2023 was applied; Figure 1). With the former breakpoint applied, the MIC_{50} and MIC_{90} did not change, but the overall resistance prevalence was only 24% (n=220). Pirlimycin showed a total resistance prevalence of 29% (n=263), with an increase from 14% (n=12) in 2013 to 34% (n=24) in 2022 ($P < 0.01$; Figure 1). For marbofloxacin and amoxicillin-clavulanate, an average resistance of 19% was observed in each case (n=175 and n=172, respectively).

In vitro resistance to multiple antimicrobials

Figure 2 shows the number of antimicrobials that MRSA isolates were *in vitro* resistant to between 2013 and 2022. Since oxacillin resistance was implied and almost all isolates were resistant to penicillin and cefoperazone, most isolates showed resistance to 3 to 8 antimicrobial agents (Figure 2). This is consistent with the definition of multidrug resistance by Magiorakos et al. [27]. Over the 10-year period, there was no clear trend regarding resistance to multiple antimicrobials. The most common resistance combination overall was to oxacillin, penicillin, and cefoperazone, since 13% (n=120) of all MRSA isolates exhibited this resistance pattern. Also very frequent was a resistance combination against all tested β-lactams (11%, n=96), as well as resistance to β-lactams and erythromycin (8%, n=74). This did not change noteworthily over the ten years, and other combined resistances were not observed.

DISCUSSION

Unlike previous publications, this study included a large number of samples and farms over a 10-year period. However, this difference also limits the comparability to other studies about the *in vitro* resistance of MRSA: Each of the publications included fewer than 53 MRSA isolates [7, 8, 14, 29, 30, 31, 32, 33, 34, 35, 36] and none was specifically for the region of Southern Germany.

The comparison of resistance patterns from different studies was further complicated by varying sample selections, as some studies only included isolates from clinical or subclinical cases [7, 14, 33]. Some studies worked with MRSA isolates from several

regions [8] or countries [3], or included only isolates from a single dairy farm [31]. This might explain why our MRSA prevalence of 0.9% (n=1,341, among all submitted *S. aureus* isolates) differed from the 6% found in the German national monitoring program [14]. In our study, 99% (n=905) of MRSA isolates were considered resistant to penicillin, which was comparable to the 100% resistance found in different regions of Germany [29, 36]. Since MRSA are considered resistant to virtually all β-lactam antimicrobials [37], our few isolates that tested sensitive to penicillin could be due to applied human breakpoints, data inaccuracies or alleles that were not expressed. In contrast to the results for penicillin, a study from Northern Italy reported 89% MRSA isolates resistant against marbofloxacin [32]. This was higher than our 19% (n=175) resistant isolates. However, their classification of dairy herds as MRSA negative or positive was based on a selection of isolates identified by bulk tank analysis, which has a low sensitivity for *S. aureus* detection [38]. Similarly, Feßler et al. [29] only reported about MRSA ST398 isolates. Since we did not specify the MRSA isolates further this might explain the observed differences in resistance to pirlimycin (29% (n=263) in our study, 56% by Feßler et al. [29]) or amoxicillin-clavulanate (19% (n=172) in our study, 31% by Feßler et al. [29]).

Contrary to the CLSI recommendation to report MRSA strains as resistant to all β-lactams [39], we included amoxicillin-clavulanate, cefquinome and cefazolin in our AMR analysis. Their recommendation was originally developed from severe cases of human septicemia but could be justified in the context of bovine mastitis because of the ineffective immune response of the mammary gland in *S. aureus* infections [40] and the often subtherapeutic concentrations of antimicrobials in udder tissue [41]. Therefore, since we included other β-lactam antimicrobials than just penicillin and oxacillin in our discussion, we must point out that the respective in vitro results may not be sufficiently predictive of therapeutic outcome.

In this study, data analysis in a single laboratory over more than a decade allowed a reliable trend evaluation for the included dairy farms in the region of Bavaria. Although more sensitive laboratory techniques such as nitrocefin for ß-lactamase testing and *mec* PCR for MRSA detection have become available over time, the convenient and reliable methods employed in this study have remained consistent within the TGD, in order to ensure the consistency necessary for trend analyses.

