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Characterization of Antibiotic and Biocide Resistance Genes and Virulence Factors of *Staphylococcus* Species Isolated from California Mastitis Test-Positive Milk from Cows in Northern and Kigali Province, Rwanda

Diplomarbeit

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vorgelegt von

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1. Introduction and hypotheses (F. I. Antók and R. Mayrhofer)

The aim of this study was to characterize a collection of bovine staphylococci associated with clinical and subclinical mastitis. The bacteria were isolated from CMT positive milk samples taken from 112 crossbred milking cows during farm visits in the Northern and Kigali Province of Rwanda.

Staphylococcal species were identified to the species level by matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany) or *rpoB* gene sequencing if MALDI-TOF results were ambiguous. Antibiotic susceptibility testing was performed to detect both phenotypic and genotypic resistance patterns. Resistance to biocides and metals was investigated by genotypic method only. Isolates of *Staphylococcus* (*S.*) *aureus* were examined for the presence of virulence-associated genes, genotyped by *spa* typing and phenotypically characterized using Fourier Transform Infrared (FTIR) spectroscopy. Based on FTIR results, 22 *S. aureus* isolates were selected and further characterized employing DNA microarray, multi-locus sequence typing (MLST) and whole genome sequencing. *mecA* positive staphylococci were also genotyped using *dru* typing.

In Rwanda, the most frequently used antibiotics for the treatment of every kind of illness are penicillin/ampicillin, tetracycline and gentamicin [1,2]. Moreover, biocides are widely used. In consequence, previous studies reported a high prevalence of resistance to these antimicrobials, particularly among coagulase-negative *Staphylococcus* spp. (CoNS) [3-5].

The hypotheses of the study are:

a) *S. aureus* is the predominant *Staphylococcus* species and the majority of isolates are resistant to penicillinase-labile penicillin and tetracycline
b) biocide resistance genes are more common in *S. aureus* than in CoNS

1.1. Description of the study area (F. I. Antók and R. Mayrhofer)

The first part of the study, the collection and preparation of the samples was conducted in the Northern and Kigali Province in Rwanda. Rwanda is a relatively small country in East Africa, bordered by Tanzania, Uganda, Democratic Republic of Congo, and Burundi. It covers an

area of 26,338 square kilometres and the entire country's elevation span range between 1000 m and 4500 m above sea level. Geographically, it is dominated by hills and mountains in the western and northern part and by savanna in the eastern region, with numerous lakes throughout the country. The climate is temperate to semitropical, with two rainy and two dry seasons each year. Rwanda is divided into five Provinces: Eastern (Districts: Nyagatare, Rwamagana, Gatsivo, Kayonza etc.), Northern (Districts: Musanze, Gicumbi, Burera etc.), Western (Districts: Nyabihu, Rubavu and Rutsiro etc.), Southern (Districts: Nyanza, Huye, Ruhango etc.) and Kigali (Districts: Gasabo, Kicukiro, Nyarugene). The Districts are again divided into Sectors (total 416 Sectors) [6].

Rwanda's economy suffered heavily during the genocide in 1994, but has strengthened since then [6]. The demand for livestock products is rapidly increasing, which is probably driven by a population growth from three million to twelve million in the last 60 years [7,8]. In 2000, the government of Rwanda started a development program, called Rwanda Vision 2020. The main goals were to transform the country into a knowledge-based middle-income country and to modernize its agriculture and livestock production [7]. The government now funds for public veterinary services provided by district and sector veterinary officers, however, with limited capacity to support dairy farmers [1].

The cattle population in Rwanda is dominated by the indigenous long-horned Ankole cattle and crossbred Holstein Friesian. The Holstein Friesian breed has been imported to improve dairy productivity. While Ankole cattle are highly adapted to local environmental conditions, Holstein Friesian are more susceptible to develop mastitis [7,9].

Three recent studies conducted in Rwanda showed a high prevalence of subclinical mastitis in the Nyagatare District (52 %), the Musanze District (62 %) and in peri-urban areas of Kigali (76,2 %) [9-11]. Coliform bacteria, CoNS and *S. aureus* were reported to be the most prevalent pathogens [9,11].

In a study by Iraguha et al. several risk factors associated with mastitis in Rwanda have been identified including cow dirtiness, production system, breed, teat-end conditions and lactation stage [9]. Hand milking is common in Rwanda and mastitis may be transmitted through contaminated hands, clothing, and other materials [9].

For farmers in Rwanda veterinary drugs such as antibiotics are supplied by local pharmacies [1]. In a cross-sectional survey, a high usage of antibiotics in farm animals (97,4 %) and the

use of non-prescribed antibiotics by more than half of the farmers (55,6 %) were observed [1]. The same study revealed that penicillin-streptomycin and tetracycline are the most commonly used antibiotics in farm animals. Although policies and laws regulating the use of antibiotics in humans and animals exist in Rwanda, antibiotics are often purchased without any prescription [1]. Consequently, it is of high importance to raise the awareness of appropriate use of antibiotics, especially in farm animals.

To improve knowledge in animal welfare and to provide clinical and laboratory veterinary services, the first private animal clinic 'New Vision Veterinary Hospital (NVVH)' has been established in Musanze District in 2015. Since the beginning, NVVH strives to provide education to local vets, students and farmers by collaborating with local and foreign universities and organizations. There is also a close cooperation between the University of Veterinary Medicine in Vienna and the University of Rwanda.

1.2. *Staphylococcus* spp. (F. I. Antók and R. Mayrhofer)

Staphylococci are gram-positive, non-motile, cocci-shaped bacteria, which often form grape-like clusters. They are facultative anaerobes and their cell wall contains peptidoglycan and teichoic acids [12]. Most of the staphylococcal species are commensals or opportunistic pathogens that colonize the skin and mucous membranes [13]. In humans and animals, staphylococci may be cause of several diseases such as pneumonia, endocarditis and mastitis [14].

The genus *Staphylococcus* currently consists of 53 species (http://www.bacterio.net/staphylococcus.html) and is divided into two groups based on the ability to form coagulase, an enzyme that promotes clotting of blood. The coagulase-positive staphylococci (CoPS) group includes the main species *S. aureus*, which is considered to be a major contagious pathogen, while the CoNS group is highly heterogeneous and its members have been determined as minor pathogens [12,15].

1.2.1. Coagulase-negative *Staphylococcus* spp. and their role in bovine mastitis

CoNS may cause bovine mastitis and have traditionally been determined as minor pathogens, especially in comparison with major pathogens such as *S. aureus,* streptococci and coliforms [15-17]. They are usually associated with a moderate intramammary infection, which often

remains subclinical [18,19]. In some cases, they may also cause persistent intramammary infections [18]. Subclinical mastitis caused by CoNS is associated with a decrease in milk production and an increase in somatic cell count (SCC) in milk [17,20]. CoNS are part of the normal skin flora and may enter the teat canal and penetrate the secretory tissues, which may lead to infection [17]. Knowledge on the identity of the species present in a herd or farm is important, since different species exhibit different pathogenic properties and also antimicrobial resistance patterns may vary among species [15,17].

CoNS species such as *S. epidermidis, S. chromogenes, S. simulans* and *S. haemolyticus* have been isolated from the skin, the teat canal or vagina [15,21]. They have shown to be able to produce biofilms [16,17,22], which may contribute to antimicrobial resistance, immune evasion and the severity of intramammary infections [16,22]. Within CoNS, the five members of the *S. sciuri* group (*S. sciuri, S. lentus, S. vitulinus, S. fleurettii* and *S. stepanovicii*) have been isolated from the skin of humans and animals and from the environment [12,14,23]. One characteristic of this group is the presence of species-specific *mecA* homologues that do not confer resistance to methicillin, although highly similar to *mecA* carried by methicillin-resistant *S. aureus* (MRSA) [12].

Another CoNS species, *S. xylosus*, has been isolated from the environment but also from milk and extramammary tissues [15,23]. The ability to produce biofilm has also been proven for this pathogen [16]. Overall, *S. chromogenes, S. haemolyticus, S. epidermidis, S. simulans and S. xylosus* are among CoNS the most frequently isolated species from bovine mastitis cases [24,25].

Studies conducted in East Africa have revealed that CoNS are also commonly associated with bovine mastitis in this area, but isolates have not been further characterized [26-29].

1.2.2. *Staphylococcus aureus* and its role in bovine mastitis

S. aureus, a CoPS species, is considered to be a major contagious pathogen that can cause clinical mastitis or recurrent subclinical mastitis, even in well-managed dairy herds. Symptoms of clinical mastitis include visible changes of the milk (e.g. changes in color and consistency), inflammation of the udder (e.g. swelling, heat, pain) and general signs such as fever and lethargy, whereas subclinical mastitis is asymptomatic [15,30]. *S. aureus* is also part of the normal bacterial flora of the cow and the primary mode of transmission is cow-to-cow

[15]. Also humans can be a vector and therefore transmit bacteria, for example by hand milking.

S. aureus produces coagulase, an enzyme which converts serum fibrinogen to fibrin and stimulates clotting [13]. Differentiation from other coagulase-positive species (*S. intermedius, S. hyicus*) may be achieved by growth on P agar supplemented with acriflavin or the β-galactosidase test [31].

Several potential virulence factors including extracellular toxins (α, β, γ and δ hemolysins, enterotoxins), enzymes (staphylokinase, lipase, esterase, protease, nuclease), cell-wall associated proteins (protein A, collagen-binding protein, fibronectin-binding protein, elastin-binding protein), capsular polysaccharides and slime have been described in *S. aureus* [15,32]. *S. aureus* can colonize mammary epithelial cells and extracellular matrix components and may invade mammary epithelial cells, where it is found enclosed in membrane-bound vacuoles in the cytoplasm. Recurrent subclinical infection may be the result of intracellular localization, where bacteria are protected from host defenses and effects of antibiotics [15].

DNA sequence-based approaches such as MLST have been widely used to characterize *S. aureus* isolates and to gain knowledge on the population structure of this staphylococcal species [33,34]. MLST allows assignment of *S. aureus* isolates to different clonal complex (CC) groups [35]. Livestock-associated strains have often been assigned to non-human clonal complexes like CC97, CC398, CC9 and CC151 [36,37]. However, CC97 which is known to be common in ruminants [38-40], has also been isolated from humans and from other animals (e.g. pigs) [40,41]. Strains of human origin mainly belong to the six clonal complexes CC1, CC5, CC8, CC22, CC30, and CC45 [35,42]. The assignment of strains isolated from companion animals (horses, dogs and cats) to human-associated clonal complexes CC1, CC8, CC22 and CC45 indicates transmission between owners and animals due to close contact [36].

