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PII: S1438-4221(16)30411-8
DOI: http://dx.doi.org/doi:10.1016/j.ijmm.2017.05.003
Reference: IJMM 51124

To appear in:

Received date: 13-12-2016
Revised date: 11-5-2017
Accepted date: 14-5-2017

Please cite this article as: Fernandez, Silvina, Ledo, Camila, Lattar, Santiago, Llana, Mariángeles Noto, Bertelli, Andrea Mendoza, Gregorio, Sabrina Di, Sordelli, Daniel O., Gómez, Marisa I., Mollerach, Marta E., High virulence of methicillin resistant Staphylococcus aureus ST30-SCCmecIVc-spat019, the dominant community-associated clone in Argentina.International Journal of Medical Microbiology http://dx.doi.org/10.1016/j.ijmm.2017.05.003

High Virulence of Methicillin Resistant *Staphylococcus aureus* ST30-SCC*mec*IVc-*spa*t019, the Dominant Community-Associated Clone in Argentina

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Abstract

Community-acquired methicillin resistant *Staphylococcus aureus* emerged as a worldwide health problem in the last few years. In Argentina, it is found in 70% of skin and skin structure infections in previously healthy adult patients and causes severe invasive diseases. The ST30-SCC*mec*IVc-*spa*t019 clone is predominant in adult infections and has displaced the previously prevalent ST5-SCC*mec*IVa-*spa*t311 clone in community settings. In the present work we compared the virulence of both clones in order to explain the displacement, and found that ST30-IVc is associated with invasive infections in adult patients from Argentina and possesses a different virulence-

associated genes profile compared to ST5-IVa. A representative strain of ST30 lineage has a more aggressive behavior in animal models of infection and expresses higher level of Fibronectin binding protein A coding gene, which could enhance the bacterial invasion capacity.

Keywords: Community-acquired methicillin resistant *Staphylococcus aureus.* ST30-SCC*mec*IVc. Virulence.

Introduction

The virulence of *Staphylococcus aureus*, especially community-associated strains, is due to a large variety of virulence factors including secreted toxins which damage host cell membranes and trigger an inflammatory response, or enzymes that degrade host proteins (Otto, 2014). Moreover, *S. aureus* can produce a large set of surface molecules that allow the bacterial binding to host specific receptors (Johannessen et al., 2012), and has developed several mechanisms to evade the host immune system (Zecconi and Scali, 2013) including the ability to form biofilms (Archer et al., 2011). Most of our current knowledge of these *S. aureus* factors and mechanisms was obtained from research using experimental animal models of infection.

Community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) emerged as a relevant public health problem during the 1990s when numerous cases of infections began to be communicated, usually in young and previously healthy individuals away from nosocomial institutions. The strains involved were genetically distinct from healthcare associated MRSA (HA-MRSA). However, strains that are prevalent causes of CA-MRSA infection are now replacing traditional HA-MRSA strains in hospitals (Mediavilla et al., 2012). Most CA-MRSA isolates are obtained from skin and skin structure infections (SSSIs), and thus CA-MRSA is the major bacterial pathogen associated with this type of disease worldwide. SSSIs can present as an

uncomplicated or a complicated infection (Stryjewski and Chambers, 2008), and may be the primary focus for invasive disease (Fernandez et al., 2013; Klevens et al., 2007; Skinner and Keefer, 1941). CA-MRSA is now well established as an important pathogen associated with several invasive infections, such as endocarditis, osteomyelitis and pneumonia (Deleo et al., 2010).

CA-MRSA strains belong to different genetic lineages that can be classified by molecular typing methods. Geographic distribution of CA-MRSA lineages is dynamic, with some predominant clones: ST1-SCC*mec*IV (USA400), ST8-SCC*mec*IV (USA300), ST30-SCC*mec*IV (South West Pacific clone), ST59 (Taiwan clone), ST80 (European clone), ST93-IV and ST72-IV (Bal et al., 2016; Deleo et al., 2010). Some of them have a certain continent specificity while others like ST30-SCC*mec*IV and ST8-SCC*mec*IV are considered pandemic as they have been isolated repeatedly from every continent (Mediavilla et al., 2012). In two multicenter epidemiological studies that were conducted in Argentina between March 2010 and December 2011, with the participation of 19 hospitals and health care centers, we studied 218 patients with SSSIs due to CA-MRSA and 55 patients with invasive infections due to CA-MRSA. The strains recovered were genotyped by pulsed field gel electrophoresis (PFGE), *spa* typing and Multilocus Sequence Typing (MLST), and the clone found predominantly both in SSSIs and invasive infections was CA-MRSA ST30-SCC*mec*IVc-*spa*t019-PVL positive (Fernandez et al., 2013; Lopez Furst et al., 2013). This clone replaced in Argentina the previously prevalent CA-MRSA clone ST5-SCC*mec*IVa-*spa*t311-PVL positive (Gardella et al., 2008; Sola et al., 2008). This observation led us to hypothesize that the emerging ST30-SCC*mec*IVc-*spa*t019-PVL positive clone could have different pathogenic capacity compared to the ST5-SCC*mec*IVa-*spa*t311-PVL positive clone,

perhaps by having advantages in surviving in the infection site or a better ability for dissemination.

In order to explore this hypothesis, the objectives of this work were to study the association of ST30 and ST5 clones with invasive disease, to compare the pathogenicity of representative isolates in three animal models of infection and to analyze the presence and expression of virulence-associated genes.

Material and methods

Microorganisms

Bacterial isolates came from a CA-MRSA collection of two multicenter prospective epidemiological studies conducted at nineteen hospitals and health-care centers in Argentina, between March 2010 and October 2011. The collection contains a total of 117 ST30-IVc isolates and 37 ST5-IVa isolates. The studies included patients ≥14 years old diagnosed with Skin and Skin Structure Infection (SSSI) (Lopez Furst et al., 2013) or invasive infection due to MRSA (Fernandez et al., 2013) without any of the following contacts with the healthcare system within the previous 12 months: hospitalization, dialysis, surgery, presence of catheters or percutaneous medical devices, or residence in a long-term care facility.

Virulence associated genes were analyzed in the all ST5 and ST30 strains. A total of 40 isolates belonging in the predominant clonal types found in both studies (ST30 and ST5) were chosen for biofilm production assay.