Over the 10 years, the prevalence of MRSA-positive herds among *S. aureus*-positive herds increased minimally, as did the quarter prevalence of MRSA among *S. aureus*-positive quarters. This trend was not surprising, since control of MRSA in dairy herds is challenging and frequent use of β-lactam antimicrobials may further facilitate MRSA selection [42, 8]. However, this mild increase in MRSA prevalence should not be of great relevance, because it was observed only in the evaluation of samples containing *S. aureus* and not in all quarter milk samples submitted to the TGD. The high percentage of β-lactamase positive isolates in our study might have introduced some bias to prevalence analysis. 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* [43]. However, it is important to note that the MRSA prevalence from our study largely matched those reported in other publications from Germany and Europe, as demonstrated below. Furthermore, while our study population may not be entirely representative for herd-level observations, it nevertheless offers reliable insights into resistance trends.

In the study period, the *in vitro* resistance prevalence of MRSA isolates from bovine quarter milk samples remained high or increased for some of the tested antimicrobials (e.g., penicillin, cefoperazone, cefquinome, cefazolin, kanamycin-cefalexin; Figure 1). However, AMR prevalence remained below 35% for pirlimycin, marbofloxacin and amoxicillin-clavulanate and showed a notable drop for erythromycin. Therefore, the overall proportion of resistant MRSA isolates moderately decreased between 2013 and 2022. This is in accordance with previous findings in Germany, as Tenhagen et al. described a high but slightly decreasing proportion of resistant MRSA isolates between 2010 and 2019 [8].

Consequently, successful therapy of bovine mastitis caused by MRSA remains unlikely. Treatment with β-lactam antimicrobials is probably ineffective, as resistance against these agents was high or even increased already *in vitro*. Resistance to non-β-lactam antimicrobials (e.g., erythromycin, marbofloxacin, pirlimycin) may be less frequent, but only very few of these agents are approved for treating intramammary infections in dairy cows in Germany [5]. Since MRSA eradication may not be achieved by culling infected animals alone, treatment might be an option for individual, newly infected, and primiparous cows [4]. However, while there are currently no studies that confirm this hypothesis, there are reports about successful

eradication programs that have effectively reduced the prevalence of β-lactamase producing *S. aureus* through the removal of afflicted dairy cows from their herds [44].

The AMR trends in our study were compared with veterinary antimicrobial sales and usage data in Germany. However, it is important to consider potential bias due to ecological fallacy [45]. The overall antimicrobial sales decreased by 65% from 2012 to 2021 [46]. The constantly low average therapy frequency from 2013 to 2020 [47] may account for the observed decline in AMR prevalence. Particularly the substantial reduction (-73%) in erythromycin sales [46] and the relative increase in use of penicillin and aminoglycosides [47] aligned with resistance trends in our study (Figure 1). The observed overall reduction of antimicrobial sales, use, and resistance prevalence could be due to the changed Veterinary Pharmacies Prescription Regulation (TÄHAV) from 2018. It obliged susceptibility testing for critically important antimicrobials prior to use, and limited treatment duration as well as change of antimicrobial agents during therapy of individual cases. A more specific consequence of this could be the drop in sales of critically important antimicrobials from 2018 onwards [46]. This may have resulted in interim higher usage of β-lactams [47], potentially contributing to increased resistance to these agents, as we observed for cefquinome and cefazolin in our study (Figure 1).

