Aus dem Institut für Mikrobiologie und Tierseuchen

der Freien Universität Berlin

Characterization of equine *Staphylococcus aureus* isolates with particular reference to their oxacillin and sulfamethoxazole/trimethoprim susceptibility

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Anissa Deborah Scholtzek
Tierärztin
aus Solingen

Berlin 2020
Journal-Nr.: 4236

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Science makes people reach selflessly for truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist.

Lise Meitner

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List of abbreviations

aa	amino acid
AST	antimicrobial susceptibility testing
BAC	benzalkonium chloride
BMD	broth microdilution
BORSA	borderline oxacillin-resistant *Staphylococcus aureus*
BST	biocide susceptibility testing
CA-MRSA	community-associated methicillin-resistant *S. aureus*
CHX	chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
CC	clonal complex
DD	agar disk diffusion
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GLU	glutardialdehyde
HA-MRSA	hospital-associated methicillin-resistant *S. aureus*
LA-MRSA	livestock-associated methicillin-resistant *S. aureus*
majE	major error
minE	minor error
MIC	minimal inhibitory concentration
MLST	multi locus sequence typing
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-susceptible *S. aureus*
PBP	penicillin binding protein
PVL	Panton-Valentin-Leukocidin
QAC	quaternary ammonium compound
QC	quality control
SCC*mec*	staphylococcal cassette chromosome *mec*
Scin	staphylococcal complement inhibitor
spa	*Staphylococcus* protein A gene
ST	sequence type
SUL	sulfonamide
SXT	sulfamethoxazole/trimethoprim
TMP	trimethoprim
vmajE	very major error
WGS	whole genome sequence

1 Introduction

The development of antimicrobial resistance in bacterial pathogens is an important public health issue. The first antimicrobial agents introduced into clinical use were sulfonamides in 1935, followed by penicillin in 1940/41 [1]. The development and use of antimicrobial agents quickly led to resistance among the target bacteria, which motivated the discovery and development of many new antimicrobial substances, a trend that declined in the 1990s [1]. In parallel to human medicine, antimicrobial agents were introduced into veterinary medicine. The widespread use of antimicrobial agents in veterinary medicine raised concerns about the development of resistance. Bacterial isolates from animals could acquire resistance against antimicrobial agents, which are commonly used to treat human infections. This resistance could then be transferred between bacterial isolates of human and veterinary origin that are involved in infections [2]. One example of a zoonotic bacterial species with a large number of acquired resistance properties is *Staphylococcus aureus*. *S. aureus* causes infections in humans and animals, and it can be resistant against methicillin – an antimicrobial developed against β-lactamase-carrying bacteria – which poses a threat, especially in nosocomial settings. These strains are commonly referred to as methicillin-resistant *S. aureus* (MRSA). Humans can get colonized or infected through contact with colonized and infected humans, a contaminated environment, vectors such as flies, the food chain, or through close contact especially with companion animals like horses, cats and dogs [3-8]. The transfer of MRSA between humans and animals has been described previously. In a study by Nienhoff et al., two cases of human-to-animal transmission of MRSA were evaluated. In one case, a veterinarian colonized by a livestock-associated MRSA (ST398) passed the pathogen on to his dog, in the other case, a relative of the owner passed her ST225 MRSA strain to the family dog [9]. A Danish study investigated two cases of farmers, that shared the same ST130 MRSA strains with their cow and sheep [10]. When resistant bacterial isolates are exchanged between humans and animals in either direction, the transferred bacteria can exchange their resistance genes with the indigenous microbiota of the new host.

As the selection pressure imposed by the antimicrobial use is considered as the major driving force in resistance development and spread among bacteria, counteractive measures currently focus on an overall reduction of antimicrobial use and the implementation of antibiotic stewardship concepts to avoid an overuse or imprudent use of antimicrobial agents [11]. Therefore, numerous programs were established to monitor the current status and development of antimicrobial resistance in bacteria [12-17]. Some of these programs focus on veterinary samples, since the use of relevant antimicrobial agents in food-producing animals

and companion animals is assumed to lead to resistant human pathogens via co- and cross-resistance.

To ensure prudent use of antimicrobial agents, antimicrobial susceptibility testing (AST) is used to determine the antimicrobial resistance patterns of clinical isolates in veterinary and human medicine and thereby help the clinician to choose a treatment option with highest probability of success.

1.1 *Staphylococcus aureus* as a zoonotic pathogen

S. aureus is usually a harmless commensal on the healthy skin and mucosal surfaces of animals and humans but can cause diverse infections ranging from mild skin diseases to life-threatening septicemia [18, 19]. Because of its zoonotic potential, humans can also get infected via close contact to infected or colonized animals and vice versa. This is especially true for people who work with animals or people keeping pets for companionship, e.g. dogs, cats and horses [6, 7, 20, 21]. Most studies focused mainly on those zoonotic pathogens that can cause severe infections in humans, such as MRSA, known to lead to infections that are difficult to treat, especially in a nosocomial setting [6, 22, 23]. With regard to equine medicine, the respective studies dealt mainly with bacterial infections of horses and their caretakers, showing that caretaker and horse may harbor the same bacterial strain [6, 8]. A Belgian study sampled also healthy horses and their caretakers and detected colonization with the same MRSA strains, but to a very low extent [24]. Sieber et al. conveyed a long-time study in an equine clinic, in which they detected *S. aureus* strains, frequently present in the patients, the environment and the veterinary personnel. The initial, methicillin-susceptible, strains were at one timepoint replaced by a MRSA clone [25]. Vincze et al. investigated *S. aureus* isolates from wound samples of companion animals and showed, that 41.3 % of the detected isolates were MRSA [26]. Cuny et al. found 19.5 % of veterinary personnel colonized with LA-MRSA types known to colonize and infect horses [23]. These studies show that transmission between horses and humans take place and may pose a great threat to both, veterinary and human medicine. Of special concern in zoonotic transmission of bacteria are wound and surgical site infections of horses, because the veterinarian or caretaker usually is in close contact with the wound when applying the wound dressing. *S. aureus* is one of the most common bacterial species associated with surgical site and wound infections [27, 28], therefore, there is a high possibility, that transmission of these isolates takes place.

1.2 General characteristics of *Staphylococcus aureus*

S. aureus is a Gram-positive, coccoid bacterium, with a size of about 1.0 µm, a member of the order *Bacillales* and the family *Staphylococcaceae*. It is immobile and non-spore-forming,

facultatively anaerobic and is not fastidious in its growth conditions [29]. *S. aureus* harbors the virulence factor coagulase, which is also used for diagnostics. It has a capsule which serves as protection against phagocytosis. In addition, protein A in the bacterial cell wall binds the Fc part of immunoglobulins and thereby also exerts an anti-phagocytic effect. It harbors proteins associated with adhesion, like the fibronectin-binding proteins FnbA and FnbB or the collagen adhesin Cna. The clumping factors ClfA and ClfB bind the γ-chain or the α- and β-chain of fibrinogen, respectively. On sheep blood agar *S. aureus* typically presents as eponymous golden yellow colonies often accompanied by variable zones of hemolysis. In general, staphylococci can produce four types of hemolysins, which vary in their host affinity and their effect on erythrocytes [29]. The α-hemolysin effects predominantly erythrocytes of rabbits, sheep and cattle and has dermonecrotic and neurotoxic properties. At temperatures of 37° C, the β-hemolysin has a high affinity to erythrocytes of sheep, cattle and humans. However, this β-hemolysis is incomplete and completes only at lower temperatures (hot-cold-lysis). The γ-hemolysin is a bi-component toxin, known to lyse not only erythrocytes, but also neutrophils and macrophages. The δ-hemolysin causes membrane damage in various mammalian cells, including erythrocytes, and has pro-inflammatory effects. All four hemolysins can coexist in a single isolate [29, 30].

Besides hemolysins, there are further pore forming toxins, like leukotoxins, which have differing cell specificities. One well-known example is the Panton-Valentine-Leukocidin (PVL), a toxin that is mostly isolated from severe infections with pathogenic *S. aureus* isolates and is capable of causing deadly infections, especially pneumonia and skin and soft tissue infections in previously healthy people, due to its affinity to leukocytes and neutrophils.

Another major group of toxins in *S. aureus* are exfoliative toxins. They constitute of very specific serine proteases, destroying desmosome cadherins in the superficial skin layers and thus resulting in blister formation and skin-peeling of the host, either at a defined area or throughout the body [31].

The last of the three main toxin groups of *S. aureus* are the superantigens (SAgs). One of the respective toxins is the toxic shock syndrome toxin 1 (TSST1), which activates T-cells, resulting in massive cytokine release and the classic shock symptoms. Other superantigens include enterotoxin-like-proteins and enterotoxins, which are mostly linked with food poisoning and are often very stable to heat, making them a potential risk for food poisoning even if the food has been heated [30, 31].

S. aureus is a highly clonal bacterium and is differentiated into sequence types (STs) via multi locus sequence typing (MLST). STs are further grouped in clonal complexes (CCs). Via sequencing of the *Staphylococcus* protein A gene (*spa*), *spa* types can be identified. For MRSA, further typing can be performed for the staphylococcal cassette chromosome *mec* (SCC*mec*) types [32] or the direct repeat unit (*dru*) types [33]. MRSA isolates are also

categorized, depending on their origin; there are hospital-associated (HA-MRSA), community-associated (CA-MRSA) and livestock-associated (LA-MRSA) lineages. These classifications and assignments help to understand origins of colonization and infections in animals and humans [32, 34].

1.3 Important antimicrobial resistance properties of *Staphylococcus aureus*

S. aureus exhibits a wide range of antimicrobial resistance properties, for which numerous resistance genes and/or mutations have been identified. Many of these can be detected among isolates from both, human and animal origin and they are often located on mobile genetic elements, such as plasmids and transposons, which allows a wider spread of these resistance genes [18].

The *blaZ* gene confers resistance to β-lactams via enzymatic inactivation, but the most relevant genes regarding β-lactam resistance are the *mec* genes, which are responsible for methicillin resistance via target site replacement. Resistance to tetracyclines is mediated by the *tet* genes which lead to either active efflux (*tet*(K), *tet*(L)) or target site protection (*tet*(M), *tet*(O)). The *cat* genes inactivate nonfluorinated phenicols, while *fexA* causes active efflux of all phenicols. Regarding aminoglycosides, the resistance genes (*aacA-aphD*, *aadD*, *aphA3* and *aadE*) cause enzymatic inactivation, while each gene is effective against a different set of aminoglycosides. Lincosamides are inactivated through an enzyme encoded by the genes *lnu*(A) and *lnu*(B). The *mph*(C) gene mediates resistance to macrolides. Next to resistance genes conferring resistance to one class of antimicrobial agent, there are also genes and/or mutations mediating multidrug resistance. Via target site modification, *erm* genes, such as *erm*(A), *erm*(B), *erm*(C) or *erm*(T), confer resistance not only to macrolides, but also to lincosamides and streptogramin B antibiotics. The genes *vga*(A), *vga*(C), *vga*(E), *lsa*(E) and *sal*(A) code for ABC-F proteins that protect the target sites of lincosamides, pleuromutilines and streptogramin A antibiotics. Via target site methylation, isolates harboring the *cfr* gene gain resistance to all phenicols, lincosamides, oxazolidinones, pleuromutilines and streptogramin A antibiotics. The multidrug resistance genes *optrA* and *poxtA* [35] confer resistance to oxazolidinones and phenicols or oxazolidinones, phenicols and tetracyclines, respectively, via target site protection [18, 36].

This is just a small collection of the overall known and published resistance genes for *S. aureus*. In some cases, not a resistance gene, but a single or double mutation in a target gene can cause resistance to the respective agent, e.g. for fluoroquinolones, where mutations in the DNA gyrase and topoisomerase genes (*gyr* and *grl* genes) or for rifampicin, where mutations in the *rpoB* gene result in resistant isolates [18, 37].

1.3.1 Oxacillin and oxacillin resistance in *S. aureus*

The first semisynthetic, penicillinase-resistant β-lactam was methicillin. In 1962, oxacillin was introduced as its successor. These antimicrobial agents are made up of a thiazolidine ring, a β-lactam ring and a side chain that differs between the agents; in the case of oxacillin, it is an acyl side chain (Figure 1).

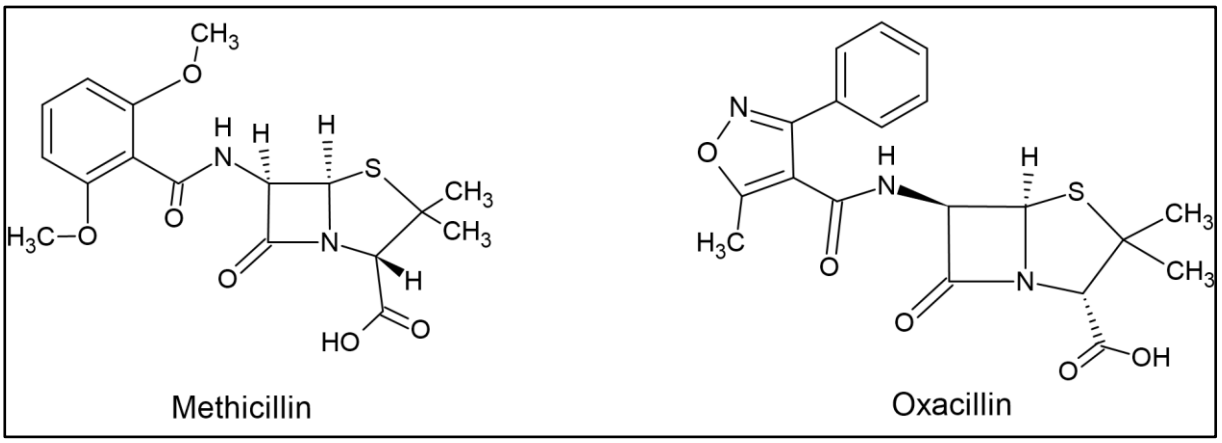

Figure 1 Chemical structures of methicillin and oxacillin

β-Lactams are bactericidal, they bind to penicillin binding proteins (PBPs) – enzymes involved in the crosslinkage between peptide chains in the bacterial cell wall synthesis – interfering with proper cell wall formation [38]. Oxacillin has a narrow spectrum of activity against Gram-positive bacteria. It is recommended as treatment for infections of the musculoskeletal system, skin infections and in general infections with oxacillin-susceptible bacteria in cats, dogs, and horses. For cattle, it is also used to treat mastitis [39, 40]. Next to its clinical usage, the agent also serves the diagnostic purpose, to identify methicillin resistance. According to standards of the Clinical and Laboratory Standards Institute (CLSI), a staphylococcal isolate classified as resistant to oxacillin, must be interpreted as resistant to virtually all β-lactams, except newer cephalosporins with activity against MRSA (e.g. ceftaroline and ceftobiprole), which are not licensed for veterinary use [41-43].

Staphylococcal resistance to oxacillin is usually conferred by expression of altered PBP variants with low affinity to penicillins (e.g. PBP2a), encoded by *mec* genes (*mecA* and *mecC*), located on mobile genetic elements, the SCC*mec* [44]. Recently, a third *mec* gene, *mecB*, was identified on a plasmid [45]. Since the acyl side chain protects the β-lactam ring of oxacillin, β-lactamases encoded by the genes *blaZ* and bla_{ARL} do not confer resistance to this agent.

In the past decade, a new phenomenon, borderline oxacillin-resistant *S. aureus* (BORSA) occurred. These isolates are defined by their elevated oxacillin minimum inhibitory concentration (MIC) of 1 – 4 mg/L, while lacking a *mec* gene. The possible underlying

mechanisms are (i) a hyperproduction of β-lactamases leading to slow inactivation of oxacillin, (ii) a decreased expression of the factors essentially for methicillin resistance *femA*, *femB, femX*, *femC* or *femD,* or (iii) mutations in further genes, involved in PBP expression (e.g. *gdpP* and *yjbH*) [18, 44, 46].

1.3.2 Potentiated sulfonamides and the respective resistance in *S. aureus*

Sulfonamides (SULs) were the first antimicrobial agents, introduced in the 1930s. About 40 years later they were potentiated via the combination with trimethoprim (TMP). Their structures are shown in Figure 2.

There are three groups of SULs, according to their half-life: short-acting (up to 8 hours), intermediate-acting (8 – 16 hours), and long-acting ones (more than 16 hours). However, this classification is based on pharmacokinetic studies in humans and in general the half-life of SULs in animals is shorter [47]. Both components (the SUL and TMP) alone have a bacteriostatic effect, only together they can be bactericidal, if combined in the right concentrations and ratio in vivo. They affect subsequent steps in the folate synthesis of bacterial cells. SULs compete with *p*-amino -benzoic -acid and therefore block dihydrofolic acid synthesis, while TMP inhibits the following dihydrofolate reductase [48]. Together, they have a broad spectrum of activity against Gram-positive and Gram-negative bacteria. For most bacterial species, the maximum synergistic effect in humans is achieved by a TMP:SUL ratio of 1:20. To reach this ratio in human plasma, preparations have a fixed ratio of 1:5, when an intermediate-acting SUL is used. Unfortunately, so far this has not been proven for animals. In most animals, however, TMP has a much shorter half-life, than the SUL compound [49], therefore the combination with even intermediate-acting SULs might not lead to a sufficient dose of the combined agents at the animal's body site [50].

The combination is recommended for use in animals for a variety of infections, including infections of the respiratory tract, the central nervous system, joints, skin and soft tissues, the prostate and the abdomen [39, 40]. It is not recommended for treating abscesses or infections involving anaerobic bacteria, since necrotic tissue might have an inactivating effect on the combination. In Germany, the combination of SULs and TMP account for 12 % of the used antimicrobial agents in horses, in Belgium and the United Kingdom, even a quarter of prescribed antimicrobial agents for horses are SUL/TMP combinations [51]. Since the gastro-intestinal intake seems sufficient, oral administration of the agents is possible [49].

