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Diversity of *Staphylococcus aureus* associated with mastitis from dairy cows in Rwanda

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ABSTRACT

Objectives: The objective of the present study was to examine the diversity of *Staphylococcus aureus* from mastitis milk samples of cows in Rwanda.
Methods: A total of 1080 quarter milk samples from 279 dairy cows were collected in 80 different farms from all five provinces of Rwanda. In total, 135 *S. aureus* isolates were obtained and subjected to genotyping (*spa* typing, DNA microarray, whole-genome sequencing (WGS)), antimicrobial susceptibility testing (AST) and phenotypic profiling by Fourier Transform Infrared (FTIR) spectroscopy (including capsular serotyping).
Results: Resistance to penicillin and/or tetracycline was most frequently observed. Ten sequence types (STs) (ST1, ST151, ST152, ST5477, ST700, ST7110, ST7983, ST7984, ST8320, ST97) belonging to seven clonal complexes (CCs) (CC1, CC130, CC152, CC3591, CC3666, CC705, CC97) were detected. The Panton–Valentine leukocidin (PVL) genes (*lukF-PV/lukS-PV*), the bovine leukocidin genes (*lukM/lukF-P83*) and the human and bovine toxic shock syndrome toxin gene *tst-1* variants were detected. FTIR-based capsular serotyping showed CC-specific differences. Most CC97 (*cap*5 allele) isolates were primarily nonencapsulated (82%), whereas isolates of CC3591 and CC3666 (*cap8* allele) were mostly encapsulated (86.4% and 57.8%, respectively). Our results underline the widespread global distribution of cattle-adapted CC97.

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Conclusion: The presence of CC3591 and CC3666 in bovine mastitis suggests an important role in cattle health and dairy production in Rwanda. The results of the present study support the need for a rigorous One-Health Surveillance program of the bovine–human interface.

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1. Introduction

Bovine mastitis is one of the most prevalent diseases in dairy cattle and influences dairy production worldwide by decreasing the quantity and quality of milk [1,2]. Interaction between animals, environment, microorganisms, and physical or chemical trauma can be the origin of this multifactorial inflammatory disease. A distinction is made between clinical mastitis and subclinical mastitis (SCM). While cows with clinical mastitis show the typical symptoms of an inflammation of the udder together with changes in the composition and physical properties of their milk, cows with SCM do not present any apparent clinical symptoms or changes of their milk. However, cows suffering from SCM and those suffering from clinical mastitis have a lower milk yield [2]. The California Mastitis Test (CMT) on-site can easily detect the increased somatic cell count [3] of such animals.

Clinical mastitis and SCM can be due to *Staphylococcus aureus*, typically causing recurrent or chronic mastitis even in dairy cows from farms with good milking practices [4]. The primary route of transmission of *S. aureus* is cow-to-cow, but also via contaminated equipment used during the milking process. The prevalence of SCM in Rwanda is more than 50% [5]. Aside from reduced milk yields and increased veterinary costs, contaminating bacteria and antimicrobial residues also significantly impact the farmer's economic losses [6]. Although veterinarians and farmers in Rwanda must follow laws and regulations concerning the purchase and usage of veterinary medicine, more than 55% of the farmers reported having purchased and used over-the-counter medication, including antimicrobial agents. Most farmers in this study justified the use of antimicrobial agents to prevent disease and increase the growth of the animals [7].

Bovine mastitis is an important disease in Rwanda. Due to the historical and cultural significance of cows and milk, the economic losses caused by mastitis have even greater importance [8]. Rwanda Vision 2020, a development program recently launched by the government of Rwanda, aimed at transforming the country into a knowledge-based middle-income country by improving agriculture and livestock production [9].

In Rwanda, milk is often consumed and processed raw which increases the risk of milk as a source of diverse infections in humans [8,10,11].

A recent study by our working group characterised antimicrobial resistance, biocide resistance, and virulence-associated genes of *Staphylococcus* species associated with bovine mastitis in the Northern Province of Rwanda [12]. However, information about *S. aureus*-associated bovine mastitis at a national level is still scarce. *S. aureus* is one of the main causes of bovine mastitis worldwide [2]. The aim of the present study was to examine the diversity of *S. aureus* isolates that originated from mastitis milk samples of cows living in all five Provinces in Rwanda.

2. Materials and methods

2.1. Isolation and identification of S. aureus

The study was discussed, and the sampling was approved by the institutional ethics and animal welfare committee of the Research Screening and Ethical Clearance Committee of the College of Agriculture, Animal Science and Veterinary Medicine, University of Rwanda (UR-CAVM, 003/2021/DRI 23.08.2021) in accordance with the Good Scientific Practice guidelines of the Rwandan national legislation.