When comparing the results from our study with findings from different national monitoring programs in Europe [14, 44, 48, 49], a low or even decreased MRSA prevalence in bovine dairy herds could be observed in most of them. In the monitoring program from England and Wales, there were no MRSA cases in dairy cattle from 2012 to 2020 [48], and from Sweden only 8 cows had tested positive for MRSA in 2010 to 2014 [44]. The Swedish and German monitoring programs also included AMR data of MRSA in their reports. Overall, the *in vitro* resistance of MRSA and their prevalence among *S. aureus* isolates from our study are slightly lower compared to the German monitoring program, but slightly higher than findings from the other European nations. All these countries have developed their AMR monitoring and reduction programs based on the corresponding preceding EU regulations [50]. It is an encouraging observation that in most countries that reduced antibiotic use, prevalence and/or *in vitro* resistance of MRSA also mildly decreased, even though approaches varied widely. However, the impact of successfully implemented mastitis control programs (e.g., the five-point mastitis control plan [51]) should not be underestimated.

Since they aim to prevent new infections while eliminating existing infections, they consequently contribute to reduce the spread of AMR. In the international comparison of AMR prevention and MRSA control programs, the effectiveness of the German approach can be confirmed. Nevertheless, the other European nations serve as role models and demonstrate our potential for improvement.

CONCLUSIONS

The prevalence of MRSA isolates in bovine quarter milk samples containing *S. aureus* was low and has only increased minimally during the study period. Because the overall percentage of *in vitro* resistant MRSA was high (especially against β-lactams) and only declined slightly, successful therapy of MRSA infections in Bavarian dairy cows remains unlikely.

COMPLIANCE WITH ETHICAL STANDARDS

The authors have not stated any conflicts of interest. No ethical approval under the German animal welfare law was required for sample collection.

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Table 1. Overview of methicillin-resistant *Staphylococcus aureus* (MRSA) quarter milk samples *in vitro* tested for β-lactamase activity and analyzed with broth microdilution (BMD) between 2013 - 2022. Multiple references of individual isolates possible.

				CMT[1] score and clinical outcome		
Pathogen	**Isolates (n)**	**Cows (n)**	**Herds (n)**	**CMT Negative (%)**	**Subclinical Mastitis (%)**	**Clinical Mastitis (%)**
All S. aureus	147,718	83,683	11,078	37.5%	56.9%	5.6%
MRSA	1,341	976	389	28.9%	62.3%	8.9%
BMD Analysis	**Isolates (n)**	**Cows (n)**	**Herds (n)**			
All S. aureus	21,525	19,959	7,403	7.2%	65.3%	27.5%
MRSA	910	817	364	21.7%	66.6%	11.8%

Prevalence[2] per year	**MRSA herds (n) / *S. aureus* herds**	**MRSA quarters (n) / *S. aureus* quarters**	**MRSA cows (n) / MRSA herd**
2013	2.2% (n=52)	0.6% (n=115)	2.3% (n=88)
2014	1.6% (n=40)	0.3% (n=61)	2.1% (n=52)
2015	2.3% (n=57)	0.8% (n=130)	3.4% (n=97)
2016	2.1% (n=47)	0.7% (n=95)	2.4% (n=80)
2017	2.1% (n=50)	0.7% (n=112)	3.4% (n=84)
2018	2.3% (n=63)	1.3% (n=198)	4.3% (n=140)
2019	2.7% (n=63)	1.5% (n=212)	4.6% (n=147)
2020	2.9% (n=62)	1.4% (n=163)	3.1% (n=127)
2021	2.4% (n=50)	1.3% (n=150)	4.5% (n=104)
2022	2.3% (n=40)	1.2% (n=105)	3.2% (n=83)

[1] California Mastitis Test.

[2] Prevalence was analyzed among all *S. aureus* sample submissions to the TGD.

Table 2. MIC_{50} and MIC_{90} 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.

