SCREENING OF VIRULENCE GENES IN *Staphylococcus aureus* ISOLATES FROM RABBITS

Viana D.[1], Selva L.[1], García-Quirós A.[1], Penadés M. [1], Penadés J.R. [2,3], Corpa J.M.[1]*

[1]Dept. de Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los Alimentos (Histología y Anatomía Patológica), CEU-Cardenal Herrera University, 46113 Moncada (Valencia), Spain
[2]Dept. de Ciencias Biomédicas, CEU-Cardenal Herrera University, 46113 Moncada (Valencia), Spain
[3]Centro de Investigación y Tecnología Animal. Instituto Valenciano de Investigaciones Agrarias (IVIA), Apdo. 187, Polígono La Esperanza, 100, 12400, Segorbe (Castellón), Spain
*Corresponding author: jmcorpa@uch.ceu.es

ABSTRACT

Staphylococcus aureus is a versatile pathogen that can survive in diverse host environments and produce a wide range of diseases both in humans and animals. This versatility depends on its ability to modulate gene expression and the synthesis of virulence determinants. Therefore, this study aimed to investigate the distribution of bacterial virulence determinants in the most prevalent *S. aureus* strain types causing lesions in rabbits. Sixty-nine *S. aureus* strains were isolated from rabbit does with different chronic purulent lesions from 30 Spanish industrial rabbitries. Genotyping characterization of the strains was performed based on the analysis of the polymorphic regions of the *coa*, *spa* and *clf*B genes, as well as Multylocus Sequence Typing (MLST) on one strain of each of the most frequent genotypes. The isolates were also analyzed for the presence of forty virulence genes by PCRs and Southern blot, in order to determine their relationship with the genotype and the type of infection respectively. The great majority of isolates belonging to the same genotype were related to the same virulence factors, even though certain virulence factors were variable inside a genotype. However, the type of infection could not be related to any combination of virulence factors.

Key words: *Staphylococcus aureus*, Rabbit, Virulence factors, genotype, MLST.

INTRODUCTION

Staphylococcus aureus is a versatile, opportunistic pathogen with abilities to persist and multiply in a variety of environments and cause a diverse range of diseases in both humans and animals. In rabbits, this bacterium infects dermal lesions and invades subcutaneous tissues causing from minor skin infections to severe gross lesions for which adult does from rabbitries are culled. Its ability to produce such a wide spectrum of disease has been attributed to two major mechanisms: 1) Invasion and inflammation and 2) Toxin production (Zhu, 2010).

Invasion and inflammation include mechanisms for colonization, synthesis of extracellular molecules that facilitate adherence and the ability to evade host defenses. In this first step of the pathogenesis, the ability of the bacterium to produce an array of more than 30 virulence factors that contribute effectively to the establishment and maintenance of the infection play a crucial role (Haveri *et al*., 2008).

It is well known that *S. aureus* produces exotoxins with superantigen activity that are responsible for damaging host tissue and promoting dissemination, such as toxic shock syndrome toxin (TSST), staphylococcal enterotoxins (SEs), exfoliative toxins (ETs) or Panton-Valentine Leukocidine (PVL).
As major virulence factors in *S. aureus*, TSST, ETs, and SEs have been implicated in host colonization, invasion of damaged skin and mucus, and evasion of host defense mechanisms. Therefore, it is important to determine the toxin genes profile of *S. aureus* strains from different lesions in rabbits. Therefore, it seems reasonable to postulate that *S. aureus* strains associated with rabbit infections have variable combinations of pathogenic determinants and the presence or the expression of these combinations varies depending on the genotype and the type of the infection.

MATERIALS AND METHODS

Bacterial isolates

A total of 69 *S. aureus* strains isolated from rabbit does (*Oryctolagus cuniculus*) with different chronic purulent lesions were tested. The animals came from 30 industrial rabbitries located on the Spanish Mediterranean coast. Molecular typing of the isolated *S. aureus*, based on the analysis of the polymorphic regions of the *coa*, *spa* and *clfB* genes, was performed as previously described by Viana *et a*l. (2007).

Multilocus Sequence Typing (MLST)

One strain of the most frequent genotypes (Viana *et al*., 2007) was further analyzed by MLST, using the primers and PCR conditions specified at the MLST website (http://www.mlst.net). PCR products were directly sequenced by the Service of Sequenciation of the Institute of Molecular and Cellular Biology of Plants of the Polytechnical University of Valencia (IBMCP-UPV), using an ABI 377 (PE Biosystems; Foster City, California).

Evaluation of bacterial virulent determinants

Forty determinants were examined for the presence of the gene by PCR and Southern Blot. The oligonucleotides designed for this study were based on gene sequences available from GenBanK and were obtained from Invitrogen, and the appropriate different thermocycler programs and positive controls based on previous references.