One representative strain of each lineage (03-P-018 for ST30, and 16-P-013 for ST5) was selected from the SSSI CA-MRSA collection for comparative analysis in three animal models of infection and gene expression analysis. The main features of these strains, renamed as Sa30 and Sa5, are described in Table 1. Strains were conserved frozen at -20°C in Trypticase soy broth (TSB) supplemented with glycerol 20% and routinely cultured on Trypticase soy agar (TSA) for 24 h at 37°C.

Table 1. Features of the two representative *S. aureus* strains utilized in animal experiments and RT-qPCR.

Staphylococcus aureus strains		
Name	Sa5	Sa30
Clonal complex	CC5	CC30
Sequence type	ST5	ST30
spa type	t311	t019
agr	II	III
SCC*mec*	IVa	IVc
Virulence coding genes	PVL, *fnbA, fib, clfA,clfB, eno, ebpS, sdrE, sea, hlg, sei, seg, icaA,*	PVL, *fnbA, fnbB, clfA,clfB, bbp, cna, eno, ebpS, hlg, sei, seg, icaA,*
cap specificity	*cap5*	*cap8*
Susceptibility to erythromycin	R	R
Source of isolation	SSSI	SSSI
Original strain names	16-P-013	03-P-018

R: resistant

SSSI: Skin and Skin Structure Infection

PVL: Panton-Valentine leucocidin

PCR amplification of virulence-associated genes

Detection of toxin coding genes (*sea, seg, sec, sei, seb, sej, tst, hlg*) (Kumar et al., 2009; Nashev et al., 2004), adhesins coding genes (*fnbA, fnbB, fib, clfA, clfB, sdrE, bbp, cna, eno, ebpS*) (Kumar et al., 2009; Tristan et al., 2003), the *arcA* gene, part of the arginine catabolic mobile element (ACME) (Goering et al., 2007) and genes involved in biofilm formation (*icaA*) was carried out by PCR strategies described previously. The strains NRS384, NRS110, NRS174, NRS158, NRS113 and NRS111 from Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) collection were included as positive controls for PCR reactions. Genomic DNA extraction was performed as previously described (von Specht et al., 2006).

Biofilm production assay

Biofilm development was assessed by measuring the accumulation of biomass on the surface of sterile 96-well flat-bottom polystyrene plates (Deltalab). Briefly, 200 µl of a 1/40 dilution of an overnight culture in TSB supplemented with sterile glucose 0.25% were added to wells. Following 48 h incubation at 37°C, the optical density (OD) (595 nm) was quantified to estimate bacterial growth (OD_1). The plate was then washed with PBS twice and dried at air for 2 h. The remaining attached bacteria were fixed with 200 µl of methanol 99% (v/v) per well and after 15 minutes the plates were emptied and dried under air. Afterwards the plates were stained for 20 minutes with 200 µl per well of 0.5% crystal violet. Finally, wells were washed with water, dried under air, and the dye was solubilized with 33% acetic acid solution. The optical density of each well was measured at 595 nm (OD_2). Biomass was quantified by calculating the OD_2/OD_1 quotient. *S. aureus* Newman Δ*ica* (non *ica*-dependent biofilm producer) and *S. epidermidis* NRS101 (prototype biofilm producer) were included in the assay as negative and positive controls respectively.

Mouse skin infection model

The model of *S. aureus* skin infection in mice was adapted from Malachowa *et al* (Malachowa et al., 2013). Eight to nine week-old female Balb/c mice were subcutaneously inoculated with either the Sa30 or Sa5 strains ($1x10^7$ colony forming units (CFU) under general anesthesia with ketamine (40 mg/kg) and xylazine (5 mg/kg). One day prior to inoculation mice were weighted and the dorsal or the right flank regions were shaved to enhance visualization and permit measurement of the skin lesions. The animal body weight and the lesion size were monitored daily over six days after infection. Skin lesions were measured using calipers and areas were calculated using a previously described formula (Malachowa et al., 2013). On the sixth day the

animals were euthanized by CO_2 inhalation, the abscesses were excised and homogenized using a rotor-stator homogenizer. Serial dilutions of homogenates were plated on TSA supplemented with 5 µg/ml erythromycin for CFU count determination. In addition, spleens were extracted, homogenized in 1 ml of PBS and 100 µl were plated on mannitol salt agar plates to quantify the bacterial load in order to determine the bacterial invasiveness.

Rat acute osteomyelitis model

The assay was performed as previously described by Lattar *et al* (Lattar et al., 2014). Briefly, groups of female eight-nine weeks old Wistar rats were inoculated in the left tibia with 1-2 $x10^6$ CFU of the Sa30 or Sa5 strain under general anesthesia with ketamine (40 mg/kg) and xylazine (5 mg/kg). The inoculum was prepared in sterile PBS, and mixed immediately before inoculation with fibrin glue (Tissucol kit; Baxter Argentina-AG, Vienna, Austria) (Lattar et al., 2014; Spagnolo et al., 1993). Ninety six hours after infection the animals were euthanized by CO_2 inhalation and the right and left tibiae were aseptically excised. In order to quantify the macroscopic bone engrossment, the Osteomyelitic Index (OI) was determined morphometrically as described before. Subsequently, a one centimeter bone section that contained the inoculation point was crushed, homogenized and cultured onto TSA plates to perform CFU count determination.

Mouse respiratory infection model

Female eight weeks old Balb/c mice were inoculated intranasally with $1x10^8$ CFU of the Sa30 or Sa5 strain suspended in 10 µl of PBS, under general anesthesia with ketamine (40 mg/kg) and xylazine (5 mg/kg). Four hours after administration, blood

was obtained by puncturing the orbital plexus with a capillary tube, and a volume of 20 µl was cultured in 1 ml of brain heart infusion (BHI) to explore the occurrence of bacteremia. Twenty-four hours later mice were euthanized by CO_2 inhalation and both lungs were removed aseptically and homogenized in 1 ml of PBS at room temperature. Homogenates were plated on mannitol salt agar to quantify the bacterial load.

Accesory gene regulator (*agr)* locus function

The *agr* locus functionality was assessed by the delta haemolysin assay described by Traber et al (Traber et al., 2008). Haemolytic activity was determined by cross-streaking perpendicularly to *S. aureus* RN4220, which produces only *β*-haemolysin, on a sheep blood agar (SBA) plate.

