World Rabbit Science

World Rabbit Sci. 2015, 23: 185-195
doi:10.4995/wrs.2015.3961
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SCREENING OF VIRULENCE GENES IN *STAPHYLOCOCCUS AUREUS* ISOLATES FROM RABBITS

VIANA D., SELVA L., PENADÉS M., CORPA J.M.

Biomedical Research Institute (PASAPTA-Pathology group), Veterinary School, Universidad CEU Cardenal Herrera, Av. Seminario s/n, Moncada, 46113 Valencia, Spain.

Abstract: *Staphylococcus aureus* is a versatile pathogen able to cause disease in both humans and animals. In rabbits, this bacterium infects animals of different ages, producing several purulent lesions. The ability of *S. aureus* to cause disease depends on a combination of virulence factors. The aim of this study was therefore to investigate the distribution of bacterial virulence determinants in 69 *S. aureus* isolates from rabbits. Some virulence factors (7 adhesins, 1 toxin and 1 protease) were positive in all rabbit *S. aureus* isolates analysed, while others (1 adhesin and 10 toxins) were always negative. The remaining virulence factors were more variable among isolates. An association between genotype and the different profiles of virulence factors was observed, but not with the type of lesion ($P<0.05$). One strain of each genotype was further analysed by multilocus sequence typing, generating ST121, ST96 and ST2951, determining a greater number of enterotoxins in ST121 isolates compared to ST96 and ST2951 isolates, which could justify the different pathogenicity between strains.

Key Words: *Staphylococcus aureus*, rabbit, virulence factors, genotype, MLST.

INTRODUCTION

Staphylococcus aureus is a pathogen capable of infecting humans and a wide variety of animals. This bacterium affects rabbits of different ages, infects dermal lesions and invades subcutaneous tissues (Okerman *et al*., 1984), resulting in different pathologies including suppurative dermatitis, mastitis, multisystemic abscessation and pododermatitis (Corpa *et al*., 2009).

The ability of *S. aureus* to cause disease is due to a combination of virulence factors. *S. aureus* can produce more than 30 virulence factors that contribute to the establishment and maintenance of the infection (Haveri *et al*., 2008). Adherence to host tissues is crucial for the colonisation and establishment of infection. Colonisation sites provide a reservoir from which bacteria can be introduced when the host defences are breached (Gordon and Lowy, 2008). In this first step, adhesins or microbial cell surface molecules bind to host cells or to extracellular matrix molecules. Once *S. aureus* adheres to host tissues, it is able to grow and persist. During the infection, *S. aureus* produces numerous enzymes, such as proteases, lipases, and elastases, which enable it to invade and destroy host tissues and metastasise to other sites (Gordon and Lowy, 2008). Other virulence factors, such as toxins with superantigen activity, allow the persistence of *S. aureus* in the host, playing an important role in avoidance of the host immune system during an infection (Projan and Novick, 1997).

As pathogenicity and infectivity of a certain strain of *S. aureus* might depend on its repertoire of virulence factors, it seems reasonable to postulate that *S. aureus* strains associated with rabbit infections have variable combinations of pathogenic determinants and the presence or expression of these combinations varies depending on the genotype and the type of infection. So, the aim of this study was to investigate the distribution of bacterial virulence determinants

Correspondence: D. Viana, dviana@uch.ceu.es. Received *June 2015* - Accepted *July 2015*.
http://dx.doi.org/10.4995/wrs.2015.3961

in the most prevalent *S. aureus* strain types causing lesions in rabbits. These bacterial strains were analysed to determine the presence of various virulence genes by polymerase chain reaction (PCR) and Southern blot and explore their possible relationship with the genotype and type of lesions.

MATERIALS AND METHODS

Bacterial isolates

Sixty-nine *S. aureus* strains isolated from rabbits were tested. These strains were taken from 69 rabbit does, 1 strain of each animal, with different chronic purulent lesions previously described (Viana *et al*., 2007). For evaluation of virulence genes, 1 strain of each genotype and lesion was selected. The animals came from 30 industrial rabbitries situated in the Valencia province (Valencian Community), on the Spanish Mediterranean coast. The genotype of the isolated *S. aureus* strains was performed based on the analysis of the polymorphic regions of the *coa*, *spa* and *clf*B genes (Viana *et al*., 2007).