The study was conducted from November 2021 to August 2022 at the New Vision Veterinary Hospital, Northern Province, Rwanda. A total of 1080 quarter milk samples from 279 dairy cows (mix breed) were collected at 80 different farms throughout the country. Milk samples originated from Northern Province ($n = 380$), Eastern Province ($n = 264$), Western Province ($n = 324$), Southern Province ($n = 100$), and Kigali ($n = 12$). Out of 1080 quarter milk samples, 732 were CMT-positive and were used for the isolation of *S. aureus* with the following distribution: in 55 cows, one quarter, in 61 cows, two quarters, in 45 cows, three quarters, and in 105 cows all quarters were CMT-positive. They were collected as previously described by Antók et al. [12]. The milk samples were transferred to the microbiological laboratory of New Vision Veterinary Hospital (NVVH, https://nvvh.rw/), where bacteriological examinations were performed. Milk samples were cultivated on blood agar (Blood Agar Base, Rapid Labs, UK) supplemented with 5% defibrinated cow blood and on mannitol salt (MS) agar (Mannitol Salt Agar, Rabid Labs, UK). Typical *S. aureus* colonies grown on blood agar and MS agar were re-cultivated on MS agar until a pure culture was obtained. *S. aureus* was afterwards identified by classical bacteriological methods, cryoconserved at −25 °C, and sent to the Institute of Microbiology, University of Veterinary Medicine, Vienna, Austria, for species identification by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany) and further analyses.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed by agar disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) [13]. The following antimicrobial agents were tested: penicillin (PEN 10 units), cefoxitin (FOX 30 µg), ciprofloxacin (CIP 5 µg), amikacin (AMK 30 µg), gentamicin (GEN 10 µg), tetracycline (TET 30 µg), erythromycin (ERY 15 µg), clindamycin (CLI 2 µg), chloramphenicol (CHL 30 µg), trimethoprim-sulfamethoxazole (SXT 1.25/23.75 µg), nitrofurantoin (NIT 300 µg), rifampicin (RIF 5 µg), and linezolid (LZD 30 µg) (Beckton Dickinson (BD); Heidelberg, Germany). *S. aureus* ATCC® 25,923 served as a quality control strain.

2.3. Molecular characterisation of S. aureus

DNA was extracted as previously described by Loncaric et al. [14]. Detection of antimicrobial resistance and virulence genes in all isolates was conducted using a DNA microarray-based technology (INTER-ARRAY Genotyping Kit *S. aureus*, Bad Langensalza, Germany) [15], and visualization of the results was performed as previously described [14]. The *dfrG* gene present in *S. aureus* belonging to CC152 was detected by PCR [16]. All isolates were genotyped by *spa* typing [14]. Nineteen isolates were selected for whole-genome sequencing (WGS) [14] based on their assignment to different

clonal complexes and/or because isolates of the respective CCs have been poorly characterised until now. In addition, the whole genome sequenced isolates were typed with ABRicate (v1.1.0) [17]. The genomes of the isolates subjected to WGS were deposited to the BioSample database under the SubmissionID: SUB13788009 in the NCBI database.

Fourier Transform Infrared (FTIR) spectroscopy was used to subtype all isolates phenotypically, primarily based on their surface glycostructural composition that included the determination of the capsular polysaccharide (CP) expression/capsule serotype [18,19]. The *S. aureus* Reynolds prototype strain CP5 and its corresponding mutants, Reynolds CP8 and Reynolds CP- (nonencapsulated) (REF), were employed as reference samples for FTIR spectroscopic biotyping [20].

3. Results

3.1. S. aureus *isolates and antimicrobial susceptibility testing*

A total of 135 *S. aureus* isolates were obtained from 81 different cows (Table 1, Table S1), including 47 isolates from the Northern Province (26/107 cows, 24.3%), 39 from the East Province (24/66 cows, 36.4%), 37 from the West Province (24/81 cows, 29.6%), 11 from the South Province (6/25 cows; 24.0%), and one *S. aureus* isolate from Kigali (1/3 cows; 33.3%).

All isolates were susceptible to cefoxitin. The most frequently observed resistance property was combined resistance to penicillin and tetracycline ($n = 49$, 36.3%), followed by resistance to penicillin ($n = 46$, 34.1%), and resistance to tetracycline ($n = 4$, 3.0%). Thirty-six isolates were susceptible to all antimicrobial agents tested (26.7%). Genetic resistance matched the phenotypic resistances of the respective isolates. The *blaZ* gene was observed in penicillin-resistant isolates, whereas resistance to tetracycline was mediated by the *tet*(K) gene. In addition, the fosfomycin resistance gene *fosB* gene was detected in three isolates (Table 1). The phenotypic fosfomycin resistance was not examined due to the lack of clinical breakpoints. The *str* gene conferring resistance to streptomycin was detected in five isolates selected for WGS: one assigned to CC3591, one to CC3666 and the remaining three to the new sequence type (ST) 7984. The trimethoprim resistance gene *dfrG* was detected in five isolates belonging to clonal complex 152 (Table 1).

3.2. *Molecular characterization of isolates and FTIR-based capsule serotyping*

Clonal complex (CC) 97 was the most common CC ($n = 50$, 37.0%), followed by CC3666 ($n = 45$, 33.3%), CC3591 ($n = 22$, 16.3%), CC152 ($n = 8$, 5.9%), CC1, ($n = 5$, 3.7%), CC130 and CC705 (each $n = 1$, 0.7%).

Within CC97, 11 different *spa* types were identified. The *spa* type t9432 ($n = 14$) was predominant, followed by t2421 ($n = 7$), t10103 ($n = 6$), t3992 ($n = 5$), t7753 ($n = 4$), t1236 ($n = 4$), t2112 ($n = 3$), novel *spa* type t20843 ($n = 2$), t380 ($n = 2$), t9298 ($n = 2$), and t6280 ($n = 1$). Two CC97 isolates, chosen for WGS, belonged to ST97 or the new ST8320, respectively.

The second most common CC was CC3666. Within this CC, three different *spa* types were detected: t18853 ($n = 40$), novel *spa* type t20842 ($n = 4$) and novel *spa* type t20844 ($n = 1$). Two of the whole genome sequenced isolates belonged to ST5477.