Several previous reports have shown that animals may constitute a reservoir for staphylococci that may be transmitted to humans and vice versa [13,43,44]. Transmission of bacteria between host species is typically accompanied by subsequent adaptation through acquisition or loss of mobile genetic elements including phages, pathogenicity islands and plasmids. Also host-specific mutations may allow bacteria to cross host species barriers [13]. In Rwanda,

hand milking is common practice and hand-to-cow contact, clothing, and other materials may be risk factors for the transmission of bacteria [9].
Two studies conducted in East Africa (Tanzania and Kenya) revealed that *S. aureus* was the predominant *Staphylococcus* spp. isolated from bovine mastitis [28,45].

1.3. Antimicrobial resistance (F. I. Antók)

Bacteria can develop antimicrobial resistance, which means that these bacteria are non-susceptible to certain antimicrobial agents. The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the visible growth of an organism *in vitro* [46] and it determines the level of non-susceptibility. There are two types of resistance: i) Intrinsic resistance is based on species- or genus-specific attributes, e.g. the penicillin resistance of *Bordetella bronchiseptica*, which is mediated by the species-specific β-lactamase BOR-1, or the resistance of cell wall-less mycoplasmas to antimicrobials that target cell wall synthesis. ii) Acquired resistance is strain-specific and based on resistance-mediating mutations in genes or horizontal transfer of resistance genes [47].
Currently, antimicrobial therapy is still the main strategy for curing mastitis [48]. It may effectively treat intramammary infections if the causative agent is correctly identified and the appropriate antimicrobial substance is used [17]. Antimicrobial resistance is a serious problem in the treatment of mastitis and is defined as the ability of microorganisms to resist the effects of antibiotics [49]. The incorrect use of antibiotics in both human and veterinary medicine has shown to contribute significantly to the emergence of resistant strains and therapy resistance of infectious diseases. Moreover, based on the incorrect use of antibiotics in farm animals, resistant strains of animal origin may enter the food chain resulting in potential illnesses in humans [2,48,50].
Antimicrobials like penicillin and tetracycline are important antibiotics for the treatment of diseases in dairy herds, but CoNS have frequently shown to be resistant against these antibiotics [51,52]. Besides CoNS, *S. aureus* is able to acquire resistance against nearly all antimicrobial agents [53,54].

The agar disk diffusion method detects the phenotypic antibiotic resistance of bacteria. First, a bacterial suspension in 0,9 % NaCl solution at a density equivalent to 0,5 McFarland turbidity

standard (1-2 x 10^8 CFU/ml) has to be prepared. Then, small paper disks loaded with antimicrobial agents are applied on the surface of a Mueller-Hinton agar, which has been inoculated with the test organism suspension before. After incubation, the plates are controlled for bacterial growth and the zone of growth inhibition are measured. The isolates are then classified according to the criteria provided by the Clinical and Laboratory Standards Institute (CLSI, 2018).

For the agar dilution method the antimicrobial agent is included in the agar in serially diluted concentrations. Results are visible after overnight incubation and the lowest visible growth-inhibiting concentration is the MIC [46,47]. Both macro- and microdilution are two more methods to determine MIC values. For macro- and microdilution, liquid media are used instead of agar media. While macrodilution works with larger broth volumes and test tubes, microdilution is usually performed in microtiter plates. Serially diluted antimicrobials are added to liquid media in wells or tubes to obtain a gradient and inoculated by an appropriately concentrated bacterial cell suspension. After incubation MIC values are determined by turbidity [47].

A coding gene, a mutation or a combination of these two can be responsible for phenotypic resistance. Responsible genes encode for major resistance mechanisms, such as enzymatic inactivation, active efflux or protection/modification/replacement of cellular target sites of antimicrobial agents [52]. In staphylococci for example, the *blaZ* or bla_{ARL} genes encode for a penicillinase, which inactivates penicillin. The *mecA* gene, but also the *mecB* and *mecC* genes, code for an alternative penicillin-binding protein (PBP2a) with a reduced binding to β-lactam antibiotics [52]. Tetracycline resistance is commonly mediated by the genes *tet*(K), *tet*(L), *tet*(M) and *tet*(O). *tet*(K) and *tet*(L) code for membrane-associated efflux proteins, and *tet*(M) and *tet*(O) for ribosome-protective proteins [52]. In phenicols, the resistance to chloramphenicol is mediated by several genes: the *fexA* gene which is responsible for active efflux, the cat_{pC194}, cat_{pC221}, and cat_{pC223} genes encoding for enzymatic inactivation, and the *cfr* gene that induces target side modifications. The *ant(6')-Ia* and *str* genes are coding for the enzymatic inactivation of aminoglycosides. In antimicrobial resistance to macrolides, lincosamides and streptogramins several genes are involved: *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(T), *erm*(33), *erm*(43), *erm*(44) and *cfr* genes that are responsible for target side modifications, the *Isa*(B), *msr*(A), *vga*(A), *vga*(A)*32*, *vga*(C), *vga*(E), *vga*(E)*v* and *sal*(A)

genes responsible for target side protection and the *Inu*(A) gene mediating enzymatic inactivation. The *dfrA, dfrD, dfrG*, and *dfrK* genes confer resistance to trimethoprim by target site replacement [52].

1.4. Biocide and metal resistance genes (R. Mayrhofer)

Biocides (antiseptics, disinfectants) are commonly used for the control of microorganisms that are harmful to both human and animal health [3]. Some of them, including hypochlorites and (solubilized) phenols have already been introduced in the 18th and 19th century [55]. Similar to antimicrobial resistance, the frequent usage of biocides may induce resistance and insusceptibility against these chemicals [3]. Cross-resistance between biocides and antibiotics and underlying mechanisms have been described previously [56].

Quaternary ammonium compounds (QACs) are used as disinfectants and surfactants introduced in 1917 [55]. They are commonly applied in veterinary medicine and play an important role in the prevention and control of animal diseases including mastitis [57]. Bjorland et al. reported that resistance of *S. aureus* to QACs exists also in veterinary sectors [58,59]. Resistance to QACs is mediated by multidrug efflux pumps in the cell membrane, encoded by *qac* genes. These proteins transport molecules of antimicrobials out of the organism and thus increase tolerance to them. Six different QAC efflux pumps have been described in staphylococci: QacA, QacB, QacC, QacG, QacH, and QacJ. QacA and QacB belong to the Major Facilitator Superfamily (MFS), the remaining four to the Small Multidrug Resistance (SMR) family [59]. A study conducted in three African countries reported the presence of the *qacAB* gene and *smr* gene in *S. aureus* isolates [60].

Supplementary feeding with heavy metals is a widely used strategy for the prevention of gastrointestinal illnesses in farm animals [61]. Metals can also have antimicrobial effects and bacteria can develop resistance against them after long-term usage [62]. Resistance to cadmium, arsenic, zinc and copper has been detected in methicillin-resistant (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains [61-63]. The operon *czr* is responsible for the resistance to zinc consisting of the two *czr* genes, *czrA* and *czrB*. An efflux pump mediated by the *czrB* gene exports zinc out of the cell [64,65]. The *czrC* gene has been investigated in MRSA isolates revealing that this gene also encodes for cadmium resistance, especially in CC398 strains [66,67]. The *copB* gene, along with *copA*, play a role in the

copper transport and resistance system. Encoded P-type ATPases are responsible for copper uptake and efflux [68,69]. The genes *cadA, cadB, cadD* and *cadX* confer resistance to cadmium and are also mediated by efflux mechanisms [64,70]. The *ars* resistance operon contains the ATPase efflux gene *arsA*. Other genes like *arsB, arsC, arsD* and *arsR* are also part of this operon and play their role in resistance [62,68,71].

1.5. Virulence factors (R. Mayrhofer)

Virulence factors assist staphylococci to colonize and survive in the host and cause damage to host cells. Adhesion of staphylococci to mammary epithelial cells or tissues by surface components is the first step in the infection process, preventing elimination of bacteria during milking [72,73]. Next, staphylococci may survive in the host and may escape from host immune responses by forming biofilms or by the production of enzymes such as hyaluronidases, proteases, nucleases and non-enzymatic activators like coagulase and staphylokinase [54,73]. Furthermore, many staphylococcal species, CoNS and CoPS, can produce different exotoxins, which can cause damage to host cells and symptoms of disease [72,74-76].

1.5.1. Virulence factors of *Staphylococcus aureus*

S. aureus produces a notable number of different virulence factors that facilitate infection [32,74]. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) promote adhesion to components of the host's extracellular matrix [77]. These components also mediate biofilm production, which may protect the bacteria from host immune responses and antimicrobial agents [78]. Furthermore, clumping factors play an important role in attachment and colonization of *S. aureus* (clumping factor A and B, fibrinogen-binding protein, fibronectin-binding protein A and B) [54,77].

S. aureus strains may also produce one or more exotoxins including the toxic shock syndrome toxin-1 (TSST1), the staphylococcal enterotoxins (SEs) and panton-valentine leukocidin (PVL) [32,76,79,80]. SEs and TSST1 belong to a group of pyrogenic toxin superantigens (PTSAgs) that exhibit similar biological characteristics and are produced by both S*treptococcus* and S*taphylococcus* species [32]. Acting as superantigens, they promote non-specific T-cell proliferation and a cytokine release resulting in severe inflammation and toxic

shock-like syndromes [74]. The genes for these toxins are located on plasmids, bacteriophages or pathogenicity islands. As carried by mobile genetic elements they can spread among *S. aureus* strains, potentially introducing or altering their pathogenicity [32,75]. Twenty-three SEs and SE-like toxins have been identified so far in *S. aureus* (SEA-SEE, SEG-SEI, SElJ, SEK-SET, SElU, SElV, SElX, SElY) [32,81-83]. SEs are resistant to inactivation by heat and gastrointestinal proteases such as pepsin. Heat-stability may depend on the medium, pH, salt concentration and other environmental factors [84].

Certain *S. aureus* strains carry the toxic shock syndrome toxin-1 gene (*tsst-1*). Previous studies have described a bovine variant of *tsst-1* in *S. aureus* strains associated with bovine mastitis [80,85,86].

Among *S. aureus* exotoxins different leukocidin genes have been reported in strains of bovine origin such as *lukF-PV/lukS-PV* and especially *lukM/lukF-PV(P83)* [80,87]. PVL is a leukotoxin associated with invasive soft tissue- and skin-infection in humans. Consequently, its presence in bovine strains indicates human-to-cow transmission of *S. aureus* [79,80]. PVL is a potent cytotoxic factor for human neutrophils [88]. In contrast, *lukM/lukF-PV(P83)* only kills bovine neutrophils but not human neutrophils, and is common in *S. aureus* isolated from bovine mastitis [80].

The variability in the production and expression of virulence factors in *S. aureus* strains significantly contributes to the pathogen's ability to cause intramammary infection in dairy cows and the transmission of *S. aureus* to other animals including humans [74].

In CoNS virulence factors such as enterotoxins, toxins responsible for toxic shock syndrome, and factors mediating biofilm formation are not as frequently present than in *S. aureus* strains [22,72,89]. In this respect, Simojoki et al. reported that biofilm formation in CoNS isolated from mastitis cases does not play an important role in the persistence of infection in the bovine udder [22].