Multilocus sequence typing (MLST)

One strain of each genotype was further analysed by multilocus sequence typing (MLST; Enright *et al*., 2000). PCR products were directly sequenced by the Sequencing Service of the Institute of Molecular and Cellular Plant Biology of the Polytechnic University of Valencia (IBMCP-UPV), using an ABI PRISM® 377 (PE Biosystems) and MLST sequences were analysed using the MLST database (www.mlst.net).

Evaluation of bacterial virulent determinants

PCR analysis of bacterial determinants

Forty determinants, considered putative virulence factors in previous literature, were examined for the presence of gene by PCR. Sequences of the oligonucleotide primers, thermocycler programs, positive control and references are summarised in Table 1. The oligonucleotides designed for this study were based on gene sequences available from GenBanK® and were obtained from Invitrogen™.

Each amplification comprised 100 ng of DNA template, 100 pmol of each primer, 200 μM (each) deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), 1X buffer (Netzyme®, NEED), 1 mM $MgCl_2$, and 1 U of thermostable DNA polymerase (Netzyme®, NEED). Water was added to a final volume of 25 μL and thermal cycling was performed. The size of PCR products was determined by electrophoresis on 1% (wt/vol) agarose gels.

Southern blot analysis of bacterial determinants

Southern blot analysis was performed for the negative samples by PCR analysis of bacterial determinants. For Southern blot hybridisation, genomic DNA was digested with restriction endonuclease *Hind*III (Roche®)and ascertained by electrophoresis in 0.8% agarose. Fragments were transferred by alkaline capillary blotting onto nylon membranes (Hybond™-N; 0.45 mm pore-size filters; Amersham® Life Science) using standard methods. Probe labelling and DNA hybridisation were performed according to the protocol supplied with the PCR-digoxigenin DNA-labelling and chemiluminescence detection kit (Roche®).

Statistical analysis

Pearson's chi-square test was used to study the associations between virulence genes and most prevalent *S. aureus* genotypes and most frequent lesions, respectively. A *P* value of less than 0.05 was considered significant.

RESULTS

Sixty-nine *S. aureus* isolates from rabbits (1 strain of each genotype and lesion) were tested by PCR and Southern blot to analyse the presence of 40 virulence genes in *S. aureus* strains causing lesions in rabbits. Nine virulence

Table 1: Bacterial determinants examined in this study. Oligonucleotide primers and PCR programs for amplification of the genes.

Primer	Sequence (5'-3')	Positive control	PCR [a]	Reference
*clf*A				
clfA-1m	GTA GGT ACG TTA ATC GGT T	Newman	1	Peacock *et al.,* 2002
clfA-2c	CTC ATC AGG TTG TTC AGG			
*fnb*A				
fnbA-1m	CAC AAC CAG CAA ATA TAG	8325	2	Peacock *et al.,* 2002
fnbA-2c	CTG TGT GGT AAT CAA TGT C			
*fnb*B				
fnbB-1m	GTA ACA GCT AAT GGT CGA ATT GAT ACT	8325	4	Tristan *et al.,* 2003
fnbB-2c	CAA GTT CG ATA GGA GTA CTA TGT TC			
cna				
cna-1m	AGT GGT TAC TAA TAC TG	MSSA-476	2	Peacock *et al.,* 2002
cna-2c	CAG GAT AGA TTG GTT TA			
*sdr*C				
sdrC-1m	ACG ACT ATT AAA CCA AGA AC	Newman	3	Peacock *et al.,* 2002
sdrC-2c	GTA CTT GAA ATA AGC GGT TG			
*sdr*D				
sdrD-1m	GGA AAT AAA GTT GAA GTT TC	Newman	3	Peacock *et al.,* 2002
sdrD-2c	ACT TTG TCA TCA ACT GTA AT			
*sdr*E				
sdrE-1m	ATC AAG TAC TCA AAA ACA GC	Newman	3	Present study
sdrE-2c	TGG CTT GTT TCT TTA CCT GC			
bbp				
bbp-1m	AACTACATCTAGTACTCAACAACAG	RF122	2	Tristan *et al.,* 2003
bbp-2c	ATGTGCTTGAATAACACCATCATCT			
*ebp*S				
ebpS-1m	CATCCAGAACCAATCGAAGAC	Newman	2	Tristan *et al.,* 2003
ebpS-2c	CTTAACAGTTACATCATCATGTTTATCTTTG			
map/eap				
map/eap-1m	GCG AAA TAT ACA GTT AAT TT	Newman	1	Peacock *et al.,* 2002
map/eap-2c	ACT TTT TTA ATG TCA GTT GC			
ica A				
icaA-1m	CCA GAA AAT TCC TCA CCC GTA TTAG	N315	4	Present study
icaA-2c	GTG TCT GAC TTC GCT TTA ATA CAG CC			
fib				
fib-1m	GCG AAG GAT ACG GTC CAA GAG A	Newman	4	Boden W. *et al.,* 1995
fib-2c	CAA TTC GCT CTT GTA AGA CCA TT			
bap				
sasp-1m	CCC TAT ATC GAA GGT GTA GAA TTG CAC	V239	3	Trotonda *et al.*, 2005
sasp-2c	GCT GTT GAA GTT AAT ACT GTA CCT GC			
tst				
tsst-1m	CTA ATC AAA TAA TCA AAA CTG C	N315	3	Present study
tsst-2c	TTT CCA ATA ACC ACC CGT TT			
sea				
sea-1m	AAA GTC CCG ATC AAT TTA TGG CTA	MSSA-476	4	Akineden *et al.*, 2001
sea-2c	GTA ATT AAC CGA AGG TTC TGT AGA			
seb				
seb-1m	TCG CAT CAA ACT GAC AAA CG	COL	4	Akineden *et al.*, 2001
seb-2c	GCA GGT ACT CTA TAA GTG CC			