All 22 CC3591 isolates represented *spa* type t458. WGS revealed that five examined isolates belonged to the novel sequence types ST7983 ($n = 3$) or ST7110 ($n = 2$). Eight isolates belonged to CC152 and were indistinguishable by *spa* typing (t355), and four sequenced isolates represented ST152. Five isolates were assigned to CC1 and *spa* type t127. In addition, ST1 was detected in one isolate selected for WGS. Finally, two singletons belonged to either CC705, *spa* type t529 and ST151, or CC130, *spa* type t3569 and ST700. Three sequenced isolates represented ST7984 and an independent CC (Table 1, Table S1).

DNA-based microarray analyses, as well as WGS, revealed the presence of several virulence-associated genes. Within CC97, five isolates carried the toxic shock syndrome toxin 1 gene *tst-1* (human variant). All but one CC3666 isolate harboured the toxic shock syndrome toxin 1 gene *tst-1* (bovine variant). All CC3666 isolates carried the enterotoxin gene cluster (*egc*) comprising the enterotoxin genes *sei, sem, sen* (other than RF 122), *seo*, and *seu*. In addition, three isolates carried the enterotoxin B gene (Table 1, Table S1). The presence of the bovine leukocidin genes *lukF-P83/lukM* was observed in all isolates belonging to CC3591. The Panton-Valentine leukocidin (PVL) genes *lukF-PV/lukS-PV* were detected in 3 of 7 CC152 isolates. All five isolates assigned to CC1 carried the gene for enterotoxin H, and three also had the gene for enterotoxin B. The single CC130 isolate harboured the bovine leukocidin genes *lukF-P83/lukM*. The same genes were detected in the single CC705 isolate. In addition to the *lukF-P83/lukM* genes, this CC705 isolate also harboured the *tst-1* gene (bovine variant), the *egc* cluster, as well as the enterotoxin C and enterotoxin L genes. All isolates belonging to CC1 harboured *seh,* whereas three CC1 isolates carried additional *seb* genes coding for the staphylococcal enterotoxins H and B, respectively (Table 1, Table S1). Isolates belonging to all CCs harboured hemolysin *hla, hlb* and *hld* genes. Splits tree analysis of the microarray data showed clonal clustering into eight groups based on their virulence and antimicrobial resistance profile similarities (Fig. 1).

Core-genome MLST (cgMLST)-based typing, including the 19 isolates selected for WGS, was performed, and a minimum spanning tree was generated (Fig. 2). Distance calculation between all isolates revealed an allelic distance ranging between 0 and 1509 allelic differences. Based on the defined cluster threshold (CT) of 24 allelic differences, three different clusters were detected. Two clusters comprised isolates within CC152 and the other cluster isolates within CC3591.

All isolates were phenotypically subtyped for capsule polysaccharide (CP) expression using FTIR spectroscopy (Fig. 3). In total, more than half of the isolates had no capsule ($n = 75$; 55.6%), and the remaining isolates were categorised into two serotypes: serotype 8 (CP8, $n = 45$; 33.3%) and serotype 5 (CP5, $n = 12$; 8.9%). Most isolates with the *cap5* allele were nonencapsulated (78.3%), whereas isolates with *cap8* were often encapsulated CP8 (60.0%). Notably, the distribution of CP expression varied depending on the CC. CC3591 and CC3666 isolates harbouring the *cap8*-allele predominantly expressed the capsule 86.4% ($n = 22$) and 57.8% ($n = 45$), respectively. In contrast, CC97 isolates ($n = 50$) were primarily nonencapsulated (82.0%). Hierarchical cluster analysis of the FTIR spectral data revealed seven main clusters. Clusters 1 to 3 contain encapsulated isolates and can be assigned to specific CCs (Cluster 1: CC3591, CP8; Cluster 2: CC3666, CP8; Cluster 3: CC151, CP5). The two CP8-producing CCs (CC3591 and CC3666) can be clearly distinguished using FTIR spectroscopy. The remaining clusters (4 to 7) comprise mainly nonencapsulated strains, each with a mixture of various CCs, except for one cluster (cluster 7) assigned to CC97. Interestingly, the three isolates of the newly described ST7984 cluster were closely related to CC97 isolates (Fig. 3).

4. Discussion

The present study describes the characterization of *S. aureus* isolated from mastitis milk samples in Rwanda. Even though cattle play an essential role in the life of the people in Rwanda, comprehensive studies on *S. aureus*-associated mastitis considering the pheno- and genotypic characterization of the causative isolates and

Journal of Global Antimicrobial Resistance 36 (2024) 326–335

Table 1
Summarised molecular characterization, antimicrobial resistance and toxins profile of the *Staphylococcus aureus* isolates investigated.

