European Journal of Clinical Microbiology & Infectious Diseases
https://doi.org/10.1007/s10096-021-04286-3

ORIGINAL ARTICLE

Epidemiology of the *Staphylococcus aureus* CA-MRSA USA300 in Belgium

Maria A. Argudín[1,2] · Ariane Deplano[1] · Claire Nonhoff[1] · Nicolas Yin[1] · Charlotte Michel[1] · Delphine Martiny[1] · Sigrid C. J. De Keersmaecker[3] · Marie Hallin[1]

Received: 24 April 2021 / Accepted: 2 June 2021

Abstract

The methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST) 8 Panton-Valentine toxin (PVL)-positive USA300 clone has a worldwide distribution. The USA300 North American (NA) variant, harbouring the arginine catabolic mobile element (ACME), is predominant in the USA while the Latin American (LV) variant is predominant in Northern South America. Both variants have failed to become endemic in Europe. We examined here the epidemiology of the USA300 clone in Belgium from 2006 to 2019. A total of 399 clonal complex 8 PVL-positive MRSA isolates received between 2006 and 2019 by the Belgian National Reference Laboratory for *S. aureus* were investigated for the presence of ACME. Selected ACME-positive (n=102) and ACME-negative (n=16) isolates were sequenced, characterized for the presence of several resistance and virulence molecular markers and subjected to phylogenetic analysis. A total of 300 isolates were USA300-NA (ACME-positive), while only 99 were ACME-negative. Most USA300-NA interspersed in the phylogeny analysis with isolates from other countries, suggesting multiple introductions. However, two big clades were maintained and spread over a decade, peaking between 2010 and 2017 to finally decrease. Few ACME-negative isolates, mainly related to trips to South America, were identified as USA300-LV. The remaining ACME-negative isolates were ST8 SCC*mec* IVb or ST923 SCC*mec* IVa (COL923). Two clades of the USA300-NA clone have successfully spread in Belgium, but seem to currently decrease. Related South American variants have been detected for the first time in Belgium, including the emerging COL923 clone.

Keywords ACME · COMER · ST923 · WGS

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) first emerged in the early 60s and rapidly became a leading cause of nosocomial infections. Since the mid-90s, MRSA has also increasingly been identified as a cause of community-acquired (CA) skin and soft tissue infections and pneumonia, in patients lacking health-care associated risk factors [1]. CA-MRSA usually differ from hospital-acquired (HA) MRSA by the fact that they carry small staphylococcal cassette chromosome *mec* (SCC*mec*) types (type IV or V), and a phage encoded toxin called Panton-Valentine Leucocidin (PVL). Several CA-MRSA clones have been identified worldwide, mainly belonging to clonal complexes (CC) 8, 30 and 80 by multilocus sequence typing (MLST) [1]. Nevertheless, their epidemiology varies largely. In some parts of the world, CA-MRSA remained, at a relatively low prevalence, associated with small community outbreaks. In other regions, such as the USA, CA-MRSA became endemic, even spreading in the healthcare setting, blurring thereby the lines between HA- and CA-MRSA [2].

First reported in 1999, in a Mississippi jail [2], the USA300 is a particularly successful PVL-positive CA-MRSA clone that rapidly became endemic in North America (USA300-NA), accounting for up to 90% of MRSA infections in some areas [3]. This clone, originally defined on the basis of its pulsed-field gel electrophoresis (PFGE) pattern, is characterized by specific genetic features: *spa*-types associated to the

✉ Maria A. Argudín
info@mrsa.be; maria.argudin@uclouvain.be

[1] Department of Microbiology, Laboratoire Hospitalier Universitaire de Bruxelles (LHUB-ULB), CNR Staphylocoques, Route de Lennik 808, 1070 Brussels, Belgium

[2] Current address: Molecular Biology Laboratory, Tour R. Franklin, Cliniques Universitaires Saint Luc UCL, Brussels, Belgium

[3] Transversal activities in Applied Genomics, Sciensano, Brussels, Belgium

Published online: 23 June 2021

ST8/CC8, possession of SCC*mec* IVa, PVL, the arginine catabolic mobile element (ACME), a specific mutation in capsular polysaccharide gene *cap5E* (leading to the lack of a functional capsular polysaccharide) and the pathogenicity island SaPI5 (carrying the enterotoxin-like genes *selk* and *selq*) [2, 4]. In 2005, a variant called USA300 Latin American Variant (LV), as opposed to the original USA300-NA emerged in the Northern region of South America (Colombia). USA300-LV shares common genetic features with USA300-NA such as *spa*-types associated to ST8/CC8 and the possession of PVL. Nevertheless, the USA300-LV carries SCC*mec* IVc and, instead of the ACME, harbours a copper and mercury resistance mobile element known as COMER [2, 5].

Epidemiological data revealed that USA300 has a great potential for household transmission [6] and is very successful at spreading rapidly [2]. Despite its success in Northern America, and the fact that USA300-NA isolates were reported across Europe (Austria, Denmark, England, France, Germany and Switzerland) as a result of multiple introductions, USA300-NA spread remained limited in the general European population [7–9]. Similarly, USA300-LV has only been detected sporadically in Europe (Switzerland [10, 11], Germany [8]). CA-MRSA epidemiology in Europe is characterized by a high heterogeneity, the "European" ST80-SCC*mec* IV being the predominant clone [1]. In Belgium, the first PVL-positive MRSA were reported in 2002. The proportion of USA300-NA among PVL-positive MRSA raised from 0% in 2005 to 33% in 2009 in Belgium, when USA300-NA became as frequent as the European clone [12]. More recently, USA300-NA has been responsible of an outbreak in a Belgian hospital [13]. The objective of the present study was to update these Belgian data by following the demographic characteristics and molecular epidemiology of CC8 PVL-positive CA-MRSA reported cases from 2006 to 2019.

Methods

Bacteria collection and identification of ST8-PVL isolates

Belgian microbiology laboratories (n~250) are invited by the National Reference Laboratory for *S. aureus* (NRLS) to refer all their CA-MRSA strains (defined as strains isolated from ambulatory patients or during the 48 first hours of hospitalization) for detection of exotoxin genes. Each isolate has to be sent with an anonymized case report form describing demographic and clinical mandatory information: sex and age and postal code of the patient, clinical site and date of sampling. Additional optional information fields include history of hospitalization or travel during the year preceding the sampling and if the case is sporadic or part of a cluster.

All *S. aureus* isolates received are prospectively analyzed by PCR for identification (16S rRNA gene region specific for the *Staphylococci* genus and *nuc*) and for detection of *mecA* and PVL genes [12]. MRSA PVL-positive isolates are further subjected to PCR for detection of the *arcA* gene, used as a marker of the presence of the ACME locus, and to *spa*-typing [12]. The *spa*-types are analyzed by the BURP (based upon repeat pattern) algorithm with Ridom StaphType software (now available as Ridom SeqSphere+) using default parameters [14]. We selected PVL-positive MRSA isolates received from 2006 to 2019 assigned by this algorithm to CC8. These CC8 PVL-positive isolates were classified by the *arcA* gene PCR as (1) USA300-NA if *arcA*-positive, (2) CC8 PVL-positive ACME-negative if *arcA*-negative.

Antimicrobial susceptibility is also prospectively determined using the dis diffusion method for the following antibiotics: cefoxitin, chloramphenicol, clindamycin, ciprofloxacin, erythromycin, fusidic acid, gentamicin, kanamycin, linezolid, minocycline, mupirocin, rifampicin, tetracycline, tobramycin and trimethoprim/sulfamethoxazole. As interpretation is done according to available EUCAST breakpoints at the time [15], antibiograms of strains isolated before 2012 were re-interpreted according current EUCAST breakpoints for this study.