Overnight cultures of 10 representative strains of each CA-MRSA clone and *S. aureus* RN4220 were streaked on SBA plates, after adjustment of optical density equal to 0.5 McFarland standard in saline buffer.

RT-qPCR

The expression of two adhesin genes (*clfB* and *fnbA*) in Sa30 and Sa5 strains was evaluated by RT-qPCR using primers designed for this study (*fnbA*-Forward: 5´GTGGATAGCGAAGCAGGTCA-3´, *fnbA*-Reverse: 5´-CCACCACCTGGGTTTGTATC-3´, *clfB*-Forward: 5´-CCACATCAGTAATAGTAGGGGC-3´, *clfB*-Reverse: 5´-CGGAATCTGCACTTGCATTAT-3´). RNA was isolated from bacterial cultures in BHI broth, until they reached mid-exponential phase (DO_{620nm}= 0.5-0.7) and late exponential phase of growth (DO_{620nm}=1.2) by triplicate. Bacterial pellets were treated with lysozyme 15 mg/ml (Sigma-Aldrich), for one hour at 37°C and RNA was extracted using TRIZOL® Reagent (Invitrogen) with the Pure Link® RNA Mini Kit (AMBION,

USA) according to manufacturer's recommendations. RNA was quantified using NanoDrop™ 1000 EspectroPhotometer (Thermo Scientific) and treated with DNAse (3U/µl, 1 hour at 37°C, RQ1 RNase free DNase, Promega). Reverse transcription was performed using 500 ng of RNA, 200U of M-MLV™ Reverse Transcriptase (Invitrogen, USA) and 50 µM Random primers (Invitrogen, USA) according to manufacturer's recommendations. The qPCR reaction was carried out using a 1/100 dilution of cDNA, SYBR® Select Master Mix (Applied Biosystems, USA) and the primers previously described in a 7500 Real-Time PCR System (Applied Biosystems, USA). The combination of *gyr*B and *pta* were used as reference genes in mid-exponential phase of growth and reference genes *gyrB* and *gap* were used combined in late exponential phase of growth as previously described (Valihrach and Demnerova, 2012). Cq values were converted into Normalized Relative Quantities (NRQ) values using normalization to the geometrical average of the reference genes and the specific PCR amplification efficiency for each gene (Hellemans et al., 2007; Valihrach and Demnerova, 2012; Vandesompele et al., 2002). The complete RT-qPCR protocol is described in supplemental material.

Statistical analysis

GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses. Medians of CFU in each infection model and OI in acute osteomyelitis model were compared with the Mann-Whitney U test. OD median values from biofilm production assay were compared with Kruskal-Wallis test followed by Dunn's multiple comparisons test. Skin lesion areas were compared day by day with the Mann-Whitney U test. NRQ values from RT-qPCR experiments were compared with Student´s t-test or Mann-Whitney U test when corresponded. Categorical variables were

analyzed by Fisher´s exact test. In all cases a *P* value <0.05 was considered statistically significant.

Ethics statement

The animals were housed at the research animal care facility of the Instituto de Microbiología y Parasitología Médica (IMPaM-UBA-CONICET). Animal care was in accordance with the Institutional Committee for Use and Laboratory Animal Care of the University of Buenos Aires and internationally accepted guidelines (ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments). All protocols using animals were evaluated and approved by the "Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio" (CICUAL), Facultad de Medicina, Universidad de Buenos Aires.

Results

CA-MRSA ST30-IVc clone is associated with invasive disease

The CA-MRSA collection from two multicenter prospective epidemiological studies performed previously was analyzed focusing in the source of isolation and the genotypic features of each isolate recovered. Considering all the CA-MRSA isolates genotyped belonging to the predominant clones (ST30-IVc and ST5-IVa), a statistically significant association was observed between the isolation of the CA-MRSA ST30-IVc clone and the occurrence of invasive disease (Fig 1).

Virulence associated gene pattern

All ST30 and ST5 isolates carried toxin coding genes *sei* and *seg*, and adhesin coding genes *clfA, clfB, eno* and *ebpS*, and the biofilm associated *icaA* gene. Adhesin coding

gene *sdrE* was positive only in the ST5 strains whereas *fnbB, bbp* and *cna* were only detected in ST30 strains. The distribution of *hlg, sea, fib* and *fnbA* was variable (Table 2).

+: All strains were positive

-: All strains were negative

Biofilm production

Biofilm production *in vitro* by CA-MRSA ST5-IVa and ST30-IVc did not show a significant difference between clones (Fig 2). Both CA-MRSA clones and *S. epidermidis* NRS101 produced more biofilm than *S. aureus* Newman Δ*ica*.

***S. aureus* Sa30 strain exhibited higher virulence in the skin and soft tissue infection model**

All mice developed visible skin lesions the day after subcutaneous inoculation with either Sa30 or Sa5 *S. aureus* strains. The lesion areas were larger in mice inoculated with *S. aureus* Sa30 and this difference was statistically significant at the first, second and fifth day post infection (Fig 3A). Representative photographs of abscesses are shown in Fig 3B. Moreover, the number of CFU recovered from skin lesions of mice inoculated with the Sa30 strain were higher than those recovered from mice inoculated with the Sa5 strain (Fig 3C). In mice inoculated with the Sa30 strain the skin lesion contained a median of $1.7x10^8$CFU (IQR $1.2x10^8$-$3.1x10^8$) which is close to 20 times the size of the original inoculum, indicating that the subcutaneous niche is favorable for *S. aureus* Sa30 replication. Conversely, skin lesions generated by the Sa5 strain contained a median of $8.5x10^6$ CFU (IQR $2.8x10^6$-$8.3x10^7$) under identical experimental conditions. Additionally, a group of mice that was challenged with the

Sa30 strain in a lower inoculum ($1x10^6$ CFU) developed skin lesion containing a median bacterial load of $4.5x10^7$ CFU (IQR $5.8x10^6$-$1.8x10^8$), more than one order of magnitude higher than the initial inoculum. Mice from both groups lost weight after inoculation (Fig 3D).

Sa30 strain demonstrated a greater ability to escape from the primary infection site and to replicate into other tissues in this model. This was evidenced by a trend of higher spleen colonization rate for the Sa30 group as the number of animals with spleen colonization was higher for the Sa30 group (7/8 vs. 4/9) (Fig 4A). A significantly increased number of CFU was recovered from spleens of mice colonized with the Sa30 strain compared with those colonized with the Sa5 strain (Fig 4B).