1.6. Typing methods (F. I. Antók)

In the present study, different geno- and phenotyping methods have been performed in order to characterize *S. aureus* isolates recovered from bovine mastitis in Rwanda.

Advantages and disadvantages of certain PCR-based typing methods used for the characterization of *S. aureus* including amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), repetitive element sequence-based PCR (rep-PCR), and accessory gene regulator (*agr*) typing have been described previously [35]. AFLP was shown to be labor-intensive, time-consuming and expensive. RAPD and AP-PCR were easy to perform and inexpensive but exhibited low reproducibility. Rep-PCR was shown to be a simple method and expressed better performance in reproducibility. The *agr* typing method was shown to be an excellent typing method combining speed, accuracy and low running costs [35].

Pulsed-field gel electrophoresis (PFGE) was introduced in 1984 by Schwartz and Cantor [90]. With this method, digested bacterial DNA fragments are separated in a specific electric field, in which the DNA movement direction changes periodically. Only this pulsed orientation allows the fragments to be separated effectively by their size [35]. PFGE is a popular typing technique for *S. aureus* because of its discriminatory power, however it is time-consuming and special equipment and expertise is required [35,91].

1.6.1. Phenotyping methods

FTIR spectroscopy was re-introduced by Naumann and his colleagues in 1991 [92]. By this method differentiation, classification, identification and large-scale screening at the subspecies level can be achieved. The overall chemical composition of a sample is measured and specific whole-cell fingerprints are created. The fingerprint reflects the balance of compositional, conformational and quantitative differences of biochemical compounds in the cells and is analyzed by pattern recognition algorithms [93-95]. Beside identification at the subspecies level, FTIR spectroscopy also allows identification of capsular serotypes in *S. aureus* [93].

1.6.2. Genotyping methods

The *spa* typing method has become popular after it was first described in 1996 as a more useful method for the characterization of *S. aureus* than phage typing [96]. This rapid, easy to perform and cost-effective method is based on polymorphisms in the X-region of the protein A gene (*spa*) of *S. aureus*, which contains a varying number of tandem repeats. Although it is

a single-locus sequence typing (SLST) method, *spa* typing combines sequence typing with variable number of tandem repeat (VNTR) analysis enabling accurate discrimination of *S. aureus* isolates [35,97]. Each identified repeat is designated with letters and numbers and the order of the repeats determines the resulting numeric *spa* types. In a central *spa* database (https://www.spaserver.ridom.de/, http://www.seqnet.org/) data are collected from laboratories and software, which allows semi-automated sequence analysis and type assignment [35].

MLST is a typing method that analyzes sequences of 7-8 housekeeping genes, which are indispensable for the function of the cells in living organisms and are not exposed to direct evolutionary pressure. Enright et al. have developed a specific MLST protocol for *S. aureus* in 2000 [98]. With this method unique sequences are assigned to allele numbers combined into an allelic profile that determines a numerical sequence type (ST). Strains with the same allelic profile belong to the same ST and if there are differences in 1-3 loci they belong to the same CC [35,99]. The isolates of the same clonal complex are genetically related since they are carriers of the most prevalent ST of the group in the population. A freely accessible online MLST database (https://pubmlst.org/saureus/) allows widespread data comparison [35,98]. A disadvantage of MLST is that it is expensive and time-consuming [35].

Multiple-locus variable-number tandem repeat analysis (MLVA) is a typing method applied for *S. aureus* based on the determination of tandem repeat unit numbers present in different gene loci. The number of repeats in each locus results in the unique MLVA profile, which can be stored and compared in a freely accessible online database that includes *S. aureus* (http://mlva.eu). MLVA is not as expensive as MLST and allows a less difficult clustering than *spa* typing. The method exhibits acceptable reproducibility but since multiple loci have to be analyzed, it is time-consuming due to the different PCR conditions required for each locus [91,100].

Whole-genome sequencing (WGS) employing next-generation sequencing allows the identification of genome-wide variations and DNA diversity. It is a highly effective method, which will probably be the first choice typing method in the near future, once the associated costs have further decreased [35,101].

DNA microarrays are useful and efficient tools for the rapid characterization of microorganisms including *S. aureus*. The use of commercialized *S. aureus* specific DNA

microarrays enables the simultaneous detection of more than 300 specific resistance genes, virulence factors and typing markers [35,102]. However, the use of this technique is currently not affordable for routine diagnostic laboratories [35].

Direct repeat unit (*dru*) typing represents a simple and inexpensive method for the typing of methicillin-resistant staphylococci, performed similar to *spa* typing. With this method, PCR amplification of the polymorphic *dru* region is followed by sequence analysis of the *dru* amplicon and identification of the *dru* repeat [103]. A prefix, dr' (*dru* repeat), combined with numbers that identify specific 40-bp long repeat sequences of the *mecA* associated *dru* region is applied. The order of the *dru* repeats defines the *dru* type and a different prefix dt' (*dru* type), combined with numbers, is used to identify specific repeat combinations [103,104]. An online database exists, which allows comparison with *dru* sequences and types (http://dru-typing.org) [103].

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is a useful method to characterize and distinguish MRSA strains, even if they belong to the same ST. SCC*mec* is a mobile genetic element carrying the *mecA* gene, which encodes for a penicillin binding protein (PBP2a) with low affinity to β-lactam antibiotics such as methicillin. Up to date, 13 different SCC*mec* types have been described that are identified by the use of different PCRs. No single PCR exists for typing, only duplex-PCRs are commonly used, which are, however, limited to SCC*mec* types I to V [35].

2. Characterization of Antibiotic and Biocide Resistance Genes and Virulence Factors of *Staphylococcus* Species Associated with Bovine Mastitis in Rwanda

antibiotics

MDPI

Article

Characterization of Antibiotic and Biocide Resistance Genes and Virulence Factors of *Staphylococcus* Species Associated with Bovine Mastitis in Rwanda

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Abstract: The present study was conducted from July to August 2018 on milk samples taken at dairy farms in the Northern Province and Kigali District of Rwanda in order to identify *Staphylococcus* spp. associated with bovine intramammary infection. A total of 161 staphylococcal isolates originating from quarter milk samples of 112 crossbred dairy cattle were included in the study. Antimicrobial susceptibility testing was performed and isolates were examined for the presence of various resistance genes. *Staphylococcus aureus* isolates were also analyzed for the presence of virulence factors, genotyped by *spa* typing and further phenotypically subtyped for capsule expression using Fourier Transform Infrared (FTIR) spectroscopy. Selected *S. aureus* were characterized using DNA microarray technology, multi-locus sequence typing (MLST) and whole-genome sequencing. All *mecA*-positive staphylococci were further genotyped using *dru* typing. In total, 14 different staphylococcal species were detected, with *S. aureus* being most prevalent (26.7%), followed by *S. xylosus* (22.4%) and *S. haemolyticus* (14.9%). A high number of isolates was resistant to penicillin and tetracycline. Various antimicrobial and biocide resistance genes were detected. Among *S. aureus*, the Panton–Valentine leukocidin (PVL) genes, as well as bovine leukocidin (LukM/LukF-P83) genes, were detected in two and three isolates, respectively, of which two also carried the toxic shock syndrome toxin gene *tsst-1* bovine variant. t1236 was the predominant *spa* type. FTIR-based capsule serotyping revealed a high prevalence of non-encapsulated *S. aureus* isolates (89.5%). The majority of the selected *S. aureus* isolates belonged to clonal complex (CC) 97 which was determined using DNA microarray based assignment. Three new MLST sequence types were detected.

Keywords: *Staphylococcus* species; *Staphylococcus aureus*; bovine mastitis; antibiotic resistance; *spa* typing; FTIR spectroscopy; capsule serotyping; MLST; whole-genome sequencing; *dru* typing

1. Introduction

Bovine mastitis is an important disease that affects the dairy sector and is one of the economically most important diseases worldwide [1]. In Rwanda, it has a significant relevance because livestock production is rapidly increasing [2]. One reason is that milk consumption and the demand for dairy products are increasing with the rapid growth of the human population, from 3 million to 12 million people [3] in the last 60 years.

Mastitis is an inflammation of the udder tissue and the mammary gland. It is usually caused by bacteria invading through the teat canal. There are two types of mastitis: clinical and subclinical. While cows with clinical mastitis show severe symptoms (e.g., fever, hot, painful and swollen udder) and have visible changes in their milk (e.g., change of colour and consistency), cows with subclinical mastitis produce less milk and have higher somatic cell counts in their milk [1]. The California Mastitis Test (CMT) is a useful onsite method to confirm a bovine intramammary infection [4].

Staphylococci are the leading cause of mastitis [5,6], with *S. aureus* considered to be a major pathogen associated with clinical mastitis and often-recurrent subclinical mastitis, even in well-managed dairy herds. The primary mode of transmission is from cow-to-cow [1]. Coagulase-negative *Staphylococcus* spp. (CoNS) are a heterogeneous group and are also known as common pathogens involved in bovine mastitis. CoNS are primarily derived from the environment and are usually associated with a moderate infection [1].

In Rwanda, udder infections are associated with contamination via hand-to-cow contact, clothing, and other materials because hand milking is common. Poor milking hygiene is a risk factor not only for mastitis, but also for teat-end damage [7]. Reduced milk production, high veterinary costs, as well as prolific bacterial and antimicrobial contamination are the consequences of mastitis which can result in significant economic losses for the farmers [8]. Recently, the Government of Rwanda launched a development program, called Rwanda Vision 2020, with the main goal of transforming the country into a knowledge-based middle-income country by modernizing its agriculture and livestock production [2]. Public veterinary services in Rwanda are provided by district and sector veterinary officers. They have a limited capacity to support dairy farmers. Often, veterinary service workers receive poor training in dairy management and are not equipped with adequate transportation to visit farms (approximately 3200 cattle/veterinary officers). Overall, access to veterinary services in rural areas is less developed compared to urban areas [9].

In 2015, the first private animal clinic was established in the district of Musanze, called the New Vision Veterinary Hospital (NVVH), to improve animal welfare and to provide veterinary services (clinical and laboratory) as well as education based on collaboration with local and foreign universities and organizations.

Nevertheless, the farmers' access in Rwanda to veterinary drugs including antibiotics is possible through local pharmacies [9]. A recent report explained that in parts of the country, high usage of antibiotics in farm animals has become a common practice [9]. In a cross-sectional survey, the use of antibiotics in farm animals was declared by the majority of respondents (97.4%), mainly for disease prevention and growth promotion. More than half of the farmers (55.6%) were reported to use non-prescribed antibiotics in animals. Although policies and laws regulating the antibiotic use in humans and animals exist in Rwanda, antibiotics can be purchased without any medical or veterinary prescription [9]. The irrational use of antibiotics in humans and animals is highly related to the increase of antibiotic-resistant bacteria worldwide, including many classes of antimicrobial agents used in the veterinary field [10].