Whole genome sequencing (WGS)

One hundred and twenty-six isolates were selected for WGS analysis, including 102 isolates categorized as USA300 (MRSA CC8 PVL-positive ACME-positive) and 16 MRSA CC8 PVL-positive ACME-negative isolates. The selection was made to encompass to the maximum the diversity observed in the NRC collection regarding the following criteria: (i) antimicrobial resistance profile; (ii) geographical origin (11 Belgian regions); and (iii) isolation year (2006 (n=1), 2007 (n=2), 2008 (n=8), 2009 (n=12), 2010 (n=13), 2011 (n=7), 2012 (n=12), 2013 (n=13), 2014 (n=12), 2015 (n=13), 2016 (n=8), 2017 (n=13), 2018 (n=4)). Additionally, 14 isolates were selected as representative isolates of eight known outbreaks (one or two isolates per outbreak - per year, see Table S1). Finally, eight CC8 PVL-negative isolates from the NRLS collection were also subjected to WGS, acting as out-group (Table S2). DNA extraction was performed by the semi-automated MagCore platform. Sequencing libraries were constructed using the Illumina Nextera XT DNA sample preparation kit and subsequently sequenced on a MiSeq instrument with a 250-bp paired-end protocol (MiSeq v3 chemistry) according to the manufacturer's instructions. All raw reads have been deposited in the National Center for Biotechnology Information (NCBI) under BioProject accession number PRJNA639672. De novo assembly was performed with the SPAdes algorithm [16] by using the calculation engine of the BioNumerics software (version 7.6; Applied Maths).

Table 1 *spa*-types, demographics and antimicrobial resistances observed among 399 MRSA CC8 PVL-positive isolates collected in Belgium from 2006 to 2019

Trait	Characteristic or origin	USA300-NA (MRSA CC8 PVL-positive ACME-positive) N (%) (n=300)	MRSA CC8 PVL-positive ACME-negative N (%) (n=99)	*p*-value[a]
spa-types	t008	233 (77.7)	62 (62.6)	<0.0001
	t024	3 (1.0)	10 (10.1)	
	t068	3 (1.0)	1 (1.0)	
	t121	41 (13.7)	2 (2.0)	
	t334	6 (2.0)	-	
	t955	2 (0.7)	1 (1.0)	
	t3082	-	8 (8.1)	
	Others[b]	12 (4.0)	15 (15.2)	
Patient sex	Masculine	159 (53.0)	49 (49.5)	0.16
	Feminine	127 (42.3)	49 (49.5)	
	Unknown	14 (4.7)	1 (1.0)	
Patient age (years)	0–5	78 (26.0)	12 (12.1)	0.02
	6–14	25 (8.3)	11 (11.1)	
	15–24	34 (11.3)	22 (22.2)	
	25–44	95 (31.7)	32 (32.3)	
	45–69	43 (14.3)	10 (10.1)	
	> 70	9 (3.0)	3 (3.0)	
	Unknown	16 (5.3)	6 (6.1)	
Contact/trip to foreign country	Yes	37 (12.3)	14 (14.1)	0.09
	No	95 (31.7)	20 (20.2)	
	Unknown	168 (56.0)	65 (65.7)	
Origin of the sample	Blood	8 (2.7)	2 (2.0)	0.42
	Screening	34 (11.3)	11 (11.1)	
	Skin and soft tissue infections	172 (57.3)	61 (61.6)	
	Pus and internal fluids	49 (16.3)	9 (9.1)	
	Others or unknown	37 (12.3)	16 (16.2)	
Antimicrobial agent (percentage of resistance)	Cefoxitin	300 (100)	99 (100)	1.000
	Ciprofloxacin	150 (50.0)	14 (14.1)	<0.0001
	Chloramphenicol	3 (1.0)	-	1.000
	Clindamycin[c]	27 (9.0)	24 (24.2)	0.0002
	Erythromycin	280 (93.3)	32 (32.3)	<0.0001
	Fusidic acid	6 (2.0)	2 (2.0)	1.0000
	Gentamicin	2 (0.7)	1 (1.0)	0.5760
	Kanamycin	236 (78.7)	7 (7.0)	<0.0001
	Linezolid	1 (0.3)	-	1.000
	Minocycline	2 (0.7)	-	1.000
	Mupirocin	4 (1.3)	-	0.5760
	Rifampicin	-	-	-
	Tetracycline	23 (7.7)	11 (11.1)	0.3016
	Tobramycin	3 (1.0)	1 (1.0)	1.000
	Trimethoprim/sulfamethoxazole	-	3 (3.0)	0.0149

[a] Chi-square test was applied to categorical data. Fisher's exact test (two-tailed) was used to determine statistical significant differences on antimicrobial resistance data. [b] *spa*-types assigned only to 3 or less USA300-NA (t460, t574, t622, t656, t1313, t1354, t1578, t1767, t2849, t3361, t4712) or CC8 PVL-positive and ACME-negative (t148, t190, t197, t211, t304, t1610, t1635, t4919, t5326, t17261, t17320) isolates. [c] Inducible resistance included. *N*, number of isolates

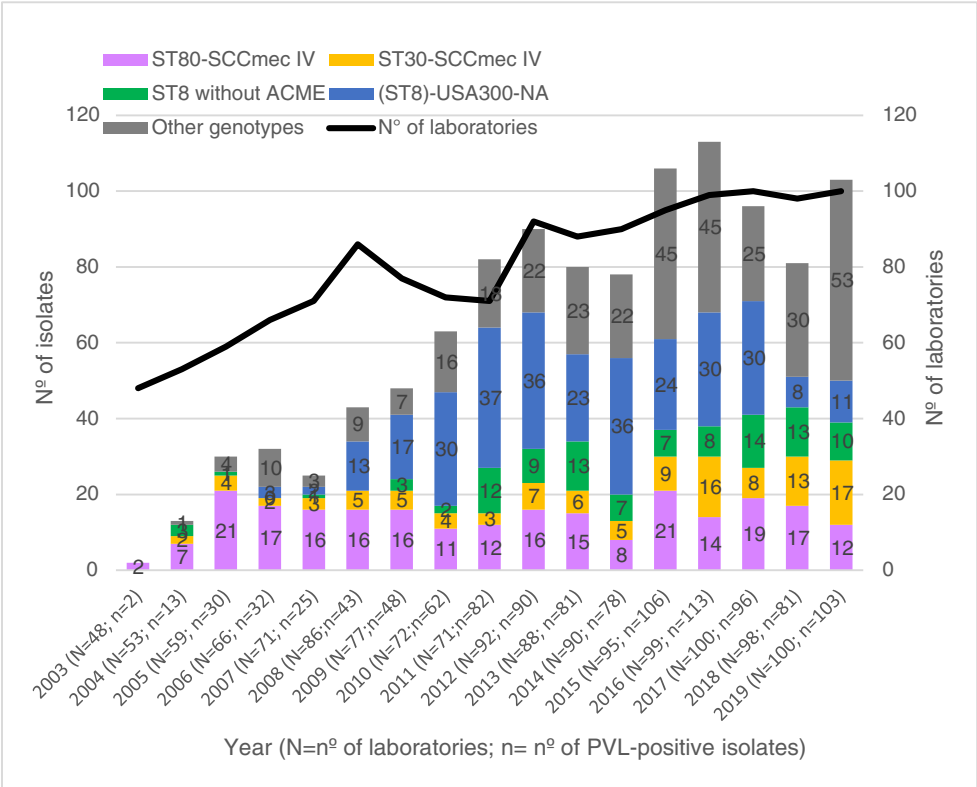


Fig. 1 Evolution of major genotypes recovered at the National Reference Laboratory for *S. aureus* (NRLS) of PVL-positive CA-MRSA in Belgium, 2003-2019. The X-axis indicates for each year the no. of laboratories that sent strains to the NRLS (N) and the no. of PVL-positive isolates received (n)

Phylogenetic analysis

Trees were generated from whole-genome single-nucleotide polymorphisms (SNPs). Sequence reads were mapped against the reference chromosome of the USA300-NA TCH1516 (accession number: NC_010079) using BioNumerics. Default strict filtering procedures were used to remove artifactual SNPs as previously described [17]. A rooted maximum likelihood tree (MLT) of the Belgian CC8 strains against a global ST8 sequences collection (n=121, Table S3) selected from different studies [6, 8, 11, 18, 19] was inferred using PHyML v3.0 [20] with Smart Model Selection [21] under the Bayesian Information Criterion with 100 bootstrap replicates and visualized with the Interactive Tree of Life (iTOL) web service [22]. Minimum spanning trees (MST) based on wgSNPs of (1) closely related Belgian USA300-NA using TCH1516 as reference chromosome; and (2 and 3) non-USA300-NA by using reference chromosomes CA12 (accession number: NZ_CP007672) and V2200 (accession number: NZ_CP007657) were constructed using BioNumerics. The epidemiological cut-off value of 20 SNPs proposed by Goyal et al. [23] was used as a marker for identity clusters.