The Sa30 strain displayed increased survival and virulence in the acute osteomyelitis model

Wistar rats were inoculated in the left tibia with $1\text{-}2x10^6$ CFU of *S. aureus* Sa30 strain or Sa5 strain. There was a significant difference in virulence in both outcome measures: severity of the bone lesion measured by OI and CFU bone load. The macroscopic analysis of bone engrossment revealed that OIs were significantly higher in animals inoculated with the Sa30 strain, compared with those challenged with the Sa5 strain, indicating that a major damage of the tibiae was inflicted by *S. aureus* of the ST30 lineage (Fig 5A). The bacterial load was significantly increased in bones infected with the Sa30 strain compared with tibiae from rats infected with the Sa5 strain (Fig 5B). In previous own experiments where the strain *S. aureus* NRS384, (representative of USA300 clone) was inoculated in rat tibiae following the same experimental design we obtained a median bacterial count of $1.4x10^5$ CFU (IQR $3.6x10^4$-$3.5x10^5$) and a median OI of 0.3 (IQR 0.2-0.5). These values are not significantly different from those

obtained with Sa30 strain, indicating a similar capacity of both clones to survive in bone niche. Moreover, Sa30 produced more bone damage than USA300 (data not shown).

Sa30 and Sa5 exhibited the same behavior after intranasal infection

Using the respiratory infection model no significant differences were observed in the number of CFU recovered from the lungs 24 h after bacterial challenge (Fig 6A). However, the number of animals that suffered bacteremia four hours after intranasal inoculation was higher for the group challenged with the Sa30 strain, indicating a trend towards increased capacity for dissemination from the lung of this strain (Fig 6B).

Accesory gene regulator function

Increased activity of the δ - haemolysin was observed in all ST5 strains tested, indicating greater activation of *agr* system when compared to those of ST30. The decreased *agr* activity exhibited by ST30 lineage could benefit adhesion factors expression. The hemolytic activity of the representative Sa30 and Sa5 strains is shown in Figure 7. The production of α-haemolysin is also evidenced in this assay.

Virulence associated-genes expression

Since the strains of ST30 and ST5 lineages harbored several adhesin coding genes equally, two of them were selected for the analysis of expression by RT-qPCR in Sa30 and Sa5 strains. The Fibronectin-binding protein A coding gene (*fnbA*) was of special interest because FnBPA is involved in *S. aureus* invasion of host cells (Fraunholz and Sinha, 2012) and clumping factor B coding gene (*clfB*) was also selected because ClfB protein promote nasal colonization in both mice (Schaffer et al., 2006) and humans (Wertheim et al., 2008). Both strains expressed similar amounts of

the *clfB* gene regardless the growth phase analyzed (Figs 8A and 8C). By contrast, the expression of *fnbA* was higher in Sa30 strain compared with Sa5 strain in both mid-exponential (Fig 8B) and late exponential phase (Fig 8D).

Discussion

The progress in the understanding of *S. aureus* infections and disease has expanded by the development of appropriate animal models that were used to reveal virulence determinants and to support efficacy studies with vaccine antigens. In this study we utilized previously validated animal models to compare the virulence of the two predominant CA-MRSA clones described in Argentina. Taken together, our data suggest that the emerging CA-MRSA ST30-SCC*mec*IVc clone has a more aggressive behavior than the CA-MRSA ST5-SCC*mec*IVa. The first evidence is that a higher proportion of CA-MRSA invasive infection cases in adult patients from Argentina were due to the ST30 clone.

The skin and soft tissue infection model was selected because it approximates the most frequent disease presentation associated with CA-MRSA clones, and was the source of isolation of the strains used in this work. These infections commonly constitute the primary source for invasive staphylococcal diseases. Indeed in a previous study we identified skin infections as the initial source in almost 50% of invasive disease included in a multicenter study of CA-MRSA invasive infections performed in Argentina (Fernandez et al., 2013). In the present study, we observed an increased capacity of ST30 *S. aureus* to survive and replicate in the subcutaneous niche and these findings were strengthened by the observation that the bacterial load recovered from skin lesions due to Sa30 was more than ten-fold higher than the initial inoculum. Second, we detected that Sa30 exhibits a greater tendency to escape from skin abscess

and migrate toward other sites, and an advantage to replicate in a different organ such as the spleen. This invasion capacity is concordant with our observation of the significant association between the isolation of ST30 CA-MRSA and invasive disease described herein (Fig 1).

As it was demonstrated by Chua et al, the ST30 lineage can display the same virulence phenotype as CA-MRSA USA300. These authors compared the virulence of the CA-MRSA ST30-IV-PVL positive isolate (JKD6177), the well-known virulent pandemic USA300 clone (David and Daum, 2010; Diep et al., 2006) and other common Australian clones in a mouse skin infection assay (Chua et al., 2011). It is interesting to highlight that the ST30 strain produced similar levels of Hla and PVL compared with the USA300 clone and exhibited a similar behavior in the mouse skin infection model (Chua et al., 2014; Chua et al., 2011). Although genomic dissimilarities between the JKD6177 strain and Sa30 strain included in our study could not be disregarded, the results mentioned above, taking together with the results obtained herein, emphasize that the circulation of ST30 lineage in our country is an issue of concern.

The osteomyelitis model explores the capacity to colonize and survive in the bone niche, and this type of infection is a frequent presentation of CA-MRSA invasive disease. In the present work, animals infected with Sa30 presented a more severe bone pathological involvement (bigger bone engrossment of the infected tibiae) than Sa5. It is noteworthy that both the CA-MRSA ST30 clone and MRSA USA 300 share a similar ability to survive in the bone niche in these experimental settings (data not shown).

It is estimated that 15-36% of world population is colonized with *S. aureus* in the anterior nares, and nasal carriage is considered an important risk factor for the development of a *S. aureus* infection (Verhoeven et al., 2014). The respiratory infection model analyzed the capacity of the strain to develop a lung infection via intranasal

inoculation. In the context of the respiratory infection model, Sa30 and Sa5 behaved similarly, as they were recovered in equal CFU number from the mice lungs. This observation, opposite to what has been observed in the other two models performed, could provide an insight into the specific virulence factors playing a significant role in the establishment of infection through different inoculation routes. Possibly the expression of *clfB* gene provides both clones an equal ability to persist and develop infection via intranasal inoculation in mice. Further investigations are needed to better understand these observations.