Isolates	ID alias	Province*	CC**	ST***	*spa*	Antimicrobial resistance profile		Superantigens	Leukocidins
						Phenotype	Genes detected		
151FL, 168FR		W	CC97		t380	nr****			*lukF/S, lukD/E*
167FL, 167FR, 167RL, 168FL, 169FL		W	CC97		t3992	nr			*lukF/S, lukD/E*
188RR		N	CC97		t6280	nr			*lukF/S, lukD/E*
219RR		W	CC97		t7753	nr			*lukF/S, lukD/E*
220FR, 227FL, 227FR		W	CC97		t7753	nr			*lukF/S, lukD/E*
111FL	510,580–22	S	CC97	ST8320	t9432	PEN	*blaZ*	*tst-1* (human)	*lukF/S, lukD/E*
111RR, 111FR, 115RL, 116bfR		S	CC97		t9432	PEN	*blaZ*	*tst-1* (human)	*lukF/S, lukD/E*
128FL		E	CC97		t2421	PEN	*blaZ*		*lukF/S, lukD/E*
12RRb		N	CC97		t9432	PEN	*blaZ*		*lukF/S, lukD/E*
134RR		E	CC97		t9432	PEN	*blaZ*		*lukF/S, lukD/E*
135RL		E	CC97		t2112	PEN	*blaZ*		*lukF/S, lukD/E*
14FL		N	CC97		t1236	PEN	*blaZ*		*lukF/S, lukD/E*
144FR		W	CC97		t1236	PEN	*blaZ*		*lukF/S, lukD/E*
151RL, 151RR		W	CC97		t9298	PEN	*blaZ*		*lukF/S, lukD/E*
84FL, 84RL, 88FL		E	CC97		t10103	PEN	*blaZ*		*lukF/S, lukD/E*
123FR, 123RL, 125RR, 127FL, 127RL, 129RR		E	CC97		t2421	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
137FL		E	CC97		t9432	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
140FL, 142RL		E	CC97		t2112	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
147RL, 251FR		W	CC97		t10103	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
149FL, 149RL		W	CC97		t20843	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
150FL, 15RR		W	CC97		t9432	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
153RR		W	CC97		t10103	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
191RL		N	CC97		t1236	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
225RR		W	CC97		t1236	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
7FL2, 7FRa		N	CC97		t9432	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
7RL	510,581–22	N	CC97	ST97	t9432	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
9FL1		N	CC97		t9432	PEN, TET	*blaZ, tet*(K)		*lukF/S, lukD/E*
35FR, 15FL, 15RL		N	CC3666		t18853	nr		*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
185FR, 192RR, 210FL, 12FL, 15RR, 12RRa		N	CC3666		t18853	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
186RL		N	CC3666		t20844	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
222RL		W	CC3666		t18853	PEN	*blaZ*	egc, sei, selm, seln (other than RF122), *selo, selu*	*lukF/S, lukD*
226RL, 254RR		W	CC3666		t18853	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
227RR	510,578–22-WH	W	CC3666	ST5477	t18853	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
65FR, 87FL		E	CC3666		t18853	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
70FL, 70FR, 72FL, 72FR		E	CC3666		t20842	PEN	*blaZ*	*tst-1* (bovine), *seb, egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
85RR		E	CC3666		t18853	PEN	*blaZ*	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
107FL, 117FL, 117FR, 117RR		S	CC3666		t18853	PEN, TET	*blaZ, tet*(K)	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
136RL2		E	CC3666		t18853	PEN, TET	*blaZ, tet*(K)	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
152RL, 152RR/FL		W	CC3666		t18853	PEN, TET	*blaZ, tet*(K)	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*
16FR	510,577–22-WH	N	CC3666	ST5477	t18853	PEN, TET	*blaZ, tet*(K)	*tst-1* (bovine), *egc, sei, selm, seln* (other than RF122), *selo, selu*	*lukF/S, lukD*

(*continued on next page*)

Table 1 (*continued*)

Isolates	ID alias	Province*	CC**	ST***	*spa*	Antimicrobial resistance profile		Superantigens	Leukocidins
						Phenotype	Genes detected		
207FL, 207RL, 207RR, 208RL, 50FR, 6RR, 7FL1, 8FRb, 9RR		N	CC3666		t18853	PEN, TET	*blaZ*, *tet*(K)	*tst-1* (bovine), *egc*, *sei*, *selm*, *seln* (other than RF122), *selo*, *selu*	*lukF/S*, *lukD*
56FR, 56RR, 58FR, 65FL, 76FR, 90FL		E	CC3666		t18853	PEN, TET	*blaZ*, *tet*(K)	*tst-1* (bovine), *egc*, *sei*, *selm*, *seln* (other than RF122), *selo*, *selu*	*lukF/S*, *lukD*
69FL		Kigali	CC3666		t18853	PEN, TET	*blaZ*, *tet*(K)	*tst-1* (bovine), *egc*, *sei*, *selm*, *seln* (other than RF122), *selo*, *selu*	*lukF/S*, *lukD*
131FR, 133FL, 133RR, 135RR		E	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
150RL, 172RR		W	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
200FL, 207FLa		N	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
220FL		W	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
254FL	510,574–22	W	CC3591	ST7983	t458	nr			*lukF/S*, *lukF-P83/lukM*
27FL	510,575–22	N	CC3591	ST7983	t458	nr			*lukF/S*, *lukF-P83/lukM*
27RR, 29FL, 29RR, 7RR, 8FRa, 7FRb		N	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
10RL	510,582–22	N	CC3591	ST7983	t458	nr			*lukF/S*, *lukF-P83/lukM*
6FR		N	CC3591		t458	nr			*lukF/S*, *lukF-P83/lukM*
116aFR		S	CC3591		t458	TET	*tet*(K)		*lukF/S*, *lukF-P83/lukM*
133RL	510,572–22	E	CC3591	ST7110	t458	TET	*tet*(K)		*lukF/S*, *lukF-P83/lukM*
134FR	510,573–22	E	CC3591	ST7110	t458	TET	*tet*(K)		*lukF/S*, *lukF-P83/lukM*
100FL		S	CC152		t355	PEN	*blaZ*		*lukF*
218RL		W	CC152		t355	PEN	*blaZ*		*lukF*
240RL		W	CC152		t355	PEN	*blaZ*, *dfrG*		*lukF*, *lukF-PV/lukS-PV*
240RR	510,568–22	W	CC152	ST152	t355	PEN	*blaZ*, *dfrG*		*lukF*, *lukF-PV/lukS-PV*
247RL		W	CC152		t355	PEN	*blaZ*, *dfrG*		*lukF*, *lukF-PV/lukS-PV*
34FL	510,570–22	N	CC152	ST152	t355	PEN	*blaZ*, *dfrG*		*lukF*, *lukF-PV/lukS-PV*
35RL	510,571–22	N	CC152	ST152	t355	PEN	*blaZ*, *dfrG*		*lukF*, *lukF-PV/lukS-PV*
248FR	510,569–22	W	CC152	ST152	t355	TET	*tet*(K), *dfrG*		*lukF*, *lukF-PV/lukS-PV*
183FL	510,566–22 -WH	N	CC1	ST1	t127	PEN	*blaZ*	*seb*, *seh*	*lukF/S*, *lukD/E*
183FR, 183RL		N	CC1		t127	PEN	*blaZ*	*seb*, *seh*	*lukF/S*, *lukD/E*
184FL, 188FL		N	CC1		t127	PEN	*blaZ*	*seh*	*lukF/S*, *lukD/E*
136RL1	510,584–22	E	CC (in.)§	ST7984	t21056	PEN	*blaZ*, *tet*(K), *str*		*lukF/S*, *lukD/E*
127FR/RR	510,583–22	E	CC (in.)	ST7984	t20974	PEN, TET	*blaZ*, *tet*(K), *str*		*lukF/S*, *lukD/E*
76FL	510,585–22	E	CC (in.)	ST7984	t21056	PEN, TET	*blaZ*, *tet*(K), *str*		*lukF/S*, *lukD/E*
211RL	510,567–22	N	CC130	ST700	t3569	nr			*lukF/S*, *lukF-P83/lukM*, *lukD*
58FL	510,579–22	E	CC705	ST151	t529	nr		*tst-1* (bovine), sec, *sel*, *egc*, *seg*, *sei*, *selm*, *seln*, *seln* (other than RF122), *selo*, *selu*	*lukF/S*, *lukF-P83/lukM*, *lukD/E*