Characterization of isolates subjected to WGS

STs were assigned in silico by uploading de novo assembled genomes to the MLST 2.0 tool of the Center for Genomic Epidemiology (CGE) [24, 25]. SCC*mec* types, virulence genes, chromosomal mutations related to antibiotic resistances, acquired antimicrobial resistance genes and replicon types were identified by uploading de novo assembled genomes to CGE tools based in nucleotide sequences: SCCmecFinder 1.2 (database: referenced, threshold for ID: 90%; minimum length: 60%), VirulenceFinder 2.0 (threshold for ID: 85%; minimum length: 60%), ResFinder 3.2 (threshold for ID: 80%; minimum length: 60%) and PlasmidFinder 2.1 (threshold for ID: 80%; minimum length: 60%) [24, 26–28]. Mutations at *cap5E* were detected by comparing de novo assemblies with *S. aureus* strain Newman (accession number: AP009351.1) using BioNumerics. The BacMet database [29, 30] and the NCBI were used to create a custom database of metal and biocide resistance, *agr* (types I, II, III and IV) and ACME/COMER (*abiα*, *abiC*, *copXL*, *kdp*, *merAB*, *oop3*, *speG*) related genes and regions (Table S4). De novo assembled genomes were uploaded to the CGE MyDbFinder tool (threshold for ID: 80%; minimum length: 60%) [24] to determine the presence of genes compiled in this custom database. Manual inspection of the sequences was performed in those sequences missing key genetic elements of USA300-NA isolates. A heatmap for indicating presence/absence of genes was performed by using the free web tool Heatmapper [31]. CC8 isolates were classified as (1) USA300-NA if harbouring ACME type I, SCC*mec* IVa and

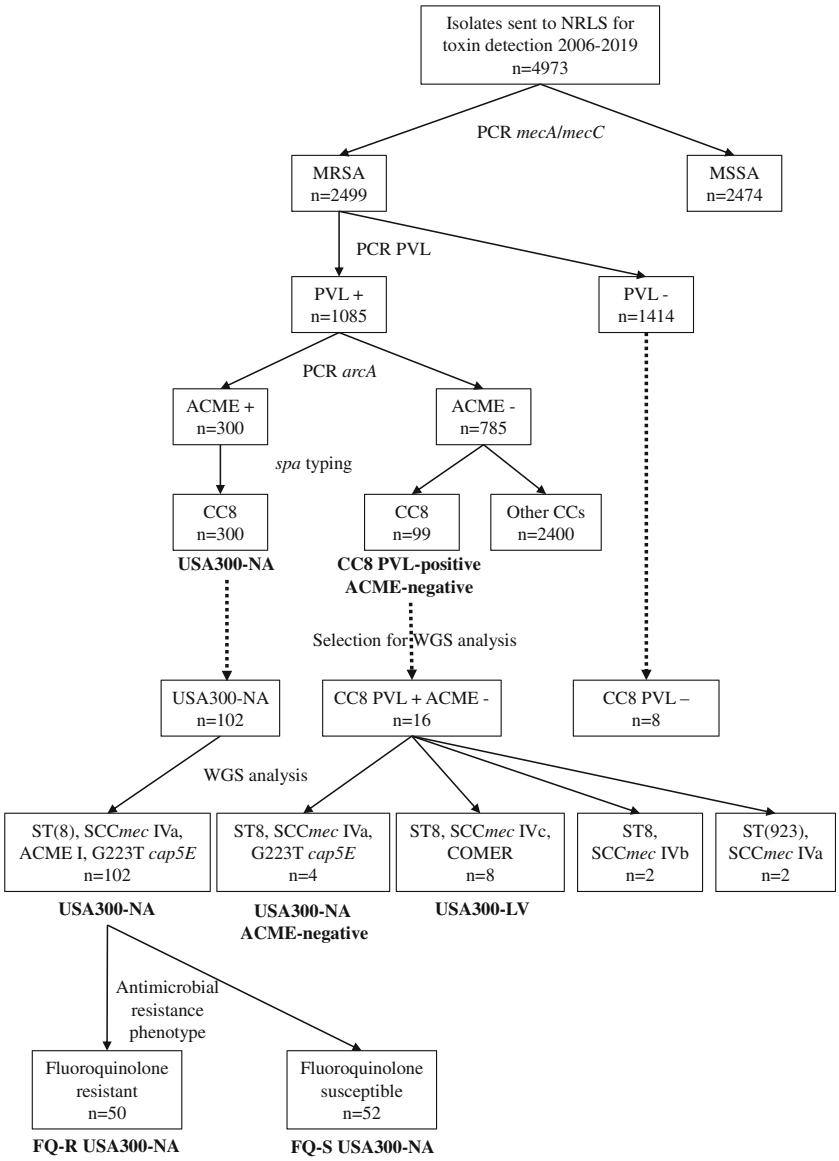


Fig. 2 Classification of the isolates based on PCR and WGS results. Sequence types (ST) with the number in brackets correspond to a mix of the sequence type indicated and closed related ones. CC, clonal complex; N, number of isolates; NRLS, National Reference Laboratory for *S. aureus*; WGS, whole genome sequence

G223T *cap5E* mutation, or (2) USA300-LV if harbouring of SCC*mec* IVc and COMER.

Statistical methods

Chi-squared test was applied to categorical data. Fisher's exact test (two-tailed) was used to determine statistical significant differences (p value) on antimicrobial resistance data. p value < 0.05 was considered statistically significant.

Results

MRSA CC8 PVL-positive population in Belgium

Among the 4973 *S. aureus* isolates received by the NRLS for toxin detection between 2006 and 2019, 2499 isolates were CA-MRSA, of which 1085 were PVL positive. PVL-positive CA-MRSA belonged to a wide range of genotypes, USA300-NA (n=300) being the most frequent between 2010 and 2017 (Fig. 1, Fig. 2). The USA300-NA clone represented 12% of the total number of CA-MRSA, and nearly 30% of the PVL-positive MRSA isolates. By contrast, only 9.5% of CA-MRSA isolates (n=99) were CC8 PVL-positive ACME-negative. CA-MRSA CC8 PVL-positive isolates showed 29 different *spa*-types, t008 being the most frequent among both USA300-NA (78%) and CC8 PVL-positive ACME-negative isolates (62%) (Table 1).