One of the main strategies exhibited by *S. aureus* during the infection process is the ability to adhere to extracellular matrix and plasma proteins, which is a crucial factor in the colonization and dissemination of *S. aureus* throughout the host. In the present study, a comparative analysis of the virulence gene profile of two CA-MRSA clones demonstrated that the differences are restricted to certain key adhesin coding genes. In fact *fnbB, bbp* and *cna* were only detected in ST30 isolates while *sdrE* was positive only in the ST5 clone. The importance of our findings is strengthened by other reports describing that only genes encoding bone sialoprotein binding protein *(bbp)* and fibronectin binding protein B *(fnbB)* are significantly associated with hematogenous osteomyelitis/arthritis and native-valve endocarditis, suggesting their involvement in hematogenous tissue infections (Tristan et al., 2003). The presence of the two fibronectin-binding proteins coding genes is associated with strains recovered from invasive disease (endocarditis or primary septic arthritis and/or osteomyelitis) in comparison with nasal carriage strains (Peacock et al., 2000). The expression of FnBPA and FnBPB have been described as the most important factor for host cell invasion including non-professional phagocytes as osteoblasts, keratinocytes, fibroblasts and endothelial cells (Shinji et al., 2011) and, in a septic mouse infection model, Shinji *et al*.

demonstrated that cooperation between the two FnbPs is indispensable for the induction of severe infection resulting in septic death (Shinji et al., 2011). Both clones analyzed herein possess several adhesin coding genes; however, the specific repertoire of the ST30 clone could represent an advantage for the establishment of a localized infection and the dissemination to other tissues. For example, the increased capacity of invasion in animal models could be due to the presence of the two FnbPs coding genes and a possible higher production of fibronectin-binding protein A demonstrated in Sa30 strain.

The adherence to polystyrene plates did not show significant differences between clones; nevertheless this result may not accurately reflect the tissue adherence *in vivo.*

Expression of capsular polysaccharide (CP) appears to be essential for dissemination of *S. aureus* from the primary infection site, and loss of CP production is associated with *S. aureus* persistence and the development of chronic infections. *S. aureus* strains producing type 5 (CP5) and type 8 (CP8) capsules account for about 80% of human clinical isolates (Tuchscherr et al., 2010).

The expression of CP5 was found to be negatively correlated with the adherence to endothelial cells, probably by masking cell wall adhesins (Pohlmann-Dietze et al., 2000). In a recent communication, Liu *et al*. showed a difference between CP8 and CP5 behavior *in vivo* and *in vitro*. On the one hand, serotype 8 *S. aureus* clinical isolates released soluble CP8 during culture while little soluble CP5 was released by the serotype 5 isolates. On the other hand, CP8 was detected in the plasma of bacteremic mice at higher levels and more consistently than CP5; and antibodies against CP8 were unable to protect mice against experimental bacteremia provoked by clinical CP8 *S. aureus* strains. As suggested by the authors, released CP8 would compete and interfere with the opsonic function of capsular antibodies (Liu et al., 2016).

Both clonal lineages analyzed in the present work are characterized by possession of different capsular genes. In the light of the results mentioned above, the production of CP8 by ST30 lineage could probably increase its adhesion and virulence.

CA-MRSA ST30-IV-PVL positive belongs in the clonal complex 30 (CC30) and is thought to have evolved from a common ancestor with the phage type 80/81 penicillin-resistant pandemic *S. aureus* (DeLeo et al., 2011), a clone which emerged throughout 1950s and became a recognized virulent strain that caused a high number of skin lesions, sepsis, and pneumonia in children and young adults in hospitals and the community. CC30 is one of the most frequent lineages distributed worldwide and in recent descriptive studies it has been associated with an increased risk for infective endocarditis (Nienaber et al., 2011) and persistent *S. aureus* bacteremia (Xiong et al., 2009). More specifically, the MRSA ST30-IV clone was responsible for the large outbreak occurred in Uruguay in 2003 when at least 12 deaths were registered in previously healthy patients (Ma et al., 2005). Recently, two clinical cases from different geographic areas were communicated describing severe invasive disease associated with this clone (Dhanoa et al., 2012; Fernandez et al., 2015).

We recognize certain potential limitations to our study. The amplification of specific sequences for virulence genes may not fully correlate with conserved functionality of these genes. Therefore, phenotypic expression of selected target genes needs to be assessed to allow definitive conclusions on the contribution of these determinants to virulence. Since the infection studies were performed in experimental animal models of disease using a single representative strain of each lineage, further studies may be required to extend the conclusions to the rest of the ST30-IVc strains and to explore the relevance of our observations to human patients. It would also be

interesting to compare the cytolytic capacities of both clonal lineages because it contributes substantially to CA-MRSA virulence.

In conclusion, the present data support our hypothesis that CA-MRSA ST30-SCC*mec*IVc-*spa*t019-PVL positive clone, which has become the most prevalent clone in the Argentine community settings, is more virulent than the displaced ST5-SCC*mec*IVa-*spa*t311-PVL positive clone. Future studies are required to elucidate the molecular basis responsible for the difference between the emerging and the displaced CA-MRSA clone in the region under investigation.

Acknowledgments

This work was supported in part by grants from Universidad de Buenos Aires, Argentina to MM (UBACyT 20020130100381BA 2014-2017), DOS (UBACyT 20020100100347; 2014-2017) and Agencia Nacional de Promoción de la Ciencia (ANPCyT) to MM (PICT 2012/2362), to DOS (PICT 2013-0941), to MIG (PICT 2011-2263 y PICT 2013-1233). DOS, MM and MIG are members of the Research Career, CIC-CONICET Argentina. We thank Lorena Medina and Monica Pomerantz for her dedicated technical assistance.

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Figure captions

Fig 1. Association between lineages of CA-MRSA isolates and type of infection.

CA-MRSA clone ST30-IVc, the predominant clone described in SSTIs and invasive infections in adults from Argentina was significantly associated with invasive disease, compared with cases of CA-MRSA infections due to ST5-IVa clone. **P*<0.05, Fisher´s exact test.