A recent study conducted in a hospital in Kigali, Rwanda assessing the antimicrobial susceptibility patterns of bacteria from human patients, showed a high prevalence of antimicrobial resistance, also among *Staphylococcus* spp. [11]. However, there is very limited information on the antimicrobial susceptibility pattern of bacteria isolated from milk samples obtained from cases of bovine mastitis in Rwanda. Recently, two studies showed a high prevalence of mastitis in the Northern Province and the peri-urban areas of Kigali [12,13], but characterization of causative agents and antimicrobial susceptibility testing, both phenotypic and genotypic, have not been performed. Thus, the present study aims to fill these gaps by fully characterizing a collection of bovine staphylococci associated with clinical and subclinical mastitis from the Northern Province and Kigali the District of Rwanda.

2. Results

From 303 CMT-positive milk samples collected from 112 crossbred milking cows, 161 non-repetitive staphylococcal isolates comprising 14 staphylococcal species were recovered: *S. aureus* ($n = 43$), *S. xylosus* ($n = 36$), *S. haemolyticus* ($n = 24$), *S. sciuri* ($n = 14$), *S. chromogenes* ($n = 10$), *S. saprophyticus* ($n = 9$), *S. epidermidis* ($n = 8$), *S. succinus* ($n = 5$), *S. capitis* ($n = 3$), *S. hominis* ($n = 2$), *S. devriesei* ($n = 2$), *S. auricularis* ($n = 2$), *S. equorum* ($n = 2$), and *S. simulans* ($n = 1$).

2.1. Antimicrobial Susceptibility Testing

All 161 isolates were susceptible to rifampicin, linezolid, and gentamicin. All but two were susceptible to cefoxitin and chloramphenicol. A high number of the isolates was resistant to penicillin ($n = 73$, 45.3%) and tetracycline ($n = 63$, 39.1%) (Tables 1 and 2). Twenty-three isolates were resistant to clindamycin, ten to erythromycin, and six isolates were resistant to trimethoprim-sulfamethoxazole (Tables 1 and 2).

The detection of resistance genes confirmed the phenotypic resistance profiles of the respective isolates, detecting *blaZ* ($n = 73$, 45.3%), *tet*(K) ($n = 45$, 71.4%), both *tet*(K) and *tet*(L) ($n = 17$, 27.0%) and all three *tet*(K), *tet*(L) and *tet*(O) ($n = 1$, 1.6%). Clindamycin-resistant isolates carried the following resistance genes: *erm*(C) ($n = 8$, 34.8%), *vga*(A) ($n = 2$, 8.7%), *erm*(44) ($n = 2$, 8.7%), *sal*(A) ($n = 2$, 8.7%), both *vga*(A) and *sal*(A) ($n = 2$, 8.7%), both *erm*(C) and *sal*(A) ($n = 1$, 4.3%), both *sal*(A) and *erm*(44) ($n = 1$, 4.3%) and all three *vga*(A), *sal*(A) and *lnu*(A) ($n = 2$, 8.7%). In the erythromycin-resistant isolates, two macrolide resistance genes were present: *erm*(C) ($n = 6$), and *msr*(A) ($n = 4$), whereas the trimethoprim-sulfamethoxazole-resistant isolates carried both *dfrA* (also known as *dfrS1*) and *dfrD* genes ($n = 1$), both *dfrD* and *dfrG* genes ($n = 3$) and all three *dfrA*, *dfrD* and *dfrG* genes ($n = 2$). Two isolates were resistant to chloramphenicol, which was associated with the presence of *fexA* in a *S. xylosus* and cat_{pC221} in a *S. saprophyticus* isolate. The streptomycin resistance gene *str* was detected in all 161 isolates, but its presence was not always associated with a higher MIC value (i.e., >8 mg/L) [14] (Tables 1 and 2).

The *mecA* gene was detected in cefoxitin-resistant *S. hominis* and *S. sciuri*, whereas the *mecC* gene could not be identified. One *dru* type (dt10cz) was detected in a *S. hominis* isolate, but the other *mecA*-positive isolate was not *dru*-typeable.

None of the tested isolates carried the genes *erm*(A), *erm*(B), *erm*(F), *erm*(T), *erm*(43), *erm*(33), *lsa*(B), $vga(A)_v$ *vga*(C), *vga*(E), *vga*(E)*v*, *dfrK*, *tet*(M), *ant(6′)-la*, *cfr*, cat_{pC194}, or cat_{pC223}.

Table 1. Summarized molecular characterization, antimicrobial resistance and toxins profile of Coagulase-negative *Staphylococcus* isolates investigated.

Isolates	Species	Origin [1]	Antimicrobial Resistance Profile			Biocide and Metal Resistance Genes
			Phenotype [2]	MIC [3] of Streptomycin	Genes Detected	
2FR	*S. chromogenes*	M 1		32 [4]	*str*	
3RL	*S. haemolyticus*	M 1	ERY, CLI	32	*erm*(C), *str*	
4FR	*S. epidermidis*	M 1	PEN, TET	32	*blaZ*, *tet*(K), *tet*(L), *str*	*copB*, *qacAB*, *smr*
4RR1	*S. hominis*	M 1	BLA, FOX, ERY, TET, CIP	‹4	*blaZ*, *mecA*, *msr*(A), *tet*(K), *tet*(L), *str*	*cadD*, *arsA*, *qacAB*, *smr*
4RR2	*S. capitis*	M 1	PEN	‹4	*blaZ*, *str*	*cadD*, *arsA*, *qacAB*, *smr*
7FL	*S. chromogenes*	M 2	ERY, CLI	‹4	*erm*(C), *str*	
7RR	*S. epidermidis*	M 2	PEN, ERY, CLI, TET	32	*blaZ*, *erm*(C), *tet*(K), *tet*(L), *str*	*cadD*, *arsA*, *smr*
8RL	*S. haemolyticus*	M 2	ERY, CLI	32	*erm*(C), *str*	
13FLg	*S. xylosus*	M 3	PEN	‹4	*blaZ*, *str*	*cadD*, *copB*
13FLw	*S. xylosus*	M 3	PEN, TET	32	*blaZ*, *tet*(K), *tet*(L), *str*	*cadD*, *arsA*, *smr*
13FLw wh	*S. xylosus*	M 3	ERY	‹4	*msr*(A), *str*	
13RR	*S. xylosus*	M 3	ERY, CLI, CHL	‹4	*msr*(A), *fexA*, *str*	
14FL1	*S. equorum*	M 3		‹4	*str*	
17RR	*S. equorum*	M 4		‹4	*str*	*smr*
18RLw1	*S. epidermidis*	M 4	PEN, TET	32	*blaZ*, *tet*(K), *tet*(L), *str*	*cadD*, *arsA*, *qacAB*, *smr*
18RLw2	*S. haemolyticus*	M 4	PEN, TET	32	*blaZ*, *tet*(K), *tet*(L), *str*	*cadD*, *arsA*, *qacAB*, *smr*
18RLg	*S. haemolyticus*	M 4		‹4	*str*	*cadD*, *copB*, *arsA*, *smr*
18FL	*S. auricularis*	M 4		16	*str*	*copB*
24RLw	*S. xylosus*	M 5		‹4	*str*	*cadD*, *smr*
24RLg	*S. haemolyticus*	M 5		32	*str*	
25FLw	*S. hominis*	M 5	PEN	‹4	*blaZ*, *str*	*cadD*, *arsA*, *qacAB*, *smr*
25FLg	*S. xylosus*	M 5	PEN	‹4	*blaZ*, *str*	
25FL3	*S. xylosus*	M 5		‹4	*str*	*cadD*
25RR	*S. epidermidis*	M 5	PEN, TET	32	*blaZ*, *tet*(K), *str*	
25RRg	*S. sciuri*	M 5	CLI	‹4	*sal*(A), *str*	
26RL1	*S. xylosus*	M 6		‹4	*str*	*cadD*
26RRw	*S. xylosus*	M 6		‹4	*str*	
26RRg	*S. xylosus*	M 6		‹4	*str*	
27RLg	*S. xylosus*	M 6		‹4	*str*	
28FRg	*S. xylosus*	M 7		‹4	*str*	
30FL	*S. devriesei*	M 8	TET	16	*tet*(K), *str*	*arsA*
30RL	*S. devriesei*	M 8	PEN, TET	32	*blaZ*, *tet*(K), *str*	*arsA*
30FR	*S. chromogenes*	M 8	PEN, TET	‹4	*blaZ*, *tet*(K), *str*	
32FR	*S. chromogenes*	M 8		32	*str*	
33RL	*S. chromogenes*	M 8	PEN, TET	32	*blaZ*, *tet*(K), *str*	
33FR	*S. haemolyticus*	M 8		32	*str*	
34RLw	*S. haemolyticus*	M 9		32	*str*	*cadD*
35FR	*S. haemolyticus*	M 9		16	*str*	*arsA*
35RRg	*S. haemolyticus*	M 9		16	*str*	*arsA*
36FL	*S. haemolyticus*	M 9	TET	32	*tet*(K), *tet*(L), *str*	
38FL	*S. auricularis*	M 9		‹4	*str*	*cadD*