More than half of the CC8 PVL-positive isolates (n=233, 58.4%) were obtained from skin and soft tissue infections (SSTIs) (Table 1). There was no difference regarding the site of infection (p-value = 0.42) and the sex (p-value = 0.16) of the patients carrying an USA300-NA isolate compared to patients carrying an ACME-negative isolate. There was a

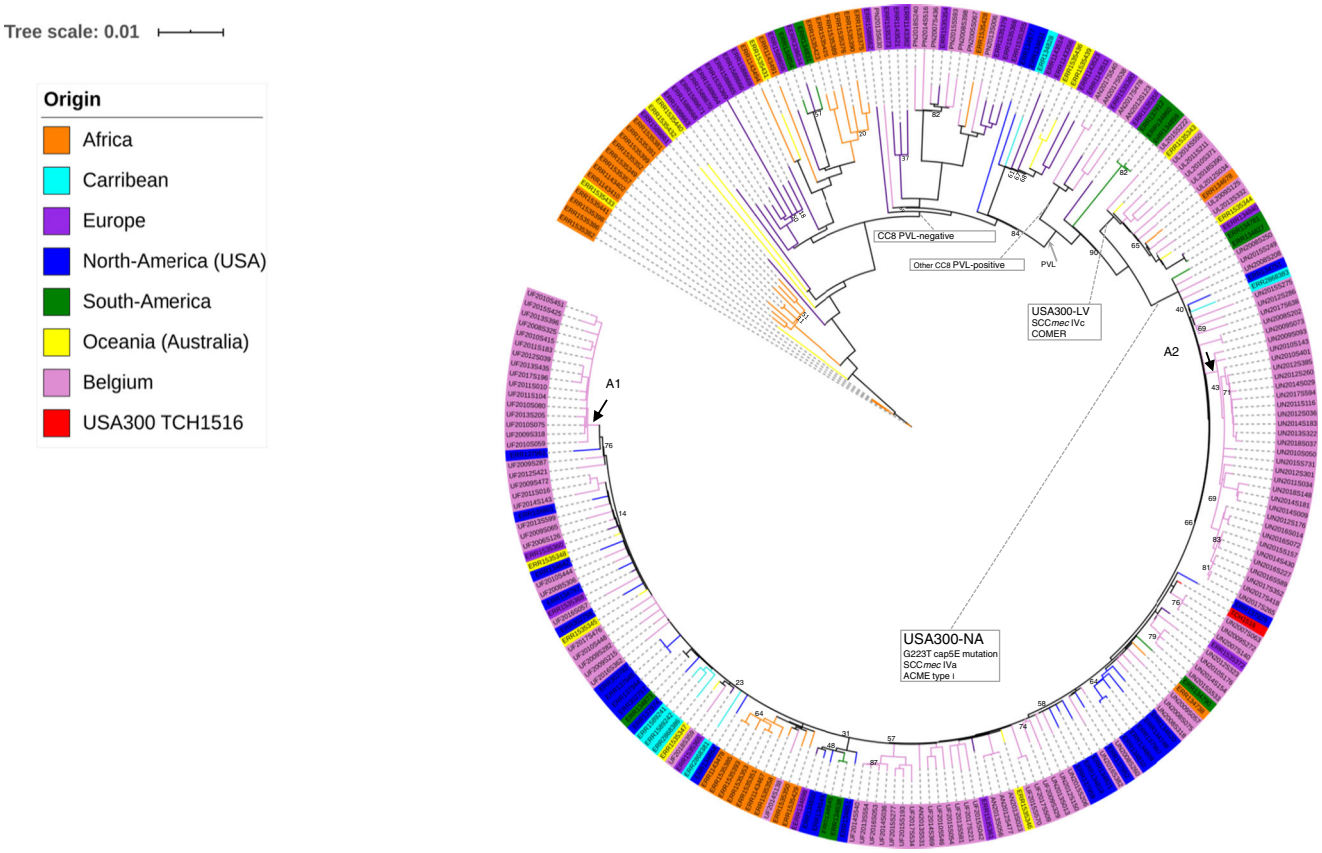


Fig. 3 Maximum-likelihood phylogeny of *S. aureus* CC8 isolates from Belgium (n=126) and from a ST8 global representative collection (n=121) based on 9548 wgSNPs. The USA300-NA TCH1516 strain (in red) was used as reference genome. The scale bar at the figure indicates the substitution rate. Bootstraps values are indicated by numbers. Colours depict geographic region of origin. Arrows indicated clades of Belgian-related strains with less than 100 SNPs of difference. Dashed lines connect branches with isolate numeration and/or accession numbers. Abbreviations before Belgian isolate accession numbers classify the isolates as follows: USA300-NA (UN), USA300-NA with mutations conferring fluoroquinolone resistance (UF), USA300-LV (UL), CC8 PVL-positive ACME-negative (AN), CC8 PVL-negative (PN)

statistical difference (p-value = 0.02) regarding the age distribution of the two populations as the proportion of 0 to 5 years children among NA-USA300-positive patients (26%) was about twice the one observed in ACME-negative positive patients (12%) (Table 1). Nevertheless, median age was for both groups (~25 years).

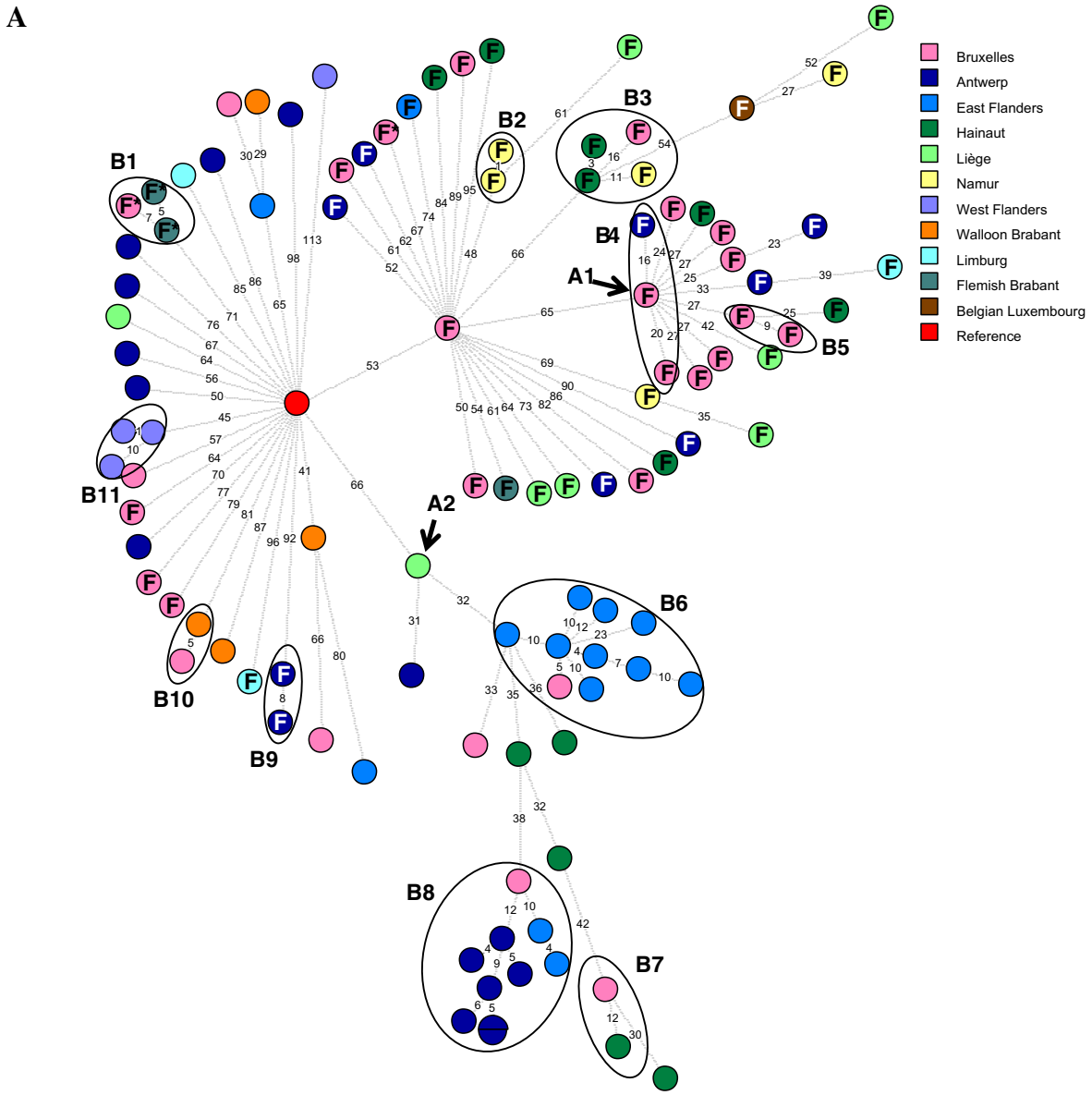
Most USA300-NA isolates (>75%) were resistant to erythromycin and kanamycin (n=119), and half (n=114) were ciprofloxacin resistant (Table 1, see also Table S1). Only 17 (5.7%) USA300-NA isolates were resistant to four or more antimicrobial groups other than ß-lactams. In contrast, most (85%) CC8 PVL-positive ACME-negative did not show any additional resistance (n=51) or carried only one (n=33). Interestingly, resistance to clindamycin (a frequently prescribed antibiotic for CA-MRSA infections) was significantly lower in the USA300-NA than in CC8 PVL-positive ACME-negative strains.