Antimicrobial	**MIC in µg/ml**	**2014**	**2016**	**2018**	**2020**	**2022**
Oxacillin	MIC_{50}	>4	>4	>4	>4	>4
	MIC_{90}	>4	>4	>4	>4	>4
Penicillin	MIC_{50}	>8	>8	>8	>8	>8
	MIC_{90}	>8	>8	>8	>8	>8
Cefoperazone	MIC_{50}	8	16	16	16	16
	MIC_{90}	>16	>16	>16	>16	>16
Cefquinome	MIC_{50}	2	2	4	4	4
	MIC_{90}	4	≥8	≥8	≥8	≥8
Cefazolin	MIC_{90}	≤4	≤4	8	≤4	8
	MIC_{90}	16	32	>32	>32	>32
Erythromycin	MIC_{50}	1	0.5	0.25	0.5	0.5
	MIC_{90}	>4	>4	>4	>4	>4
Kanamycin-cefalexin	MIC_{50}	≤4/0.4	≤4/0.4	≤4/0.4	≤4/0.4	≤4/0.4
	MIC_{90}	≤32/3.2	≤32/3.2	≤32/3.2	≤32/3.2	≤32/3.2
Pirlimycin	MIC_{50}	2	≤1	≤1	≤1	≤1
	MIC_{90}	>4	>4	>4	>4	>4
Marbofloxacin	MIC_{50}	0.5	0.5	0.5	≤0.25	≤0.25
	MIC_{90}	>2	>2	>2	>2	1
Amoxicillin-clavulanate	MIC_{50}	≤4/2	≤4/2	≤4/2	≤4/2	≤4/2
	MIC_{90}	8/4	8/4	8/4	8/4	8/4

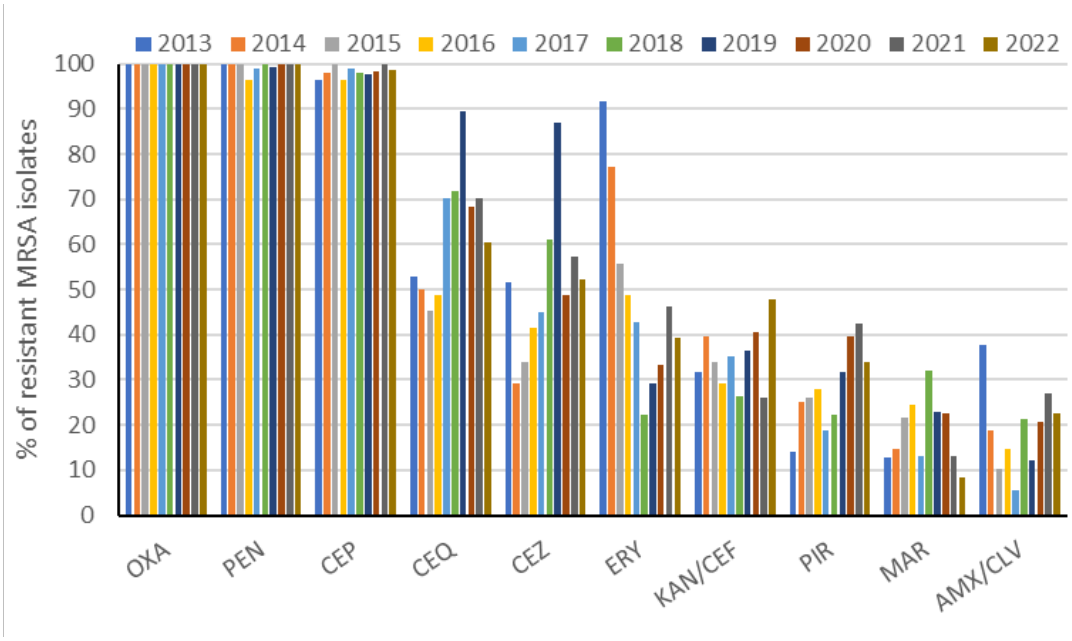

Figure 1. Percentage of resistant MRSA (methicillin resistant *Staphylococcus aureus*) isolates by year and antimicrobial substance based on breakpoint method. For kanamycin-cefalexin the breakpoint of CLSI Vet01S 2023 was applied. ERY = erythromycin, PEN = penicillin, CEP = cefoperazone, PIR = pirlimycin,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