(Table 1 continues on next page)

(Table 1; continued from previous page)

Primer	Sequence (5'-3')	Positive control	PCR [a]	Reference
sec				
sec-1m	GAC ATA AAA GCT AGG AAT TT	N315	4	Akineden *et al.*, 2001
sec-2c	AAA TCG GAT TAA CAT TAT CC			
sed				
sed-1m	CTA GTT TGG TAA TAT CTC CT	FRI1151m	4	Akineden *et al.*, 2001
sed-2c	TAA TGC TAT ATC TTA TAG GG			
see				
see-1m	TAG ATA AGG TTA AAA CAA GC	FRI326	4	Akineden *et al.*, 2001
see-2c	TAA CTT ACC GTG GAC CCT TC			
seg				
seg-1m	AAT TAT GTG AAT GCT CAA CCC GAT C	N315	4	Akineden *et al.*, 2001
seg-2c	AAA CTT ATA TGG AAC AAA AGG TAC TAG TTC			
seh				
seh-1m	CAA TCA CAT CAT ATG CGA AAG CAG	MSSA-476	4	Akineden *et al.*, 2001
seh-2c	CAT CTA CCC AAA CAT TAG CAC C			
sei				
sei-1m	CTC AAG GTG ATA TTG GTG TAG G	N315	4	Akineden *et al.*, 2001
sei-2c	AAA AAA CTT ACA GGC AGT CCA TCT C			
selk				
selk-1m	ATG GCG GAG TCA CAG CTA CT	COL	4	Holtfreter *et al.*, 2004
selk-2c	TGC CGT TAT GTC CAT AAA TGT T			
sell				
sell-1m	CAC CAG AAT CAC ACC GCT TA	N315	4	Holtfreter *et al.*, 2004
sell-2c	TCC CCT TAT CAA AAC CGC TAT			
selm				
selm-1m	CTA TTA ATC TTT GGG TTA ATG GAG AAC	N315	4	Jarraud *et al.*, 2001
selm-2c	TTC AGT TTC GAC AGT TTT GTT GTC AT			
seln				
seln-1m	ACG TGG CAA TTA GAC GAG TC	N315	4	Jarraud *et al*, 2001
seln-2c	GAT TGA TCT TGA TGA TTA TGA G			
selo				
selo-1m	GAG AGT TTG TGT AAG AAG TCA AGT G	N315	4	Smyth *et al.*, 2005
selo-2c	GAT TCT TTA TGC TCC GAA TGA GAA			
selp				
selp-1m	CTG AAT TGC AGG GAA CTG CT	N315	4	Holtfreter *et al.*, 2004
selp-2c	ATT GGC GGT GTC TTT TGA AC			
selq				
selq-1m	GAA CCT GAA AAG CTT CAA GGA	N315	4	Holtfreter *et al.*, 2004
selq-2c	ATT CGC CAA CGT AAT TCC AC			
selu				
selu-1m	TAA AAT AAA TGG CTC TAA AAT TGA TGG	N315	4	Letertre *et al*, 2003
selu-2c	ATC CGC TGA AAA ATA GCA TTG AT			
eta				
eta-1m	CTA GTG CAT TTG TTA TTC AA	2020	4	Akineden *et al.*, 2001
eta-2c	TGC ATT GAC ACC ATA GTA CT			
etb				
etb-1m	ACG CGT ATA TAC ATT CAA TT	2020	4	Akineden *et al.*, 2001
etb-2c	TCC ATC GAT AAT ATA CCT AA			