WGS and phylogenetic analysis

The 102 ACME-positive isolates selected for WGS had all the characteristics of USA300-NA: ST8 (n=98) or a closely related ST (n=4), SCC*mec* IVa, ACME type I and G223T *cap5E* mutation (Fig. 2, Table S1). In the global phylogeny analysis, the Belgian USA300-NA isolates interspersed with USA300-NA isolates recovered from other countries (Fig. 3). Nevertheless, in both MLT and MST trees, there were two clades of related (less than 100 SNPs) Belgian strains (Fig. 3, Fig. 4 A). The first one (clade A1) grouping 16

Fig. 4 Minimum spanning trees (MSTs) of the following: (A) USA300-NA and related isolates from Belgium (n=106) based on 2948 wgSNPs. The USA300-NA TCH1516 (in red) was used as reference genome. Each circle represents a strain coloured on the basis of the Belgian region of origin. Fluoroquinolone-resistant isolates are indicated with an "F" inside the corresponding circle. ACME-negative isolates are indicated with a "*" symbol inside the corresponding circle. Arrows indicated the "root"-isolate of the clades (A1, A2) of strains with less than 100 SNPs of difference. Clusters (B1 to B11) with <20 SNPs of difference are encircled. A table with additional characteristics (year of isolation, number of laboratories and region) of the isolates belonging to these clusters is included. (B) MST of USA300-LV isolates from Belgium based on 614 wgSNPs. The USA300-LV CA12 was used as reference genome. (C) MST of non-USA300 CC8-PVL isolates from Belgium based on 393 wgSNPs. The ST923 V2200 was used as reference genome

A



Subcluster (N)	Year of isolation (N)	N° laboratories	Regions (N)
B1 (3)	2012, 2013 (2)	2	Brussels, Flemish Brabant (2)
B2 (2)	2009, 2011	1	Namur
B3 (4)	2013, 2014 (2), 2016	4	Brussels, Hainaut (2), Namur
B4 (3)	2009, 2010 (2)	3	Antwerp, Brussels (2)
B5 (2)	2012, 2013	2	Brussels (2)
B6 (9)	2010, 2011, 2012 (3), 2013, 2014 (2), 2018	6	Brussels, East Flanders (8)
B7 (2)	2014, 2018	2	Brussels, Hainaut
B8 (10)	2012, 2014, 2015, 2016 (4), 2017 (3)	5	Antwerp (7)*, Brussels, East Flanders (2)
B9 (2)	2015, 2017	1	Antwerp (2)
B10 (2)	2008, 2009	2	Brussels, Walloon Brabant
B11 (3)	2007 (2), 2009	2	West Flanders (3)

N, number of isolates when more than one; *these 7 isolates were selected a part of 2 known outbreaks (Table S1).

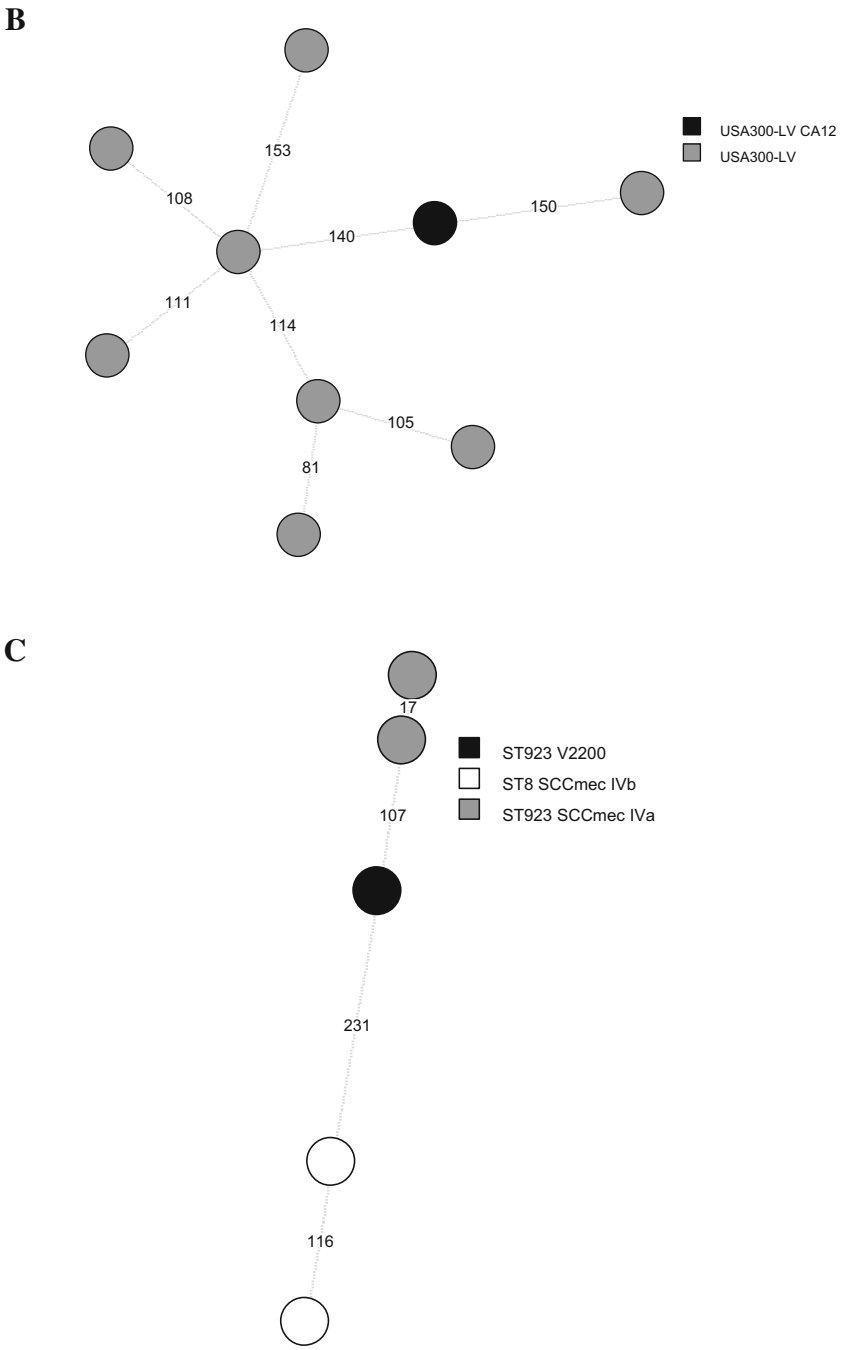


Fig. 4 continued.

phenotypically fluoroquinolone resistant (FQ-R) USA300-NA with 9 to 74 SNPs difference that were recovered from 2008 to 2017, mainly (n=9) from the Brussels area. The second clade (A2), grouped 29 fluoroquinolone susceptible (FQ-S) isolates recovered from 2009 to 2018, with 4 to 95 SNPs difference. The majority (n=19) of these isolates were from Flanders. Using the epidemiological cut-off value of ≤20 SNPs defined by Goyal et al. [23] for identifying outbreaks, we identified eleven clusters (B1 to B11) of strains that were indeed related in time and/or in space. Clusters B4 and B5 were part of the A1 clade, and clusters B6 to B8 to the A2 clade (Fig. 4 A).

Four ST8 isolates, which interspersed in the phylogeny analysis with USA300-NA isolates (Fig. 3, Fig. 4A), had genetic traits that corresponded to this clone (SCC*mec* IVa, G223T *cap5E* mutation) but were ACME-negative (confirmed by PCR and visual inspection of the sequences), and were thus classified as "ACME-negative USA300-NA" isolates.