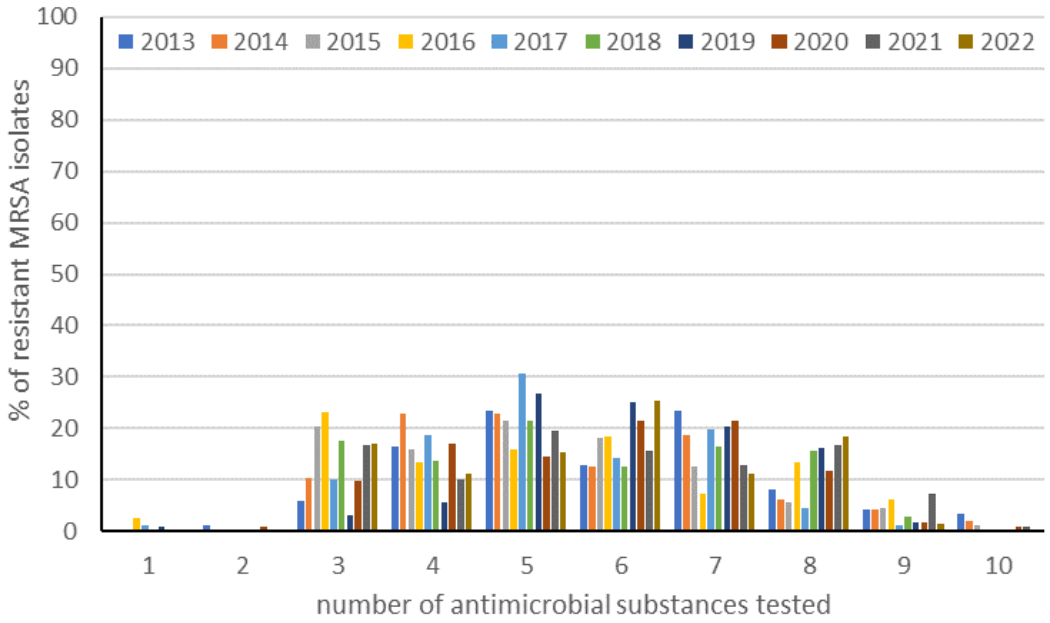

Figure 2. Number of antimicrobial substances that MRSA (methicillin-resistant *Staphylococcus aureus*) isolates tested *in vitro* resistant to by year. For kanamycin-cefalexin the breakpoint of CLSI Vet01S 2023 was applied.

IV. DISCUSSION

1. General aspects and comparability

This retrospective case series allowed an overview of AMR trends of both *S. aureus* and MRSA from Bavarian dairy herds between 2012 (2013 for MRSA) and 2022. The in vitro susceptibility to the most common antimicrobial agents for intramammary therapy was determined and changes over the study period were evaluated within a national and international context.

Because of submission bias and the selection criteria of a diagnostic laboratory, our study population is not an unbiased representation of all Bavarian dairy farms. Consequently, no prevalence for *S. aureus* was calculated, and the MRSA prevalence was derived only from *S. aureus* isolates and *S. aureus*-positive herds that submitted samples to this laboratory. Therefore, the selection criteria and study setup have to be considered when comparing the results of this study to the AMR of *S. aureus* and MRSA of other studies.

However, the large study population, the high count of quarter milk samples and farms per year, and consistent laboratory methods ensured the consistency necessary for this AMR trend analysis. As a result, this study included the most *S. aureus* and MRSA ever isolated in Germany.

2. *S. aureus* susceptibility testing

The methodology for susceptibility testing was mostly in accordance with the recommendations of the CLSI (CLSI, 2023b). Only the pre-selection of isolates by β-lactamase testing did not use the most recently recommended nitrocefin-based test or penicillin zone-edge test. This was because the udder health laboratory of the TGD used the iodometric method recommended by Rosselet et al (1977) and Gedek (1978) for decades, and reliable trend analysis was only ensured by consistent laboratory methods. The same argument, i.e. a well-established method, applies for MRSA identification by growth on Brilliance MRSA-2 agar (Oxoid), while the newer *mec* PCR method would be more sensitive, but was not suitable for the high quantity of samples passing through this laboratory.