Fig 2. Biofilm production by isolates of the ST30 and ST5 lineages.

Each box shows the median $OD_{595\ nm\ crystal\ violet}/OD_{595nm\ bacterial\ growth}$ and the interquartile range compared with Kruskall-Wallis test (*P*<0.0001). Individual groups were compared using the Dunn´s multiple comparison test showing no difference in biofilm production between ST30-IVc and ST5-IVa clones. Both clones produced a higher amount of biofilm than *S. aureus* Newman Δ*ica*. ** *P*<0.001, *** *P*<0.0001, Dunn´s Multiple comparison test.

Fig 3. Virulence comparison in the skin infection model.

(A) Daily measurements of the skin lesion size after subcutaneous inoculation with the indicated *S. aureus* strain. The data indicate the median values and the interquartile range of the medians. **P < 0.05* Mann-Whitney U test. (B) Photographs correspond to representative skin lesions produced by the two strains tested. (C) Bacterial load in the

skin lesion. Each dot represents the determination made on each individual mouse and the lines indicate the median of each group. ** $P < 0.01$, Mann-Whitney U test. (D) Weight loss is represented as the percentage of weight loss compared with day 0.

Fig 4. Bacterial dissemination from primary infection foci.

(A) Spleen colonization is presented as the percentage of animals with positive cultures. (B) The boxes represent the CFU recovered from each spleen in the two groups evaluated. * $P < 0.05$, Mann-Whitney U test.

Fig 5. Virulence comparison in the rat osteomyelitis model.

Dots represent the determination made on each individual rat, and the lines indicate the median of each group. * $P < 0.05$, Mann-Whitney U test (A**)** Osteomyelitic indexes (OIs) determined as an estimation of tissue damage. (B) Bacterial load recovered per bone expressed as $\log_{10}$ numbers of CFU.

Fig 6. Virulence comparison in the airway infection model.

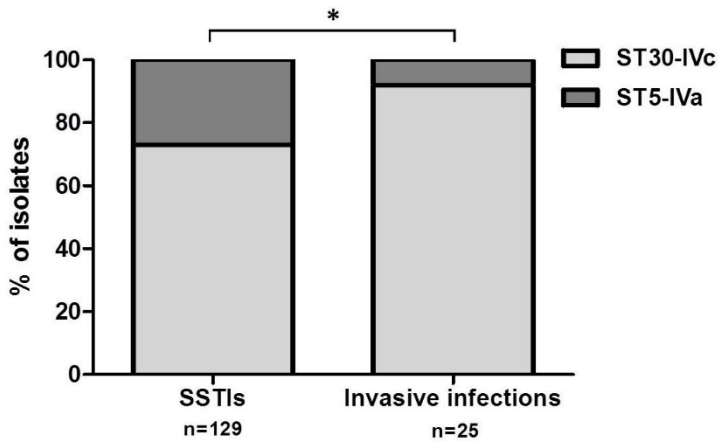
(A) Bacterial counts measured in lungs 24 hours after intranasal inoculation. Each dot represents a determination made in a single animal and the line indicates the median of each group. (B) Rate of bacteremia determined at four hours after intranasal inoculation.

Fig 7. Hemolytic activity of Sa30 and Sa5 against RN4220 in SBA plates.

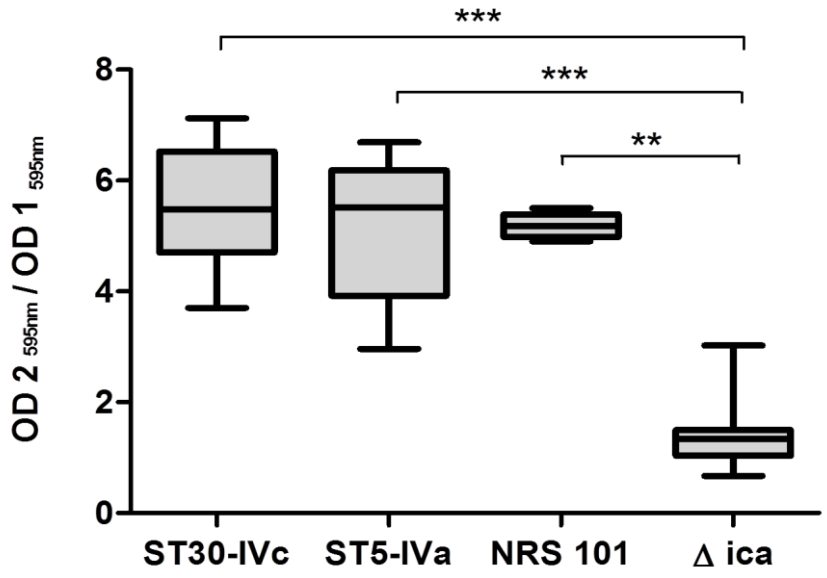
Fig 8. Expression of virulence genes.

Level of expression of *clfB* (A) and *fnbA* (B) genes in mid-exponential phase of growth normalized to reference genes *gyrB* and *pta*. The bars represent the mean and SEM of logarithm of normalized relative quantities (NRQ) values compared with Student´s t test (A) or median and interquartile range of (NRQ compared with Mann-Whitney U test (B). Level of expression of *clfB* (C) and *fnbA* (D) genes in late-exponential phase of

growth normalized to reference genes *gyrB* and *gap*. Each bar represents the mean and SEM of logarithm NRQ values.* $P < 0.05$, Students´t test.