Table 2 Chromosomal mutations and acquired resistance genes among 118 MRSA CC8 PVL-positive isolates subjected to WGS analysis

Resistance	Resistance phenotype observed	Gene(s)	USA300-NA (ST8-IVa PVL-positive A C M E -positive) (n=102) N (%)	ST8-IVa PVL-positive A C M E -negative[a] (n=4) N (%)	USA300-LV (ST8-IVc PVL-positive A C M E -negative) (n=8) N (%)	ST8-IVb PVL-positive A C M E -negative (n=2) N (%)	ST923-IVa PVL-positive A C M E -negative (n=2) N (%)
Aminoglycoside	Gen-Kan-Tob	*aac(6')-aph(2")*	1 (1.0)	-	-	-	-
	-	*Δant(6)-Ia*	95 (93.1)	-	-	-	-
	Kan	*aph(3')-III*	94 (92.2)	-	-	-	-
	ND	*ant(9)-Ia*	1 (1.0)	-	-	-	-
Fluoroquinolone	(Cip)[b]	*grlA/parC* S80F	2 (2.0)[a]	-	-	-	-
	(Cip)[b]	*grlA/parC* S80Y	51 (50)[a]	4 (100)	-	-	-
	Cip	*grlB* D432N	1 (1.0)	-	-	-	-
	Cip	*grlB* P585S	1 (1.0)	-	-	-	-
	Cip	*gyrA* S84A	2 (2.0)	-	-	-	-
	Cip	*gyrA* S84L	48 (47.1)	4 (100)	-	-	-
Fosfomycin	ND	*fusB*	1 (1.0)	-	-	-	-
Fusidic acid	Fus	*fusA L461F*	1 (1.0)	-	-	-	-
	Fus	*fusA* L461S	1 (1.0)	-	-	-	-
Mupirocin	Mup(I)	*ileS* V588F	1 (1.0)	-	-	-	-
MLS	Ery	*erm*(A)	1 (1.0)	-	-	-	-
	Ery-Cli	*erm*(C)	3 (2.9)	-	2 (25.0)	-	-
	(Cli)[c]	*lnu*(A)	2 (2.0)	-	-	-	1 (50)
	Ery(-Cli)[d]	*mph*(C), *msr*(A)	99 (97.1)	-	-	-	2 (100)
Phenicol	Chl	*cat*	2 (2.0)	-	-	-	-
Tetracycline	(Tet)[e]	*tet*(K)	6 (5.9)	-	1 (12.5)	-	-
	Tet	*tet*(M)	1 (1.0)	-	-	-	-
Trimethoprim	ND	*dfrG*	-	-	2 (25.0)	-	-
Biocides	ND	*lmrS*, *mepA*, *norAB*, *perR*	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
	ND	*qacA*	1 (1.0)	-	-	-	-
	ND	*qacC/qacD/smr*	5 (4.9)	4 (100)	-	-	-
Metals	ND	*asrCΔR*	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
	ND	*cadD*	101 (99.0)	4 (100)	8 (100)	2 (100)	2 (100)

[a] Isolates presenting all the characteristics of USA300-NA, except the ACME. [b] Two strains with *grlA* p.S80Y mutation and one strain with *grlA* p. S80F did not show ciprofloxacin resistance by disc diffusion, but the diameters were close to the resistance limit. [c] Two strains carrying *lnuA* did not show in vitro clindamycin resistance. [d] Five strains carrying *mph*(C) and *msr*(A) had in vitro clindamycin resistance. [e] One strain carrying *tetK* did not show tetracycline resistance by disc diffusion. *N*, number of isolates; *ND*, not determined

Eight ACME-negative isolates were classified as USA300-LV (SCC*mec* IVc, COMER) (Fig. 2). They all were referred at different years between 2009 and 2018, by 6 different laboratories. These isolates had between 81 and 174 SNPs difference (Fig. 4 B) and in 6 cases, the information provided to the NRLS indicated that they were related to trips or contacts with South America. Two ACME-negative isolates belonged to ST8 SCC*mec* IVb and were sent at different times by two different laboratories located in different regions. The remaining two *arcA*-negative isolates belonged to ST923 and a new ST, differing from ST923 by one nucleotide in the *aroE* gene, and both harboured SCC*mec* IVa. These two isolates were only 17 SNPs apart (Fig. 4 C) and were related to a small intra-familial outbreak (Table S1). PVL-negative Belgian CC8 isolates were distantly separated (353 SNPs) from PVL-positive CC8 isolates (Fig. 4, Figure S1).

Virulence, antibiotic, biocide and metal resistance genes among MRSA CC8 PVL-positive isolates

The four ACME-negative USA300-NA (all FQ-R) and the FQ-R USA300-NA isolates (n=50) mainly carried mutations S80Y at *grlA* and S84L at *gyrA* (Table 2). Three

Table 3 Virulence-related genes and replicon types among 118 MRSA CC8 PVL-positive isolates subjected to WGS analysis

Type	Gene(s)	USA300-NA (ST8-IVa PVL-positive ACME-positive) (n=102) N (%)	ST8-IVa PVL-positive ACME-negative[a] (n=4) N (%)	USA300-LV (ST8-IVc PVL-positive ACME-negative) (n=8) N (%)	ST8-IVb PVL-positive ACME-negative (n=2) N (%)	ST923-IVa PVL-positive ACME-negative (n=2) N (%)
agr type	*agr1*	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
Exoenzyme	*aur*	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
	splAB	102 (100)	4 (100)	8 (100)	-	-
	splE	101 (99.0)	4 (100)	8 (100)	-	-
Toxins	*hlgAB*, *lukED*, *lukFS-PV*	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
	hlgC	101 (99.0)	4 (100)	8 (100)	2 (100)	2 (100)
	sea	-	-	-	-	2 (100)
	sed, selj, ser	-	-	-	2 (100)	2 (100)
	selk, selq	66 (64.7)	4 (100)	6 (75)	2 (100)	2 (100)
Immune evasion	*sak, scn*	102 (100)	4 (100)	7 (87.5)	2 (100)	2 (100)
Replicon	*rep7a*	10 (9.8)	-	1 (12.5)	-	-
	rep7c	102 (100)	4 (100)	8 (100)	2 (100)	2 (100)
	rep10	4 (4.0)	-	2 (25.0)	-	-
	rep13	3 (2.9)	1 (25.0)	-	-	1 (50.0)
	rep15	1 (1.0)	-	-	-	-
	rep16	100 (98.0)	4 (100)	-	-	-
	rep19	94 (92.2)	4 (100)	-	-	-
	rep20	1 (1.0)	-	7 (87.5)	2 (100)	2 (100)
	rep21	45 (44.1)	4 (100)	6 (75.0)	2 (100)	2 (100)
	repUS21	-	-	1 (12.5)	-	-

[a] Isolates presenting all the characteristics of USA300-NA, except the ACME. *N*, number of isolates

ciprofloxacin susceptible USA300-NA isolates carried a single mutation at *grlA*. Most kanamycin-resistant USA300-NA (92%) carried the *aph*(3′) III gene and most erythromycin-resistant USA300-NA (97%) carried macrolide resistance *mph*C and *msr*A genes, and this was regardless their fluoroquinolone resistance profile. In a few cases, the resistance gene profile did not correspond to the phenotypic resistance profile observed (see Table 2, Figure S2 and Table S1): two clindamycin susceptible strains carrying *lnuA*, one tetracycline susceptible strain carrying *tetK*. All sequenced MRSA CC8 PVL-positive isolates carried also the same biocides and metal (different from the COMER element) resistance genes.

The distribution of virulence genes was similar between the different CC8 PVL-positive populations, the main difference being the exoenzymes and enterotoxins content (Table 3, Figure S2). The four isolates not related to USA300 carried the enterotoxins *sea*, *sed*, *selj* and *ser*. Most FQ-R USA300-NA (n=45, 90%), all ACME-negative USA300-NA and the three ciprofloxacin susceptible USA300-NA presenting a single *grlA* mutation, carried the enterotoxin genes *selk* and *selq* (indicating the presence of SaPI5). Most (63.3%, 31 out of 49) of the FQ-S USA300-NA (Fig. 4 A) did not carry these SaPI5 enterotoxin genes. Interestingly, some replicons types (*rep16*, *rep19*) were only related to USA300-NA isolates (Table 3, Figure S2).