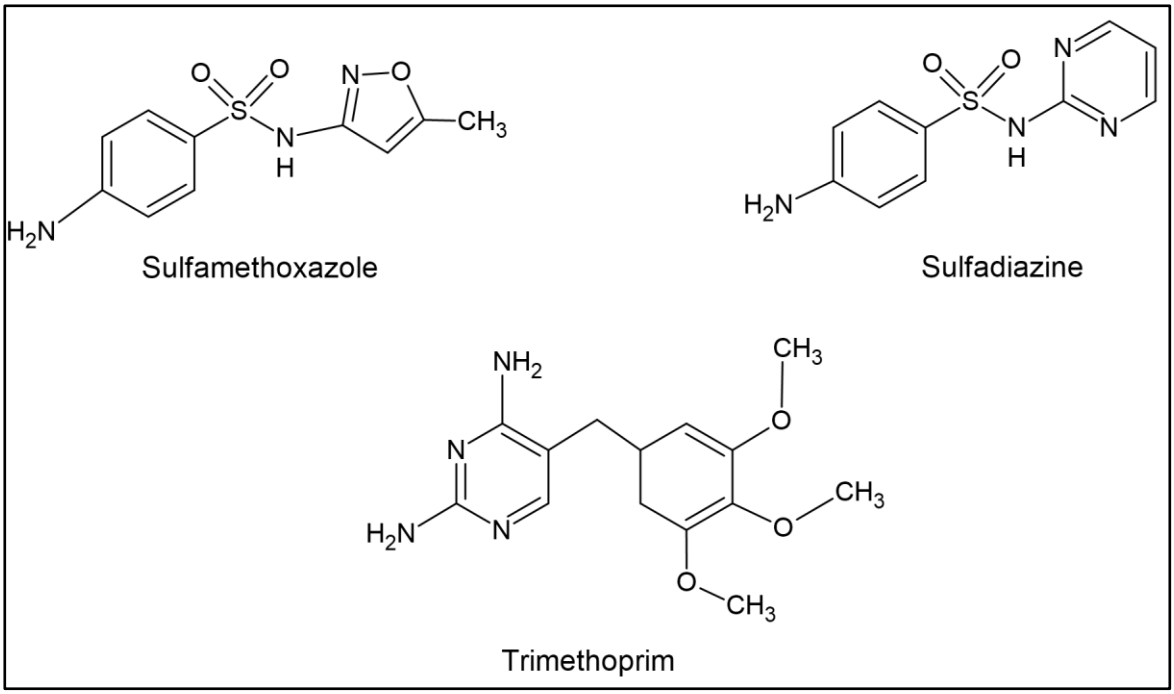

Figure 2 Chemical structures of the sulfonamides sulfamethoxazole and sulfadiazine, and trimethoprim

Resistance to TMP in staphylococci is usually mediated by TMP-resistant dihydrofolate reductases encoded by the genes *dfrS1* (= *dfrA*), *dfrD*, *dfrG* or *dfrK*. Even though resistance to SULs has been described in staphylococci, so far the genetic basis remains unknown. Different mechanisms might result in resistance, like mutations in the dihydropteroate synthase genes, hyperexpression of susceptible ones or increased *p*-amino -benzoic -acid production [18].

1.4 Bacterial resistance to biocides

Antimicrobial agents are drugs with activity against bacteria and used to treat and prevent bacterial infections in humans and animals by either killing the bacterium (bactericidal effects) or inhibiting its growth (bacteriostatic effects). They usually interact with a very specific target in the bacterial cell. Biocides also have antimicrobial effects, but are used for disinfection in health care settings and food production, for conservation in the food industry, as preservatives in consumer products like cosmetics, cleaning agents for surfaces, in pharmaceutical goods, treatment of wastewater and during manufacturing processes of all kinds [52]. In contrast to antimicrobial agents, biocides usually do not attack one specific structure in bacterial cells, but multiple target sites. Ever since antimicrobial-resistant bacteria have become a major concern of the public health and the general public got more informed about hygiene and the connection to bacterial contamination and infection, biocide usage

increased in all sectors [52]. Alongside with the use of biocides, the question of bacterial resistance towards them and a possible linkage to antimicrobial resistance arose. Based on the definition of resistance to antimicrobial agents, “resistance” to biocides would occur when a bacterial isolate survives in a biocide (product) concentration, which should be lethal. “Reduced susceptibility” to biocides occurs, if a bacterial isolate is able to withstand higher concentrations of the biocide, than the majority of the species’ population [52].

Our knowledge of the detailed mechanisms of biocides, especially when used in low or subinhibitory concentrations remain poor. There are three levels of interactions with bacterial cells, namely interactions with (i) outer cellular elements (e.g. changing the hydrophobicity of the cell), (ii) the cytoplasmic membrane (e.g. interfering with membrane located enzymatic systems) and (iii) cytoplasmic components (e.g. inhibiting ribosomal functions). One biocide can operate on more than one level, which is why the bacterial answer to these agents is also more general than specific [53]. There are different classes of biocides, like quaternary ammonium compounds (QAC), biguanides and aldehydes, that differ in their spectrum of activity.

Benzalkonium chloride (BAC) is a QAC with bactericidal activity against Gram-positive and, to a lesser extent, Gram-negative bacteria. Its mechanism of activity is mainly the destabilization of the bacterial membrane. Resistance mechanisms involve changes in membrane composition and the expression of *qac* genes, which encode inducible QAC efflux pumps [54, 55]

Chlorhexidine (CHX) belongs to the bis-biguanides and has a bacteriostatic effect in low concentrations and a bactericidal effect in high concentrations. Its spectrum includes in particular Gram-positive, but also Gram-negative bacteria and mycobacteria. It damages the cell membrane and interferes with membrane located enzyme systems. Acquired resistance is achieved via multidrug efflux pumps and changes in the cell membrane that limit the uptake of the biocide [56].

Glutardialdehyde (GLU) is a member of the aldehydes, with a broad spectrum of bactericidal activity via inhibition of bacterial protein, DNA and RNA biosynthesis and interference with various enzymes. Bacterial resistance involves the modification of porins to reduce the biocide influx and the upregulating of multidrug efflux pumps [57].

1.5 Bacterial susceptibility testing

Bacterial susceptibility testing is used to determine the susceptibility to antimicrobial agents (via AST) or biocides (via biocide susceptibility testing, BST) among bacterial isolates. AST and BST need to use standardized protocols that yield reproducible results and include guidelines for quality control (QC). In order to act according to antibiotic stewardship concepts,

the most appropriate antimicrobial agent needs to be selected for the treatment of bacterial infections. Therefore, AST protocols were established, that give essential information to clinicians of both, veterinary and human medicine, and scientists. Optimally, there is a link between the AST result and the expected clinical outcome. For human isolates, those protocols were established in the 1960s, while the first comparable protocols for veterinary medicine were available in the 1990s. Since veterinary medicine deals with a great variety of species, where pharmacokinetic and -dynamic processes differ vastly, the establishment of standardized protocols is difficult and time consuming [58]. To this day, the most commonly used AST protocols for veterinary pathogens worldwide are the CLSI standards [42, 59-61]. Nevertheless, the VetCAST group, a subdivision of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), is currently developing interpretive criteria for AST results of veterinary pathogens [62]. Regarding biocides, for a long time, scientists used a range of different methods to access biocide susceptibility. This variety of protocols renders a comparison of the results obtained in different studies difficult. In 2018, we published a protocol for a broth macrodilution method, which allows a harmonization of BST [63].

1.5.1 Methods used for antimicrobial susceptibility testing

AST methods can be divided into two groups: methods that provide qualitative results and methods that provide quantitative results. Qualitative methods result only in a classification of the bacterial isolate as “susceptible”, “intermediate” or “resistant”, while quantitative methods provide MICs, that tell the veterinarian how susceptible or how resistant the bacterial isolate in question is. The MICs can be compared with specific drug concentrations at the site of infection [64], and thereby allow a better judgement of the suitability of the antimicrobial agents considered for therapeutic interventions [65]. The most common qualitative method is the agar disk diffusion (DD). Here, the isolate is streaked on an agar plate and disks containing a defined amount of the respective antimicrobial agent are positioned on the agar plate. After incubation, an inhibition zone appears around the disk, the zone diameter is measured and refers to the classifications susceptible, intermediate or resistant [41, 42, 59].

Agar dilution is a quantitative method. Here, each agar plate is prepared containing a different test concentration of the antimicrobial agent. The bacterial inoculum is applied as droplets containing a specific amount of bacteria. This allows testing of many isolates on one plate. After incubation, the plate with the lowest concentration without visible growth defines the MIC [66].

There are two broth dilution methods, which differ in the final test volume, namely broth macrodilution and broth microdilution (BMD) but the test principle is the same. A specific amount of bacteria is inoculated in a two-fold dilution series of the respective antimicrobial

agent, and - after incubation - the lowest concentration without visible growth is considered the MIC. However, for some combinations of bacterial species and antimicrobial agents “trailing endpoints” occur. This is a phenomenon, where a growth reduction of ≥ 80 % is defined as the MIC, e.g. when testing staphylococci against chloramphenicol, erythromycin, tetracycline and sulfamethoxazole/trimethoprim (SXT) [41, 59]. Broth macrodilution is carried out in tubes, with volumes of 2 milliliters [59], while BMD works with a final volume of 100 – 200 microliters and is mostly conducted in 96-well microtiter plates. BMD is often used, since it is less media- and time-consuming and there are commercial plates available. Broth macrodilution takes a lot of time to prepare and needs more media, therefore it is not suited for a high throughput, and the manual preparation of the antimicrobial dilution series increases the possibility of errors [64]. The principle of broth dilution methods was adopted for automated testing systems, which offer a higher throughput since shorter incubation times are used. After inoculating the respective, antimicrobial dilution series containing, panels, the system detects turbidity and/or oxidation-reduction indicators repeatedly in the early growth phase of the bacteria, to calculate the estimated MIC, which is then categorized according to the system’s software [67].

1.5.2 Methods used for biocide susceptibility testing

Since standardized methods were missing, there was a wide range of protocols used to access biocide susceptibility [68-70]. Usually a broth dilution method, similar to those in AST was used. In 2018, we published a harmonized broth macrodilution protocol for BST [63]. Here, the final test volume is 2 mL, and the MICs should be read after 24h incubation at 37°C. Based on this broth macrodilution protocol, we developed a BMD protocol, which is carried out in final volumes of 200 µL [71].

1.5.3 Quality Control ranges

For all methods described, QC is needed. Thus, for specific bacterial reference strains from the strain collections, QC ranges are established by interlaboratory trials. These QC strains are tested in parallel to the clinical or environmental isolates. Whenever the QC strains are within their acceptable range, published with the test protocols, the test results are considered as valid. If they are not within the acceptable range, something failed in the protocol execution, which makes all results obtained with this passage invalid, and the test needs to be repeated [58].So far, QC ranges for BST are missing and need to be established.

1.5.4 Clinical Breakpoints

After AST, the resulting MIC or zone diameter values need to be interpreted. Depending on the background of the isolates and the research purpose, two kinds of interpretative criteria

can be applied: clinical breakpoints or epidemiological cut-off values (ECOFFs). Clinical breakpoints are used to predict the clinical outcome of a bacterial infection, treated with the respective antimicrobial agent. In veterinary medicine, clinical breakpoints refer to a defined antimicrobial agent or a combination of antimicrobial agents (e.g. SXT), defined target bacteria and a defined body site within a defined animal species. To develop clinical breakpoints, MIC distribution of a target bacteria, pharmacokinetic and pharmacodynamic data of the antimicrobial agent in the respective animal host as well as clinical outcome data need to be considered [62, 72, 73]. In contrast, ECOFFs are determined by mathematical calculation and do not take into account the aforementioned clinical aspects. ECOFFs allow a classification of subpopulations as “wildtype” (usually low MICs) and “non-wildtype” (usually higher MICs). Isolates of the “non-wildtype” population commonly show aquired resistance mechanisms. However, the classification of an isolate as “non-wildtype” does not necessarily mean that it is also classified as clinically resistant when clinical breakpoints are applied.

In veterinary medicine, pharmacokinetic and pharmacodynamic data is an especially crucial factor for the establishment of clinical breakpoints, since the various animal species have different pharmacokinetic and pharmacodynamic processes and antimicrobial agents are not always approved for every species and route of administration. Clinical outcome data, which consists of clinical studies comparing the clinical outcome with the MICs of the respective isolates, is the third factor considered for clinical breakpoints. For veterinary bacterial pathogens, there are still many clinical breakpoints missing [58]. In the veterinary documents of the CLSI, some clinical breakpoints from human medicine are listed and shall be used, wherever species-specific breakpoints are missing [41]. However, since pharmacological processes might differ vastly between humans and animal species, these values should be used with caution.

1.6 Aims of the study

Between the years 2015 and 2017, a total of 19 *Staphylococcus aureus* isolates, that caused mainly surgical site infections in patients of a veterinary clinic for horses, were collected because they showed unusual resistance properties regarding oxacillin and the combination SXT. The aims of this project were to characterize the isolates with particular reference to:

(1) their antimicrobial and biocide resistance properties, as well as their relatedness, taking into account the available background information on the equine patients,
(2) their reduced susceptibility to oxacillin and the identification of the underlying mechanism via genetic and biochemical approaches, and
(3) their phenotypic and genotypic SXT resistance, considering diagnostic difficulties and investigating resistance properties for the single substances sulfisoxazole and TMP.

2 Publications

2.1 Publication I

Scholtzek AD, Hanke D, Walther B, Eichhorn I, Stöckle SD, Klein KS, Gehlen H, Lübke-Becker A, Schwarz S, Feßler AT. 2019

Molecular characterization of equine *Staphylococcus aureus* isolates exhibiting reduced oxacillin susceptibility

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Toxins (Basel). 13;11(9)

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Article

Molecular Characterization of Equine *Staphylococcus aureus* Isolates Exhibiting Reduced Oxacillin Susceptibility

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Abstract: The detection of borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) represents a challenge to both, veterinary and human laboratories. Between 2015 and 2017, 19 equine *S. aureus* with elevated minimal inhibitory concentrations for oxacillin were detected in routine diagnostics. The aim of this study was to characterize these isolates to identify factors possibly associated with the BORSA phenotype. All *S. aureus* were subjected to antimicrobial susceptibility testing and whole genome sequencing (WGS). A quantifiable β-lactamase activity assay was performed for a representative subset of 13 isolates. The WGS data analysis of the 19 BORSA isolates identified two different genomic lineages, sequence type (ST) 1 and ST1660. The core genome multilocus sequence typing (cgMLST) revealed a close relatedness of all isolates belonging to either ST1 or ST1660. The WGS analysis identified the resistance genes *aadD*, *dfrG*, *tet*(L), and/or *blaZ* and *aacA-aphD*. Phenotypic resistance to penicillins, aminoglycosides, tetracyclines, fluoroquinolones and sulfamethoxazole/trimethoprim was observed in the respective isolates. For the penicillin-binding proteins 1–4, amino acid substitutions were predicted using WGS data. Since neither transglycosylase nor transpeptidase domains were affected, these alterations might not explain the BORSA phenotype. Moreover, β-lactamase activity was found to be associated with an inducible *blaZ* gene. Lineage-specific differences regarding the expression profiles were noted.

Keywords: borderline oxacillin resistance; BORSA; susceptibility testing; MSSA; whole genome sequencing; *blaZ*

Key Contribution: This study characterizes two lineages of *S. aureus* causing infections in an equine clinic and analyzes the genetic basis of their borderline oxacillin resistance.

1. Introduction

Staphylococcus aureus can be a harmless commensal residing on the skin and mucosal surfaces of healthy animals and humans [1], but it can also cause a broad spectrum of diseases, ranging

from relatively mild skin infections to life-threatening pneumonia, sepsis and endocarditis [2,3]. The infections caused by antimicrobial-resistant staphylococci are a major threat in human and veterinary medicine, since treatment options are often limited [3–5]. A broad range of different staphylococcal species has been detected in horses, including *S. aureus* and *Staphylococcus delphini* as coagulase-positive species, but also a variety of coagulase-negative staphylococci [6–10]. Several studies on equine *S. aureus* and, in particular, equine methicillin-resistant *S. aureus* (MRSA) have been performed in recent years [11–15].

Besides MRSA, also borderline oxacillin-resistant *S. aureus* (BORSA) gained particular attention [16]. Commonly, BORSA were initially defined by their reduced susceptibility to oxacillin with minimal inhibitory concentrations (MICs) of 1–2 mg/L, but some BORSA strains can also exhibit oxacillin MICs of 4–16 mg/L [16]. These isolates lack an additional penicillin-binding protein (PBP), encoded by either *mecA* or *mecC*, which are located on mobile genetic elements, the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) in MRSA [1,16] or *mecB* carried on a plasmid, as recently described [17].

S. aureus isolates are assigned to clonal complexes (CC) based on their multi locus sequence types (STs). Since 2005, livestock-associated MRSA (LA-MRSA), in Europe attributed to CC398, became a public health issue, colonizing animals and humans. In addition, LA-MRSA were described for human cases of wound infection, deep abscess, cellulitis, necrotizing fasciitis and bacteremia [18,19]. In Europe, CC398 with *spa* type t011, but also other *spa* types such as t034 and t6867, is the most common clonal complex among MRSA in horse clinics [6,11,13,20–27]. However, several studies also observed a low number of isolates (MRSA and methicillin-susceptible *S. aureus* (MSSA)) attributed to CC1 with *spa* type t127 [6,11,21,25,26]. This clonal complex is known to be also associated with infections in humans, causing the majority of staphylococcal bacteremia in Denmark [28]. In other countries, such as England [29], Spain [30], Norway [31], Turkey [32] and Italy [33], CC1 is detected at lower frequencies. Other studies focusing on equine clinics found isolates with *spa* types t549 [6,25,26] and t3034 [21,26], which belong to ST1660. This ST has not been assigned to a clonal complex so far.