Table 1. *Cont.*

Isolates	Species	Origin [1]	Antimicrobial Resistance Profile			Biocide and Metal Resistance Genes
			Phenotype [2]	MIC [3] of Streptomycin	Genes Detected	
42FR	*S. haemolyticus*	M 11	TET	32	*tet*(K), *tet*(L), *str*	
43FRw	*S. xylosus*	M 11	TET	‹4	*tet*(K), *str*	*copB*
44FL	*S. xylosus*	M 11		‹4	*str*	
46FR	*S. epidermidis*	M 11	PEN	32	*blaZ*, *str*	*cadD*
47RRg	*S. chromogenes*	M 12		32	*str*	*qacAB*, *smr*
50RL	*S. sciuri*	M 12	CLI	‹4	*erm*(44), *str*	
50RR	*S. sciuri*	M 12	CLI	‹4	*erm*(44), *sal*(A) *str*	
51RR	*S. xylosus*	M 12	TET	‹4	*tet*(K), *str*	
52FL	*S. haemolyticus*	K	PEN, CLI, TET	32	*blaZ*, *erm*(C), *tet*(K), *tet*(L), *str*	*cadD*, *copB*, *qacAB*, *smr*
52FR	*S. haemolyticus*	K		‹4	*str*	*cadD*, *copB*, *arsA*
53FL	*S. haemolyticus*	K	PEN, CLI, TET	32	*blaZ*, *tet*(K), *str*	*copB*
53RL	*S. haemolyticus*	K	CLI, TET	32	*vga*(A), *sal*(A), *lnu*(A), *tet*(K), *tet*(L), *str*	*qacAB*, *smr*
53RR	*S. haemolyticus*	K	CLI, TET	32	*vga*(A), *sal*(A), *lnu*(A), *tet*(K), *tet*(L), *str*	*qacAB*, *smr*
54FR	*S. haemolyticus*	K	CLI	32	*vga*(A), *str*	
54RRw	*S. haemolyticus*	K	PEN, CLI, SXT, TET	32	*blaZ*, *dfrA*, *dfrD*, *tet*(K), *str*	
54RRg	*S. xylosus*	K		32	*str*	*smr*
55RR1	*S. epidermidis*	K	PEN, TET	‹4	*blaZ*, *tet*(K), *str*	*copB*, *arsA*, *qacAB*, *smr*
55RR2	*S. capitis*	K	PEN	‹4	*blaZ*, *str*	*copB*, *arsA*, *smr*
56RL	*S. sciuri*	K	CLI	‹4	*vga*(A), *sal*(A), *str*	
57FLw	*S. capitis*	K	PEN, TET	‹4	*blaZ*, *tet*(K), *tet*(L), *str*	*cadD*, *smr*
57FRw	*S. haemolyticus*	K	CLI, TET	32	*tet*(K), *tet*(L), *str*	*copB*, *smr*
58FL	*S. haemolyticus*	K	CLI, TET	32	*erm*(C), *sal*(A), *tet*(K), *tet*(L), *str*	*smr*
58FR	*S. haemolyticus*	K	CLI, TET	32	*vga*(A), *tet*(K), *tet*(L), *str*	
58RR	*S. xylosus*	K		‹4	*str*	
61RR	*S. xylosus*	K	SXT, TET	‹4	*dfrA*, *dfrD*, *dfrG*, *tet*(K), *tet*(L), *str*	*smr*
61RL	*S. xylosus*	K	TET	32	*tet*(K), *str*	*copB*, *smr*
62FR	*S. xylosus*	K		‹4	*str*	*copB*
62RR	*S. haemolyticus*	K		‹4	*str*	*cadD*
63RL	*S. sciuri*	K	PEN	‹4	*blaZ*, *str*	
64RR	*S. epidermidis*	K	PEN, SXT, TET	32	*blaZ*, *dfrA*, *dfrD*, *dfrG*, *tet*(K), *tet*(L), *tet*(O), *str*	*copB*, *arsA*, *smr*
65RL	*S. haemolyticus*	K	PEN, ERY, SXT, TET	32	*blaZ*, *msr*(A), *dfrD*, *dfrG*, *tet*(K), *str*	*cadD*, *copB*, *arsA*
66RL	*S. xylosus*	K	PEN, TET	‹4	*blaZ*, *tet*(K), *str*	*qacAB*
66RR	*S. epidermidis*	K	PEN, TET, TEC	32	*blaZ*, *tet*(K), *str*	*cadD*, *smr*
67RL	*S. chromogenes*	K		32	*str*	
68RL	*S. chromogenes*	K		32	*str*	
68RR	*S. xylosus*	K	PEN	‹4	*blaZ*, *str*	
70RLw	*S. simulans*	K	PEN	32	*blaZ*, *str*	*copB*
70FR	*S. sciuri*	K	FOX	‹4	*mecA*, *str*	

Table 1. *Cont.*

Isolates	Species	Origin [1]	Antimicrobial Resistance Profile			Biocide and Metal Resistance Genes
			Phenotype [2]	MIC [3] of Streptomycin	Genes Detected	
1stCowFL	*S. chromogenes*	M 13		‹4	*str*	
2ndCowRL	*S. xylosus*	M 13	TET	‹4	*tet*(K), *str*	
73RL	*S. sciuri*	M 14	PEN	‹4	*blaZ*, *str*	
73RR	*S. xylosus*	M 14		‹4	*str*	
78FR	*S. xylosus*	M 17		‹4	*str*	
78RL	*S. sciuri*	M 17	CLI	‹4	*vga*(A), *sal*(A), *str*	
81 RR	*S. haemolyticus*	M 18	PEN	‹4	*blaZ*, *str*	*cadD*
82RL	*S. sciuri*	M 18	CLI	‹4	*erm*(44), *str*	
82RR	*S. saprophyticus*	M 18	TET, CHL	4	*tet*(K), cat_{pC221}, *str*	
84RR	*S. saprophyticus*	M 18	TET	‹4	*tet*(K), *str*	*copB*
85FR	*S. xylosus*	M 19	TET	8	*tet*(K), *str*	
85FL	*S. saprophyticus*	M 19	TET	8	tet(K), *str*	*copB*, *arsA*, *qacAB*
86FR	*S. saprophyticus*	M 19		‹4	*str*	*copB*
87FL	*S. saprophyticus*	M 19	TET	4	*tet*(K), *str*	*copB*
89FR	*S. sciuri*	M 20		‹4	*str*	
89RR	*S. xylosus*	M 20	PEN	‹4	*blaZ*, *str*	
94RR	*S. succinus*	M 21	PEN	‹4	*blaZ*, *str*	*copB*
94RL	*S. sciuri*	M 21	PEN	‹4	*blaZ*, *str*	
95FR	*S. xylosus*	M 21		‹4	*str*	
95RR	*S. xylosus*	M 21		‹4	*str*	
96FR	*S. xylosus*	M 21	TET	‹4	*tet*(K), *str*	*qacAB*
96RR	*S. xylosus*	M 21		‹4	*str*	
97RL	*S. sciuri*	M 21		‹4	*str*	
97RR	*S. xylosus*	M 21	SXT	‹4	*dfrD*, *dfrG*, *str*	
98RR	*S. succinus*	M 21	PEN	‹4	*blaZ*, *str*	*cadD*
99FR	*S. xylosus*	M 22		‹4	*str*	
99RL	*S. xylosus*	M 22		‹4	*str*	*copB*
103RR	*S. chromogenes*	M 22	PEN	32	*blaZ*, *str*	
104RR	*S. succinus*	M 23		‹4	*str*	*smr*
104RL	*S. succinus*	M 23	PEN	‹4	*blaZ*, *str*	*cadD*, *arsA*, *smr*
105RL	*S. succinus*	M 24		‹4	*str*	*cadD*, *smr*
106FL1	*S. saprophyticus*	M 24		‹4	*str*	*copB*
107RL	*S. saprophyticus*	M 25	PEN	16	*blaZ*, *str*	*copB*
108FL	*S. saprophyticus*	M 25	SXT	‹4	*dfrD*, *dfrG*, *str*	*arsA*
110RL	*S. xylosus*	M 26		‹4	*str*	*copB*, *smr*
110RR1	*S. saprophyticus*	M 26		‹4	*str*	*copB*, *arsA*
110RR2	*S. xylosus*	M 26		‹4	*str*	*copB*
111RL	*S. sciuri*	M 26	PEN, CLI	‹4	*sal*(A), *blaZ*, *str*	
113RL	*S. sciuri*	M 26		16	*str*	

[1] Origin: M = Musanze Farm, K = Kigali Farm.[2] Phenotype: PEN = penicillin; CIP = ciprofloxacin; CHL = chloramphenicol; CLI = clindamycin; ERY = erythromycin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; FOX = cefoxitin, TEC = teicoplanin. [3] mg/L. [4] 32 or higher (mg/L).

Table 2. Summarized molecular characterization, antimicrobial resistance and toxin profile of the *Staphylococcus aureus* isolates investigated.

Isolates	Origin [1]	CC [2]	ST [3]	*spa*	Antimicrobial Resistance Profile			Biocide and Metal Resistance Genes	Capsule Serotype [7]	*cap* gene (*cap* 8)	*cap* gene (*cap* 5)	Hemolysins	Leukocidin (luk) Components	Biofilm-Associated Genes	Adhesion Factors	Superantigens
					Phenotype [4]	MIC [5] of Streptomycin	Genes Detected									
1FR *	M 1		ST97	t1236	PEN	32 [6]	*blaZ, str*		not tested	NEG [8]	POS [8]	*hla, hlb, hld*	NEG	*icaC, icaD*	*clfA, fib, fnbA, fnbB, sasG*	
6RR *	M 2	CC152	ST152	t458	ERY, CLI	32	*erm*(C), *str*		CP5	NEG	POS	*hla, hlb, hld*	*lukS-PV/lukF-PV*	*icaA, icaD*	*clfA, clfB, cna, fnbA, fnbB*	
11RR *	M 3		ST97	t1236	PEN	›4	*blaZ, str*	*smr*	nt	NEG	POS	*hla, hlb, hld*	NEG	*icaC, icaD*	*clfA, fib, fnbA, fnbB, sasG*	
24RR *	M 5	CC3666	ST5477	t1236	PEN, TET	32	*blaZ, tet*(K), *tet*(L), *str*		nt	POS	NEG	*hla, hld*	*lukD*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	*tsst-1, sei, sem, sen, seo, seu*
26FR	M 6			t1236	PEN	32	*blaZ, str*		not tested	not tested	not tested	not tested	not tested	not tested	not tested	
26FL	M 6	CC97		t1236	PEN	16	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
26RL2	M 6			nt [7]	PEN	32	*blaZ, str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
27FL	M 6			t1236	PEN	32	*blaZ, str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	*sec*
27RLw	M 6	CC97		t1236	PEN	›4	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
27RR	M 6			t1398	TET	4	*tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
36RR	M 9	CC97		t1236	PEN	32	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
37RR	M 9			t9432	PEN, TET	›4	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
39FR	M 10	CC97		t2112	PEN	8	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
40FL	M 10	CC97		t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
43RL	M 11	CC97		t18835	PEN, TET	32	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
44RR	M 11	CC3591		t458		›4	*str*	*smr*	CP8	POS	NEG	*hla, hlb, hld*	*lukM/lukF-PV (P83)*	*icaA, icaC, icaD*	*clfA, clfB, fib, cna, fnbA*	
63FL	K	CC152	ST152	t355	ERY, CLI	32	*erm*(C), *str*		not tested	NEG	POS	*hla, hlb, hld*	*lukS-PV/lukF-PV*	*icaA, icaD*	*clfA, clfB, cna, fnbA, fnbB*	
71FL	M 14	CC3591	ST5475	t355	TET	32	*tet*(K), *str*		nt	POS	NEG	*hla, hlb, hld*	NEG	*icaA, icaC, icaD*	*clfA, clfB, fib, cna, fnbA*	*sem, seo*
74FL	M 14			t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
75FR	M 15	CC97		t10103	PEN, TET	32	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
75FL	M 15			t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
76RR	M 16	CC3591	ST5476	t458		›4	*str*		CP8	POS	NEG	*hla, hlb, hld*	*lukM/lukF-PV (P83)*	*icaA, icaC, icaD*	*clfA, clfB, fib, cna, fnbA*	
77RR	M 17	CC3591		t458		16	*str*		CP8	POS	NEG	*hla, hlb, hld*	*lukM/lukF-PV (P83)*	*icaA, icaC, icaD*	*clfA, clfB, fib, cna, fnbA*	
78FL	M 17	CC97		t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
80RL	M 18			t380	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	*sec*
82FL	M 18			t380	PEN, TET	›4	*blaZ, tet*(K), *str*		not tested	not tested	not tested	not tested	not tested	not tested	not tested	
83RL	M 18	CC97		t380	PEN	32	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
84RL	M 18			t380	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
85RR	M 19	CC97		t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
85RL	M 19			t10103	PEN, TET	32	*blaZ, tet*(K), *str*	*qacAB*	nt	not tested	not tested	not tested	not tested	not tested	not tested	
86FL	M 19			t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
87FR	M 19			t10103	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
87RL	M 19			t1236	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
90FR	M 20			t9432	PEN, TET	32	*blaZ, tet*(K), *str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
90FL	M 20			t9432	PEN, TET	32	*blaZ, tet*(K), *str*		not tested	not tested	not tested	not tested	not tested	not tested	not tested	
91FL	M 20	CC97		t9432	PEN	32	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
100RR	M 22	CC97		t1236	PEN, TET	›4	*blaZ, tet*(K), *str*	*qacAB*	nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
100RL	M 22	CC97		t1236	PEN, TET	›4	*blaZ, tet*(K), *str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
101RR	M 22	CC97		t10103	PEN	32	*blaZ, str*		nt	NEG	POS	*hla, hlb, hld*	*lukD, lukE*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	
101RL	M 22			t10103	PEN	32	*blaZ, str*		nt	not tested	not tested	not tested	not tested	not tested	not tested	
103FR	M 22	CC3666		t18853	PEN, TET	32	*blaZ, tet*(K), *str*	*smr*	nt	POS	NEG	*hla, hlb, hld*	*lukD*	*icaA, icaC, icaD*	*clfA, clfB, fib, fnbA, fnbB, sasG*	*tsst-1, sei, sem, sen, seo, seu*
104FR	M 23			t1236	PEN, TET	32	*blaZ, tet*(K), *str*	*smr*	nt	not tested	not tested	not tested	not tested	not tested	not tested	
106FL2	M 24			t18835	PEN, TET	32	*blaZ, tet*(K), *str*	*qacAB*	nt	not tested	not tested	not tested	not tested	not tested	not tested	