Figr-1



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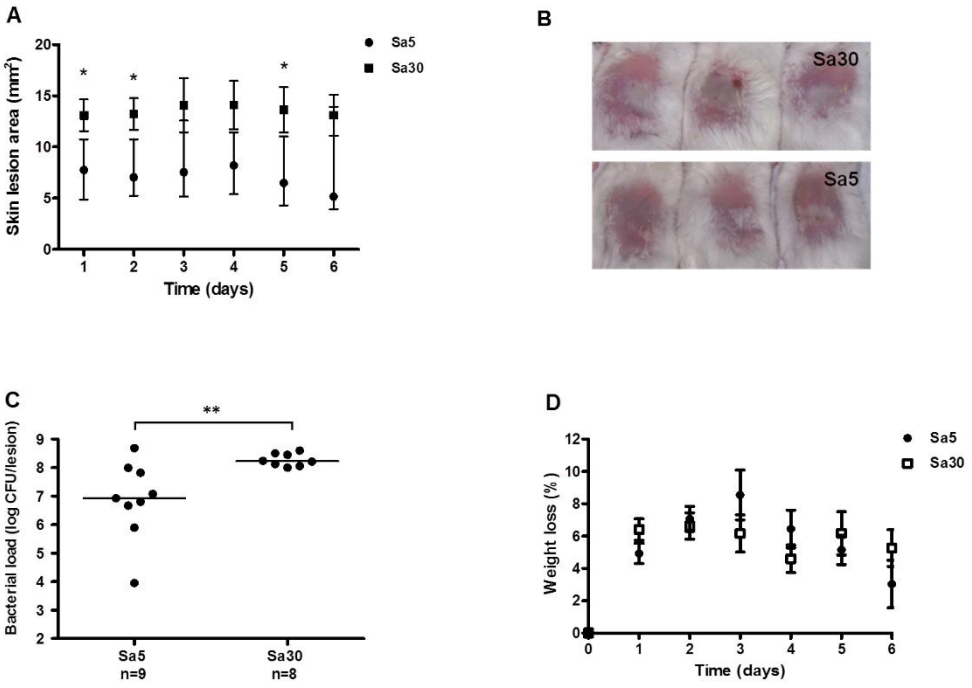

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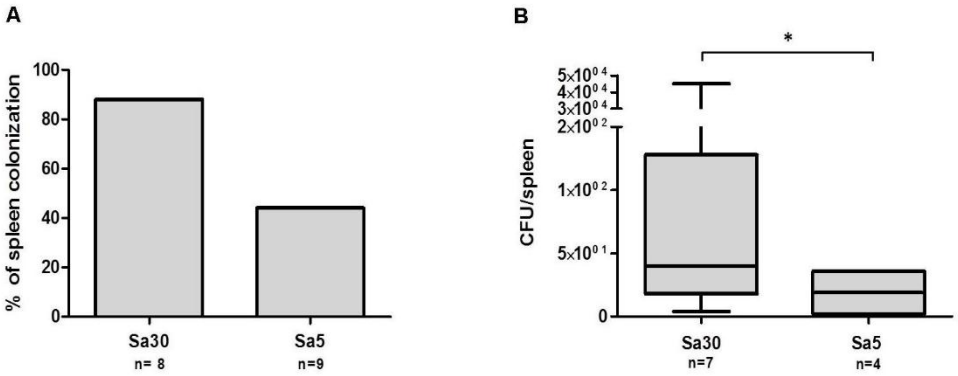

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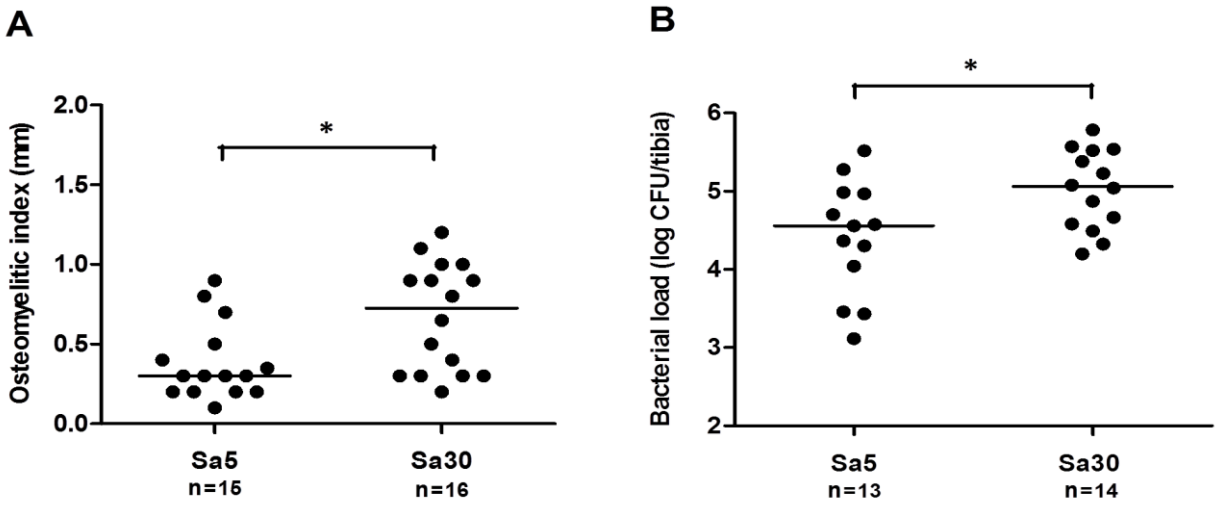
Figr-5



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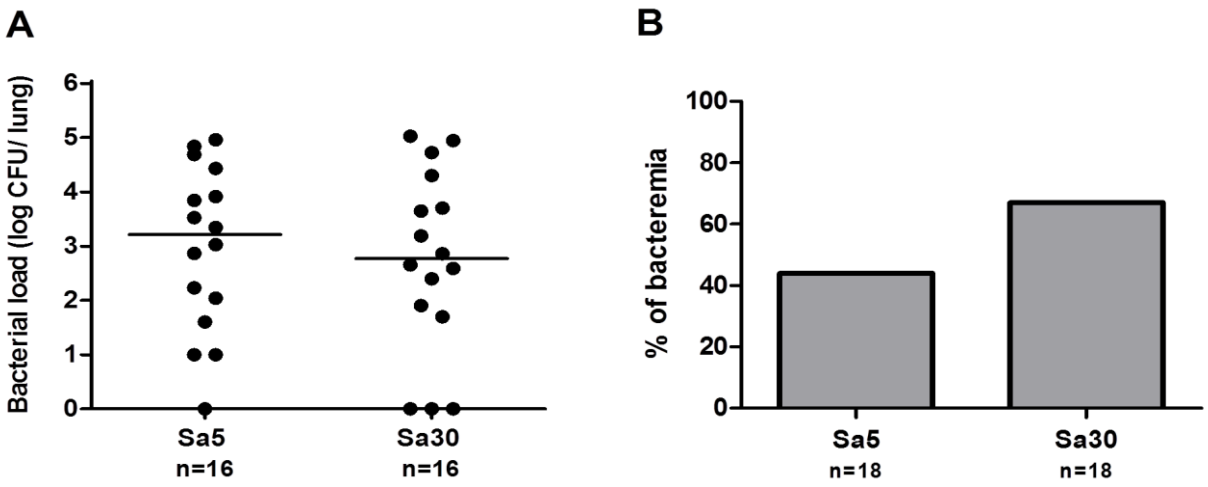