Susceptibility testing by broth microdilution using the breakpoint method was analyzed by applying official breakpoints from standards in effect at the time (e.g., NCCLS M31-A3, CLSI Vet01, CLSI M100). However, due to the incomplete breakpoints for the indication *S. aureus* mastitis in cattle, the respective MIC_{50} and MIC_{90} were provided for each antimicrobial across all years. The further development of official breakpoints for this indication is urgently needed.

3. Resistance trends in a national context

Overall, the percentage of in vitro resistant isolates from bovine *S. aureus* mastitis cases was low and decreased over the study period. Similarly, the prevalence of MRSA isolates in bovine quarter milk samples containing *S. aureus* was low and has only increased minimally. The overall proportion of resistant MRSA isolates was high and decreased only slightly between 2013 and 2022.

When comparing the results from this study to data from the German AMR monitoring program (BVL, 2021), similar findings were observed for both *S. aureus* and MRSA: between 2009 and 2019, the overall percentage of in vitro resistant *S. aureus* isolates from bovine mastitis cases was low and even mildly decreased. Furthermore, the prevalence of MRSA isolates among all *S. aureus* was low.

Furthermore, the trends in AMR in this study mostly agreed with the results of the German monitoring programs about antimicrobial sales (BMG et al., 2011) and antimicrobial use (Kasabova et al., 2021) in German veterinary medicine. The decreased overall antimicrobial sale (by 65% from 2012 to 2021) and the constantly low average therapy frequency (2013 to 2020) may account for the observed decline in AMR prevalence of *S. aureus* and MRSA. Particularly the substantial reduction (-73%) in erythromycin sales until 2014 and the relative increase in use of penicillin and aminoglycosides aligned with resistance trends in this study. The observed trends could be the result of the changed Veterinary Pharmacies Prescription Regulation (TÄHAV) from 2018, which obliged susceptibility testing for critically important antimicrobials prior to use, and limited treatment duration as well as change of antimicrobial agents during

therapy of individual cases. However, data about antimicrobial use in the study population as well as specific usage data for different livestock species in the monitoring programs would be necessary to substantiate this hypothesis.

4. Resistance trends in an international context

The slightly decreasing resistance prevalence of *S. aureus* and MRSA from dairy cows found in this study was similar to the results of various European monitoring programs (Korsgaard et al., 2020; Swedres-Svarm, 2014; UK-VARSS, 2021). These programs observed a low or even decreased MRSA prevalence in bovine dairy herds. However, the United Kingdom and Sweden in particular had hardly any MRSA-positive samples in dairy cattle, which can probably be attributed to the much stricter control methods (that include the mandatory removal of cows with β-lactamase producing *S. aureus*).

All these European AMR monitoring programs were developed based on the corresponding preceding EU regulations (Naranjo-Lucena & Slowey, 2023). Although the approaches varied widely, it was observed that in most nations that reduced antibiotic use, prevalence and/or in vitro resistance of *S. aureus* and MRSA also mildly decreased. While the effect of German AMR prevention strategies can be seen, the other European countries serve as role models and demonstrate the potential for even greater improvement.

5. Conclusion and perspective

These quite positive developments of the AMR situation of *S. aureus* and MRSA lead to the following conclusions concerning treatment and control: Penicillin has been in use against *S. aureus* mastitis for 50 years. Nevertheless, the percentage of in vitro resistant isolates from bovine *S. aureus* mastitis cases was still low and further decreased for some antibiotics over the study period. Therefore, penicillin should remain the first-choice antibiotic in *S. aureus* mastitis therapy in Bavaria, if antimicrobial therapy is considered at all due to poor cure rates in chronic cases. However, successful therapy of bovine mastitis caused by MRSA remains unlikely, because the proportion of resistant MRSA isolates was high and decreased only slightly.

In conclusion, this work did provide insightful results for the prevalence and impact of changes in antimicrobial usage on the AMR of *S. aureus* and MRSA

isolates from quarter milk samples of cattle in Bavaria.