[1] Origin: M = Musanze Farm, K = Kigali Farm. [2] clonal complex. [3] sequence type. [4] Phenotype: PEN = penicillin; CIP = ciprofloxacin; CHL = chloramphenicol; CLI = clindamycin; ERY = erythromycin; SXT = trimethoprim-sulfamethoxazole; TET = tetracycline; FOX = cefoxitin, TEC = teicoplanin. [5] mg/L. [6] 32 or higher (mg/L); [7] Capsule serotype: nt = non-typable; CP5 = Serotype 5; CP8 = Serotype 8. [8] NEG = negative, POS = positive. * analysed by whole-genome sequencing.

2.2. Metal and Biocide Resistance Testing

Biocide resistance profiling revealed that 33 isolates carried the *smr* gene, most frequently the species *S. haemolyticus* ($n = 7$), *S. epidermidis* ($n = 6$), *S. xylosus* ($n = 6$) and *S. aureus* ($n = 4$). Seventeen isolates carried the *qacAB* gene, where the predominant species were *S. haemolyticus* ($n = 4$), *S. epidermidis* ($n = 3$), *S. aureus* ($n = 3$), *S. xylosus* ($n = 2$) and *S. hominis* ($n = 2$). Furthermore, the presence of the following metal resistance genes was confirmed: *cadD* ($n = 25$), *copB* ($n = 27$) and *arsA* ($n = 21$). The most prevalent species, which carried the *cadD* gene, was *S. haemolyticus* ($n = 8$), followed by *S. xylosus* ($n = 5$) and *S. epidermidis* ($n = 4$). A significant carriage rate of *copB* was shown by *S. saprophyticus* ($n = 7$) and *S. xylosus* ($n = 7$). The *arsA* gene was mostly detected in the species *S. haemolyticus* ($n = 6$), *S. epidermidis* ($n = 4$) and *S. saprophyticus* ($n = 3$). However, none of the isolates carried the *czrC* gene (Tables 1 and 2) and all *S. aureus* isolates were negative for metal resistance genes.

2.3. Additional Characterization of S. aureus Isolates

Among *S. aureus*, the *lukS-PV* and *lukF-PV* genes coding for the Panton–Valentine leukocidin (PVL) were detected in two isolates, the bovine leukocidin gene *lukM/lukF-P83* was present in three isolates. The *tsst-1* gene was detected in two isolates and was solely found in combination with enterotoxin genes. The enterotoxin genes *sei* ($n = 2$), *sem* ($n = 3$), *sen* ($n = 2$), *seo* ($n = 3$) and *seu* ($n = 2$), that belonged to the *egc* cluster, and *sec* ($n = 2$) were detected. Staphylococcal enterotoxin genes *sea*, *seb*, *sed*, *see*, *seg*, *seh*, *sej*, *sek*, *sel*, *seq*, *ser* and the gene for the enterotoxin like protein CM14 could not be detected in the *S. aureus* isolates (Table 2).

Ten different *spa* types were identified among the tested isolates. The *spa* type t1236 ($n = 18$) was predominant, followed by t10103 ($n = 5$), t380 ($n = 4$) and t9432 ($n = 4$), t458 ($n = 4$), t355 ($n = 2$) and singletons t2112 and t1398. Two new *spa* types were detected: t18835 ($n = 2$, repeat order 26-23-34-34-34-34-33-34) and t18853 ($n = 1$, repeat order 04-20-17-24-17).

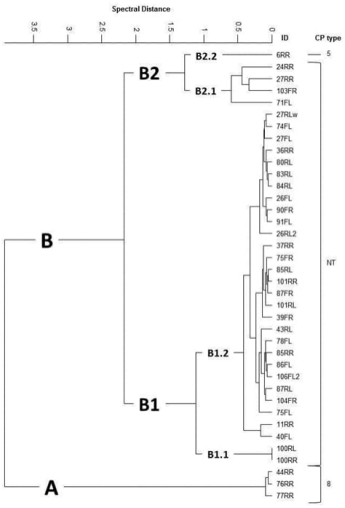

Figure 1. FTIR spectroscopy-based cluster of *S. aureus* isolated from quarter milk samples of cows with mastitis. CP = capsule type; NT = none typeable.

FTIR-based capsule serotyping revealed a high prevalence of non-encapsulated *S. aureus* isolates ($n = 34$; 89.5%) and the remaining isolates produced a capsule of either serotype 8 (CP8, $n = 3$) or 5 (CP5, $n = 1$). Hierarchical cluster analysis of spectral FTIR data grouped the *S. aureus* isolates into two main

clusters (A; *n* = 3 and B; *n* = 35; Figure 1). Cluster A could be assigned to CP 8 while non-typeable (NT) isolates were grouped into the main cluster B, except one isolate assigned to CP5 (B2.2). All NT isolates were found to harbour either the *cap8*- (B2.1, *n* = 4) or *cap5*-specific allele (B1.1, *n* = 2 and B1.2, *n* = 28). No association between the origin of the samples and the FTIR cluster alignment was detectable.

Among the selected *S. aureus* isolates examined using DNA microarray and whole-genome sequencing, different resistance genes (*blaZ*, *erm*(C), *tet*(K)) and virulence genes (*hla*, *hlb*, *hld*, *lukD*, *lukE*, *lukM*, *lukF-P83*, *icaA*, *icaC*, *icaD*, *bap*, *clfA*, *clfB*, *fib*, *can*, *fnbA*, *fnbB*, *sasG*) could be found (Table 2). Four different clonal complexes (CC) were identified. Here, the CC97 isolates (*n* = 14) clustered into FTIR cluster B1, the CC3591 isolates (*n* = 4) into clusters A and B2.1, the CC3666 isolates (*n* = 2) into cluster B2.1 and one isolate of CC152 into cluster B2.2. Three *S. aureus* that were selected for MLST revealed the new sequence types ST5475 (199-805-44-430-447-192-733), ST5476 (199-806-741-2-447-192-734) and ST5477 (6-55-45-2-109-14-741).

3. Discussion

Clinical and subclinical mastitis can be one of the serious consequences of poor milking hygiene [5,7]. Previous studies have shown that the prevalence of mastitis within the East African region is high and that CoNS are common pathogens in bovine mastitis [5,15–17]. This finding was also confirmed in this study.

In the present study, *S. aureus* was the predominant *Staphylococcus* spp., which is in accordance with studies from other countries in that region, such as Tanzania, and Kenya [16,18]. Another study from Uganda showed that the predominant *Staphylococcus* spp. were from the CoNS group, but they were not further characterized to the species level [15]. Among CoNS, *S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *S. simulans* and *S. xylosus* are usually the most common isolated species associated with bovine mastitis [19,20]. However, distribution of CoNS species has shown to be herd-specific and influenced by different management practices that can vary between countries [1,20].

Penicillin resistance is probably the best known antimicrobial resistance property of *S. aureus* and its frequency in the current study is in accordance with other studies that examined antibiotic susceptibility patterns of staphylococci isolated from cases of bovine mastitis in other parts of Africa as well as in Germany and Finland [16,21–24]. Penicillin is a routinely used antimicrobial agent for the prevention and treatment of mastitis in dairy cows in Rwanda [9] and the *blaZ* gene was present in all 73 penicillin-resistant *Staphylococcus* spp. isolates (100%) in the current study. This gene encodes a narrow-spectrum β-lactamase which confers penicillin resistance [10,25].

Tetracycline belongs to the broad-spectrum antimicrobial agents and is also an often-used antimicrobial agent in farm animals in Rwanda [9]. Resistance to tetracyclines is frequently mediated by the genes *tet*(K) and *tet*(L), which code for active efflux mechanisms, and occasionally by *tet*(M) and *tet*(O), which encode ribosome-protective proteins [10]. In the present study, *tet*(K) was found in all tetracycline-resistant staphylococci (100%), followed by *tet*(L) (28.6%) and *tet*(O) (1.6%), while *tet*(M) was not detected in any of the tetracycline-resistant isolates. In a study from Tunisia, 10.3% of the staphylococcal isolates (*n* = 68) showed resistance to tetracycline and this resistance was always encoded by the *tet*(K) gene [26]. In another study from Germany, the *tet*(M), *tet*(K) and *tet*(L) genes were investigated among resistant *S. aureus* sisolates, originating from cases of bovine clinical mastitis (*n* = 25) and from farm personnel (*n* = 2), and *tet*(M) was found in 100%, *tet*(K) in 92.6% and *tet*(L) in 40.7% of the isolates [23].

Two *S. haemolyticus* and one *S. xylosus* isolate exhibited phenotypic resistance to clindamycin although a corresponding resistance gene was not detected. Whole genome sequencing of these isolates in a future study will hopefully clarify the genetic basis for the observed lincosamide resistance. Another problem detected in this study was the phenotypic assessment of streptomycin resistance. All isolates carried the resistance gene *str*, but MICs to streptomycin varied between ≤4 and 32 mg/L. Neither CLSI, nor EUCAST provide clinical breakpoints for streptomycin and staphylococci. The sequenced *str* amplicons obtained from staphylococcal isolates with low streptomycin MICs as well as from those

with high streptomycin MICs did not differ in their sequences (author's own observation). Again, whole genome approaches may help to clarify the situation.