(Table 1 continues on next page)

(Table 1; continued from previous page)

Primer	Sequence (5'-3')	Positive control	PCR [a]	Reference
lukS,F-PV				
pvl-1m	ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A	RN6390	4	Lina *et al.,* 1999
pvl-2c	GCA TCA AGT GTA TTG GAT AGC AAA AGC			
hlg				
hlg-1m	GCC AAT CCG TTA TTA GAA AAT GC	Newman	4	Lina *et al.,* 1999
hlg-2c	CCA TAG ACG TAG CAA CGG AT			
*ssp*A				
ssp-1m	CAA GTG CTG CAG GTC AAG TTG	COL	4	Present study
ssp-2c	CCG TGC GTA GCA TCT ACG ACG TG			
cap5				
cap5-1m	CAT AAT TCG AGG GTT TGG TC	Newman	4	Present study
cap5-2c	CTA ATT GCC GCT GGA ACT GCC			
cap8				
cap8-1m	GTT TGG TCT TGT TAT TTG TGG	RF122	4	Present study
cap8-2c	CTT CTA ACG AAT GAC TCT TCC G			
*agr*I				
agrI-1m	CAC TTA TCA TCA AAG AGC C	RN6734	3	Strommenger *et al.,* 2004
agrI-2c	CCA CTA ATT ATA GCT GG			
*agr*II				
agrII-1m	GTA GAG CCG TAT TGA TTC	RN6607	3	Strommenger *et al.,* 2004
agrII-2c	GTA TTT CAT CTC TTT AAG G			
*agr*III				
agrIII-1m	TAT ATA AAT TGT GAT TTT TTA TTG	RN8465	3	Peacock *et al.,* 2002
agrIII-2c	TTC TTT AAG AGT AAA TTG AGA A			
*agr*IV				
agrIV-1m	GTT GCT TCT TAT AGT ACA ATG TT	RN4850	3	Peacock *et al.,* 2002
agrIV-2c	CTT AAA AAT ATA GTG ATT CCA ATA			

PCR program[a]: 1: 94°C for 3 min; 35 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 120 s; 72°C for 5 min. 2: 94°C for 3 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 120 s; 72°C for 5 min. 3: 94°C for 3 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s; 72°C for 5 min. 4: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; 72°C for 5 min.

determinants were positive in all the rabbit *S. aureus* isolates analysed in this study: 7 virulence factors were adhesins (*fnb*A, *clf*A, *sdr*C, *ebp*S, *map/eap, ica*A and *fib*), 1 virulence factor was a toxin (*hlg*C) and 1 virulence factor was a protease (*ssp*A) (Table 2). On the other hand, 11 virulence factors could not be detected using the oligonucleotide

Table 2: Virulence factors positive in all *S. aureus* analysed isolates.

Virulence factor	Protein	Function
Adhesins		
*fnb*A	FnBPA	Fibronectin-binding protein
*clf*A	ClfA y ClfB	Fibrinogen-binding protein
*sdr*C	SdrC	Adhesin
*ebp*S	EbpS	Elastin-binding protein
map/eap	Map/Eap	Analogous, fibrinogen-binding protein
*ica*A	IcaA	*ica* locus, bacterial polysaccharide synthesis
fib	Fib	Fibrinogen-binding protein
Toxin		
*hlg*C	γ-haemolysin	Leukocidin
Protease		
*ssp*A	V8 protease	Serine protease

Table 3: Virulence factors negative in all *S. aureus* analysed isolates.