Previous studies showed an increase of both, MRSA and BORSA in equine clinics [20,21]. Moreover, there has been evidence that, once resistant staphylococcal isolates enter a human or veterinary clinic [11,34,35], the number of nosocomial infections—in particular wound infections [34,35]—increases. Nosocomial settings provide a selective pressure to bacterial pathogens since antimicrobial agents and also biocides are widely used [36,37]. While horse clinics have been identified as "hot spots" for pathogens accumulating antibiotic resistance in the recent past, little is known about frequency and dimension of biocide resistance. Consequently, more research is needed in this particular field and biocide susceptibility of field isolates should be monitored. However, studies determining MICs for biocides often lack an approved method and are therefore difficult to compare [37].

The aim of this study was to thoroughly characterize *S. aureus* of equine origin with elevated oxacillin MICs ranging from 0.5 mg/L to ≥4 mg/L via VITEK2 while lacking known methicillin resistance genes, such as *mecA* and *mecC*.

2. Results

2.1. Molecular Typing of Equine BORSA Isolates

The investigation of the whole genome sequences (WGSs) of the 19 isolates revealed two multi locus sequence (MLS) types, ST1 (n = 3, allelic profile: 1-1-1-1-1-1-1) and ST1660 (n = 16, allelic profile: 6-79-6-47-89-70-61). All ST1 isolates had *spa* type t127 (repeats: 07-23-21-16-34-33-13), whereas the ST1660 isolates displayed three different *spa* types, namely t549 (n = 1, IMT41899), t2484 (n = 1, IMT39637) and t3043 (n = 14). A comparison of these *spa* types revealed only differences in the number of the terminal repeats (Figure 1):

t549	(*n*=1)	04-20-69-31-70-13-17-16-16-16
t2484	(*n*=1)	04-20-69-31-70-13-17-16-16
t3043	(*n*=14)	04-20-69-31-70-13-17-16-16-16-16

Figure 1. Comparison of the *spa* types, obtained for the ST1660 isolates.

2.2. Phylogenetic Analysis

The core genome multilocus sequence typing (cgMLST) analysis revealed 14 different allelic profiles and two different clusters for the 19 BORSA isolates (Figure 2). Cluster 1 represented the ST1 sequences and consisted of two closely related allelic profiles differing only in one target gene. Cluster 2 included the ST1660 isolates assigned to 12 distinct, but also closely related allelic profiles differing in 0 to 7 target genes only. The phylogenetic relationship of the two clusters was considerably distant, mirrored by the differences in 1697 of 1744 alleles included in the comparison.

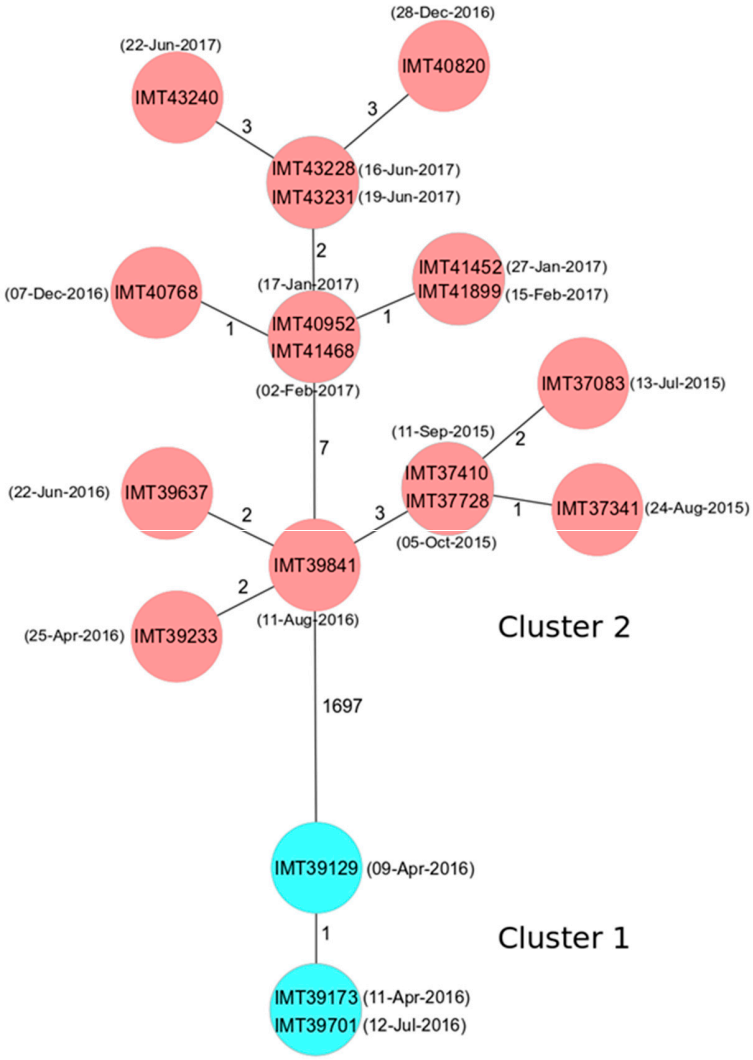

Figure 2. The minimum spanning tree showing the clonal relationship of 19 borderline oxacillin-resistant *S. aureus* (BORSA) isolates based on a core genome multilocus sequence typing (cgMLST) analysis including 1744 genes using the SeqSphere+ software. Each circle represents an allelic profile and the connecting lines display the number of different alleles between the distinct profiles. The individual isolate IDs are shown within the circles, the collection date is given in brackets while the sequence types (STs) types are indicated by colot. ST1 in light blue, ST1660 in red.

2.3. Virulence Factors of the 19 Equine BORSA Isolates

The molecular characterization based on WGSs revealed that all isolates harbored genes encoding the *S. aureus* bicomponent gamma-hemolysins (HlgA, HlgB and HlgC), alpha-hemolysin (Hla) and delta-hemolysin (Hld). Moreover, all ST1 isolates were positive for a β-hemolysin (Hlb) converting phage, which functionally inactivated the *hlb* gene (Table 1).

Table 1. Virulence genes of the 19 equine *S. aureus* isolates.

ST	Isolate	*hlb*	*fnbA*	*fnbB*	*ica*	*sei*	*selm*	*seln*	*selo*	*ϕent2*	*selq*	*seh*	*lukD/E*	*lukP/Q*
ST1	IMT39129	-	+	+	+	-	-	-	-	-	-	+	+	+
ST1	IMT39173	-	+	+	+	-	-	-	-	-	-	+	+	+
ST1	IMT39701	-	+	+	+	-	-	-	-	-	-	+	+	+
ST1660	IMT39637	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT37083	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT37341	+	-	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT37410	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT37728	+	+	+	+	-	+	+	+	+	+	-	+	+
ST1660	IMT39233	+	+	+	+	+	+	+	-	+	+	-	+	+
ST1660	IMT39841	+	-	+	+	-	+	+	+	-	+	-	+	+
ST1660	IMT40768	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT40820	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT40952	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT41452	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT41468	+	+	+	+	-	+	+	+	+	+	-	+	+
ST1660	IMT43228	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT43231	+	+	+	+	+	+	+	+	+	+	-	+	+
ST1660	IMT43240	+	+	+	+	-	+	+	+	+	+	-	+	+
ST1660	IMT41899	+	-	-	+	-	+	+	+	+	+	-	+	+

+ = gene present, - = gene absent or deleted

All isolates harbored a similar set of genes associated with adherence, including *ebpS* (encoding the elastin binding protein) and *efb* (encoding the extracellular fibrinogen binding protein). All isolates, except isolates IMT37341, IMT39841 and IMT41899, were positive for the fibronectin binding protein gene *fnbA*. Isolate IMT41899 was also negative for the gene *fnbB*. All isolates harbored the intercellular adhesion gene cluster (*ica*), which is associated with biofilm formation.

The ST1660 isolates carried genes for staphylococcal enterotoxins (SE) and SE-like toxins associated with the *egc* enterotoxin gene cluster, namely *sei*, *selm*, *seln*, and *selo*. Moreover, pseudogene *ϕent2* was present in all but one [IMT39841] and *selq* was present among all ST1660 isolates, while the ST1 isolates harbored the enterotoxin gene *seh*. All isolates harbored the leukotoxin-encoding genes *lukD* and *lukE*. All isolates were positive for the bacteriophage Saeq1 (acc. no. LT671578) harboring genes (*lukP* and *lukQ*) which code for a further leukocidin (Table 1). For the staphylococcal complement inhibitor (Scin)—also encoded on this phage—96% amino acid (aa) identity compared to the reference, bacteriophage Saeq1, was observed. All isolates were negative for the genes encoding the toxic shock syndrome toxin 1 (*tst*) and the Panton-Valentin leucocidin (PVL) genes *lukF-PV* and *lukS-PV*.

2.4. Antimicrobial Resistance Properties

Antimicrobial susceptibility testing was performed via broth micro- and macrodilution according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [38,39]. The MIC value distribution and their classification into the categories susceptible, intermediate (if available) or resistant is displayed in Table 2. Penicillin MIC values from 2 to ≥64 mg/L were determined for the 19 isolates reported. Hence, all isolates were classified as resistant [38,39] and harbored the β-lactamase gene *blaZ*.

Table 2. Distribution of minimal inhibitory concentrations (MIC) values of the 19 different equine *S. aureus* isolates.

	No. of Isolates with MIC (mg/L)																Susceptible		Intermediate		Resistant	
Antimicrobial Agent(s)	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**no.**	**%**	**no.**	**%**	**no.**	**%**
Oxacillin	-	-	-	-	2	1	9	7	-	-	-						19	100	-	-	-	-
Penicillin	-	-	-	-	-	-	-	3	-	-	-	-	16				-	-	-	-	19	100
Ampicillin		-	-	-	-	-	1	2	-	-	-	-	1	15								
Amoxicillin/clavulanic acid [a]		-	-	-	3	-	9	7	-	-	-	-	-	-								
Imipenem	12	7	-	-	-	-	-	-	-	-	-	-	-									
Ceftiofur		-	-	-	-	1	11	6	1	-	-	-	-	-								
Cefquinome	-	-	-	-	-	8	11	-	-	-	-	-	-									
Cefalothin			-	-	3	16	-	-	-	-	-	-	-	-	-							
Cefotaxime	-	-	-	-	-	-	-	11	8	-	-	-	-									
Cefoperazone			-	-	-	-	-	3	8	8	-	-	-	-	-							
Erythromycin	-	-	-	-	10	8	1	-	-	-	-	-	-				18	94.7	1	5.3	-	-
Tylosin tartrate			1	-	-	-	11	7	-	-	-	-	-	-	-							
Tulathromycin			-	-	-	-	-	-	3	12	4	-	-									
Tilmicosin			-	-	-	-	12	7	-	-	-	-	-	-	-							
Clindamycin		-	-	15	4	-	-	-	-	-	-	-	-	-			19	100	-	-	-	-
Pirlimycin		-	-	-	-	13	6	-	-	-	-	-	-	-								
Tiamulin		-	-	-	-	4	15	-	-	-	-	-	-	-								
Ciprofloxacin	-	-	-	9	6	1	2	1	-	-	-	-					18	94.7	1	5.3	-	-
Enrofloxacin	-	1	4	10	1	2	1	-	-	-	-	-					15	78.9	1	5.3	3	15.8
Marbofloxacin	-	-	-	-	14	2	3	-	-	-	-	-										
Nalidixic acid			-	-	-	-	-	-	-	1	15	-	3	-								
Gentamicin				-	-	-	-	-	-	-	-	7	11	1	-	-	-	-	-	-	19	100
Kanamycin						-	-	-	-	-	-	-	5	14								
Streptomycin					-	-	-	-	-	14	5	-	-	-	-	-						
Neomycin				-	-	11	5	-	-	2	1	-	-	-								
Tetracycline				-	16	-	-	-	-	-	-	1	2	-	-	-	16	84.2	-	-	3	15.8
Doxycycline			-	10	6	-	-	-	1	2	-	-	-	-	-		10	52.6	6	31.6	3	15.8
Sulfamethoxazole/trimethoprim[a]	-	-	-	1	-	7	8	-	3	-	-	-	-				16	84.2	-	-	3	15.8
Florfenicol				-	-	-	-	-	18	1	-	-	-	-	-	-						
Linezolid		-	-	-	-	-	2	14	3	-	-	-	-	-			19	100	-	-	-	-
Vancomycin	-	-	-	-	-	8	11	-	-	-	-	-	-				19	100	-	-	-	-
Quinupristin/dalfopristin	-	-	-	-	10	9	-	-	-	-	-	-	-				19	100	-	-	-	-

Grey shading indicates concentrations not included in the test panel. Isolates with growth throughout the panel have MIC values equal to or larger than the highest concentration tested and are, therefore, displayed in the next higher concentration with grey shading; classification as susceptible, intermediate or resistant, according to CLSI [38,39] is indicated by black vertical bars; species-specific clinical breakpoints for horses were applied for penicillin, enrofloxacin and doxycycline, the remaining breakpoints were adopted from human medicine [38,39]. [a] amoxicillin and trimethoprim MIC values were used for the combinations amoxicillin/clavulanic acid (2:1) and sulfamethoxazole/trimethoprim (19:1), respectively.

Broth microdilution revealed comparatively low oxacillin MICs of 0.25 or 0.5 mg/L for the ST1 isolates, and, as expected, the WGSs lacked the methicillin resistance genes *mecA*, *mecB* or *mecC*. Resistance to gentamicin and kanamycin, tetracyclines, and the combination sulfamethoxazole/trimethoprim was observed. The respective aminoglycoside resistance genes *aacA-aphD* and *aadD*, the tetracycline resistance gene *tet*(L) as well as the trimethoprim resistance gene *dfrG* were detected in the WGSs. The enrofloxacin MICs of 0.5 or 1 mg/L classified the respective isolates as resistant and the analysis of the quinolone resistance determining regions (QRDR) of GyrA/GyrB and GrlA/GrlB [40,41] revealed that the isolates displayed the same aa alteration Ser80Tyr in GrlA compared to a wildtype sequence (acc. no. D67075.1).

The ST1660 isolates had comparably higher MICs for oxacillin of 1 or 2 mg/L. Resistance to gentamicin and kanamycin was found in all isolates, which corresponded well to the presence of the *aacA-aphD* gene. The neomycin MIC values of 0.5 or 1 mg/L were lower than those obtained for the ST1 isolates with MICs of 8 or 16 mg/L, which might be caused by the absence of the *aadD* gene, which confers kanamycin/neomycin resistance. A single isolate was classified as intermediate to erythromycin. In comparison to the ST1 isolates, the ST1660 isolates displayed lower enrofloxacin MICs of 0.03 to 0.25 mg/L, resulting in the classification of one isolate as intermediate based on its MIC of 0.25 mg/L. However, a single aa exchange Glu434Asp in GrlB was observed in the QRDRs. Six isolates with doxycycline MICs of 0.25 mg/L were classified as intermediate.

The results of the quality control strain *S. aureus* ATCC® 29213 were always within the respective QC ranges [38,39].

2.5. Investigation of the Reduced Susceptibility to Oxacillin

Agar disk diffusion for penicillin resulted in zone diameters of 8–18 mm, which classified the isolates as resistant [38,39]. The ST1 isolates displayed remarkably larger zone diameters (16–18 mm) than the ST1660 isolates (8–12 mm) (Table 3). A similar situation was observed for oxacillin, ampicillin, ampicillin-sulbactam and amoxicillin-clavulanic acid, whereas the amoxicillin zone diameters were comparable for the isolates of both STs and measured up to 8 mm (Table 3). Comparing the results for ampicillin, and ampicillin-sulbactam as well as for amoxicillin and amoxicillin-clavulanic acid, increased zone diameters (of ≥5 mm) were detected for the β-lactamase inhibitor containing compounds. The results obtained with the quality control strains *S. aureus* ATCC® 25923 and *Escherichia coli* ATCC® 35218 were always within the respective QC ranges [38,39].

Table 3. Zone diameter values of the 19 *S. aureus* isolates.

ST [a]	Isolate	Zone Diameter (mm) [b,c]					
		PEN (10 U)	OXA (1 µg)	AMP (10 µg)	SAM (10/10 µg)	AMX (20/10 µg)	AMC (10 µg)
ST1	IMT39129	**18**	20	18	26	7	30
ST1	IMT39173	**18**	19	18	22	8	30
ST1	IMT39701	**16**	19	17	26	8	30
ST1660	IMT39637	**10**	8	9	18	no zone	22
ST1660	IMT37083	**12**	14	12	19	7	22
ST1660	IMT37341	**10**	12	10	16	no zone	20
ST1660	IMT37410	**10**	12	10	18	no zone	20
ST1660	IMT37728	**10**	12	10	18	no zone	20
ST1660	IMT39233	**10**	14	10	18	no zone	22
ST1660	IMT39841	**8**	14	10	16	no zone	20
ST1660	IMT40768	**9**	14	9	16	no zone	21
ST1660	IMT40820	**10**	14	10	17	no zone	20
ST1660	IMT40952	**11**	14	10	18	no zone	21
ST1660	IMT41452	**9**	14	10	18	no zone	20
ST1660	IMT41468	**10**	15	10	17	7	20
ST1660	IMT43228	**10**	14	11	18	no zone	21
ST1660	IMT43231	**10**	14	8	17	7	22
ST1660	IMT43240	**12**	15	12	20	7	24
ST1660	IMT41899	**11**	14	11	19	7	22

[a] ST = sequence type; [b] PEN = penicillin, OXA = oxacillin, AMP = ampicillin, SAM = ampicillin-sulbactam, AMX = amoxicillin, AMC = amoxicillin-clavulanic acid [c] Bold numbers indicate resistance according to CLSI [38].