* W, Western; N, Northern, E, Eastern, S, Southern.
** CC, clonal complex.
*** ST, sequence type.
**** nr, nonresistant, PEN, penicillin, TET, tetracycline.
§ (in.), independent.

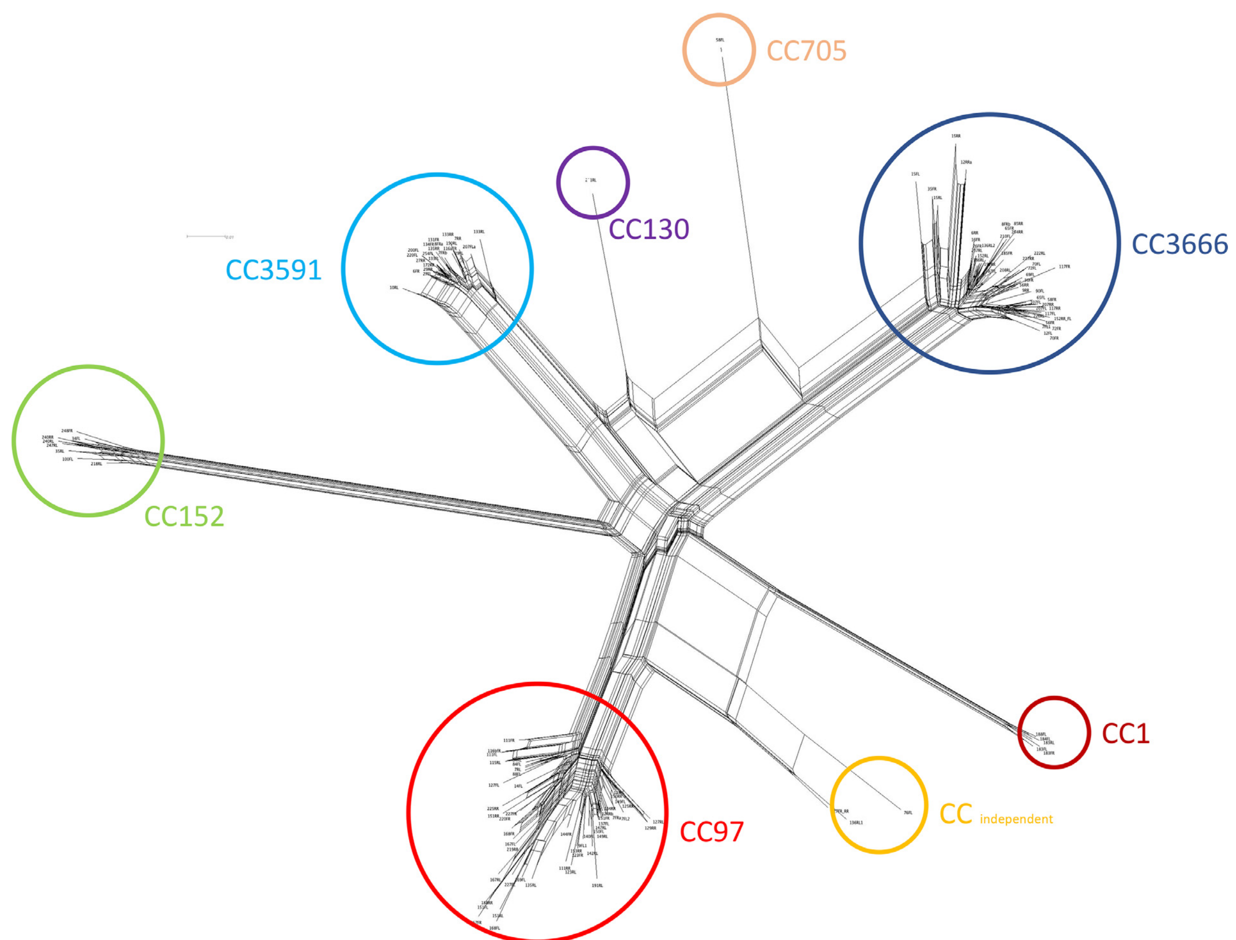

Fig. 1. SplitsTree network of *Staphylococcus aureus* isolates examined.