Virulence factor	Protein	Function
Adhesin		
bap	Bap	Formation of bacterial biofilm
Toxins		
tst	TSST-1	Exotoxin superantigens
sea, seb, sec, see, selp, selq	Enterotoxina A, B, C, E, P, Q.	Exotoxin superantigens
eta y etb	Toxina exfoliativa A y B	Exotoxin superantigens
lukS, F-PV	Leucocidina Panton-Valentine	Leukotoxin

primers available, as none of the analysed strains harboured genes encoding *bap, tst, sea, seb, sec, see, selp, selq*, *eta, etb and lukS, F-PV* (Table 3).

The rest of the virulence factors were more variable between the analysed isolates. Table 4 summarises a comparison of the distribution of the virulence factors obtained. Type 8 capsule (one of the capsular polysaccharides involved in the inhibition of opsonophagocytosis) was the most frequent one in rabbit isolates (76.8%, Table 4). In addition, the global regulator *agr*, remarkably responsible for the expression of major virulence factors, was analysed. In this case, the most frequent *agr* subgroup was type IV (51%, Table 4).

Generally, enterotoxins are associated in different gene combinations. In the present study, the most frequent enterotoxin association was gene cluster type 2 (*egc*-2), which contains 5 superantigen genes *seg*, *sei*, *selm*, *seln*, *selo* and *selu* (Table 5).

Presence of virulence factors in the most prevalent S. aureus genotypes

After investigating the distribution of bacterial virulence determinants in *S. aureus* isolates from rabbits, the most prevalent genotypes (9 genotypes from 56 different isolates, Table 6) were associated with different profiles of virulence factors. Genotypes A1/II1/δ, A1/II1/ε, A1/II1/η and A1/III1/δ generated the ST121, genotypes B1/I1/α, B1/IV1/α, B1/IV2/β and C1/I1/β generated the ST96 and genotype D1/IV2/α generated the ST2951 (Table 6). Each

Table 4: Comparison of the distribution of virulence factors in rabbit *S. aureus* isolates analysed in this study

Virulence factor	Protein	Function	Positive isolates (%)
Adhesins			
cna	Cna	Collagen-binding protein	65 (94.2)
*sdr*D	SdrD	Adhesins	62 (89.9)
*sdr*E	SdrE	Adhesins	54 (78.3)
bbp	Bbp	Bone sialoprotein-binding protein	52 (75.4)
*fnb*B	FnBPB	Fibronectin-binding protein	41 (59.4)
Toxins			
sed	Enterotoxin D	Exotoxin superantigen	13 (18.8)
seg	Enterotoxin G	Exotoxin superantigen	39 (56.5)
seh	Enterotoxin H	Exotoxin superantigen	10 (14.5)
sei	Enterotoxin I	Exotoxin superantigen	42 (60.9)
selk	Enterotoxin K	Exotoxin superantigen	11 (15.9)
sell	Enterotoxin L	Exotoxin superantigen	10 (14.5)
selm	Enterotoxin M	Exotoxin superantigen	42 (60.9)
seln	Enterotoxin N	Exotoxin superantigen	40 (58)
selo	Enterotoxin O	Exotoxin superantigen	40 (58)
selu	Enterotoxin U	Exotoxin superantigen	48 (69.6)
Capsular polysaccharides			
*cap*5	Cap5	Capsular polysaccharide	5 (7.2)
*cap*8	Cap8	Capsular polysaccharide	53 (76.8)
Global regulator			
*agr*I	Agr type I	Global regulator	7 (10)
*agr*II	Agr type II	Global regulator	0 (0)
*agr*III	Agr type III	Global regulator	27 (39.1)
*agr*IV	Agr type IV	Global regulator	35 (51)

identified genotype was associated with diverse profiles of adhesin genes (Table 6), with some exceptions: *sdr*D, *sdr*E and *fnb*B were variable in 3 genotypes (A1/II1/δ, A1/II1/η and C1/I1/β). Genotype B1/IV2/β had the minimal number of adhesins needed to cause injury (lacking of *sdr*D, *sdr*E and *bbp*). Furthermore, only genotype D1/IV2/α (ST2951) belonged to *agr* subgroup type I and capsular polysaccharide serotype 5 (Table 6).