Regarding the reduced susceptibility to oxacillin, the Fem (factors essential for methicillin resistance) proteins [42] were further investigated, including the aa sequences of FemA, FemB, FemX, FemC and FemD. Compared to the β-lactam susceptible *S. aureus* reference strain ATCC® 25923 (acc. no. CP009361.1), the following situation was observed. Within the FemA protein sequence, all isolates displayed the aa alteration Glu234Asp, while the ST1 isolates had an additional difference, Tyr195Phe. Moreover, the ST1 isolates displayed the aa alteration Arg199Ser in the FemB sequence. Within the FemX protein sequence, all isolates had an Asn18His alteration. While all ST1 isolates exhibited only one additional aa exchange (Ile51Val), the ST1660 isolates showed two additional exchanges (Asn155Thr and Thr262Lys) in the FemX protein sequence. There were no differences regarding the aa sequences of FemC and FemD.

All isolates had aa differences in the catabolite control protein A (CcpA), a global transcriptional regulator, shown to be associated with oxacillin resistance in staphylococci [43,44]. The following differences were observed: Lys171Glu and Ser207Gly, and the ST1 isolates showed an additional Ala197Glu exchange.

All ST1660 isolates had two aa differences (Ile456Val and Asp561Glu) in the phosphodiesterase GdpP, for which is known, that mutations can lead to elevated oxacillin MICs [45,46]. The position Ile456Val is located in the so-called desert hedgehog (DHH) domain [47,48].

Being the primary targets of β-lactam antibiotics, the penicillin binding proteins (PBPs) [42] were also comparatively investigated. The PBP aa sequences of the analyzed isolates were compared to the respective ones of the reference strain *S. aureus* ATCC® 25923 (acc. no. CP009361.1). Differences could be detected in all investigated PBP aa sequences. All ST1 isolates showed two aa exchanges in PBP1 (Asp118Asn, Val617Met), three in PBP3 (Gly167Arg, Lys504Arg, Asp563Glu) and one in PBP4 (Thr189Ser). The ST1660 isolates displayed a single variation in PBP2 (Val102Met), four differences in PBP3 (Gly167Glu, Ala330Ser, Lys504Arg, Asp563Glu) and one in PBP4 (Glu398Ala). Moreover, all isolates had an elongation of eleven aa at position 717 in the PBP2 aa sequence.

Furthermore, the aa sequences of the additional proteins that have been shown to be involved in methicillin resistance, like the efflux pump regulator MgrA [49] and the multiple peptide resistance factor MprF [50] involved in cell wall synthesis, showed no differences to the respective aa sequences of *S. aureus* ATCC® 25923.

2.6. Overexpression and Induction of blaZ

The representative isolates (all three ST1 and ten ST1660) were subjected to β-lactamase activity testing using a nitrocefin assay. The induction with ampicillin revealed a 5.05–19.10-fold increase of β-lactamase production for the ST1660 isolates, while the ST1 isolates showed only a moderate increase (3.17–3.40-fold) (Figure 3a). The Mann-Whitney-U-Test revealed a significant difference ($U = 0.0$; $p = 0.007$) between the ST1 and the ST1660 isolates. Susceptibility testing after induction resulted, for all but one isolate, in oxacillin MICs that were one to two dilution steps higher than the corresponding values of the uninduced isolates (Figure 3b). The isolate IMT37083 (ST1660) showed no change in its oxacillin MIC of 2 mg/L. For the remaining nine ST1660 isolates tested, oxacillin MICs increased to 4 mg/L, thus they were classified as resistant. Even though the ST1 isolates showed increased oxacillin MICs of 1 mg/L, they were still classified as susceptible according to the CLSI standards [38,39].

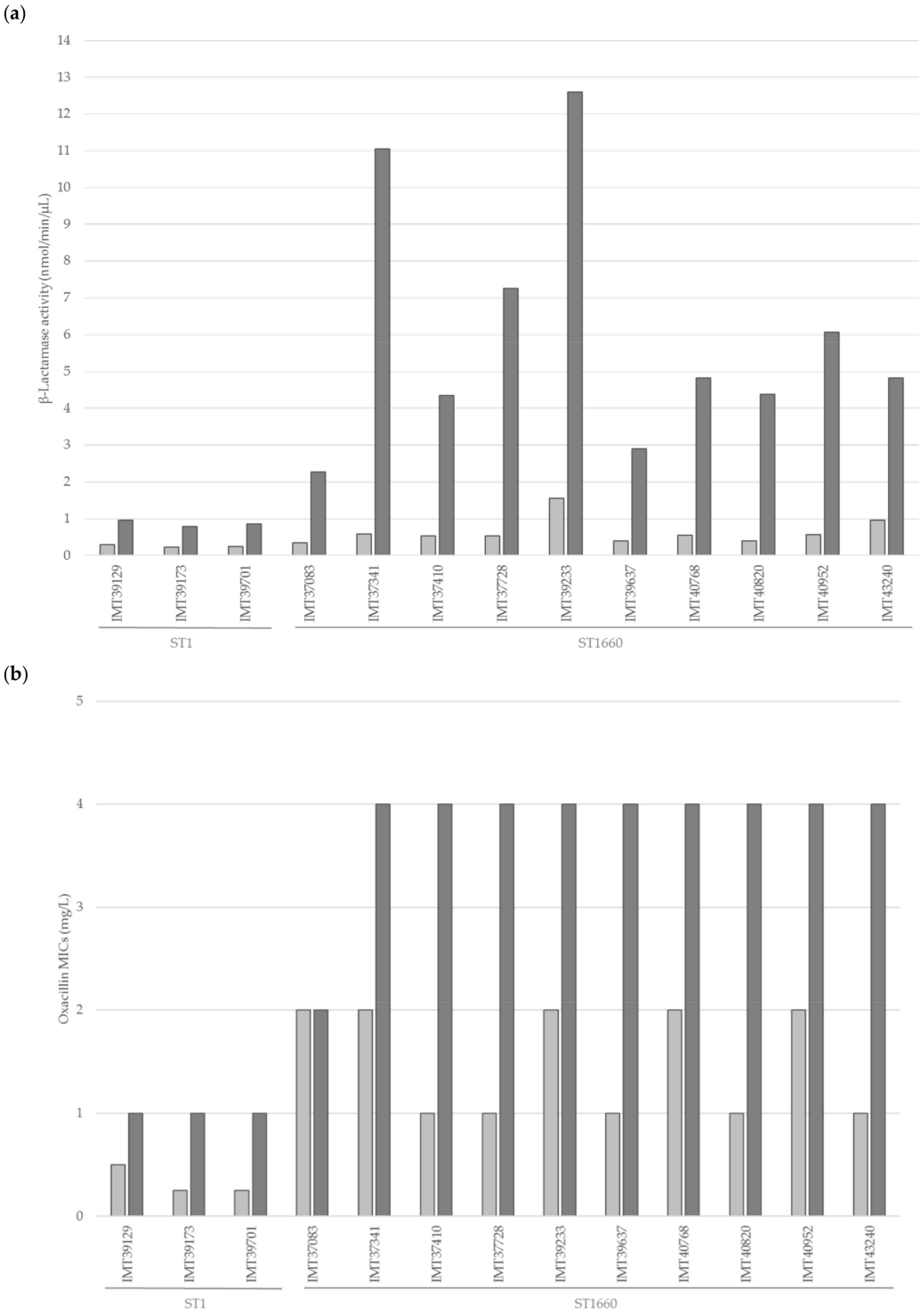

Figure 3. (**a**) Mean β-lactamase activity (nmol/min/µL) obtained by testing in duplicate and (**b**) oxacillin MIC values of selected isolates before and after induction. Light grey = before induction, dark grey = after induction.

The investigation of the β-lactamase operon containing genes encoding the proteins BlaZ, BlaR1 and BlaI revealed the differences between the sequence types. The aa sequences were compared to the originally described sequence of *S. aureus* transposon Tn*552* (acc. no. X52734.1) [51]. For the repressor BlaI, only one aa alteration, Gly21Asp, was observed which was present in all ST1 isolates and the ST1660 isolate IMT37083. The BlaZ proteins of the ST1 isolates had seven aa exchanges (Ser22Pro,

Val86Ile, Glu145Gly, Tyr220Cys, Val9Ala, Ala112Glu and Pro217Ser). The aforementioned ST1660 isolate IMT37083 shared the latter three aa exchanges (Val9Ala, Ala112Glu and Pro217Ser) with the three ST1 isolates and harbored the additional aa exchange Lys169Arg. In the BlaR1 sensor protein, all ST1 isolates showed 32 aa exchanges, with 19 of them also occurring in the aforementioned ST1660 isolate IMT37083 (Figure 4). The remaining ST1660 isolates had four aa exchanges in BlaR1 (Ala91Thr, Ser106Cys, Asp447Asn, and Phe491Leu).

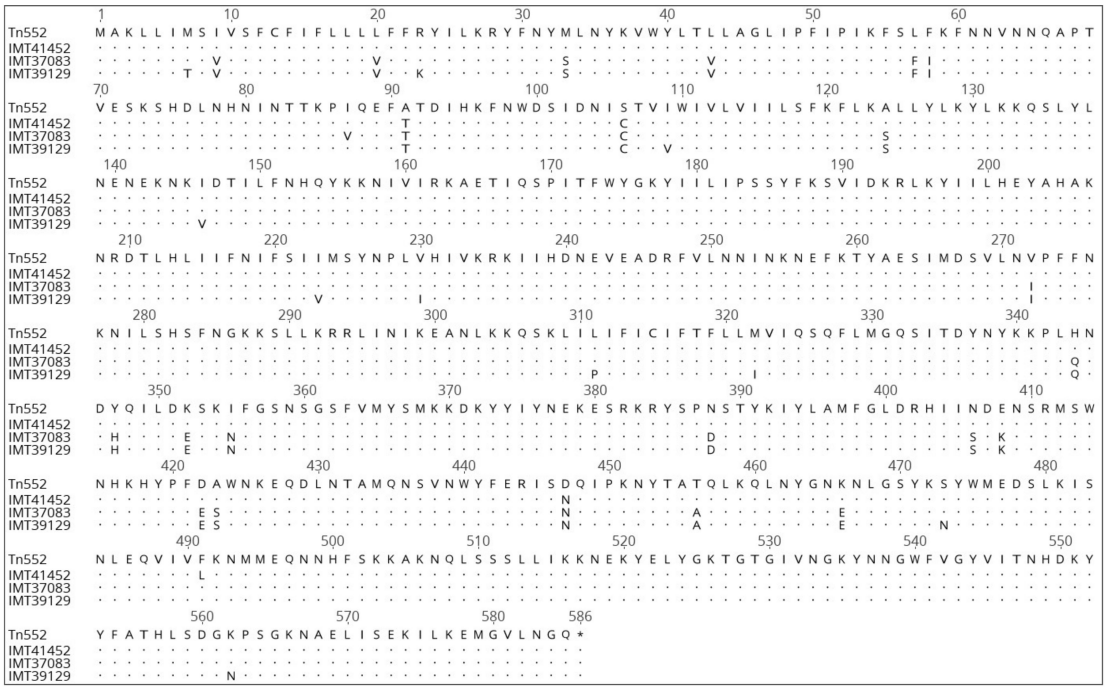

Figure 4. Comparison of the aa sequences of BlaR1 of IMT41452, which represents the majority of the ST1660 isolates, except the differing ST1660 isolate IMT37083, and IMT39129, which represents the ST1 isolates. The transposon Tn*552* of *S. aureus* NCTC 9789 (acc. no. X52734.1) served as a reference. The dots indicate the identity of aa, while letters indicate aa exchanges.

2.7. Susceptibility to Biocides

Comparative biocide susceptibility testing via broth macrodilution revealed MICs of 0.00006–0.0005% for benzalkonium chloride (BAC), 0.125–0.5% for glutardialdehyde (GLU) and 0.00006–0.00025% for chlorhexidine (CHX). The broth microdilution results were 0.000125–0.0005% (BAC), 0.25–0.5% (GLU) and 0.000125–0.00025% (CHX) (Table 4). Overall, the results vary between three to four dilution steps in broth macrodilution and between two to three dilution steps in broth microdilution. The biocide MIC values of the ST1 and ST1660 isolates did not show major differences. Only a slight difference could be seen regarding the CHX MIC values. The *S. aureus* reference strain ATCC® 6538 was tested for comparative reasons and showed comparable results to previous studies [37].

Table 4. MIC distribution obtained with broth micro- and macrodilution.

Method [a] and ST	BAC [b] (MIC in %)			GLU (MIC in %)				CHX (MIC in %)		
	0.00006	0.000125	0.00025	0.0005	0.125	0.25	0.5	0.00006	0.000125	0.00025
Micro ST1				3	1	2				3
Macro ST1			3			2	1			3
Micro ST1660		1	11	4		13	3		9	7
Macro ST1660	1	1	5	9	3	12	1	4	12	

[a] Micro = broth microdilution, Macro = broth macrodilution [b] BAC = benzalkonium chloride, GLU = glutardialdehyde, CHX = chlorhexidine. Numbers indicate the numbers of isolates showing the respective MIC.

3. Discussion

Between 2015 and 2017, BORSA isolates caused infections in horses of a German equine clinic. These isolates were initially noticed as they showed elevated MICs for oxacillin using VITEK2. However, these MICs were classified as susceptible.

An analysis of whole genome sequencing (WGS) data revealed two lineages being associated with the conspicuous oxacillin phenotypes, ST1-t127 and ST1660-t3043, -t2484 and -t549. ST1 is attributed to CC1, which is known to be a livestock-associated putative pathogen, causing zoonotic infections [18]. The isolates with similar characteristics to our isolates (ST1-t127, ST1660-t549 and ST1660-t3043) have been previously described as MRSA and MSSA in equine samples [6,11,21,25,26] and have also been detected in samples obtained from humans in different European and non-European countries [28–33]. In addition, MSSA ST1660 with other *spa* types, t2484 for *S. aureus* from a horse in Germany [52] and t3043 for an isolate obtained from a donkey in Tunisia [53] were described. The isolates attributed to ST1-t127 belong to the three most prevalent lineages of MRSA in the Italian pig industry [54,55] and were also detected in cattle in Italy and China, [56,57] and wild boars in Germany [58].

The two clusters obtained by cgMLST were in accordance with the multi locus sequence typing (MLST) and could further differentiate the isolates. Interestingly, the four ST1660-t3043 isolates obtained in 2015 cluster very closely together, showing 0-2 allelic differences only, while the isolates obtained in other years were more distantly related (Figure 2). The correlation of the cgMLST and the *spa* types revealed that the 14 isolates with *spa* type t3043 could be assigned into 11 allelic profiles by cgMLST. However, it should be noted that one allelic profile was shared by two isolates with different *spa* types, namely t549 and t3043. A former study on the relatedness of *S. aureus* outbreak isolates using the SeqSphere+ cgMLST approach revealed that genomes with 0 to 8 allelic differences should be considered as related, while those with 9 to 29 allelic differences seemed to be possibly related, and those with 30 or more differences were rated as unrelated [59]. However, a lack of epidemiological metadata concerning possible relatedness of individual cases forbids further speculation here. Moreover, a recent study on equine MRSA obtained from horses directly at hospital admission revealed a very limited number of genomic differences for unrelated equine ST398 MRSA as well [13].

All isolates were negative for PVL and the toxic shock syndrome toxin 1, a T-cell activating superantigen (SAg). The ST1660 isolates carried further genes encoding SAgs, including enterotoxins and enterotoxin-like proteins, which are beyond others associated with *egc*. However, only non-*egc* encoded SAgs have been implicated in toxin-mediated diseases [60]. Thus, the role of *egc*-encoded SAgs in equine *S. aureus* requires further investigation. At present, there is an ongoing discussion about the impact of enterotoxins on colonization abilities of *S. aureus.* The ST1 isolates harbored the enterotoxin gene *seh*, which is usually attributed to CC1 and the corresponding protein is known for its binding affinity to human major histocompatibility complex class II [61]. The ST1 and ST1660 isolates were positive for the leukocidin genes *lukP/Q* located on a bacteriophage most similar to Saeq1 (acc. no. LT671578) [62]. Very recently, this phage was reported for MRSA-ST398 isolated from horses in the same area [13]. LukPQ preferentially kills equine neutrophils, but it also showed activity towards human neutrophils at high concentrations [62]. Moreover, an important immune-modulating factor, a variant of Scin, is also located on that phage. Previous research indicated a C3-inhibiting activity of *eq*SCIN in plasma of a much broader range of hosts, including horses, humans, and pigs [63]. According to Monecke et al., most isolates of CC1 harbor a β-hemolysin converting phage, which is supported by our findings [64].

Regarding the antimicrobial resistance properties, the ST1 isolates were resistant to penicillins, aminoglycosides, enrofloxacin, sulfamethoxazole/trimethoprim and tetracyclines and accordingly classified as multiresistant, based on their resistance to three or more classes of antimicrobial agents [65]. Similar resistance profiles were also detected among BORSA isolates of ST1 and ST1660 and MRSA CC398 from horses in equine clinics [13,21]. The ST1660 isolates were resistant to penicillins, and aminoglycosides. It should be mentioned, that except for penicillin, enrofloxacin and doxycycline, no equine-specific clinical breakpoints for the tested antimicrobial agents were available [38].