their antimicrobial resistance properties are still rare. In 2019, our working group (Antok et al., 2019) identified *S. aureus* belonging to four CCs (CC97, CC3591, CC3666, CC152). *S. aureus* isolates belonging to these CCs were also detected in a recent study dealing with the genetic characterization of isolates using WGS originating from dairy cows in Rwanda [21].

CC97 was found to be the predominant clonal lineage comprising 50 isolates. Humans were presumably the original host of this CC [22]. After the human-to-cattle host jump, CC97 became the most prevalent bovine *S. aureus* clone globally [22], but it is also known to switch back into humans potentially [22,23]. CC97 is strongly associated with bovine mastitis [22]. The majority of the CC97 isolates in the present study were resistant to penicillin (*blaZ*) in combination with tetracycline (*tet*(K), which is in accordance with our previous study (Antok et al., 2019) and the recent study of Ndahetuye et al. [21]. Resistance to penicillin and/or tetracycline is also common in CC97 isolates originating from bovine mastitis in other countries [24,25]. Five CC97 isolates belonging to *spa* type t9432 harboured a human variant of the toxic shock syndrome toxin 1 gene *tst-1*. The presence of the *tst-1* gene in CC97 of bovine origin is uncommon [25,26]. The *tst-1* genes in CC97 isolates were not associated with superantigen genes, such as the staphylococcal enterotoxin C and L genes, which are part of the pathogenicity island SaPIbov carrying sec/*tst-1*/*sel* toxin genes [27]. All CC97 isolates harboured the leukocidin genes *lukD/E*, which are prevalent in CC97 of bovine origin [24,26,28], but their role in the pathogenesis of bovine mastitis is still not entirely known [2,28]. Confirming previous studies, we could not detect capsular expression in most CC97 isolates. CC97 strains (*cap5-agrI*) are considered to be able to survive and persist in the intracellular niche [29].

The second most prevalent clonal complex was CC3666. To the author's best knowledge, *S. aureus* belonging to CC3666 was isolated solely from bovine mastitis cases in Rwanda (Antok et al., [21]) and in the neighbouring country Tanzania [30]. As for CC97, most CC3666 isolates were resistant to penicillin or the combination of penicillin and tetracycline due to the presence of *blaZ* and *tet*(K) genes. This is in accordance with previous studies (Antok et al., [21,30]). In contrast to CC97, isolates belonging to CC3666 are much less diverse. All but five isolates belonged to the same *spa* type t18553, and amongst the remaining five, two novel *spa* types, t20842 and t20844, were observed. The main characteristic of the CC3666 isolates is that all but one isolate carried a bovine variant of the toxic shock syndrome toxin 1 gene *tst-1*, which is common amongst mastitis isolates [12,31]. In addition, all isolates harboured the enterotoxin genes *sei, sem, sen, seo,* and *seu*, which belonged to the *egc* cluster, and all isolates were positive for the *lukD* gene but negative for the *lukE* gene. These characteristics were observed in CC3666 from our previous study as well as those from Tanzania (Antok et al., [30]). Ndahetuye et al. [21] did not report virulence-associated genes, but after comparing their whole genome sequences, the same characteristics could be detected. Data on the prevalence of capsular expression of the clonal complexes CC3666 and CC3591 have not yet been published. We found the majority of CC3666 isolates were encapsulated. Since this clonal complex can be assigned to the *agr* type II (*agr*II), it is tempting to speculate that CC3666 isolates, like CC705, are better adapted to the extracellular niche in the udder environment