Quaternary ammonium compounds (QACs)-based antiseptics are frequently used worldwide and this prevailing usage can lead to bacterial resistance against these substances [27,28]. In the current study, the antiseptic resistance genes *qacAB* and *smr* were examined. The *smr* gene was found more frequently than the *qacAB* genes. These results were similar to those of a study from Norway assessing the resistance to QACs in bacteria from milk samples obtained from 127 dairy cattle herds and 70 goat herds, where the *smr* gene was present in 64.2% and the *qacAB* gene in 28.5% of the isolates (n = 42) [28]. Studies about the bacterial resistance to QACs in staphylococci originating from bovine milk in Africa are scarce. One study from three African countries (Angola, São Tomé and Príncipe, Cape Verde), where a total of 301 *S. aureus* isolates were investigated, reported an intermediate prevalence for the *qacAB* gene (40.5%) and a low prevalence for the *smr* gene (3.7%) [29].

Many other substances with antimicrobial effects, including metal-containing compounds, are used in food-animal production, where they can contribute to the selection of isolates among staphylococcal species [30]. According to a study from 2017 on cattle production in East Rwanda, only 3.6% (n = 13) of the farmers practiced supplementary feeding [2]. However, in the present study, conducted in Northern parts of Rwanda and Kigali, 51 (31.5%) of the bacterial isolates carried at least one heavy metal resistance gene. Heavy metal resistance genes occurred most frequently in *S. haemolyticus* (n = 12) followed by *S. xylosus* (n = 11) and *S. saprophyticus* (n = 8). In another study, *S. haemolyticus* and *S. epidermidis* carried the most heavy metal resistance genes [31], but the isolates in the current study did not show a high rate of heavy metal resistance genes, which is possibly explained by the different geographical collection sites.

The vast majority of the collected *S. aureus* mastitis isolates in this study were non-encapsulated as shown by spectroscopic capsule serotyping. This is in concordance with several previous reports showing a high prevalence of non-encapsulated mastitis isolates in Argentina, USA and Austria [32–34]. Moreover, non-encapsulation was associated with high within-herd prevalence of *S. aureus*-based persistent, contagious bovine intramammary infections [35]. Indeed, this study provides further evidence that loss of capsule expression is a key phenotypic feature associated with bovine mastitis, a primarily chronic infection [36]. Out of the 38 FTIR-typed isolates, 22 were selected for clonal complex (CCs) identification using DNA microarray-based technology and three of them (two CC3591 and one CC3666) were genotyped by MLST. The four CCs (CC97, CC3591, CC3666, CC152) identified were relatively distinctive for one of the FTIR clusters, also seen by Kümmel et al. in 2016 [34], though no connection to one particular farm could be found. Most isolates were assigned to the common bovine lineage CC97, indicating predominance of this cattle-adapted clone, which has already been reported from bovine mastitis cases worldwide including Europe, Japan, Algeria, and South Africa [37–40].

The most predominant *spa* type among *S. aureus* in the present study was t1236. This is a *spa* type within ST97 and associated with CC97 along with the other *spa* types t2112, t380, and t10103, commonly found among *S. aureus* from neighbouring Uganda [41]. The *spa* type t1236 has also been detected among *S. aureus* from bovine milk in Japan, reported as ST97 [38]. The *spa* type t458, which was found in four isolates in the current study, has been detected in *S. aureus* from a case of bovine mastitis in China [42] and from bovine milk in Japan [38]. Many African studies (Democratic Republic of the Congo, Gabon, Ghana, Kenya, Nigeria and Uganda) reported the presence of *spa* type t355 in *S. aureus* from humans [43–48], which was also identified in three isolates in the current study.

Five *S. aureus* isolates carried PVL genes, which is of interest due to the common association with soft tissue and skin infections and the reported human to cow transmission of *S. aureus* [49,50]. The PVL genes code for proteins which are responsible for cytotoxic activity, especially leukocytes are affected [51]. The *lukS-PV* and *lukF-PV* genes (PVL genes) were mainly detected in *S. aureus* of human origin [52], but have also been reported in isolates from bovine mastitis cases in Africa suggesting human to cow transmission of the respective isolates [41,50]. These human-associated genes were also detected in two *S. aureus* ST152 isolates obtained from two cows kept in two different farms in this study (Table 2). The LukM/LukF-PV(P83) protein only kills bovine neutrophils and is common

in *S. aureus* isolated from bovine mastitis [51,52]. In a study from North-Western Ethiopia, however, this bovine-related leukocidin was detected in a low percentage (4%) and the isolates did not belong to the common ST97 [50]. This was in line with the results of the current study where this gene was only present in three of the further selected *S. aureus* isolates, which belonged to ST5476 and to CC3591. Previous reports demonstrated that isolates belonging to ST97 may also be negative for the bovine-related leukocidin [38,53].

In the present study, the *tsst1* gene was detected in two isolates and further classified as bovine variant of *tsst1* which has been described in previous studies dealing with *S. aureus* associated with bovine mastitis [39,50–55].

4. Materials and Methods

4.1. Isolation and Identification of Staphylococci

Isolation of *Staphylococcus* spp. was conducted from July to August 2018 from CMT-positive milk samples originating from 112 crossbred dairy cows kept on 28 farms in the Northern Province and the Kigali District of Rwanda. Farms were selected for sampling based on farmers' reports on decreased milk production of multiple cows. Before sampling, a short clinical check was performed on each selected cow, including palpation of the udder, examination of the milk and measuring the body temperature. Afterwards, CMT was performed, which can indicate the presence of mastitis [4]. Collected milk samples were transported to the microbiological laboratory of NVVH, and bacteriological analyses were performed. Milk samples were cultivated on blood agar (Blood Agar Base, Rapid Labs, UK) supplemented with 5% of defibrinated sheep blood. After incubation at 37 °C for 24 h, each colony representing a distinct colony morphotype, but showing typical staphylococcal colony appearance, was regrown on the same medium. Pure staphylococcal cultures were stored at 4 °C until they were transported to the diagnostic laboratory of the Institute of Microbiology at the University of Veterinary Medicine, Vienna for further examination. All isolates were regrown on BD Columbia III agar plates with 5% Sheep Blood (Becton Dickinson, Heidelberg, Germany), and identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany). If MALDI-TOF MS yielded ambiguous results, *rpoB* gene sequencing was performed [56].

4.2. Antimicrobial Susceptibility Testing and Detection of Resistance Genes and SCCmec-Associated Direct Repeat Unit (dru) Typing

Antimicrobial susceptibility testing was performed by agar disk diffusion according to CLSI standards (CLSI, 2018) for the following antimicrobial agents (µg/disk): tetracycline (30), ciprofloxacin (5), erythromycin (15), clindamycin (2), penicillin (10 IU), cefoxitin (30), chloramphenicol (30), gentamicin (10), rifampicin (5), linezolid (30), and trimethoprim-sulfamethoxazole (1.25 + 23.75). In addition, minimum inhibitory concentrations (MICs) of streptomycin were established by the agar dilution method on Mueller–Hinton agar in serial twofold dilutions (4, 8, 16, and 32 µg/mL) in accordance with the CLSI document M7-A9 (CLSI, 2012).

Staphylococcal DNA was extracted as described previously [57]. PCR was used to detect the presence of the following antibiotic resistance genes: *blaZ* (confers resistance to penicillins except isoxazolyl-penicillins) [25]; *mecA*, *mecC* (confer resistance to all penicillins and cephalosporins approved for veterinary use) [58]; *erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(T), *erm*(33), *erm*(43), and *erm*(44) (confer resistance to macrolides, lincosamides, and streptogramin B), *vga*(A), *vga*(A)$_V$, *vga*(C), *vga*(E), *vga*(E)*v* and *sal*(A) (confer resistance to streptogramin A, lincosamides and pleuromutilins); *Isa*(B) and *Inu*(A) (confer elevated MICs or resistance to lincosamides) [23,59–68]; *msr*(A) (confers resistance to macrolides and streptogramin B) [57]; *cfr* (confers resistance to all phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A) [69]; *fexA* (confers resistance to all phenicols) [69]; cat_{pC194}, cat_{pC221}, and cat_{pC223} (confer resistance to non-fluorinated phenicols, e.g., chloramphenicol) [70]; *ant*(6′)-*Ia* and *str* (confer resistance to the aminoglycoside streptomycin) [14]; *dfrA*, *dfrD*, *dfrG*, and *dfrK*

(confer resistance to trimethoprim) [57,71]; *tet*(K) and *tet*(L) (confer resistance to tetracyclines except minocycline and glycylcyclines) [57]; *tet*(O) and *tet*(M) (confer resistance to tetracyclines, including minocycline, but excluding glycylcyclines) [72].

PCRs targeting *qacAB* (confers high-level resistance to antiseptics) and *smr* (confers low-level resistance to antiseptics) genes were performed as previously described [27]. Furthermore, PCRs were performed for detecting the presence of the following heavy metal resistance genes: *cadD*, *copB*, *arsA* and *czrC* [30,31].

The *mecA*-positive isolates were further examined by SCC*mec*-associated direct repeat unit (*dru*) typing [73].

4.3. Additional Characterization of S. aureus Isolates

All *S. aureus* isolates were examined by different PCRs targeting Panton–Valentine Leukocidin (PVL) genes, staphylococcal enterotoxins (SE), and the toxic shock syndrome toxin 1 (TSST1) as previously described [58]. Furthermore, *S. aureus* were genotyped by *spa* typing [57].

Using Fourier Transform Infrared (FTIR) spectroscopy, all isolates were further phenotypically subtyped based on their surface glyco structural composition that included the determination of the capsular polysaccharide (CP) expression [74,75]. On FTIR based clustering, 22 *S. aureus* isolates were selected and further analysed using DNA microarray-based technology to detect over 300 different target sequences including antimicrobial resistance and virulence-associated genes, species-specific genes, and SCC*mec*-associated genes [76]. Three isolates were genotyped using MLST as previously described [57]. In addition, whole-genome sequencing, as well as contig assembly and annotation, and comparative genomics were conducted as previously described using Seqsphere+ (Ridom, Münster, Germany) [77–79]. The same software was used for cgMLST [77]. The genomes of four *S. aureus* isolates were submitted under SUB6695668 in the NCBI BioProject database.

5. Conclusions

The present study is the first investigating not only the phenotypic but also the genotypic resistance to antimicrobial agents and biocides in *Staphylococcus* spp. isolated from cases of bovine mastitis in Rwanda. It improves our knowledge about the high diversity of *Staphylococcus* spp., their occurrence in the study area and about the presence of resistance genes.