Regarding the reduced oxacillin susceptibility, the VITEK2 results were compared with the broth microdilution results. The broth microdilution results were generally lower than the results obtained by VITEK2. Moreover, higher MICs were determined for the ST1660 isolates compared to the ST1 isolates.

All isolates lacked the known *mec* genes, encoding an additional PBP and causing resistance to virtually all β-lactams, except specific anti-MRSA compounds [38]. Moreover, no gene with considerable homology to *mec* genes could be detected in the wholegenome sequences so far. Therefore, other potential causes were analyzed. An analysis of the PBPs, compared to the susceptible *S. aureus* ATCC® 25923 (acc. no. CP009361.1) revealed aa alterations in PBP1, PBP2, PBP3 and PBP4, even though none of these differences were within the functional transglycosylase or transpeptidase domains [1,2,66–68]. Since Morroni et al. compared the PBPs of ceftobiprole-resistant MRSA isolates with the vancomycin-resistant *mecA*-carrying MRSA Mu50 [69], a comparison of our isolates with Mu50 (acc. no. NC_002758.2) was performed. Some of the mutations present in Mu50 compared to *S. aureus* ATCC® 25923, were also present in our collection, including the eleven aa terminal elongation in PBP2, but also the aa differences Gly167Arg, Lys504Arg and Asp563Glu in PBP3, as well as Thr189Ser (ST1) in PBP4. Since Mu50 is an MRSA isolate with an alternative PBP2a, it cannot be stated whether these aa changes are involved in the generation of elevated oxacillin MICs.

The mutations in the *gdpP* gene, which encodes a phosphodiesterase that regulates gene expression, have also been described in association with borderline oxacillin resistance [45]. The GdpP protein has two functional domains, GGDEF and DHH. The GGDEF domain contains a diguanylate cyclase, conferring the capacity to synthesize the second nucleotide messenger cyclic di-GMP, and the DHH domain contains the phosphodiesterase characteristic catalytic DHH motif, mediating hydrolysis of cyclic di-AMP, which is, besides others, involved in cell wall homeostasis [47,48]. Only one aa difference of the ST1660 isolates (Ile456Val) is located in the DHH domain. The fact that one aa alteration is located in a functional domain might indicate a possible contribution to the reduced oxacillin susceptibility of the ST1660 isolates. These alterations were only present among the ST1660 isolates and, therefore, did not explain the BORSA phenotype of the ST1 isolates. However, the comparatively lower oxacillin MICs confirmed for the ST1 isolates versus the ST1660 isolates could possibly be in accordance with this finding. A study by Griffiths and O'Neil revealed that neither a mutation Asp418Ala within the DHH domain nor a truncation of GdpP to 370 aa, causing a deletion of this domain, contributed to oxacillin resistance [45]. Therefore, the aa changes in the GdpP protein reported here for ST1660 isolates might have little or no effect on the phenomenon of elevated oxacillin MICs in this study.

In the case of MRSA, some genes, e.g., those of the *fem* family, are important for methicillin resistance [70]. However, the respective Fem proteins were found in the WGSs of susceptible and resistant *S. aureus* isolates. In MRSA, the inactivation of these factors results in a Fem-specific reduced resistance to oxacillin in the corresponding strains, ranging from slightly decreased MICs to complete hypersusceptibility to β-lactam antibiotics [42,71]. Thus far, studies only showed a reduction of oxacillin resistance in usually resistant MRSA isolates, when these factors were altered or deleted [42,70,72]. Consequently, the involvement of the Fem alterations in the increase of oxacillin MICs is not likely.

In line with this, the deletion or inactivation of the carbon catabolite protein CcpA was only shown to increase the β-lactam susceptibility in *S. aureus*, including MRSA, *Staphylococcus epidermidis* as well as different streptococci [43,44,73,74]. Therefore, the detected aa differences did not seem to play a role in the reduced oxacillin susceptibility.

Among staphylococci, the resistance to penicillins is commonly mediated by the *blaZ* gene. This is in accordance with this study, since all isolates were classified as penicillin-resistant by broth microdilution and agar disk diffusion and carried the *blaZ* gene. As expected, the β-lactam compounds containing a β-lactamase inhibitor revealed larger zone diameters than the respective β-lactam compound alone. All isolates showed zone diameter differences of ≥5 mm for the combinations with a β-lactamase inhibitor, indicating the presence of an active β-lactamase. This is in accordance with the results from previous studies. Maalej and colleagues revealed the differences of at least 5 mm for oxacillin zone diameters with and without clavulanic acid [75].

Borderline oxacillin resistance can be caused by the overexpression of the β-lactamase gene *blaZ* [16]. β-Lactamase hyperproduction was evaluated as an underlying mechanism, since oxacillin is usually no target for β-lactamases. Using a nitrocefin assay for the selected isolates, inducible β-lactamase production was detected in all 13 isolates tested. Interestingly, the ST1660 isolates showed a higher degree of induction, which might explain the higher oxacillin MICs (Figure 3). Subsequent susceptibility testing after induction with ampicillin, revealed that all isolates but one showed higher oxacillin MICs than without induction. While all ST1 isolates were still classified as susceptible, nine of ten tested ST1660 isolates reached oxacillin MICs of 4 mg/L and were classified as resistant. While all other ST1660 isolates displayed almost no differences to the originally described BlaZ-BlaR1-BlaI proteins of transposon Tn*552* of *S. aureus* strain NCTC 9789 (acc. no. X52734.1), ST1660 isolate IMT37083 shared 19 of the 32 aa differences within the respective proteins with the ST1 isolates. Since BlaR1 is the sensor protein for extracellular β-lactam antibiotics [51,76–78], it is possible, that these aa changes result in a comparatively lower expression of *blaZ* in ST1 isolates and IMT37083 in the presence of β-lactam antibiotics. The observed increase of the oxacillin MICs after induction of all but one isolate (IMT37083) tested points towards borderline oxacillin resistance due to β-lactamase hyperproduction [16,75].

Biocide susceptibility testing was performed for BAC, GLU, and CHX using broth macro- and microdilution as an additional characterization of the isolates [37,79]. In the equine clinic, only BAC is used, as a compound of a floor disinfectant (7.6 g BAC per 100 g disinfectant). The MICs for GLU and CHX of the isolates in this study were below the standard concentrations used and did not differ remarkably from the MICs of the *S. aureus* reference strain ATCC® 6538. BAC is often used as an additive and not as a single antibacterial agent. Regarding BAC, the highest MIC of the isolates corresponds to the lowest used concentration of this agent.

In conclusion, in a two-year period, two closely related lineages of *S. aureus*, causing infections in an equine clinic, were identified. These isolates were attributed to sequence types/clonal complexes (including MRSA) that are commonly isolated from equine samples, but have also a zoonotic potential [21–26]. Here, borderline oxacillin resistance seems to be associated with the hyperproduction of the β-lactamase BlaZ. The detection and correct classification of isolates expressing the BORSA phenotype is of major importance since the effectiveness of β-lactams is limited and therapy failure might occur when these isolates cause infections.

4. Materials and Methods

4.1. Background Information and Bacterial Isolates

During the years 2015-2017, routine diagnostics identified 19 *S. aureus* isolates with elevated MICs for oxacillin via VITEK2. These isolates originated from 17 equine patients of a veterinary clinic (Table 5). One sample was from an injured horse and one from respiratory disease. All other samples were from surgical site infections. Most of the cases (eleven samples from nine patients) were from orthopedic procedures. Four samples were from colic patients and two from surgeries of the genito-urinary tract. All surgical patients were treated with a combination of amoxicillin and gentamicin, either as single shot therapy before surgery or up to six days after surgery. The injured patient and four with surgical site infections were additionally treated with the combination of sulfamethoxazole/trimethoprim from three up to 13 days. One of these patients had to undergo a second surgery where a single shot dose of amikacin was injected.

Table 5. Background data for the *S. aureus* isolates.

Isolate	Date	History	Surgery	Sample Material	Age	Sex
IMT39129 [a]	9 April 2016	arthritis	yes	synovial fluid	5 years	gelding
IMT39173 [a]	11 April 2016	arthritis	yes	tissue sample	5 years	gelding
IMT39701	12 July 2016	fracture	yes	wound sample	<1 year	stallion
IMT39637	22 June 2016	trauma	no	wound sample	n.k.	gelding
IMT37083	13 July 2015	fracture	yes	wound sample	14 years	mare
IMT37341	24 August 2015	colic surgery	yes	abscess	8 years	stallion
IMT37410	11 September 2015	castration	yes	wound sample	1 year	gelding
IMT37728	5 October 2015	colic surgery	yes	wound sample	11 years	gelding
IMT39233	25 April 2016	rupture of the urinary bladder	yes	wound sample	2.5 weeks	stallion
IMT39841	11 August 2016	wound healing disorder	yes	wound sample	14 years	gelding
IMT40768	7 December 2016	fracture	yes	wound sample	4 years	mare
IMT40820	28 December 2016	colic surgery	yes	wound sample	1 year	mare
IMT40952	17 January 2017	colic surgery	yes	TBS	8 years	gelding
IMT41452	27 January 2017	wound healing disorder	yes	wound sample	15 years	gelding
IMT41468	2 February 2017	sinusitis	yes	wound sample	5 years	gelding
IMT43228 [b]	16 June 2017	fracture	yes	wound sample	7 years	mare
IMT43231 [b]	19 June 2017	fracture	yes	wound sample	7 years	mare
IMT43240	22 June 2017	colic surgery	yes	wound sample	6 years	gelding
IMT41899	15 February 2017	fracture	yes	wound sample	14 years	mare

Isolates originating from the same patients are indicated with the same superscript letters. TBS = tracheobronchial secretion, n.k. = not known.

4.2. Characterization of the Isolates

The DNA extraction for WGS was performed using the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) with some adaptations for staphylococci. Before starting the official protocol, the cells were mixed with 25 µL lysostaphin solution (0.1 mg/mL) and incubated for 25 min at 37 °C. Then, 75 µL TE buffer and 25 µL proteinase K (0.1 mg/L) were added and the cells were incubated for 25 min at 37 °C. Then, 75 µL PBS and 2 µL RNAse A (2 µg/µL) were added and slightly mixed. After this, the protocol for the kit was followed starting with the addition of AL buffer. The libraries for WGS were prepared using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The 2 × 300 bp paired-end sequencing in 30-fold multiplexes was performed on the Illumina MiSeq platform. The genome sequences were *de novo* assembled using Newbler (Roche, Basel, Switzerland) and SPAdes v3.12.0 [80]. The nucleotide sequences were analyzed with Geneious v11.1.4 (Biomatters Ltd., Auckland, NewZealand) and annotated with the subsystem technology server (RAST) [81] and Prokka [82] which were compared with BLAST (National Center for Biotechnology Information, Rockville Pike, USA) [83] results. Further investigations were performed by using ResFinder [84] of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). The virulence factors were identified by using VFanalyzer of VFDB (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi?func=VFanalyzer) and checked using Geneious v11.1.4. The associated mobile genetic elements including pathogenicity islands and phages were determined using Geneious v11.1.4 software. The STs were derived from the pubmlst database (https://pubmlst.org) and *spa* types were deduced using the Ridom Spa Server (http://www.spa.ridom.de).

Whole genome sequences were analyzed for the proteins known to play a role in oxacillin resistance: GdpP, FemA, FemB, FemC (GlnR), FemD (GlmM), MgrA, CcpA and MprF as well as the penicillin binding proteins (PBP) 1, 2, 3 and 4. The sequences of these proteins were compared with *S. aureus* ATCC® 25923, an oxacillin-susceptible reference strain (acc. no. CP009361.1). The *blaZ-blaI-blaR1* operon was compared to the respective region of the originally described transposon Tn*552* (acc. no. X52734.1) [51]. The analyses were carried out with Geneious v11.1.4.

4.3. Phylogenetic Analysis

The molecular epidemiology was investigated using the previously generated WGSs as FASTA files for the *S. aureus* core genome multilocus sequence typing (cgMLST) approach. For this, the software SeqSphere+ version 6.0.2 (Ridom GmbH, Münster, Germany) was used [85,86]. To illustrate

the clonal relationship between the different isolates, a minimum-spanning tree was built based on a distance matrix of the core genome allelic profiles including 1744 of 1861 possible target genes, using the "pairwise ignoring missing values" option of the software.

4.4. Antimicrobial Susceptibility Testing

The routine diagnostics performed antimicrobial susceptibility testing via VITEK2 according to the manufacturer's instructions. Additional susceptibility testing to 31 antimicrobial agents was performed by broth microdilution according to the CLSI recommendations [38,39] using sensititre™ microtiter plates. *S. aureus* ATCC® 29213 was used for quality control. The antimicrobial susceptibility testing by broth microdilution was repeated for the β-lactam antibiotics using selected isolates (all three ST1 and ten ST1660 isolates), after the induction with ampicillin [0.25 µg/mL (ST1) or 32 µg/mL (ST1660) ampicillin (Roth®, Karlsruhe, Germany)], to investigate the effects of induced β-lactamase production. The inoculum for the induction testing was prepared using the growth method, where the isolates were incubated with the respective amount of ampicillin for 4 h in cation-adjusted Mueller-Hinton broth (CAMHB) and then, the bacterial suspension was adjusted to McFarland 0.5. Additionally, the susceptibility to kanamycin (Roth®, Karlsruhe, Germany) was tested via broth macrodilution [38,39].

4.5. Investigation of β-Lactamase-Production

Agar disk diffusion [39] was performed using BBL™ Sensi Discs for penicillin (10 IU), oxacillin (1 µg), ampicillin (10 µg) and amoxicillin/clavulanic acid (20/10 µg) and Oxoid™ discs for amoxicillin (10 µg) and ampicillin/sulbactam (10/10 µg) on Mueller-Hinton agar (MHA) plates. *S. aureus* ATCC® 25923 and *E. coli* ATCC® 35218 were used for quality control purposes, according to CLSI standard [39]. The results of agar disk diffusion for β-lactam antibiotics with and without a β-lactamase inhibitor were comparably investigated.

Using a nitrocefin-based β-Lactamase Activity Assay Kit (Sigma-Aldrich®, Munich, Germany), the β-lactamase activity of all ST1 isolates (n = 3) and selected ST1660 isolates (n = 10) were quantitatively investigated. Therefore, the isolates were cultured overnight in brain-heart-infusion (BHI, Oxoid, Wesel, Germany) at 37 °C. The next day, 5 mL BHI with and without 0.25 µg/mL (ST1) or 32 µg/mL (ST1660) ampicillin (Roth®, Karlsruhe, Germany) were inoculated with 200 µL of the overnight cultures and incubated for 4 h at 37 °C. Following the manufacturer's instructions, the reaction was prepared in duplicate with a sample volume of 20 µL per isolate and the absorbance was measured at a wavelength of 490 nm, every 60 s for one hour. The standard curves were evaluated for every microtiter plate and β-lactamase activity was calculated, according to the manufacturer's instructions.

4.6. Biocide Susceptibility Testing

Comparative biocide susceptibility testing was performed using a broth macrodilution method [37] and a broth microdilution method, which has been developed in this research group [79]. *S. aureus* ATCC® 6538 was tested for comparative reasons and the results were compared to those of a previous interlaboratory trial [37]. The optical densities were adjusted according to German Veterinary Medical Society (DVG) standards for biocide efficacy testing [87]. For broth microdilution, twofold dilution series were prepared in 100 µL per well on a 96 well plate. The biocide solutions were prepared in deionized water and the inoculum was prepared in tryptic soy broth (TSB). The inoculum was added to a final testing volume of 200 µL and the results were read after incubation for 24 h at 37 °C. The MIC was defined as the first well concentration without visible growth. The tested biocides were BAC (Roth®, Karlsruhe, Germany), GLU (Roth®, Karlsruhe, Germany) and CHX (Sigma®, Munich, Germany). The test ranges were prepared in twofold dilution series; 0.000008–0.004% for BAC, 0.008–4% for GLU and 0.00001–0.0005% for CHX.

4.7. Statistical Analysis

The statistical analysis was performed using IBM®SPSS® Statistics Version 25. To compare the β-lactamase inducibility of the isolates, non-parametric Mann-Whitney-U-test was performed.

4.8. WGS Submitted to GenBank

This Whole Genome Shotgun project has been deposited at GenBank under the accession numbers VSYP00000000 (IMT41899), VSYQ00000000 (IMT43240), VSYR00000000 (IMT43231), VSYS00000000 (IMT43228), VSYT00000000 (IMT41468), VSYU00000000 (IMT41452), VSYV00000000 (IMT40952), VSYW00000000 (IMT40820), VSYX00000000 (IMT40768), VSYY00000000 (IMT39841), VSYZ00000000 (IMT39233), VSZA00000000 (IMT37728) VSZB00000000 (IMT37410), VSZC00000000 (IMT37341), VSZD00000000 (IMT37083), VSZE00000000 (IMT39637), VSZF00000000 (IMT39701), VSZG00000000 (IMT39173) and VSZH00000000 (IMT39129).

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2.2 Publication II

Scholtzek AD, Hanke D, Eichhorn I, Walther B, Lübke-Becker A, van Duijkeren E, Köck R, Schwarz S, Feßler AT. 2020

Heterogeneity of antimicrobial susceptibility testing results for sulfamethoxazole/trimethoprim obtained from clinical equine *Staphylococcus aureus* isolates using different methods

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2.3 Unpublished data

Publication I deals with the characterization of the *S. aureus* isolates, including MLST, *spa* types, virulence factors and phenotypic and genotypic resistance properties, emphasizing on the expressed BORSA phenotype and its underlying resistance mechanism. It also comprises a short overview of the origin of the isolates. **Publication II** focuses on the isolates' SXT susceptibilities and their diagnostic challenges. To give further information on the treatment and the clinical outcome of the 17 equine patients, the unpublished Table 1 was added.