Similarly, the different genotypes identified were associated with diverse profiles of enterotoxin genes with more variability than adhesin genes (Table 7). *sed, seh, selk, sell* and *selu* were variable in 7 different genotypes (A1/II1/δ, A1/II1/η, A1/III1/δ, B1/I1/α, B1/IV1/α, C1/I1/β and D1/IV2/α). Four isolates were lacking enterotoxins: 3 isolates belonging to genotype B1/IV2/β and 1 isolate belonging to genotype D1/IV2/α.

Although some virulence factors were variable in the same genotype, the distribution of all virulence factors analysed was related to the type of *S. aureus* genotype ($P<0.05$).

Presence of virulence factors in the most frequent lesions

After investigating the diverse distribution of bacterial virulence determinants in *S. aureus* isolates from rabbits, the most prevalent lesions (5 type of lesions, 44 isolates, Table 8 and 9) were associated with different profiles of virulence factors. However, the type of lesion could not be related with any virulence factors ($P<0.05$) (Tables 8 and 9).

Table 5: Rabbit isolates proved positive to different enterotoxin combinations

Gen combinations	Positive isolates (%)
sec, sell, egc1	1 (1.5)
sell, egc1	2 (2.9)
sed, seh, selk, egc2	1 (1.5)
sed, selk, egc2	1 (1.5)
selk, egc2	12 (17.4)
egc2	23 (33.3)
sed, seh, sell, selu	1 (1.5)
sed, sell, selu	1 (1.5)
sed, seh	2 (2.9)
sed, sell	2 (2.9)
sed, selu	3 (4.4)
sed	2 (2.9)
seh, sell	1 (1.5)
seh, sei	1 (1.5)
seh, selu	2 (2.9)
seh	2 (2.9)
sei, selm, selu	2 (2.9)
selm, seln, selo, selu	1 (1.5)
sell	2 (2.9)
selu	2 (2.9)
None	5 (7.2)

DISCUSSION

Staphylococcus aureus has been found to be rather host specific (Herron-Olson *et al.*, 2007) and knowledge about the genetic variability within different *S. aureus* populations may help in the identification of the most likely source

Table 6: Rabbit isolates positive to adhesins, type of capsular polysaccharide and agr subgroup in the most prevalent *S. aureus* genotypes.

Genotype	ST	Isolates	cna	sdrD	sdrE	bbp	fnbB	cap5	cap8	agrI	agrII	agrIII	agrIV
A1/II1/δ	121	22	100%	91%	95%	100%	41%	-	100%	-	-	-	100%
A1/II1/ε	121	3	100%	100%	100%	100%	100%	-	100%	-	-	-	100%
A1/II1/η	121	6	100%	100%	100%	100%	50%	-	-	-	-	17%	83%
A1/III1/δ	121	4	100%	100%	100%	100%	100%	-	100%	-	-	-	100%
B1/I1/α	96	4	100%	100%	100%	100%	100%	-	100%	-	-	100%	-
B1/IV1/α	96	6	100%	100%	-	-	100%	-	100%	-	-	100%	-
B1/IV2/β	96	3	100%	-	-	-	100%	-	100%	-	-	100%	-
C1/I1/β	96	6	100%	83%	83%	100%	-	-	100%	-	-	100%	-
D1/IV2/α	2951	2	-	100%	100%	-	100%	100%	-	100%	-	-	-

Virulence factors with percentage values greater than 0% (-) and lower than 100% are highlighted in light grey.

Table 7: Rabbit isolates positive to enterotoxins in the most prevalent *S. aureus* genotypes.

Genotype	ST	Isolates	sed	seg	seh	sei	selk	sell	selm	seln	selo	selu
A1/II1/δ	121	22	-	100%	-	100%	14%	-	100%	100%	100%	100%
A1/II1/ε	121	3	-	100%	-	100%	-	-	100%	100%	100%	100%
A1/II1/η	121	6	-	100%	-	100%	83%	-	100%	100%	100%	100%
A1/III1/δ	121	4	50%	100%	25%	100%	75%	-	100%	100%	100%	100%
B1/I1/α	96	4	100%	-	-	-	-	-	-	-	-	50%
B1/IV1/α	96	6	67%	-	17%	-	-	50%	-	-	-	50%
B1/IV2/β	96	3	-	-	-	-	-	-	-	-	-	-
C1/I1/β	96	6	33%	-	100%	-	-	-	-	-	-	33%
D1/IV2/α	2951	2	-	-	-	-	-	50%	-	-	-	-