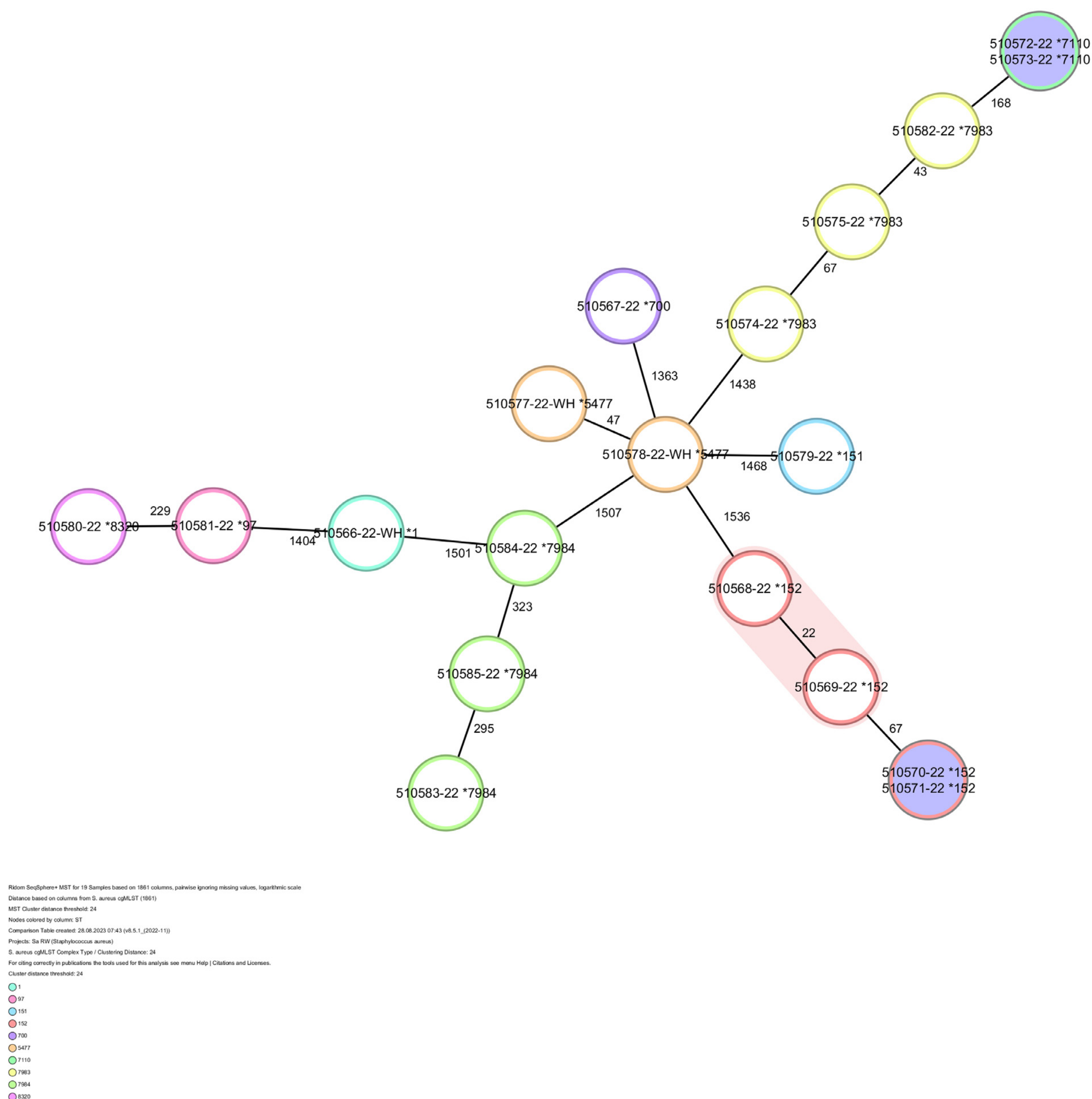

Fig. 2. Minimum spanning tree for 135 isolates based on the cgMLST of *S. aureus*. Colours correspond to the clonal complex. Each circle represents isolates with an allelic profile based on the sequence of 1861 core genome targets. Black numbers refer to the allelic differences between two isolates. Isolates with closely related genotypes were identified with a maximum of 24 allelic differences and are shaded. Each circle is marked with an alias ID and separated with "*" from the sequence type.

[29]. Since we could only detect one CC705 isolate, one might even speculate that CC3666 isolates have adopted the niche of CC705 isolates.

Due to the omnipresence of CC3666 in all provinces of Rwanda, this particular CC could emerge as an important clonal lineage associated with *S. aureus* mastitis in cattle. The same applies to *S. aureus* isolates belonging to CC3591. This clonal lineage was isolated only from bovine mastitis cases in Rwanda (Antok et al., [21]). All CC3591 isolates carried the bovine leukocidin *lukF-P83/lukM* genes, which were also observed in all analysed strains of this clonal lineage in the past (Antok et al., [21]). The *lukF-P83/lukM* genes are strongly associated with cytotoxicity to bovine neutrophils [32].

The Panton-Valentine leukocidin (PVL) genes were detected in six out of eight CC152 isolates. CC152 is a common human-associated clonal lineage from sub-Saharan Africa [33]. *S. aureus* isolates of *spa* type t355 and/or sequence type ST152 within CC152 were identified in previous studies on cows from Rwanda and Tanzania (Antok et al., [21,30]), but also across sub-Saharan Africa from humans [33]. We also observed the presence of the trimethoprim resistance gene *dfrG* in five PVL-positive isolates. As previously reported, in some CC152 isolates, *dfrG* is located in the PVL-encoding prophage φSa2 [33]. The existence of PVL and *sak/scn* genes also suggests a human origin.

Five isolates represented CC1. This CC1 is another human-associated clonal lineage with global distribution [22]. CC1 isolates are commonly found in livestock (including cases of bovine mastitis), companion animals, and wildlife [2,23]. Until present, CC1 bovine mastitis isolates have been observed in the Southern Province of Rwanda [21], whereas all five isolates of the present study were from the Northern Province.