Unpublished data Table 1 Overview of antimicrobial treatment in correlation with occurred surgery and clinical outcome of the patients

Isolate	ST	Surgery	AMX/GEN	SUL/TMP	Others	Outcome
IMT39129[a]	1	yes	single-shot[c]			euthanasia
IMT39173[a]	1	yes	single-shot			euthanasia
IMT39701	1	yes	2 d	13 d		back to owner
IMT39637	1660	no		5 d		back to owner
IMT37083	1660	yes	6 d	3 d		back to owner
IMT37341	1660	yes	4 d			back to owner
IMT37410	1660	yes	single-shot			back to owner
IMT37728	1660	yes	5 d			euthanasia
IMT39233	1660	yes	single-shot			back to owner
IMT39841	1660	yes	6 d	4 d	AMI[c]	back to owner
IMT40768	1660	yes	5 d			back to owner
IMT40820	1660	yes	5 d			back to owner
IMT40952	1660	yes	5 d			back to owner
IMT41452	1660	yes	3 d			back to owner
IMT41468	1660	yes	4 d			back to owner
IMT43228[b]	1660	yes	8 d			back to owner
IMT43231[b]	1660	yes	8 d			back to owner
IMT43240	1660	yes	5 d	3 d		back to owner
IMT41899	1660	yes	9 d			back to owner

ST = sequence type; AMX/GEN = combination of amoxicillin and gentamicin; AMI= amikacin; SUL/TMP = combination of a sulfonamide (here: sulfadiazine) and trimethoprim; the same superscript letters [a] and [b] indicate the samples originating from the same horse, [c] perioperative single-shot therapy

3 Discussion

3.1 Characterization and relatedness of the isolates

The 19 *S. aureus* isolates originated from 17 equine patients, being admitted to the Equine Clinic: Surgery and Radiology of the Department of Veterinary Medicine at Freie Universität Berlin clinic between July 2015 and June 2017. One patient came in with an injury, all other samples were from surgical site infections (**Publication I**). Whole genome sequencing allowed the identification of two lineages of pathogens. Three isolates belonged to ST1 and all had the *spa* type t127. The remaining 16 isolates were typed as ST1660 and belonged to the *spa* types t3043 (n = 14), t2484 (n = 1) and t549 (n = 1).

Horses are colonized by a great variety of staphylococcal species, including numerous coagulase-negative species – such as *Staphylococcus xylosus* and *Staphylococcus sciuri* – and coagulase-positive species, such as *S. aureus* and *Staphylococcus delphini* [74]. *S. aureus* is a highly clonal pathogen and different STs are assigned to CCs. These CCs can then be attributed to regions, host species and pathogenicity. ST1-t127 belongs to CC1, which is linked to livestock-associated isolates that cause infections in humans, horses, pigs, wild boars and cattle in Germany, Switzerland, Italy, Denmark, China and Japan [8, 23, 25, 34, 74-81]. ST1660 is not yet attributed to a CC. Isolates with this ST and all *spa* types from our study have been described before, in MSSA and MRSA of human or equine origin in Germany, Switzerland, Denmark and Tunisia [8, 25, 74, 76, 82-84]. Overall, for equine *S. aureus*, especially MRSA, a shift of the most common CCs occurred. While in 2008, the predominant clusters were CC8 and CC22 [6, 85], more recent studies report CC398 as a replacement, especially for CC8 [86, 87]. Interestingly, CC8 and CC22 are usually associated with (MRSA) infections in humans, while CC398 is associated with LA-MRSA, that also colonizes and occasionally infects humans [34]. This, again, highlights the zoonotic potential of these strains, especially since those of equine origin are often indistinguishable from those of human origin [6]. Next to these predominant CCs, other clusters and STs not attributed to CCs so far, appeared in equine isolates throughout the years. Isolates of CC1 and CC5, which are often associated with CA-MRSA and/or HA-MRSA infections in humans occurred in Germany [26], Spain [84], the UK [85], Austria [86] and Denmark [76]. The only study focusing on horses for food production was carried out by Mama et al. and the most frequent ST was ST1640 [84].

Regarding virulence factors, the three ST1 isolates harbored the same set of genes, while there were minor differences within the ST1660 isolates (**Publication I**). PVL and the toxic shock syndrome toxin 1 were absent in all isolates. All ST1 isolates were positive for the staphylococcal enterotoxin H which is able to bind to human major histocompatibility complex class II and cause a strong T cell activation [88]. The ST1660 isolates harbored different

enterotoxin and enterotoxin-like genes (*sei*, *selm*, *seln*, *selo*, *Φent2* and *selq*), some of them belonging to the enterotoxin gene cluster *egc*. Since enterotoxins and enterotoxin-like proteins are currently being discussed regarding their role in the colonization capacities of *S. aureus*, it is interesting that those associated with the *egc* do not seem to be involved in toxin-mediated diseases [89]. All isolates harbored the leukocidin genes *lukE*, *lukD*, *lukP* and *lukQ*. LukE/D targets neutrophils with high affinity to murine cells [90, 91]. LukP/Q mainly effects equine but in high concentrations also human neutrophils. It was located on a bacteriophage with high similarity to Saeq1 (acc. no. LT671578) [92]. This phage also harbored the typical equine staphylococcal complement inhibitor (*eq*Scin) which showed a 96 % amino acid (aa) identity with the reference. Interestingly, other studies had identified this phage in equine MRSA isolates from the same region [93] and in isolates from horses for human consumption in Spain [84]. The ST1 isolates harbored another phage converting the β-hemolysin gene *hlb*, and thereby inactivating its function. The literature shows that a β-hemolysin converting phage is common in *S. aureus* isolates of CC1 [34]. Usually, they harbor the genes for staphylokinase (*sak*), enterotoxin A (*sea*), the chemotaxis inhibitory protein (*chp*) and *scin*, which are associated with the immune invasion cluster in human isolates [94]. In isolates of animal origin they are much less frequent as they play a role for the colonization of human hosts [95].

The investigation of the relatedness of the strains was carried out via core genome MLST, which allowed the identification of two main groups, the ST1 and the ST1660 isolates, harboring two and twelve allelic profiles, respectively (**Publication I**). The four isolates from 2015 (all ST1660-t3043) clustered very closely, with only 0-2 allelic differences. Overall, the *spa* types correlated well with the allelic profiles with one exception. One allelic profile was shared by two isolates obtained in early 2017 with different *spa* types, namely t549 and t3043. Unfortunately, there was no background information on possible relatedness of individual cases. Cunningham et al. proposed that when using core genome MLST for investigation of the relatedness of bacterial strains, isolates with 0 to 8 allelic differences shall be considered as related, those with 9 to 29 as possibly related and those with more than 30 differences as unrelated [96]. Using these thresholds, all isolates were related within their STs (0-7 allelic differences within the STs), while the ST1 isolates were not related with the ST1660 isolates (1697 allelic differences). However, speculations about the transmission of the isolates between individuals seem to be misplaced, since a recent study identified low genetic diversity in MRSA isolated from unrelated cases directly at hospital admission [93, 97].

3.2 Resistance properties

The focus of this PhD thesis is antimicrobial resistance. Therefore, the isolates were tested for their phenotypic antimicrobial and biocide susceptibility and the genetic basis of the

detected resistance properties. Regarding antimicrobial resistance, all isolates were tested via automated test systems, BMD and DD. Regarding biocide susceptibility, we tested the isolates via BMD and broth macrodilution in parallel to investigate the comparability of the results.

3.2.1 Antimicrobial resistance

AST for screening of the isolates was carried out via BMD for 31 antimicrobial agents and broth macrodilution for kanamycin. The MIC results are presented in **Publication I**. All isolates were resistant to penicillin via the *blaZ* gene. Resistance to aminoglycosides was also detected in all isolates, with the respective genes *aacA-aphD* (both STs) and *aadD* (only ST1 isolates). The ST1 isolates were additionally resistant to tetracycline (*tet*(L)) and enrofloxacin, with a respective aa alteration in their GrlA protein. The *tet*(L) gene is not very often detected in staphylococci, but if so, it is more often seen in isolates of animal origin compared to those of human origin [18]. The most frequently detected *tet* genes in staphylococci are *tet*(K) and/or *tet*(M) [18, 25, 87]. Using the horse-specific clinical breakpoints for doxycycline [41], all ST1 isolates were classified as resistant, while the ST1660 isolates were classified as intermediate (n = 6) and susceptible (n = 10). Resistance to TMP among the ST1 isolates was mediated by the TMP resistance gene *dfrG*, which is usually found in isolates of bovine or porcine origin [18]. The ST1660 isolates harbored the trimethoprim resistance gene *dfrS1*. Multiresistance is defined as resistance to three or more classes of antimicrobial agents [73]. Using this definition, all isolates were identified as multiresistant.

Similar antimicrobial resistance phenotypes have been identified in equine *S. aureus* isolates (MRSA and MSSA) in Switzerland, Germany, France and the USA [25, 26, 98, 99]. It is important to note, that species-specific clinical breakpoints for equine isolates only exist for penicillin, enrofloxacin and doxycycline. Therefore, it is not possible to reliably predict a clinical outcome for the treatment with other antimicrobial agents.

3.2.2 Biocide resistance

Comparative BST was carried out in two replicates, via BMD and broth macrodilution, according to our protocols published by Schug et al. [71] and Feßler et al. [63] (**Publication I**). The investigated biocides BAC, CHX and GLU were used as representatives of different biocide classes, namely QACs, bis-biguanides (cationic compounds) and aldehydes, that were also used for the development of the two BST protocols. With only slight deviations of ± one dilution steps, which is the overall accepted range in AST [41, 59], the results obtained with both methods proved to be comparable. Since both methods were established using the reference strains for biocide efficacy testing, *S. aureus* ATCC® 6538 was tested in parallel with both methods, to see whether the results were reproducible. They were always within the most

common values measured for this strain (for BAC 0.000125 %; for GLU 0.125 - 0.25 % and CHX 0.000125 - 0.00025 %). The MICs of the clinical isolates were similar to those of the reference strain *S. aureus* ATCC® 6538 and revealed MICs of 0.00006 – 0.0005 % for BAC, 0,125 - 0,5 % for GLU and 0.00006 - 0.00025 % for CHX. The MICs for CHX and GLU were far below the used concentrations of these agents for all isolates. CHX is used for antiseptic washes and cremes in concentrations of 0.1 – 4 % [100, 101] and GLU is used for disinfection of medical devices in concentrations of 1.5 – 25 % [102]. Regarding BAC, the highest tested MIC corresponds to the lowest usage concentration of the agent. In these concentrations (0.0005 – 0.03 %), BAC is commonly used as a conservation agent in eye drops, nasal sprays and antiseptic mouthwashes [100, 101], and it is usually combined with other agents for synergistic effects. Since the MIC is defined as the lowest concentration inhibiting bacterial growth, even the lowest used concentration of BAC should still be able to inhibit the bacterial growth, especially when a multi-compound biocide is used. For the disinfection of surfaces, BAC is commonly used in higher concentrations. For example, in the horse clinic of our study, BAC was the component of a floor disinfectant (7.6 g BAC per 100 g disinfectant) with other QACs and isopropanol. It should be noted though, that for BST, the isolates were incubated for 24 hours in the presence of the biocide, while the actual exposure time of bacteria to the biocide for disinfection is much shorter – usually only a few minutes. Therefore, the MIC values can, in contrast to those for antimicrobial agents, not be directly correlated to practical use. Here, the MIC serves the purpose of characterizing clinical isolates and monitoring changes in susceptibility of a bacterial species. Another study used our broth macrodilution protocol [63] to test the BAC, CHX and GLU susceptibility of MRSA isolates from primates in the United States [103]. The MICs ranged from 0.0001 - 0.0008 % for BAC, from 0.00005 – 0.0001 % for CHX and from 0.06 – 0,12 % for GLU [data kindly provided by the authors]. Besides slightly differing dilution steps, these results can be compared to those of our equine isolates. Overall, the MICs of our isolates were in similar ranges: for BAC, they were slightly lower and for GLU slightly higher than the ones observed for the MRSA isolates from the primates. It should be noted that the primate isolates with the elevated BAC MICs harbored a *qacC* gene, which is known to lead to a reduced susceptibility to BAC [104]. In 2013, Couto et al. investigated antimicrobial and biocide susceptibility regarding BAC, CHX and triclosan) of equine methicillin-resistant staphylococcal isolates. Within their collection, two *S. aureus* isolates occurred with MICs of 0.5 mg/L for BAC and CHX [105]. Converting these values to percent, this corresponds to a MIC of 0.00005 %. Therefore, their values were much lower than those of our isolates. Regarding BAC, only one of our isolates, IMT37083, the first of our collection yielded a similar MIC of 0.00006 % in broth macrodilution. Couto et al. determined the biocide MICs using a BMD with Mueller Hinton broth [69], which differs from our method, using tryptic soy broth as medium. Moreover, slightly differing dilution steps and an incubation time of 18

hours instead of our 24 hours were used. Therefore, the results are not suitable for a direct comparison.

3.3 Reduced susceptibility to oxacillin

The 19 isolates were noticed because of their elevated oxacillin MICs via VITEK® 2 (**Publication I**). Except for two isolates, the MICs were still classified as susceptible, but they were higher than expected for susceptible isolates. Testing was repeated via BMD and revealed overall lower MICs, especially for the ST1 isolates. The whole genome sequences (WGSs) of all isolates were investigated and no *mec* gene or *mec* gene homologue, encoding an additional PBP resulting in oxacillin resistance, was present. The WGSs were further screened for alterations in relevant genes or proteins. Several aa changes were identified in the PBPs 1, 2, 3 and 4, when compared to the oxacillin-susceptible *S. aureus* ATCC® 25923 (acc. no. CP009361.1), but none were within the functional domains (transglycosylase or transpeptidase) [106, 107], which makes a contribution to reduced oxacillin susceptibility unlikely. Interestingly, a few of the alterations were also identified in MRSA Mu50 (acc. no. NC_002758.2) in a previous study [108], but since this strain harbors a *mecA* gene, the relevance of these alterations remains questionable. The aa sequences of the Fem (factors essential for methicillin resistance) proteins were compared to those of *S. aureus* ATCC® 25923 and all isolates had aa differences in their FemA, FemB and FemX sequences. Since these proteins are present in oxacillin-resistant and -susceptible isolates, and mutations of the respective genes usually result in a decrease of resistance up to hyper-susceptibility [109, 110], these findings should not contribute to the reduced susceptibility observed. For the carbon catabolite protein CcpA, similar reasoning can be applied: even though aa alterations were identified in the 19 isolates, a contribution to reduced oxacillin susceptibility was not reported for this protein, on the contrary, inactivating mutations of the respective gene resulted in increase of oxacillin susceptibility in staphylococci [111-114]. Another protein possibly involved in oxacillin tolerance is GdpP, a phosphodiesterase that regulates gene expression via degradation of cyclic di-AMP and cyclic di-GMP, whose inactivation can lead to overexpression of PBP4 [46, 115, 116]. The GdpP protein harbors two functional domains, GGDEF and DHH. The ST1660 isolates shared an aa exchange in the functional DHH domain, which could contribute to elevated oxacillin MICs. However, Griffiths and O'Neill stated, that neither a deletion of nor a mutation within the domain, would lead to an increase in oxacillin tolerance [117]. Contrary to this, in 2018 Chung et al. created a laboratory mutant of *S. aureus* with increased tolerance to oxacillin, without an increase of the MIC due to a prolonged lag phase before the exponential phase. An investigation of the WGSs revealed only two aa alterations: an inactivating early-stop mutation in the *atl* gene encoding a bifunctional autolysin

and a mutation in the DHH domain of the *gdpP* gene, resulting in the aa exchange A434E. Therefore, they concluded, that this latter mutation was involved in mediating tolerance to oxacillin [118]. Since the aa exchange was only present in the ST1660 isolates, this could explain the relatively higher oxacillin MICs of these isolates compared to those of ST1. On the other hand, the ST1 isolates still expressed the BORSA phenotype with elevated MICs, therefore, the aa alteration in GdpP cannot be the only underlying mechanism.