Virulence factors with percentage values greater than 0% (-) and lower than 100% are in light grey.

of an isolate (Haveri *et al*., 2008). The aim of this study was to investigate the distribution of bacterial virulence determinants in the most prevalent *S. aureus* strain types causing lesions in rabbits that could possibly explain this host specificity. In the present study, the diversity of analysed virulence genes was high, as previously described in cows (Akineden *et al*., 2001; Zecconi *et al*., 2006) and humans (Peacock *et al*., 2002). Adhesins *coa, spa, clf*A, *clf*B, *fib, sdr*C and *map/eap* were positive in all the rabbit *S. aureus* isolates analysed in this study. These adhesins are specific to *S. aureus,* secreted by all *S. aureus* isolates and can be used for the identification of this bacterium (Boden Wastfelt and Flock, 1995; Josefsson *et al*., 1998; Luczak-Kadlubowska *et al*., 2006; Hussain *et al*., 2008). Moreover, *ebp*S, *fnb*A and *ica*A were positive in all the analysed isolates. Vancraeynest *et al.* (2004) reported a prevalence for *ebp*S of 74%, which is lower than the result obtained in this study (100%), while the distribution of *ebp*S in human isolates is variable according to previous literature (58%-100%) (Peacock *et al*., 2002; Tristan *et al*., 2003). In line with other studies, all rabbit *S. aureus* strains were positive for *ica*A (Arciola *et al*., 2001; Vancraeynest *et al*., 2004); however, none of the isolates analysed in this study was biofilm-forming (data not shown). Moreover, all isolates were negative to *bap,* which has only been described to date in bovine isolates (Cucarella *et al*., 2001). The rest of the adhesins showed variable prevalence: *cna* (94.2%), *sdr*D (89.9%), *sdr*E (78.3%), *bbp* (75.4%) and *fnb*B (59.4%). The high prevalence of *cna* was unexpected, as it was remarkably higher in this study than in isolates from humans (Nashev *et al*., 2004), other animals (Zecconi *et al*., 2006) or even rabbits (Vancraeynest *et al*., 2004). Most of the isolates were positive for 3 *sdr* adhesins (78%), which is the most common result described in the bibliography (Peacock *et al*., 2002), although some isolates are only positive to *sdr*C (7%) (Sabat *et al*., 2006).

Studies comprising human and animal isolates showed that staphylococcal enterotoxin genes are very common in *S. aureus* strains (Smyth *et al*., 2005; Blaiotta *et al*., 2006). In the present study, *egc*-2 was the most frequent combination, even though 15 different combinations were also identified. Enterotoxins associated in different gene combinations are generally encoded on mobile genetic elements (MGEs) (Holtfreter *et al*., 2007). Some combinations were not consistent with current knowledge of superantigen genes carrying MGEs, indicating the existence of variants or new types of MGEs (Wang *et al*., 2009). A recent study showed that rabbit strains are characterised by few MGEs

Table 8: Rabbit isolates positive to adhesins, type of capsular polysaccharide and agr subgroup among the most frequent lesions.

Lesion	Isolates	cna	sdrD	sdrE	bbp	fnbB	cap5	cap8	agrI	agrII	agrIII	agrIV
Mastitis	15	93%	80%	67%	67%	67%	7%	80%	13%	-	53%	33%
Pododermatitis	15	88%	88%	71%	59%	71%	20%	53%	27%	-	40%	33%
Abscesses	5	100%	100%	100%	100%	60%	-	80%	-	-	20%	80%
Conjunctivitis	5	80%	100%	80%	60%	80%	20%	80%	20%	-	60%	20%
Otitis	4	100%	75%	75%	75%	25%	-	75%	-	-	75%	25%

Virulence factors with percentage values greater than 0% (-) and lower than 100% are in light grey.

Table 9: Rabbit isolates proved positive to enterotoxins in the most frequent lesions.