Due to the rising importance of the dairy production system in Rwanda, improvements in the prevention and treatment of bovine mastitis are critical to prevent misuse of antimicrobial agents and the increase of resistance to antimicrobial agents and biocides, which is in accordance with the 'one world, one health' principle [80].

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3. Summary

Bovine mastitis is an important and cost intensive disease. It affects dairy cattle worldwide and results in high economic losses. *Staphylococcus* spp. play an important role as cause of bovine mastitis.

In the present study, a total of 161 staphylococcal isolates originating from California-Mastitis-Test positive milk samples were phenotypically and genotypically characterized. Milk samples were collected from 112 crossbred dairy cows presenting mastitis and kept in farms located in the Northern and Kigali Province of Rwanda. Milk samples were cultivated on blood agar (Blood Agar Base, Rapid Labs, UK) at the laboratory of the New Vision Veterinary Hospital (NVVH) in Rwanda. Resulting cultures with typical staphylococcal colony appearance were transported to the Institute of Microbiology, University of Veterinary Medicine, in Vienna and species were identified using matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany) and *rpoB* gene sequencing. Forty-three isolates were identified as *Staphylococcus aureus* and among coagulase-negative staphylococci *Staphylococcus xylosus* and *Staphylococcus haemolyticus* were the most frequently identified staphylococcal species. Antimicrobial susceptibility testing was performed by agar disk diffusion and the agar dilution method. PCR was further used to detect the presence of antibiotic resistance genes as well as heavy metal and biocide resistance genes. A high frequency of resistance to penicillin (45,3%) and tetracycline (39,1%) was observed, which was associated with the presence of the genes *blaZ*, *tet*(K), *tet*(L) and *tet*(O). The two *mecA*-positive strains were further genotyped by direct repeat unit (*dru*) typing and *dru*-type *dt10cz* was detected in one isolate. All *Staphylococcus aureus* isolates were examined by different PCRs for the detection of virulence-associated genes. Furthermore, *Staphylococcus aureus* isolates were genotyped by *spa* typing resulting in t1236 being the predominating *spa* type. Using Fourier Transform Infrared (FTIR) spectroscopy, 38 *Staphylococcus aureus* isolates were grouped into clusters and further phenotypically typed for capsule expression. Twenty-two *Staphylococcus aureus* strains were selected based on FTIR results and their assignment to clonal complexes (CC) was determined by DNA-based microarray analysis. Three of these 22 strains were further genotyped by Multi-locus sequence typing (MLST). Using whole-genome sequencing (WGS)

further virulence genes (*hla, hlb, hld, lukD, lukE, lukM, lukF-P83, icaA, icaC, icaD, bap, clfA, clfB, fib, cna, fnbA, fnbB, sasG*) have been found. The toxic shock syndrome toxin gene (*tsst-1*) was detected in two isolates only in combination with other enterotoxin genes including *sei, sem, sen, seo* and *seu.* Two isolates carried panton-valentine leukocidin (PVL) genes (*lukS-PV, lukF-PV*). Four Clonal Complexes were identified, namely CC97, CC3591, CC3666 and CC152. The majority of the selected *Staphylococcus aureus* isolates belong to CC97 and all of them were non-encapsulated. The production of a capsule of serotype 8 was only detected in isolates belonging to CC3591 (n = 3) and capsular serotype 5 was only detected in one CC152 isolate. MLST revealed three new sequence types.

The present study is the first conducted on *Staphylococcus* spp. isolated from bovine mastitis cases in the Northern and Kigali Province of Rwanda. Findings from phenotypic and genotypic resistance testing were in accordance with the frequent use of certain antibiotics for the treatment of bovine mastitis in Rwanda. In-depth characterization of *Staphylococcus aureus* isolated from dairy cattle kept in Rwanda aimed at raising awareness for this pathogen since *Staphylococcus aureus* is not only the most important cause of bovine mastitis but also constitute a potential risk for human health.

4. Zusammenfassung

Mastitis bei Rindern ist eine wichtige und kostenintensive Erkrankung, die weltweit zu hohen wirtschaftlichen Verlusten führt. Staphylokokken spielen eine wesentliche Rolle als Verursacher dieser Erkrankung.

In der vorliegenden Studie wurden 161 *Staphylococcus*-Isolate, die aus California-Mastitis-Test -positiven Milchproben gewonnen werden konnten, phäno- und genotypisch charakterisiert. Die untersuchten Milchproben stammten von 112 an Mastitis erkrankten Milchkühen aus der Northern und Kigali Province in Ruanda. Die Milchproben wurden vorerst in Ruanda im Labor des New Vision Veterinary Hospital (NVVH) auf Blutagarplatten (Blood Agar Base, Rapid Labs, UK) kultiviert und die erhaltenen Kulturen mit typischer Staphylokokken-spezifischer Koloniemorphologie schließlich an das Institut für Mikrobiologie der Veterinärmedizinischen Universität Wien übersandt. Dort wurden die Kulturen mittels matrix-assisted laser desorption ionization – time of flight Massenspektrometrie (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany) und *rpoB*-Sequenzierung artdiagnostisch bestimmt. Dreiundvierzig Isolate konnten als *Staphylococcus aureus* identifiziert und die 118 Koagulase-negativen Staphylokokken größtenteils als *Staphylococcus xylosus* und *Staphylococcus haemolyticus* bestimmt werden.

Zur Überprüfung der Empfindlichkeit wurde der Plattendiffusionstest und die Agardilutionsmethode eingesetzt. Konnten hierbei phänotypische Resistenzen ermittelt werden, wurden die Isolate mittels PCR auf vermittelnde Resistenzgene als auch auf das Vorkommen von Schwermetall- und Biozid-Resistenzgenen überprüft. Am häufigsten konnte dabei eine phänotypische Penicillin- und Tetracyclin-Resistenz (45,3%; 39,1%) beobachtet werden, wofür die Resistenzgene *blaZ*, *tet*(K), *tet*(L) und *tet*(O) verantwortlich waren.

Bei zwei *mecA*-positiven Stämmen wurde außerdem eine direct repeat unit (*dru*)-Typisierung durchgeführt, wobei bei einem Stamm der *dru*-Typ *dt10cz* ermittelt werden konnte.

Alle *Staphylococcus aureus*-Stämme wurden auf Virulenzfaktoren mittels PCR untersucht und der *spa*-Typisierung zugeführt, wobei der *spa*-Typ t1236 am häufigsten nachgewiesen werden konnte. Achtunddreißig *Staphylococcus aureus*-Isolate konnten mithilfe der Fourier-Transform-Infrarotspektroskopie (FTIR) in Cluster eingeteilt und die verschiedenen Kapsel-Serotypen bestimmt werden. Basierend auf den Ergebnissen der FTIR-Spektroskopie wurden

22 Stämme ausgewählt und ihre Zugehörigkeit zu klonalen Komplex (CC) mittels Microarray-Methode bestimmt und schließlich drei von diesen Stämmen mittels Multilokus-Sequenztypisierung (MLST) genotypisiert. Außerdem konnten mittels Gesamtgenom-Sequenzierung weitere Virulenzfaktoren (*hla, hlb, hld, lukD, lukE, lukM, lukF-P83, icaA, icaC, icaD, bap, clfA, clfB, fib, cna, fnbA, fnbB, sasG*) ermittelt werden. So war das toxische Schocksyndrom-Toxin-Gen (*tsst-1*) immer in Kombination mit anderen Enterotoxin-Genen (*sei, sem, sen, seo* und *seu*) bei zwei Isolaten nachzuweisen. Zwei weitere Isolate trugen Panton-Valentine Leukocidin (PVL)-Gene (*lukS-PV*, *lukF-PV*). Vier klonale Komplexe konnten nachgewiesen werden, nämlich CC97, CC3591, CC3666 und CC152. Die Mehrheit der untersuchten Stämme konnte CC97 zugeordnet werden, allerdings wiesen diese Stämme keine Kapselbildung auf. Der Kapseltyp CP8 konnte lediglich bei CC3591-zugehörigen Stämmen (n = 3) und CP5 nur bei einem CC152-Isolat nachgewiesen werden. Mittels MLST konnten drei neue Sequenztypen (ST) ermittelt werden.

Im Rahmen der vorliegenden Studie konnten erstmals Mastitis-assoziierte Staphylokokken-Isolate aus der Ruandischen Northern und Kigali Province untersucht werden. Die Ergebnisse der phäno- und genotypischen Resistenz-Überprüfung bestätigen den routinemäßigen Einsatz bestimmter Antibiotika zur Behandlung von Mastitiden bei Rindern in Ruanda. Die tiefgreifende Charakterisierung von *Staphylococcus aureus* aus Ruandischen Milchkühen dient der Sensibilisierung für die von diesem Erreger ausgehenden Gefahren, wie seiner Bedeutung als wichtigster Mastitiserreger bei Rindern sowie dem potentiellen Gesundheitsrisiko für die Bevölkerung.

5. Abkürzungsverzeichnis

AFLP: amplified fragment length polymorphism

agr: accessory gene regulator

AP-PCR: arbitrarily primed PCR

CC: clonal complex

CMT: California Mastitis Test

CoNS: coagulase-negative *Staphylococcus* spp.

CoPS: coagulase-positive *Staphylococcus* spp.

dru: direct repeat unit

FTIR: Fourier Transform Infrared spectroscopy

MALDI-TOF MS: matrix-assisted laser desorption ionization – time of flight mass spectrometry

MFS: Major Facilitator Superfamily

MIC: minimum inhibitory concentration

MLST: multi-locus sequence typing

MLVA: multiple-locus variable-number tandem repeat analysis

MRSA: methicillin-resistant *S. aureus*

MSCRAMMs: Microbial surface components recognizing adhesive matrix molecules

MSSA: methicillin-susceptible *S. aureus*

NVVH: New Vision Veterinary Hospital

PBP2a: penicillin-binding protein

PFGE: pulsed-field gel electrophoresis

PTSAgs: pyrogenic toxin superantigens

PVL: panton-valentine leukocidin

QACs: quaternary ammonium compounds

RAPD: random amplification of polymorphic DNA

rep-PCR: repetitive element sequence-based PCR

S.: *Staphylococcus*

SCC: somatic cell count

SCC*mec*: Staphylococcal cassette chromosome *mec*

SEs:	Staphylococcal enterotoxins
SLST:	single-locus sequence typing
SMR:	Small Multidrug Resistance
ST:	sequence type
TSST1:	toxic shock syndrome toxin-1
VNTR:	variable number of tandem repeat
WGS:	whole-genome sequencing

6. Accomplishments

Accomplishments including sample collection and isolation of staphylococci were performed by both first authors in collaboration with the NVVH, Rwanda. MALDI-TOF MS and susceptibility testing by agar disk diffusion were performed by both first authors. All other applied methods as well as writing of the manuscript were primarily accomplished by the first authors (PCRs, writing the manuscript) or were performed by other co-authors (some specific PCRs, sequence based methods, DNA-microarray, and FTIR).

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