Resistance to β-lactams in staphylococci is usually conferred by *blaZ*. All isolates harbored this gene, resulting in penicillin-resistance throughout our collection. Since borderline oxacillin resistance can occur due to β-lactamase hyperproduction [44], respective tests were carried out. In DD, disks containing β-lactamase inhibitors produced an at least 5 mm larger zone diameter than the respective antimicrobial agents without an inhibitor. Maalej et al. stated that a difference of ≥ 5 mm is the difference necessary to identify resistance due to β-lactamases [119]. Usually, oxacillin is not a target for β-lactamases, but recently, Nomura et al. published a study where they showed that the class A -β-lactamase of *S. aureus* [120] hydrolyzed oxacillin [121]. Nonetheless, since the ST1 isolates and ST1660 isolates expressed different levels of reduced oxacillin susceptibility, hyperproduction of BlaZ was further investigated, using a nitrocefin kit. All tested isolates (all ST1 isolates and ten ST1660 isolates) revealed an inducible *blaZ*. After induction with the β-lactam ampicillin, *blaZ* expression increased for all tested isolates, especially for the ST1660 isolates, where the highest detected induction was an approximately 19-fold increase. To investigate the impact of the elevated *blaZ* expression on the oxacillin MIC, susceptibility testing was repeated after the induction with ampicillin. All but one isolates showed a 1-2 dilution step increase in their oxacillin MIC, which resulted in the classification of nine out of the ten tested ST1660 isolates as resistant (MIC = 4 mg/L). This increase in oxacillin MICs after induction of BlaZ strongly suggests a reduced susceptibility to oxacillin due to the hyperproduction of BlaZ. The only isolate that showed an increase in BlaZ production without a change of MIC was the ST1660 isolate IMT37083. To elucidate possible causes, the aa sequences of the *blaZ*-operon components BlaZ, BlaR1 and BlaI were investigated. BlaI is the repressor of BlaZ. In the absence of a β-lactam, it inhibits the transcription of *blaZ*. Whenever a β-lactam is present, the membrane located sensor domain of BlaR1 binds it, resulting in the cleavage of BlaI, which then leads to the synthesis of BlaZ [122-126]. A comparison with the originally described BlaZ-BlaR1-BlaI proteins of transposon Tn*552* of *S. aureus* NCTC 9789 (acc. no. X52734.1) revealed a high similarity between the ST1660 isolates, except IMT37083, and the original aa sequence. Interestingly, isolate IMT37083 shared 19 of 32 aa alterations within the BlaR1 protein with the ST1 isolates. Since the function of BlaR1 is to identify the presence of β-lactams and start β-lactamase synthesis by inactivating the inhibitor BlaI, it is possible, that the aa exchanges in this protein result in an impaired function and thus, in an inferior increase of β-lactamase

production. This is in accordance with an investigation of a *mecA*-positive, but oxacillin-susceptible *S. aureus* isolate, where the expression of *mecA* was hindered due to a non-functional BlaR1 [125].

In conclusion, the *S. aureus* isolates in our study all harbored an inducible *blaZ* gene, whose overexpression in the presence of β-lactam antibiotics resulted in elevated oxacillin MICs in all but one isolates. Differences between the isolates could possibly be explained by aa alterations within the BlaR1 protein, resulting in a comparatively lower expression of *blaZ*. Oxacillin, amongst other β-lactams, is used in veterinary medicine for a broad variety of bacterial infections [39, 40]. All horses who underwent surgery (n = 16) in our study, were treated with a combination of the β-lactam amoxicillin and the aminoglycoside gentamicin as single-shot therapy during surgery (**Unpublished data Table 1**). For 13 patients, treatment with this combination was prolonged for up to nine days. All but two patients, who needed to be euthanized due to the severity of their illness, could be sent back to their owners.

The resistance situation in staphylococcal isolates can change over time. For example, in the United States, resistance to oxacillin seemed to decrease in human *S. aureus isolates* sampled in a children's hospital over a time period of ten years (2005 – 2014) [127], while Conner et al. found an increase in oxacillin-resistance in canine isolates during the years 1993 – 2009 [128]. The Danish monitoring DANMAP described fluctuating oxacillin resistance in *S. aureus* isolates from human bacteremia cases, starting at 1.6 % in 2009, reaching its peak with 2.9 % in 2014 and retrieving back to 1.6 % in 2018 [13]. In Germany, from 2010 – 2015 a steady decline of oxacillin resistance from 16 % to 10 % in human *S. aureus* isolates collected by the Antimicrobial Resistance Surveillance Network was observed [129]. In the same time period, oxacillin resistance in staphylococci from pigs, changed from 60 % in 2010 to 20 % in 2015, but peaked again in 2016 with almost 70 %. In poultry, the fluctuations were similar, with about 15 % oxacillin-resistant staphylococci in 2010, 5 % in 2015 and 20 % in 2016 [130]. These findings point towards the necessity to consider host species, body sites and geographical origin when investigating staphylococci, since there might be great differences between the sample groups.

3.4 Susceptibility to the combination sulfamethoxazole/trimethoprim

Initially the isolates were tested for their antimicrobial susceptibility via VITEK® 2 and BMD with commercial sensititre™ microtiter plates. VITEK® 2 identified all but two ST1660 isolates as resistant to SXT, while BMD identified only the ST1 isolates as resistant and all ST1660 isolates as susceptible. Therefore, further tests were carried out to identify the true resistance properties of the isolates (**Publication II**), namely the automated test system BD Phoenix™, BMD with another commercial plate (Merlin, "micronaut-S anaerob") and DD. To

check for the single components of the antimicrobial combination, DD as well as a self-made microtiter plate set-up for the SUL sulfisoxazole and trimethoprim were carried out, according to CLSI standards [42, 59]. Sulfisoxazole is the class representative for SULs with available QC ranges and interpretive criteria. Resistance to this substance can be considered as resistance to any of the currently available SULs [42].

Regarding TMP, both methods identified all isolates as resistant, which is in accordance with the genotype, since all isolates harbored a TMP resistance gene, namely *dfrG* (ST1) or *dfrS1* (ST1660), which is also known as *dfrA*. Both genes have been described in staphylococcal isolates of horses and humans before [84, 131-133]. The *dfrG* gene was also identified in canine isolates in Australia [21] and was identified as the globally predominant TMP resistance gene in human isolates, via the investigation of samples from travelers across Europe [133]. The occurrence of this gene in isolates of human and animal origin points towards the possibility of zoonotic transmission. Since the TMP-insensitive dihydrofolate synthase DfrS1 is only slightly different to the TMP-sensitive dihydrofolate reductase DfrC (three aa differences: V32I, G43A and F98Y) [134], *dfrS1* is not present in the ResFinder 3.2 database (last accessed 11.06.2020). Therefore, the WGS of an isolate needs to be searched manually to identify this gene.

All ST1660 isolates were identified as susceptible to SULs via both methods, BMD and DD. The ST1 isolates were characterized as resistant via BMD and susceptible via DD. Interestingly though, the zone diameters for SULs of the ST1 isolates were significantly smaller than those of the ST1660 isolates. According to CLSI, a zone diameter of 13 - 16 mm is considered as intermediate and ≥ 17 mm as susceptible [42]. With zone diameters of 18 - 20 mm, the ST1 isolates were close to the breakpoint of intermediate classification. Up to now, no specific gene mediating SUL resistance in staphylococci has been identified, but some resistance mechanisms have been revealed. SUL resistance does not seem to be plasmid-borne [135]. In 2014, Wang et al. described the sulfonamide genes *sul1*, *sul2* and *sul3* in three *Staphylococcus* isolates from soil of pig and chicken farms for the first and only time. They used agar plates containing 60 mg/L sulfadiazine to screen for SUL resistance [136]. However, they did not elucidate whether the genes were chromosomal, or plasmid located nor if the genes were expressed and functional. Quantitative investigations were only carried out for direct soil samples and not for single isolates. They found a great variety of bacterial species within the soil samples and it is possible, that the staphylococci received a plasmid harboring the *sul* genes from accompanying bacteria, without expressing them, or that the sequenced *sul* genes belonged to other bacteria present in the sample due to slight contamination. All three *sul* genes are usually present in Gram-negative Enterobacteriaceae [18, 137]. Since SULs act as a competitor for the substrate *p*-amino -benzoic -acid of the bacterial dihydropteroate synthase, two possible resistance mechanisms are the hyperproduction of *p*-

amino -benzoic -acid or own bacterial synthesis of *p*-amino -benzoic -acid [18, 138]. Mutations in the dihydropteroate synthase gene *folP* resulting in decreased affinity to SULs have been published as a third underlying mechanism of SUL resistance [139, 140] and specific primary (F17L, S18L and T51M) and secondary mutations (E208K and KE257_dup) have been identified [141]. All three ST1 isolates harbored the primary aa exchange F17L, therefore the classification as resistant via the self-made BMD plate seems to have resulted in the correct classification (**Publications I and II**). Interestingly, only the ST1 isolates, which carried SUL and TMP resistance determinants, were consistently classified as SXT-resistant by all methods applied, while the ST1660 isolates, harboring only a TMP resistance gene, showed varying results regarding SXT. This is in accordance with a study by Nurjadi et al., who concluded that SXT resistance could only be expressed when resistance to the single compounds SUL and TMP was present [132]. Using this definition and looking at the susceptibility results of the single compounds SUL and TMP, only the ST1 isolates would be considered as SXT-resistant, while the ST1660 isolates could only be classified as TMP-resistant.

However, this is not the only possible explanation for deviating results. Hetero-resistance is defined as the occurrence of subpopulations with different susceptibilities to an antimicrobial within one isolate [142]. Coelho et al. identified hetero-resistance to SXT, TMP and sulfamethoxazole in human *S. aureus* isolates via DD. Whenever there was slight growth within the zone diameter, the isolates would be classified as hetero-resistant [131]. Using this classification, all 19 isolates would be defined as hetero-resistant to SXT (**Publication II**). The SXT-susceptible *S. aureus* RN4220 also showed slight growth within the zone diameter, but it should be noted, that both measurable margins would classify the isolate as susceptible. This is in accordance with previous studies working on hetero-resistance, stating the different MICs of the isolates' subpopulations might all be within a susceptible spectrum [142, 143]. The phenomenon of hetero-resistance could explain the different results of the varying methods.

Resistance to the combination SXT in staphylococci seems to be a growing threat in human medicine, since SXT is recommended e.g. for skin infections caused by staphylococci [144, 145]. In veterinary medicine, SXT amongst other SUL and TMP combinations is also licensed for a broad spectrum of diseases in cats, dogs, horses, cattle and poultry [39, 40]. In both, veterinary and human medicine studies focusing on the development of antimicrobial resistance in staphylococci over time, an increase in resistance to SXT, SULs and TMP was detected [127, 128, 146], pointing towards the relevance of these antimicrobial agents in both disciplines. Five patients of our study were treated with the combination of the SUL sulfadiazine and TMP (**Unpublished data Table 1**), all of those were returned to their owners. Interestingly, the patient harboring a resistant ST1 had the longest treatment period (13 days), while the remaining four patients harbored ST1660 isolates and received only 3-5 days of treatment. Since all isolates harbored a TMP resistance gene, in the respective ST1660 isolates the SUL

component of the combination seemed to inhibit bacterial growth sufficiently for a successful treatment within a few days. The prolonged treatment period for the patient with the fully SXT resistant-ST1 isolate might point towards possible treatment failure. In this case, it is likely that the patient might have recovered due to its own immunocompetence [65].

The combination SXT is usually administered in a TMP:SUL ratio of 1:5, which is based on human studies. In humans, this ensures a ratio of 1:20 for the respective body site, if an intermediate-acting SUL is used, which is the most effective concentration for most bacterial species. However, this has not been proven for animals, since TMP is metabolized much faster in the animal body, than SULs, resulting in a period where the SUL is present alone, which could lead to subtherapeutic dosages [47, 49, 50]. The patients in our study all received the combination sulfadiazine/TMP. In horses, sulfadiazine has a half-life of 3–10 hours and trimethoprim of 3 hours [47, 50]. Therefore, the time period of subtherapeutic levels might be short, given a prudent dosing interval. However, the used product is administered twice a day, probably resulting in great differences in the active substances available at the respective body site [39, 40].

3.5 Difficulties in the diagnostic of antimicrobial susceptibility

AST is not only one of the most powerful tools for the choice of antimicrobial treatment of clinicians [147] but is also used to identify rare or new antimicrobial resistance properties via surveillance systems [148]. Nonetheless, there are a number of challenges, making the interpretation of test results and their comparability difficult. Especially in veterinary medicine one major challenge is the availability of specific clinical breakpoints, for the respective animal species and indications [62, 149]. There might be major differences in the breakpoints regarding the host species. For example, if human clinical breakpoints were applied for doxycycline (resistance breakpoint ≥ 16 mg/L), none of the 19 equine *S. aureus* isolates would have been classified as resistant, while horse-specific clinical breakpoints (resistance breakpoint ≥ 0.5 mg/L) result in the classification “resistant” for the ST1 isolates, that harbor the *tet*(L) gene (**Publication I**). Therefore, it is important to carry out studies to establish clinical breakpoints for antimicrobial agents used in the respective animal species. Only then a proper treatment of bacterial infections according to susceptibility testing can be expected. Since antimicrobial resistance properties of bacteria change and new resistance mechanisms might occur, clinical breakpoints need to be adjusted over time [150], which can lead to differing classifications of the same bacterial isolate [151]. Therefore, it is important to indicate the respective breakpoints used. These changes in clinical breakpoints will ultimately result in a different prescribing behavior of clinicians in human and veterinary medicine, which should be taken into account, when interpreting results from monitoring studies.

Next to these challenges in interpretation of the results, there are possible difficulties in the performance of the tests. Regarding automated test systems, the software for the interpretation of the results needs to be up to date and should be in agreement with susceptibility testing standards to allow a comparison of the results. Most methods, except for automated test systems, rely on the manual evaluation of the test result, e.g. the CLSI states that BMD and DD results shall be read with the unaided eye [42, 59]. The laboratory personnel must be well trained to accomplish intra- and interlaboratory conformity. It is also important that the standards are followed carefully, since even slight differences e.g. in the inoculum density, can distort the test outcome (inoculum effect) [149, 152]. Regarding BMD, difficulties might appear in some antimicrobial agent and bacterial species combinations, where trailing endpoints appear. Trailing endpoints appear as slight visible growth within the wells and in these cases a reduction of ≥ 80 % of growth is defined as the MIC. Trailing endpoints have been seen when testing staphylococci for their susceptibility to chloramphenicol, erythromycin, tetracycline or SXT [41, 42, 59]. A similar situation is seen for DD of these combinations, where slight growth of ≤ 20 % within the zone diameter shall be ignored [41, 42, 59]. Another difficulty in DD can be, that the zone diameters do not always appear exactly circular, making it difficult to read a standardized result. For all test methods, there are three kinds of errors that might occur: (i) major errors (majEs), where the test classifies as resistant, when the reference method reveals a susceptible result; (ii) very major errors (vmajEs), where the test detects susceptibility, but the reference method reveals a resistant result; and (iii) minor errors (minEs), where the test identifies an isolate as susceptible or resistant, when the reference method reveals an intermediate result or vice versa. Among these errors, vmajEs are the most problematic errors, since an antimicrobial might be predicted as effective when it is not, leading to treatment failure, while majEs merely result in the unnecessary limitation of treatment options [153].

3.5.1 Oxacillin resistance

There are several methods implemented to screen for oxacillin resistance, from selective chromogenic culture media [154], to molecular based methods [155, 156], to manual or automated susceptibility testing. Within the CLSI standards, there are several recommendations regarding the detection of oxacillin resistance in staphylococci, since oxacillin and cefoxitin are used as surrogates to identify methicillin resistance [41, 42, 59]. Since the last years, novel methods are being developed, that promise faster results without the necessity of bacterial cultures. These methods involve laser light scattering, fluorescence imaging, NALFIA-based assays, and micro-/nanotechnology [157-159]. Usually, oxacillin resistance is mediated via *mec* genes, which can be identified via PCR. In some cases, as

demonstrated in this thesis, the identification of the resistance (or reduced susceptibility) mechanism is not completed with just one additional test. Whole genome sequencing can help to identify resistance determinants. A correct classification of oxacillin resistance is of high importance, especially since according to AST standards, isolates classified as oxacillin-resistant must be classified as resistant to virtually all β-lactams [41, 42, 59]. Oxacillin and other β-lactams are frequently used in human and veterinary medicine [160-163] and the imprudent use of these antimicrobial agents might (co-)select for further antimicrobial resistances. Since transmission of bacterial isolates between animals and humans was reported, especially in equine veterinary hospital settings [6, 8], oxacillin resistance should be monitored, and respective countermeasures should be implemented.

3.5.2 Sulfamethoxazole/trimethoprim resistance

As shown in chapter 3.4, different methods might yield different result for SXT, even for the same bacterial isolate. Several studies identified differing results, dependent on the test method used [153, 164, 165]. In the case of SXT, trailing endpoints appear in BMD, while in DD slight growth of ≤ 20 % should be disregarded and the more distinctive margin should be measured as the zone diameter [41, 59]. This causes a subjective reading of results, which might lead to differing results. Another challenge in DD, are high thymidine or thymine levels in the test medium, that can impair the effects of SULs and TMP. To rule out excessive amounts of these substances, *Enterococcus faecalis* ATCC® 29212 is tested for its SXT susceptibility via DD. According to CLSI, zone diameters of ≥ 20 mm without slight growth within the zone diameter indicate the right amount of thymine and thymidine within the medium [41]. All batches of media were tested with *E. faecalis* ATCC® 29212 and were within the acceptable range. Therefore, this error source could be neglected as the reason for the differing results via DD for the ST1660 isolates (**Publication II**).

Only the ST1 isolates, harboring resistance determinants for both components of the combination, were consistently identified as resistant. The ST1660 isolates, harboring only a TMP resistance gene, were classified as resistant, susceptible, or intermediate, depending on the method used (**Publication II**). Interestingly, automated testing systems seemed to identify the isolates as resistant more easily, since only two isolates were identified as susceptible via VITEK® 2 and none via BD Phoenix™. A possible explanation for this is the fact, that automated test systems detect growth markers like turbidity and/or oxidation-reduction indicators repeatedly for a shorter period of time and therefrom derive the expected growth rate [67]. Since the combination of SXT leads to trailing endpoints in broth dilution methods, which are the underlying method in automated test systems, it is possible, that the system detects initial growth, that would not go beyond an overall growth rate of ≥ 20 % and would

usually be discarded as a trailing endpoint, and considers it as full growth, resulting in a classification as resistant (majE). However, previous studies comparing error rates of automated test systems, identified majEs and vmajEs in differing proportions, when testing staphylococci to SXT, SULs and TMP. Coombs et al. published a case, were testing of MSSA and MRSA isolates with VITEK® 2 and BD Phoenix™ showed a majE rate of 68 % and 58 % respectively [153]. However, these high numbers of errors might be biased by the collection of isolates investigated, which was mostly consisting of isolates of the same clone. A study from 2003 reported quite lower error rates when testing staphylococci for their TMP (2,8 % majEs) and SXT (2,6 % majEs and 6,8 % vmajEs) susceptibility, using BD Phoenix™ [164]. Carroll et al. reported even lower rates of 1.5 % majEs for the same system when testing *S. aureus* against SXT [166]. Huh et al. investigated VITEK® 2 results of staphylococci and enterococci against SXT and identified a vmajE error rate of 9.1 % [167]. The fact, that no classification of minEs occurred in any of these studies, is explained by the lack of an intermediate category for SXT and broth dilution methods.