Lesion	Isolates	sed	seg	seh	sei	selk	sell	selm	seln	selo	selu
Mastitis	15	33%	40%	20%	47%	7%	20%	47%	40%	40%	47%
Pododermatitis	15	20%	47%	7%	53%	20%	33%	60%	53%	53%	53%
Abscesses	5	20%	80%	20%	80%	40%	-	80%	80%	80%	80%
Conjunctivitis	5	40%	20%	40%	20%	-	20%	20%	20%	20%	80%
Otitis	4	25%	25%	50%	50%	-	25%	25%	25%	25%	50%

Results equal to 0% are represented as "-".

compared to human strains (Viana *et al.*, 2015), so the study of these putative new variants might be very useful to find out more information on the evolution of the bacterium as pathogenic. The most common enterotoxins in rabbit isolates were *selu* (69.9%), *sei* (60.9%) and *selm* (62%), in agreement with previous studies in strains from the same species (Larsen *et al.*, 2002; Smyth *et al.*, 2005). In addition, all isolates proved negative for *sea, seb* and *see,* which is consistent with other available studies in which these toxins were the least distributed in animal isolates (Larsen *et al.*, 2002; Smyth *et al.*, 2005). Moreover, all isolates were negative to superantigen genes *sec, selp, selq, tst* and *pvl.* The γ-haemolysin was positive in all rabbit *S. aureus* isolates, suggesting that the *hlg* locus may be ubiquitous in rabbit isolates, as described in most bovine (Fitzgerald *et al.*, 2000; Ote *et al.*, 2011) and human *S. aureus* isolates (Prevost *et al.*, 1995). Microorganisms that cause invasive diseases commonly produce extracellular antiphagocytic capsular polysaccharides. Most clinical isolates of *S. aureus* produce either *cap*5 or *cap*8 (O'Riordan and Lee, 2004). Type 8 capsule was the most frequent one in rabbit isolates (76.8%), with low prevalence of type 5 capsule or others (7.2% and 16%, respectively). Moreover, the most frequent *agr* subgroups were type IV (51%) and type III (39.1%). Vancraeynest *et al.* (2006) also reported that the most frequent *agr* subgroup was type IV (100%). However, these results disagree with studies in human and bovine species, determining that the most frequent *agr* subgroup was type I (Buzzola *et al.*, 2007; Vautor *et al.*, 2009).

The presence of combinations of virulence factors plays an important role in the host or even in tissue specificity in *S. aureus* infections (van Leeuwen *et al.*, 2005). In spite of this, after analysing the association between the most prevalent lesions and the different profiles of virulence factors, the type of lesion could not be related to any combination of virulence factors.

Multilocus sequence typing analysis evidenced that *coa*/*spa*/*clf*B genotype is capable of distinguishing isolates belonging to the same sequence type (ST). The different MLST obtained were ST121, ST96 and ST2951, all of them previously described in rabbits (Smyth *et al.*, 2009; Agnoletti *et al.*, 2014). The main difference in the profile of virulence genes among ST121 and ST96-ST2951 strains was observed in the presence of enterotoxins. ST96 and ST2951 were lacking in *seg, sei, selk, selm, seln, selo, and* one isolated ST2951 even lacked enterotoxins. These enterotoxins exert superantigen activity and can activate very large numbers of the exposed T cell population and disturb cytokine release to evade the host immune system (Projan and Novick, 1997). In a recent study, animals infected with ST121 strains showed greater circulating granulocytes and lower lymphocytes in blood than those infected by *S. aureus* ST96 strain (Guerrero *et al.*, 2015). This different immune response could be explained by the presence or absence of these enterotoxins, although further studies are needed to confirm this hypothesis.

In conclusion, evaluating the combination of *S. aureus* virulence factors would help in the design of efficient treatments. In this study, the great majority of isolates belonging to the same genotype were related to the same virulence factors. Nevertheless, certain virulence factors were variable within the same genotype. This variability could be due to variants or new types of MGEs that can promote genetic diversity and sometimes adaptation to a new environment. ST121, ST96 and ST2951 were obtained by MLST, determining a greater number of enterotoxins in ST121 isolates compared to ST96 and ST2951 isolates, which could explain the difference in virulence among *S. aureus* strains.

Acknowledgements: This study was supported by the Inter-ministerial Commission for Science and Technology (CICYT) of the Spanish Government (AGL2011-30170-C02-02 and AGL2014-53405-C2-2-P). The fellowship support for MP from Ministry of Education, Culture and Sports. Spain (AP2010-3907) is gratefully acknowledged.

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