The role of the three isolates belonging to new *spa* types t20974 and t21056 and the new ST7984 in the pathogenesis of bovine mastitis is currently uncertain. These were only isolates carrying the fosfomycin resistance gene *fosB*. The leukocidin *lukD/E* genes

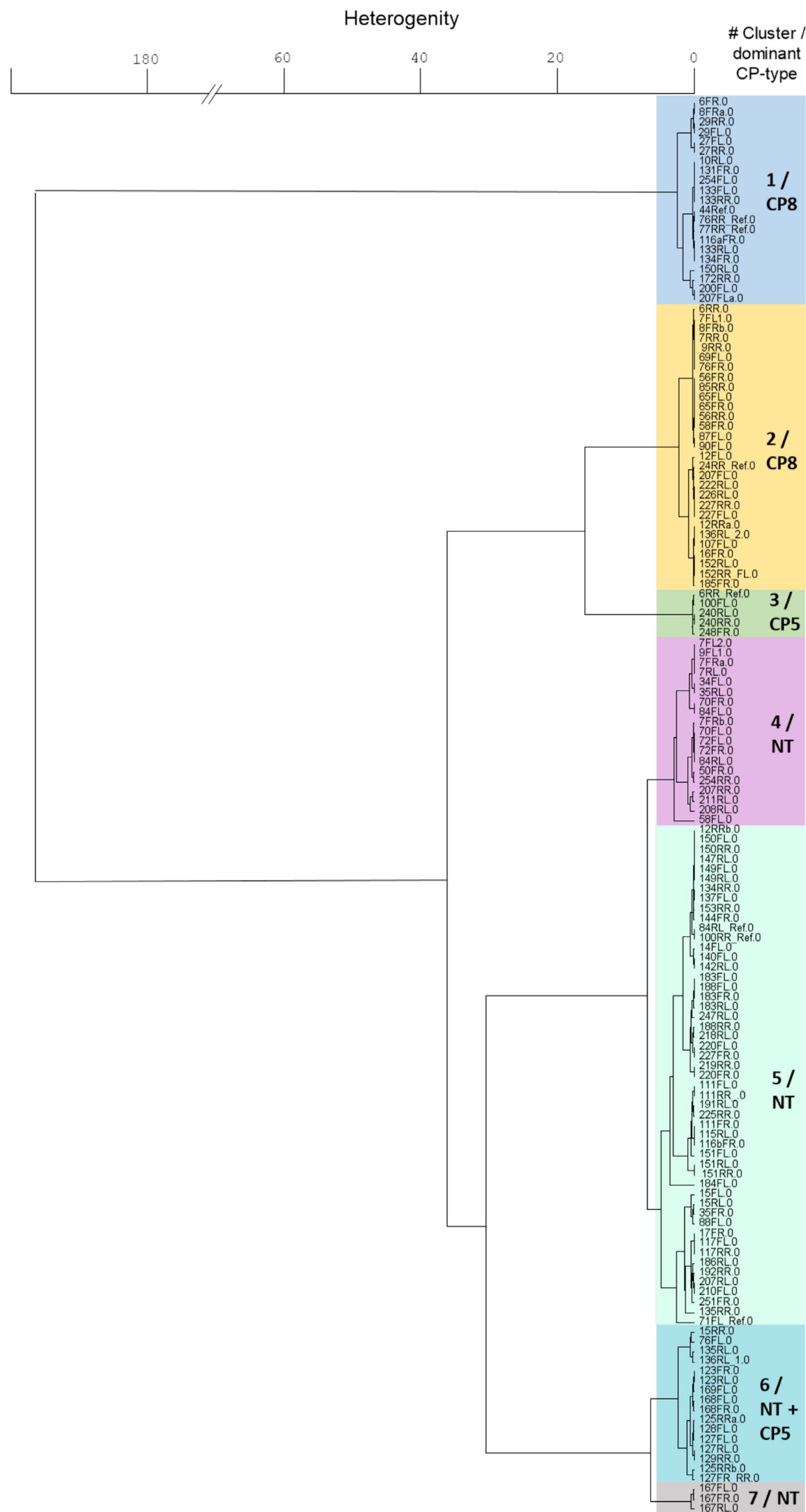

Fig. 3. FTIR spectroscopy-based dendrogram of *S. aureus* isolated from milk samples of cows with mastitis. Encapsulated: CP5 = serotype 5, CP8 = serotype 8; nonencapsulated: NT = none typeable.

were observed, but genes coding for staphylococcal enterotoxins could not be detected.

Finally, the two singletons belonging to CC130 and CC705 were observed for the first time in Rwanda in mastitis milk samples. CC130 is most likely of ovine origin with a subsequent spillover from humans into dairy cows [22]. Bovine CC130 is common in Europe and often harbours the *mecC* gene [22]. Both singletons carried *lukF-P83/lukM* genes. In addition, the worldwide common bovine CC705 was positive for the *egc* cluster, sec and *sel* genes, and *tst-1* (bovine variant), which may induce mammary inflammation [2].

5. Conclusion

In conclusion, a low diversity of bovine mastitis-associated *S. aureus* isolates was observed. More than 86.7% of all isolates belonged to CC97, CC3666 and CC3591. While bovine CC97 shows global dissemination, the data of the present study suggest that CC3666 and CC3591 either have emerged in Eastern Africa or, most likely, have been present there in the past but, due to paucity of data, remained unrepresented in the scientific literature. Whether these CCs will remain only in Eastern Africa or will become important global bovine lineages should be monitored, especially due to their virulence potential. The occurrence of human-associated CCs, like CC152, suggests interspecies transmission, which emphasizes the importance of hygiene measures and supports the need for a rigorous One-Health Surveillance program of the bovine–human interface. In addition, the presence of *S. aureus* isolates in milk is of public health concern in Rwanda, as *S. aureus*-positive tank and raw milk products could serve as vehicles for the bacterial transmission to humans.

Ethical approval: The study was discussed, and the sampling was approved by the institutional ethics and animal welfare committee of the Research Screening and Ethical Clearance Committee of the College of Agriculture, Animal Science and Veterinary Medicine, University of Rwanda (UR-CAVM, 003/2021/DRI 23.08.2021) in accordance with the Good Scientific Practice guidelines of the Rwandan national legislation.

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Competing interests: None to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2024.01.017.

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