Regarding BMD, there was no correlation between the two used commercial plates. The first set-up identified all 16 ST1660 isolates as susceptible, while the second set-up identified all isolates but one (IMT37083) as resistant. Interestingly, IMT37083 was the only isolate that was tested as susceptible to SXT by four out of five methods (**Publication II**) and is the first isolate in our collection from the clinic (**Publication I**). The horse from which IMT37083 originated, was treated with a SUL/TMP combination for three days and could be returned to the owner (**Unpublished Table 1**). Hetero-resistance could be an explanation for differing results, as was the case in the study of Coelho et al. [131]. Using the respective measures, all 19 equine isolates could be classified as hetero-resistant, since hetero-resistance does not necessarily mean one subpopulation is resistant while the other is intermediate or susceptible [142, 143]. Hetero-resistance could occur within a susceptible or within a resistant range. It is possible, that the different methods used, showed the results of respective subpopulations within the isolates.

Since SXT and other SUL/TMP combinations are frequently used in human and veterinary medicine [144, 145, 168], a correct classification is of major importance. The imprudent use of this antimicrobial combination could lead to (co-)selection of further antimicrobial resistance properties in bacterial isolates of animal and human origin. It seems obvious, that to this date, AST results for SXT of staphylococci need to be interpreted with caution. TMP resistance can be confirmed via PCR of the respective resistance genes, but since there are no specified resistance genes for SULs in staphylococci, the determination of resistance requires more effort, e.g. a whole genome sequencing approach, which is not always feasible for routine diagnostics. Therefore, further research on determination of SXT resistance and identification of SUL resistance determinants is needed.

3.6 Concluding remarks

Between the years 2015 and 2017, 19 *S. aureus* isolates from 17 patients of a veterinary clinic for horses were detected in routine diagnostics. They were noted for their elevated MICs for oxacillin via VITEK® 2 and BMD without the presence of a *mec* gene. The isolates belonged to two STs (ST1 and ST1660), which differed in their antimicrobial resistance patterns, but were all classified as multiresistant. Within the STs, the isolates seemed to be highly related, but there was no close relationship between ST1 and ST1660 isolates. Since both STs colonize and infect humans and animals, they harbor a great zoonotic potential [6, 7, 20, 74, 76]. A combination of susceptibility testing, genetic approaches and biochemical tests identified inducible hyperproduction of the β-lactamase BlaZ as the underlying mechanism for reduced susceptibility to oxacillin, a phenomenon also known as BORSA [44]. This thesis highlights the importance of the detection and correct classification of BORSA, since β-lactam antibiotics are frequently used in both, veterinary and human medicine, and offers an approach in how to accomplish that.

Another important and frequently used fixed combination of antimicrobial agent is SXT, consisting of the SUL sulfamethoxazole and TMP. This thesis revealed the challenges that occur when testing staphylococci for SXT susceptibility. Especially when isolates only harbor resistance determinants to one of the compounds or consist of hetero-resistant subpopulations, a correct classification is hardly achievable via AST, since the result highly depends on the test method used [153, 165]. This highlights the importance of complementing AST with molecular methods, like whole genome sequencing. Only the combination of both approaches helped to identify the resistance properties of the isolates. Nonetheless, further research is required to elucidate possible factors involved in SUL resistance in staphylococci, to facilitate and harmonize susceptibility testing to SULs and SXT.

Biocide susceptibility to BAC, CHX and GLU was investigated using a BMD and a broth macrodilution protocol. The two methods were carried out in two independent tests and yielded comparable and reproducible results. The MICs for CHX and GLU were far below the lowest used concentration of these agents. The highest MIC detected for BAC corresponded to the lowest used concentration of this agent. Since BAC is often combined with other biocides, and the MIC represents only the single compound, this does not necessary lead to resistance to low dosed BAC-based biocides.

4 Summary

Characterization of equine *Staphylococcus aureus* isolates with particular reference to their oxacillin and sulfamethoxazole/trimethoprim susceptibility

Staphylococcus aureus is a Gram-positive coccoid bacterium and a facultative pathogen, usually colonizing the skin and mucosal surfaces of healthy humans and animals. *S. aureus* infections range from mild skin diseases to surgical site infections, life threatening pneumonia or sepsis. *S. aureus* harbors a great zoonotic potential and humans can get infected via close contact to humans, animals or contaminated animal products or the consumption of contaminated food.

Subject of this thesis were 19 *S. aureus* isolates from 17 horses admitted to a veterinary clinic between 2015 and 2017. All but one isolate, which was sampled from an injury, originated from surgical site infections. These isolates were further investigated since they showed unusual results in antimicrobial susceptibility testing (AST). They were characterized via multi locus sequence typing (MLST), *spa* typing and phenotypic and genotypic resistance profiles against antimicrobial agents and biocides. Whole genome sequencing (WGS) allowed the in-depth investigation of resistance determinants and virulence factors.

The isolates belonged to two sequence types (STs), ST1 (n = 3) and ST1660 (n = 16), which are both common colonizers and pathogens of animals (especially horses) and humans (especially veterinary personnel). The respective *spa* types were t127 for the ST1 isolates, and t3043 (n = 14); t2484 (n = 1) and t549 (n = 1) for the ST1660 isolates. All isolates were classified as multiresistant, since all were resistant to penicillins (*blaZ*), aminoglycosides (*aacA-aphD*) and trimethoprim (*dfrG* or *dfrS1*). The ST1 isolates harbored an additional aminoglycoside resistance gene (*aadD*) and were also resistant to tetracyclines (*tet*(L)). Within the STs the isolates seemed to be closely related, but there was no relatedness between the ST1 isolates and the ST1660 isolates.

None of the isolates harbored the toxic shock syndrome toxin 1 gene *tst* or the Panton-Valentin leukocidin (PVL) genes *lukF*-PV and *lukS*-PV. All isolates were positive for staphylococcal enterotoxins or enterotoxin like proteins associated with the enterotoxin gene cluster *egc* and the leukocidin LukD/E. Saeq1, a bacteriophage harboring the leukocidin LukP/Q and the equine staphylococcal complement inhibitor eqScin, was present in all isolates.

Biocide susceptibility testing (BST) via broth macrodilution and broth microdilution (BMD) showed that both methods generated comparable and reproducible results and are suitable for future investigations of biocide susceptibility. The minimal inhibitory concentrations

(MICs) for benzalkonium chloride (BAC), chlorhexidine and glutardialdehyde did not differ vastly from those of the reference strains and were below the used concentrations of the respective agents, except for a few isolates for BAC. Since BAC is rarely used as a single agent and the MIC was merely at the lowest used concentration of the agent, this does not necessarily result in BAC resistance.

The isolates presented reduced susceptibility to oxacillin, while lacking the *mec* genes, commonly responsible for oxacillin resistance, thus being classified as borderline oxacillin-resistant *S. aureus* (BORSA). Using AST, WGS and a nitrocefin test, inducible hyperproduction of the β-lactamase BlaZ was identified as the reason for the reduced oxacillin susceptibility. Interestingly, all but one of the ST1660 isolates produced remarkably higher amounts of BlaZ than the ST1 isolates and the ST1660 isolate IMT37083. IMT37083 and the ST1 isolates shared 19 amino acid (aa) exchanges in the β-lactam sensor protein BlaR1, which could explain the lower inducibility of these isolates.

When using different test methods and set-ups to assess susceptibility to the combination sulfamethoxazole/trimethoprim (SXT), and the single compounds – a sulfonamide (SUL, here: sulfisoxazole) and trimethoprim (TMP) – varying results occurred. Again, a genetic approach was chosen to elucidate the true resistance properties. The ST1 isolates were the only ones consistently classified as resistant to SXT. Resistance to SULs (BMD) and TMP (BMD and agar disk diffusion (DD)) was also detected. These isolates harbored the TMP resistance gene *dfrG* and a mutation within their dihydropteroate synthase gene *folP*, resulting in the aa exchange F17L, which confers SUL resistance. Therefore, these isolates are truly resistant to SXT, TMP and SULs. The ST1660 isolates harbored the TMP resistance gene *dfrS1* and were consistently classified as TMP-resistant. BMD and DD identified these isolates as SUL-susceptible. Regarding SXT, the isolates were inconsistently classified as resistant, susceptible, and intermediate. There was no relevant aa alteration in their FolP proteins. This highlights the necessity on further research in this field, since the mechanisms and tangible SUL resistance determinants are barely unveiled.

This thesis also confirmed the need for further development and establishment of diagnostic procedures to identify unusual antimicrobial resistance properties and their mechanisms. AST protocols used in veterinary medicine also lack animal specific clinical breakpoints which impedes the transferability of AST results to expectable clinical outcomes and, by extension antimicrobial stewardship.

5 Zusammenfassung

Charakterisierung von *Staphylococcus aureus*-Isolaten von Pferden unter besonderer Berücksichtigung ihrer Oxacillin- und Sulfamethoxazol/Trimethoprim-Empfindlichkeit

Staphylococcus aureus gehört zu den Gram-positiven Kokken und besiedelt als fakultativ pathogener Keim Haut und Schleimhäute gesunder Menschen und Tiere. Mögliche Infektionen durch *S. aureus* reichen von milden Hauterkrankungen, über postoperative Wundinfektionen, bis hin zu lebensgefährlichen Pneumonien oder Sepsis. *S. aureus* gehört zu den Zoonoseerregern, wobei sich Menschen durch den engen Kontakt zu anderen Menschen, Tieren oder kontaminierten tierischen Produkten oder Lebensmitteln infizieren können.

In dieser Thesis wurden 19 *S. aureus* Isolate von 17 Pferden, die in den Jahren 2015 – 2017 in eine Pferdeklinik eingewiesen wurden, untersucht. Neben einem Isolat, welches von einer Verletzung auf der Weide stammte, wurden alle Isolate aus Proben postoperativer Wundinfektionen gewonnen. Die Isolate wurden untersucht, da sie in der Empfindlichkeitsprüfung in der Routinediagnostik untypische Resistenzmuster aufzeigten. Mithilfe der Bestimmung von Multi-Locus-Sequenz-Typen (MLST), *spa*-Typen, sowie phänotypischer und genotypischer Resistenz gegenüber antimikrobiellen Wirkstoffen und Bioziden, wurden die Isolate charakterisiert. Die Gesamtgenomsequenzierung ermöglichte eine detaillierte Untersuchung von Resistenz- und Virulenzfaktoren.

Die Isolate gehörten den beiden Sequenztypen (STs) ST1 (n = 3) und ST1660 (n =16) an. Beide STs wurden bereits als kolonisierende und pathogene Keime bei Tieren, vor allem Pferden, aber auch beim Menschen beschrieben. Die ST1-Isolate hatten alle den *spa*-Typ t127, während die ST1660-Isolate den *spa*-Typen t3043 (n = 14), t2484 (n = 1) und t549 (n = 1) angehörten. Da alle Isolate gegenüber Penicillinen (*blaZ*), Aminoglykosiden *(aacA-aphD*) und Trimethoprim (*dfrG* oder *drfS1*) resistent waren, wurden alle als multiresistent beurteilt. Die ST1-Isolate beherbergten zusätzlich ein zweites Aminoglykosid-Resistenzgen (*aadD*) und das *tet*(L)-Gen, welches Resistenz gegenüber Tetrazyklinen vermittelt. Innerhalb der STs zeigten die Isolate eine hohe genetische Übereinstimmung, zwischen den STs lag jedoch keine Verwandtschaft vor.

Keines der Isolate besaß das toxic-shock-syndrome-toxin-1-Gen *tst* oder die Panton-Valentin-Leukozidin (PVL)-Gene *lukF-PV* und *lukS-PV*. Alle Isolate beherbergten das Leukozidin LukD/E und Enterotoxine oder enterotoxin-ähnliche Proteine, die zum *egc* Enterotoxin-Gen-Cluster gehören. In allen Isolaten gab es den Bakteriophagen Saeq 1, welcher das Leukocidin P/Q und den pferdespezifischen Komplementinhibitor *eq*Scin beinhaltete.

Die Biozidempfindlichkeitstestung mittels Bouillon Makro- und Mikrodilution (BMD) ergab eine gute Vergleichbarkeit und Reproduzierbarkeit der Ergebnisse zwischen den Methoden. Die minimalen Hemmkonzentrationen (MHKs) der klinischen Isolate unterschieden sich für keines der getesteten Biozide – Benzalkoniumchlorid (BAC), Chlorhexidin (CHX) und Glutardialdehyd (GLU) – von denen der Referenzstämme und waren zumeist unterhalb der jeweils üblichen Gebrauchskonzentrationen. Lediglich für BAC waren die MHKs weniger Isolate im Bereich niedriger Gebrauchskonzentrationen. Da BAC jedoch meist in Kombination mit weiteren Bioziden verwendet wird, ist nicht unbedingt von einer klinischen Resistenz der Isolate auszugehen.

Die Isolate zeigten eine verminderte Oxacillin-Empfindlichkeit, ohne jedoch eines der zu erwartenden *mec*-Gene aufzuweisen. Dementsprechend wurden die Isolate als „Borderline Oxacillin-resistente *S. aureus*“ (BORSA) eingestuft. Durch Empfindlichkeitstestung, Gesamtgenomsequenzierung und einen Nitrocefin-basierten Test konnte eine induzierbare Hyperproduktion der β-Laktamase BlaZ als ursächlich für die verminderte Oxacillin-Empfindlichkeit identifiziert werden. Hierbei zeigten die ST1660-Isolate, bis auf das Isolat IMT37083, eine höhere Produktion von BlaZ, als die ST1-Isolate. Das besagte Isolat IMT37083 teilte einige Aminosäuren (AS)-Austausche im β-Laktam-Sensorprotein BlaR1 mit den ST1-Isolaten, was eine mögliche Erklärung für die niedrigeren Produktionslevel liefern könnte.

Die Testung der Empfindlichkeit gegenüber der Kombination Sulfamethoxazol/Trimethoprim (SXT), sowie ihrer Einzelkomponenten, einem repräsentativen Sulfonamid (SUL, hier Sulfisoxazol) und Trimethoprim (TMP), ergab bei der Verwendung unterschiedlicher Methoden abweichende Ergebnisse. Lediglich die ST1-Isolate wurden von allen Methoden konsequent als resistent gegenüber SXT eingestuft. Auch Resistenz gegenüber SUL (BMD) und TMP (BMD und Agar Disk Diffusion (DD)) wurde detektiert. Eine Untersuchung der Gesamtgenomsequenzen ergab, dass diese Isolate sowohl das TMP-Resistenzgen *dfrG* als auch eine Mutation in ihrem Dihydropteroatsynthase-Gen *folP* aufwiesen, welche zum AS-Austausch F17L führte. Dieser AS-Austausch vermittelt SUL-Resistenz bei Staphylokokken. Somit können die ST1-Isolate sicher als resistent gegenüber allen Komponenten und der Kombination beurteilt werden. Die ST1660-Isolate beinhalteten das TMP-Resistenzgen *dfrS1* und wurden von allen Methoden als TMP-resistent beurteilt. Bezüglich der SUL-Komponente jedoch, wiesen die Isolate keinen bekannten Resistenzmechanismus auf und wurden von BMD und DD als sensibel eingestuft. Bezüglich der Kombination SXT ergaben die verschiedenen Methoden alle Klassifizierungen von sensibel über intermediär (DD) bis resistent. Da beide Einzelkomponenten bakteriostatisch wirken und nur gemeinsam einen bakteriziden Effekt haben, ist die wahre Resistenzbeurteilung hier erschwert und weitere Studien bezüglich der korrekten

Identifizierung von Empfindlichkeiten und Resistenzen gegenüber dieser Wirkstoffkombination notwendig. Dies gilt vor allem für Staphylokokken, für die bisher kein SUL-Resistenzgen identifiziert werden konnte.

Diese Thesis erörtert die Bedeutung und Notwendigkeit der Entwicklung und Etablierung von Methoden zur korrekten Klassifizierung von ungewöhnlichen Resistenzphänomenen. Darüber hinaus fehlen tierartspezifische klinische Grenzwerte für Bakterienisolate in der Veterinärmedizin, was eine wissenschaftlich gestützte Entscheidung bezüglich einer vernünftigen Antibiotikatherapie erschwert. Auch hier sind weitere Untersuchungen und neue klinische Grenzwerte notwendig.

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Scholtzek AD, Klein KS, Stöckle SD, Eichhorn I, Walther B, Feßler AT, Hanke D, Gehlen H, Lübke-Becker A, Schwarz S: Characterization of *Staphylococcus aureus* isolates with a reduced oxacillin susceptibility causing infections in equine patients of a veterinary clinic. Proceedings of the Conference of the Deutsche Veterinärmedizinische Gesellschaft (DVG), division Bacteriology and Mycology, Hannover, Germany (2018)

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Proceedings of the 8th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Tours, France (2019)

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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 06.10.2020 Anissa D. Scholtzek

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