V. SUMMARY

Bovine mastitis caused by *Staphylococcus aureus* is one of the most prevalent and expensive diseases in the dairy industry worldwide. In addition to pathogen-specific immune evasion mechanisms, *S. aureus* isolates harboring antimicrobial resistance (like MRSA) complicate the eradication from dairy herds, since they show resistance to many antimicrobials approved for bovine mastitis therapy. This study aimed to determine the changes in antimicrobial resistance of *S. aureus*-Isolates between 2012 and 2022, and the changes of prevalence and antimicrobial resistance of MRSA-Isolates between 2013 and 2022 in quarter milk samples obtained by the Bavarian Animal Health Services (TGD) from dairy cattle.

In the study period, 167,651 quarter milk samples containing *S. aureus* from more than 90,000 cows from 12,052 Bavarian dairy farms were obtained and analyzed with the California Mastitis Test in the scope of routine mastitis diagnostics. After the identification of β-lactamase production, samples that tested positive were either transferred to Brilliance MRSA 2 agar or directly forwarded to susceptibility testing to confirm the presence of MRSA. According to routine guidelines, a selection of β-lactamase producing *S. aureus* per farm, as well as all *S. aureus* isolates from cows showing subclinical or clinical mastitis signs, from cows that received treatment, or that were selected by the farmers or their veterinarians were forwarded to antimicrobial susceptibility testing by broth microdilution. The isolates were categorized as susceptible or resistant according to breakpoints from standards in effect at the time.

From all 23,446 *S. aureus* isolates assessed with BMD, about a quarter (24%) was resistant to erythromycin, with a drop from 53% in 2012 to 8% in 2022. The second highest prevalence of in vitro resistance was to penicillin (17% of all submitted *S. aureus* isolates), which also decreased over the study period. Less than 14% of the *S. aureus* isolates were resistant to the remaining assessed antimicrobial agents (cefoperazone, pirlimycin, kanamycin-cefalexin, marbofloxacin, amoxicillin-clavulanate, cefquinome, or cefazolin, respectively). In conclusion, there was an overall trend towards fewer resistant isolates.

Since the TGD started performing MRSA diagnostics in 2013, the prevalence of MRSA among all *S. aureus* isolates from Bavarian dairy cows has been low

(0.6% in 2013) and increased only minimally (to 1,2% in 2022). Almost all of the 910 MRSA isolates examined were in vitro resistant to penicillin and cefoperazone (99% and 98%, respectively). More than half of the isolates showed resistance to cefquinome (65%) and cefazolin (53%). Furthermore, nearly half (46%) of the MRSA isolates were in vitro resistant to erythromycin, with a remarkable drop from 92% to 39% between 2013 and 2022. The overall resistance prevalence for kanamycin-cefalexin, pirlimycin, marbofloxacin, and amoxicillin-clavulanate remained below 35% for each. In the study period, the overall proportion of resistant MRSA isolates from bovine quarter milk samples was high and decreased only slightly between 2013 and 2022.

This study offered reliable insights into the AMR trends of *S. aureus* and MRSA isolates from Bavarian dairy cattle. Our findings confirm the observations of several European AMR monitoring programs, which stated that in most countries that reduced antibiotic use, prevalence and/or *in vitro* resistance of bovine *S. aureus* and MRSA also mildly decreased.

VI. ZUSAMMENFASSUNG

Die durch *Staphylococcus aureus* verursachte Mastitis der Kuh ist eine der häufigsten und teuersten Krankheiten in der Milchindustrie weltweit. Zusätzlich zu den erregerspezifischen Mechanismen zur Umgehung des Immunsystems erschweren resistente *S. aureus*-Isolate (z.B. MRSA) die Eradikation in Milchviehherden, da sie gegen viele für die Mastitistherapie bei Kühen zugelassenen Antibiotika resistent sind. Ziel dieser Studie war es, die Veränderungen der Antibiotikaresistenz von *S. aureus*-Isolaten zwischen 2012 und 2022 sowie die Veränderungen der Prävalenz und Antibiotikaresistenz von MRSA-Isolaten zwischen 2013 und 2022 in Viertelgemelksproben zu ermitteln, die durch den Bayerischen Tiergesundheitsdienst (TGD) untersucht worden waren.