Discussion

The USA300-NA is a particularly successful PVL-positive MRSA clone that became endemic in North America rapidly after its first description [3]. By contrast, studies in several European countries show great variation in the frequency of USA300-NA among PVL-positive isolates, varying from 16 to 48% [9, 11].

In Belgium, the first PVL-positive CA-MRSA isolate was reported in 2002, and the European clone ST80-SCC*mec* IV was the most frequent CA-MRSA until 2009 [12, 32]. USA300-NA was detected for the first time in 2006 and became the most frequent CA-MRSA clone between 2010 and 2017, representing from 23 to 48% of the PVL-positive isolates received. Our results show a considerable decrease of USA300-NA occurrence in Belgium since 2018, which is in

accordance with a recent suggestion made that this clone may globally decline [2]. Nevertheless, a continue surveillance is needed to further confirm this trend.

Most Belgian USA300-NA isolates were recovered from SSTIs in young individuals. As in previous studies, Belgian USA300-NA isolates showed high resistance rates to erythromycin and ciprofloxacin [19, 33], but also to kanamycin. The sequenced ciprofloxacin resistant isolates mainly carried the mutations *grlA/parC* S80Y and *gyrA* S84L. As these mutations have been described in the FQ-R USA300-NA sub-lineage that has a global distribution [7], this observation could result from its dissemination in Belgium. However, convergent evolution (de novo mutations arising in multiple lineages) cannot be ruled out. MLS and kanamycin resistance were encoded by the genes *mph*(C), *msr*(A) and *aph*(3′)-III as in previous studies [7, 19]. Additionally, some replicon types were specifically isolated from USA300-NA isolates, which is consistent with other studies that have seen a high level of plasmid identity among USA300 isolates [34]. Nevertheless, we did not check if these replicons were plasmidic or chromosome-integrated.

The 102 isolates selected to represent the USA300 population diversity observed in Belgium interspersed in the phylogeny analysis with USA300-NA isolates recovered from other countries suggesting multiple introductions as it has been seen in other European countries [7–9]. The *S. aureus* mutation rate has been estimated to be of approximately 1 SNP every 9 weeks [35] or 15 weeks [36]. The strict cut-off value of 20 SNPs proposed by Goyal et al. [23] was used as marker for cluster identification, although several authors demonstrated more than 20 SNPs of difference between intra-subject isolates [37, 38]. It allowed us to identify several clusters that were undetected using the NRLS routine typing procedure. By extending the cut-off value to less than 100 SNPs of difference, we detected two clades that seemed to maintain themselves for several years. This may suggest that, although there were multiple introductions of USA300-NA in our country, at least two clades have successfully maintained and spread over the last decade. As it has been seen in the USA [39], these two clades (one fluoroquinolone susceptible, the other, fluoroquinolone resistant) have spread in two different geographical areas (Brussels vs Flanders). The isolates from the first clade (A1) carried the *grlA/parC* S80Y and *gyrA* S84L mutations, characteristic of the known fluoroquinolone-resistant USA300-NA sub-lineage that has globally spread [7]. The second clade (A2) grouped fluoroquinolone susceptible USA300-NA that did not carry SaPI5 enterotoxin genes, although this island is considered as a molecular marker of USA300-NA [2]. It has been estimated that the SaPI5 was introduced into ST8 many years before the emergence of the epidemic USA300-NA and USA300-LV clades [5]. Nevertheless, USA300-NA isolates without SaPI5 have been detected in other recent studies [6], suggesting deletion events in some USA300-NA strains, some authors claiming that SaPI5 may not be a key feature of USA300-NA isolates after all. This A2 clade was further divided in 3 clusters. One of them (cluster B8) included CA-MRSA isolates sent between 2014 and 2015 by one laboratory located in the Antwerp region and selected here as such [13]. Epidemiological and WGS data suggest that CA-MRSA isolates collected during 2016 and 2017 by other laboratories of the same region may be related to this outbreak as well, making it wider than previously described [13].

In the present study, we found four isolates that present all characteristics of the USA300-NA clone but were ACME-negative. A recent study has shown that ACME can spontaneously excise, reporting wild-type ACME-negative USA300 clinical isolates at a low frequency [40]. The successful dissemination of USA300-NA and USA300-LV has been associated with the *copX* (also named *copB*) locus present in both ACME and COMER loci [5]. Nevertheless, the study by Wu et al. [40] did not support that the ACME element alone is a significant factor in the transmission and virulence of USA300-NA. In the present study, three out of the four "ACME-negative USA300-NA" isolates were closely related (cluster B1), but they were sent to the NRC by different laboratories at different times, suggesting a limited but successful transmission despite the lack of the ACME locus.

The eight sequenced USA300-LV were recovered at different times from 2009 to 2018, in 6 different laboratories, and were mostly related to documented trips or contacts with South America. These results are similar to other European countries data (Switzerland [10, 11], Germany [8]) where it has been sporadically described. Since our study only investigated a small selection of ACME-negative isolates, the real prevalence of USA300-LV in Belgium remains to be evaluated.

Two sequenced isolates belonged to an emerging clone, named COL923, that was first detected in Colombia around 2006–2007 [41] and currently spreading in this country [42]. It has also been detected in Ecuador and Venezuela [43]. This emerging clone has been related to paediatric infections and nasal colonization in young adults [42]. The two COL923 isolates were related to a small outbreak in 2017 involving a total of four isolates harbouring new *spa*-types t17320 or t17261. In the literature, the COL923 isolates are usually *spa*-type t1635 [44]. Interestingly, in our collection, one additional isolate, not related to the outbreak, and recovered much sooner (2014) was typed t1635. Information regarding trips was not provided for these five isolates but, as far as we know, this is the first detection of the COL923 clone outside South America.

Belgian NRLS data and isolate collection have the limitation of being voluntary based, and thus probably underestimate the true occurrence of CA-MRSA infections. Yet, they present the advantages of being continuous, prospective and nationwide. The present study shows that the USA300-NA has successfully

spread in our country: although multiple introductions probably occurred, at least two clades have successfully maintained themselves and even extended during a decade in certain regions. Interestingly, we currently observe a decrease of the USA300-NA. We also show that the USA300-LV clone, as well as the new COL923, has been detected in our country. Our results underline the importance of closely monitoring the evolving epidemiology of CA-MRSA, since USA300 strains are virulent, often multi-resistant and difficult to eradicate [13]. Furthermore, these results demonstrate the key-role WGS can play in this close epidemiological vigilance.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10096-021-04286-3.

Acknowledgements We thank Raf De Ryck, Pascal Buidin, Geneviève Hay, Nathalie Legros and Christine Thiroux for technical assistance. We thank our microbiologist colleagues for sending their staphylococcal strains to the NRC and the technicians of the service Transversal activities in Applied Genomics at Sciensano for performing the WGS.

Author contribution MAA conceived the idea and analyzed the epidemiological and NGS data. MAA, AD, CN, NY, CM, DM and MH work(ed) at the Belgian National Reference Centre of Staphylococci and therefore contributed in the generation and validation of the epidemiological data. SCJDK obtained the NGS data. MAA wrote the manuscript in consultation with MH with input from the remaining authors.

Funding This work was supported by the LHUB-ULB (no. project 485951501).

Declarations

Conflict of interest The authors declare no competing interests.