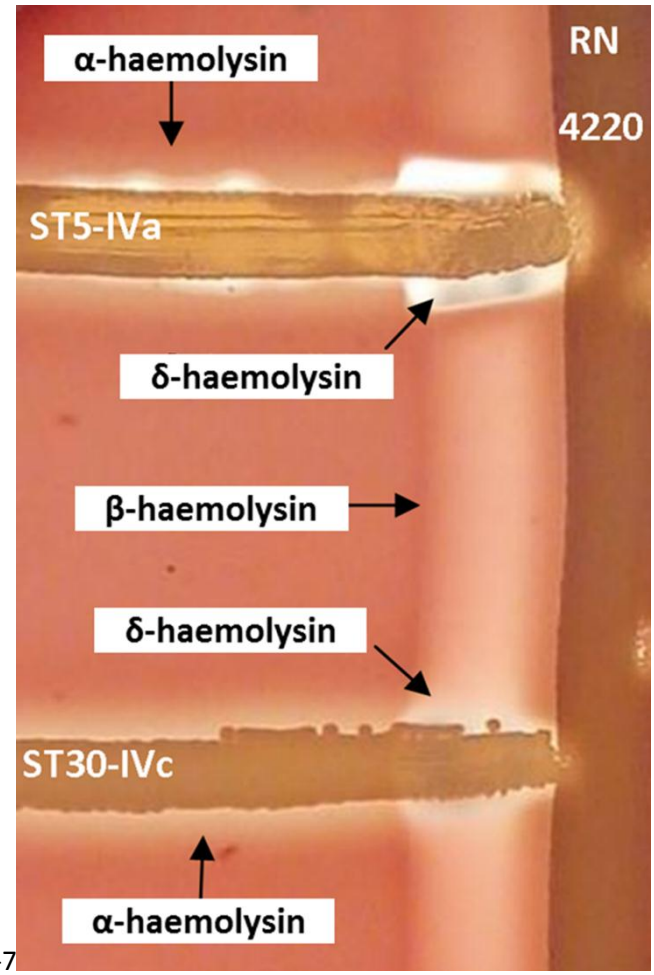

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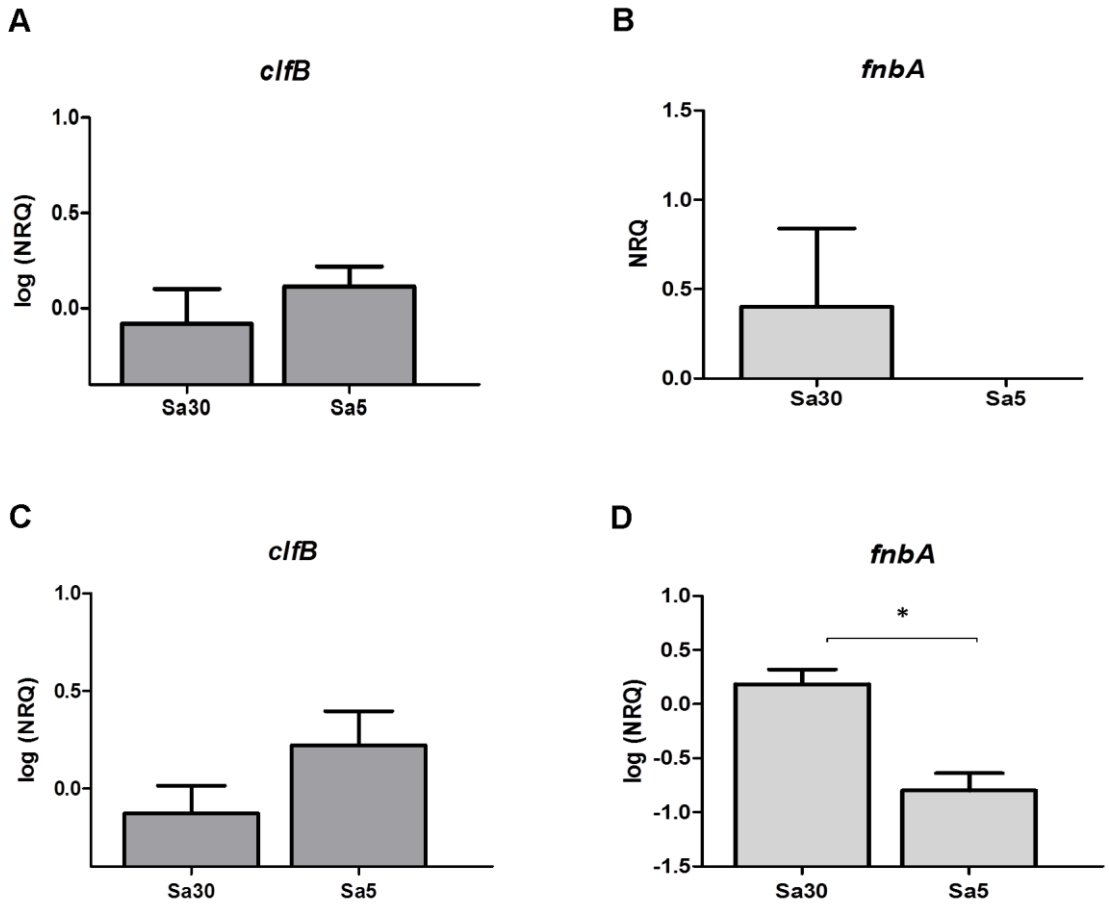

Table 2: Number and percentage of positive strains for virulence-associated genes

Gene	ST30-IVc (n:117) Positive isolates (%)	ST5-IVa (n:37) Positive isolates (%)
fnbA	102 (87,2)	35 (95,6)
fnbB	81 (69,2)	-
fib	14 (12)	+
clfA, clfB, eno, ebpS	+	+
cna, bbp,	+	-
sdrE	-	+
sec, seb, sej, seh	-	-
seg, sei	+	+
sea	15 (12,8)	+
hlg	36 (30,8)	16 (43)
icaA	+	+
tst	-	-

arcA	-	-