Im Untersuchungszeitraum wurden 167 651 *S. aureus*-haltige Viertelgemelksproben von mehr als 90 000 Kühen aus 12 052 bayerischen Milchviehbetrieben gewonnen und im Rahmen der routinemäßigen Mastitisdiagnostik mittels California Mastitis Test analysiert. Nach dem Nachweis der β-Laktamase-Produktion wurden die positiv getesteten Proben entweder auf Brilliance MRSA 2-Agar übertragen oder direkt zur Bestätigung des MRSA-Nachweises zur Empfindlichkeitsprüfung weitergeleitet. Gemäß den Routinerichtlinien wurde eine Auswahl β-Laktamase-produzierender *S. aureus* pro Betrieb sowie alle *S. aureus*-Isolate von Kühen mit subklinischen oder klinischen Mastitis-Symptomen, von Kühen, die behandelt wurden, oder die von den Landwirten oder ihren Tierärzten ausgewählt wurden, zur antimikrobiellen Empfindlichkeitsprüfung mittels Boullion-Mikrodilution weitergeleitet. Die Isolate wurden gemäß den Breakpoints der zum Zeitpunkt der Untersuchung geltenden Richtlinien als empfindlich oder resistent eingestuft.

Von allen 23 446 mit BMD bewerteten *S. aureus*-Isolaten war etwa ein Viertel (24%) resistent gegen Erythromycin, wobei ein Rückgang von 53% im Jahr 2012 auf 8% im Jahr 2022 zu verzeichnen war. Die zweithöchste Prävalenz der In-vitro-Resistenz war gegen Penicillin (17% aller eingereichten *S. aureus*-Isolate), die im Studienzeitraum ebenfalls abnahm. Weniger als 14% der *S. aureus*-Isolate waren gegen die übrigen untersuchten Antibiotika (Cefoperazon, Pirlimycin,

Kanamycin-Cefalexin, Marbofloxacin, Amoxicillin-Clavulanat, Cefquinom bzw. Cefazolin) resistent. Insgesamt war ein Trend zu weniger resistenten Isolaten festzustellen.

Seitdem der TGD im Jahr 2013 mit der MRSA-Diagnostik begonnen hatte, war die MRSA-Prävalenz unter allen *S. aureus*-Isolaten von bayerischen Milchkühen gering (0,6% in 2013) und stieg nur minimal an (1,2% in 2022). Fast alle der 910 untersuchten MRSA-Isolate waren resistent in vitro gegen Penicillin und Cefoperazon (99% bzw. 98%). Mehr als die Hälfte der Isolate wiesen eine Resistenz gegen Cefquinom (65%) und Cefazolin (53%) auf. Außerdem war fast die Hälfte (46%) der MRSA-Isolate resistent in vitro gegen Erythromycin, wobei zwischen 2013 und 2022 ein bemerkenswerter Rückgang von 92% auf 39% zu verzeichnen war. Die Gesamtresistenzprävalenz für Kanamycin-Cefalexin, Pirlimycin, Marbofloxacin und Amoxicillin-Clavulansäure blieb jeweils unter 35%. Im Untersuchungszeitraum (2013-2022) war der Gesamtanteil resistenter Isolate an allen MRSA-Isolaten aus Rinder-Viertelmilchproben hoch und ging zwischen den Jahren nur leicht zurück.

Diese Studie bot einen guten Einblick in die Entwicklung der AMR von *S. aureus*- und MRSA-Isolaten bayerischer Milchkühe. Unsere Ergebnisse bestätigen die Beobachtungen mehrerer europäischer AMR-Überwachungsprogramme, denen zufolge in den meisten Ländern, in denen der Antibiotikaeinsatz reduziert wurde, auch die Prävalenz und/oder In-vitro-Resistenz von *S. aureus* und MRSA bei Milchkühen leicht zurückging.

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