References

1. Junie LM, Jeican II, Matros L, Pandrea SL (2018) Molecular epidemiology of the community-associated methicillin-resistant *Staphylococcus aureus* clones: a synthetic review. Clujul Med 91(1):7–11
2. Planet PJ (2017) Life after USA300: the rise and fall of a superbug. J Infect Dis 215(suppl_1):S71–SS7
3. Liu C, Graber CJ, Karr M et al (2008) A population-based study of the incidence and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* disease in San Francisco, 2004-2005. Clin Infect Dis 46(11):1637–1646
4. Boyle-Vavra S, Li X, Alam MT et al (2015) USA300 and USA500 clonal lineages of *Staphylococcus aureus* do not produce a capsular polysaccharide due to conserved mutations in the *cap5* locus. mBio 6(2):e02585–e02514
5. Planet PJ, Diaz L, Kolokotronis SO et al (2015) Parallel Epidemics of community-associated methicillin-resistant *Staphylococcus aureus* USA300 infection in North and South America. J Infect Dis 212(12):1874–1882
6. Uhlemann AC, Dordel J, Knox JR et al (2014) Molecular tracing of the emergence, diversification, and transmission of *S. aureus* sequence type 8 in a New York community. Proc Natl Acad Sci U S A 111(18):6738–6743
7. Glaser P, Martins-Simões P, Villain A et al (2016) Demography and intercontinental spread of the USA300 community-acquired methicillin-resistant *Staphylococcus aureus* lineage. mBio 7(1): e02183–e02115
8. Strauss L, Stegger M, Akpaka PE et al (2017) Origin, evolution, and global transmission of community-acquired *Staphylococcus aureus* ST8. Proc Natl Acad Sci U S A 114(49):E10596–E1E604
9. Toleman MS, Reuter S, Jamrozy D et al (2019) Prospective genomic surveillance of methicillin-resistant *Staphylococcus aureus* (MRSA) associated with bloodstream infection, England, 1 October 2012 to 30 September 2013. Euro Surveill 24(4):1800215
10. Planet PJ, Diaz L, Rios R, Arias CA (2016) Global spread of the community-associated methicillin-resistant *Staphylococcus aureus* USA300 Latin American variant. J Infect Dis 214(10):1609–1610
11. Von Dach E, Diene SM, Fankhauser C, Schrenzel J, Harbarth S, Francois P (2016) Comparative genomics of community-associated methicillin-resistant *Staphylococcus aureus* shows the emergence of clone ST8-USA300 in Geneva, Switzerland. J Infect Dis 213(9): 1370–1379
12. Brauner J, Hallin M, Deplano A et al (2013) Community-acquired methicillin-resistant *Staphylococcus aureus* clones circulating in Belgium from 2005 to 2009: changing epidemiology. Eur J Clin Microbiol Infect Dis 32(5):613–620
13. Kairet K, Ho E, Van Kerkhoven D et al (2017) USA300, a strain of community-associated methicillin-resistant *Staphylococcus aureus*, crossing Belgium's borders: outbreak of skin and soft tissue infections in a hospital in Belgium. Eur J Clin Microbiol Infect Dis 36(5):905–909
14. Ridom SeqSphere+. https://www.ridom.de/seqsphere/
15. EUCAST. Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0. 2019: http://www.eucast.org
16. Bankevich A, Nurk S, Antipov D et al (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19(5):455–477
17. Heinrichs A, Argudin MA, De Mendonca R et al (2019) An outpatient clinic as a potential site of transmission for an outbreak of New Delhi metallo-beta-lactamase-producing *Klebsiella pneumoniae* sequence type 716: a study using whole-genome sequencing. Clin Infect Dis 68(6):993–1000
18. Strauss L, Ruffing U, Abdulla S et al (2016) Detecting *Staphylococcus aureus* virulence and resistance genes: a comparison of whole-genome sequencing and DNA microarray technology. J Clin Microbiol 54(4):1008–1016
19. Guardabassi L, Moodley A, Williams A et al (2019) High prevalence of USA300 among clinical isolates of methicillin-resistant *Staphylococcus aureus* on St. Kitts and Nevis, West Indies. Front Microbiol 10:1123
20. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59(3):307–321
21. Lefort V, Longueville JE, Gascuel O (2017) SMS: smart model selection in PhyML. Mol Biol Evol 34(9):2422–2424
22. Letunic I, Bork P (2019) Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res 47(W1):W256–W2W9
23. Goyal M, Javerliat F, Palmieri M et al (2019) Genomic evolution of *Staphylococcus aureus* during artificial and natural colonization of the human nose. Front Microbiol 10:1525
24. Center for Genomic Epidemiology. Available at: http://www.genomicepidemiology.org/. Accessed October 2019.
25. Larsen MV, Cosentino S, Rasmussen S et al (2012) Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol 50(4):1355–1361

26. Zankari E, Hasman H, Cosentino S et al (2012) Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644
27. Kaya H, Hasman H, Larsen J et al (2018) SCCmecFinder, a web-based tool for typing of staphylococcal cassette chromosome *mec* in *Staphylococcus aureus* using whole-genome sequence data. mSphere 3(1):e00612–e00617
28. Carattoli A, Hasman H (2020) PlasmidFinder and in silico pMLST: identification and typing of plasmid replicons in whole-genome sequencing (WGS). Methods Mol Biol 2075:285–294
29. BacMet: Antibacterial biocide and metal resistance genes database. Available at: http://bacmet.biomedicine.gu.se/. Accessed October 2019.
30. Pal C, Bengtsson-Palme J, Rensing C, Kristiansson E, Larsson DG (2014) BacMet: antibacterial biocide and metal resistance genes database. Nucleic Acids Res 42(Database issue):D737–D743
31. Heatmapper. Available at www.heatmapper.ca. Accessed January 2021.
32. Denis O, Deplano A, De Beenhouwer H et al (2005) Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium. J Antimicrob Chemother 56(6):1103–1106
33. Enstrom J, Froding I, Giske CG et al (2018) USA300 methicillin-resistant *Staphylococcus aureus* in Stockholm, Sweden, from 2008 to 2016. PLoS One 13(11):e0205761
34. Kennedy AD, Porcella SF, Martens C et al (2010) Complete nucleotide sequence analysis of plasmids in strains of *Staphylococcus aureus* clone USA300 reveals a high level of identity among isolates with closely related core genome sequences. J Clin Microbiol 48(12):4504–4511
35. Senn L, Clerc O, Zanetti G et al (2016) The stealthy superbug: the role of asymptomatic enteric carriage in maintaining a long-term hospital outbreak of ST228 methicillin-resistant *Staphylococcus aureus*. mBio 7(1):e02039–e02015
36. Worby CJ, Chang HH, Hanage WP, Lipsitch M (2014) The distribution of pairwise genetic distances: a tool for investigating disease transmission. Genetics 198(4):1395–1404
37. Popovich KJ, Snitkin E, Green SJ et al (2016) Genomic epidemiology of USA300 methicillin-resistant *Staphylococcus aureus* in an urban community. Clin Infect Dis 62(1):37–44
38. Read TD, Petit RA 3rd, Yin Z, Montgomery T, McNulty MC, David MZ (2018) USA300 *Staphylococcus aureus* persists on multiple body sites following an infection. BMC Microbiol 18(1):206
39. Challagundla L, Luo X, Tickler IA et al (2018) Range expansion and the origin of USA300 North American epidemic methicillin-resistant *Staphylococcus aureus*. MBio 9(1):e02016–e02017
40. Wu K, Conly J, McClure JA, Kurwa HA, Zhang K (2020) Arginine catabolic mobile element in evolution and pathogenicity of the community-associated methicillin-resistant *Staphylococcus aureus* strain USA300. Microorganisms 8(2):275
41. Arias CA, Rincon S, Chowdhury S et al (2008) MRSA USA300 clone and VREF–a U.S.-Colombian connection? N Engl J Med 359(20):2177–2179
42. Escobar-Perez J, Reyes N, Marquez-Ortiz RA et al (2017) Emergence and spread of a new community-genotype methicillin-resistant *Staphylococcus aureus* clone in Colombia. BMC Infect Dis 17(1):108
43. Reyes J, Rincon S, Diaz L et al (2009) Dissemination of methicillin-resistant *Staphylococcus aureus* USA300 sequence type 8 lineage in Latin America. Clin Infect Dis 49(12):1861–1867
44. Escobar JA, Marquez-Ortiz RA, Alvarez-Olmos MI et al (2013) Detection of a new community genotype methicillin-resistant *Staphylococcus aureus* clone that is unrelated to the USA300 clone and that causes pediatric infections in Colombia. J Clin Microbiol 51(2):661–664