Southern blot analysis was performed for the negative samples by PCR analysis of bacterial determinants.
Probe labeling and DNA hybridization were performed according to the manufacturer's protocol supplied with the PCR- digoxigenin DNA-labeling and chemiluminescence detection kit (Roche).

Statistical Analysis

The differences between the prevalences of the different genotypes, as well as the different lesions included in the study, were analyzed by Chi-Square analysis and Fisher's Exact p-value using. A p-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Virulence gen content in rabbit *S. aureus* isolates

The analysis of the forty virulence determinants showed that the 69 *S. aureus* rabbit strains tested contained at least 9 of those virulence factors. Seven of them were adhesins (*fnbA*, *clfA*, *sdrC*, *ebpS*, *map/eap, icaA* and *fib*), one virulence factor was a toxin (*hlg*C) and one virulence factor was a protease (*ssp*A). Vancraeynest *et al.* (2004) reported prevalence for *ebp*S of 74%, which is lower than the described in this study. On the other hand, 11 virulence factors could not be detected using the oligonucleotide primers available, as none of the analyzed strains harbored genes encoding *bap, tst, sea, seb, sec, see, selp, selq*, *eta, etb and lukS,F-PV*. Up to the moment, even though the adhesin *bap* has never been described in rabbit isolates, it has been reported in bovine (Cucarella *et al.*, 2001). The rest of virulence factors were more variable between isolates analyzed (Table 1). The high prevalence of *cna* was remarkably higher than in other *S. aureus* animals isolates or in previous rabbit studies (Vancraeynest *et al.* 2004).The majority of isolates were proved positive to three *sdr* adhesins (78%), which is the most common result described in the literature.

Staphylococcal enterotoxins genes are very common in *S. aureus* strains. In the present study, the most frequent enterotoxin association was the gene cluster type 2 (*egc*-2), which contains five superantigen genes *seg*, *sei*, *selm*, *seln*, *selo* and *selu* (Table 2). The most common enterotoxins in rabbit isolates were *selu* (69.9 %), *sei* (60.9) and *selm* (62 %), in agreement with previous studies in rabbit isolates. In addition, all the isolates were proved negative to *sea, seb* and *see,* which is consistent with previous studies where these toxins were the less distributed in animal isolates. Moreover, all the isolates were

Table 1: Distribution of virulence factors in *S. aureus* rabbit isolates analyzed.

Virulence factor	Protein	Function	% of positive isolates
Adhesins			
cna	Cna	Collagen-binding protein	94.2
*sdr*D	SdrD	Adhesins	89.9
*sdr*E	SdrE	Adhesins	78.3
bbp	Bbp	Bone sialoprotein-binding protein	75.4
*fnb*B	FnBPB	Fibronectin-binding protein	59.4
Toxins			
sed	Enterotoxin D	Exotoxin superantigen	18.8
seg	Enterotoxin G	Exotoxin superantigen	56.5
seh	Enterotoxin H	Exotoxin superantigen	14.5
sei	Enterotoxin I	Exotoxin superantigen	60.9
selk	Enterotoxin K	Exotoxin superantigen	15.9
sell	Enterotoxin L	Exotoxin superantigen	14.5
selm	Enterotoxin M	Exotoxin superantigen	60.9
seln	Enterotoxin N	Exotoxin superantigen	58
selo	Enterotoxin O	Exotoxin superantigen	58
selu	Enterotoxin U	Exotoxin superantigen	69.6

Table 2: Percentage of rabbit isolates proved positive to different enterotoxin combinations.

Gen combinations	% of positive isolates
sec, sell, egc1	1.5
sell, egc1	2.9
sed, seh, selk,egc2	1.5
sed, selk,egc2	1.5
selk, egc2	17.4
egc2	33.3
sed, seh, sell, selu	1.5
sed, sell, selu	1.5
sed, she	2.9
sed, sell	2.9
sed, selu	4.4
Sed	2.9
seh, sell	1.5
seh, sei	1.5
seh, selu	2.9
Seh	2.9
sei, selm, selu	2.9
selm, seln, selo, selu	1.5
sell	2.9
selu	2.9
None	7.2

negative to SAg genes *sec, selp, selq, tst* and *pvl.* The γ-hemolisin was positive in all rabbit *S. aureus* isolates, suggesting that the *hlg* locus may be ubiquitous in rabbit isolates, just like it has been described in bovine *S. aureus* isolates (Fitzgerald *et al.*, 2000) and in human *S. aureus* isolates. Microorganisms that cause invasive diseases commonly produce extracellular capsular polysaccharides. Most clinical isolates of S. aureus produce either cap5 or cap8. Type 8 capsule was the most frequent one in rabbit isolates (76.8%). 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.

Association of virulence genes with genotypic background

When analyzing the correlation between virulence genes profiles and the more prevalent genotypes (9 genotypes, 56 isolates out of the total of 69, table 3), each of these genotypes was also related to a certain profile of adhesin genes, with some exceptions: *sdr*D, *sdr*E and *fnb*B were variable in three genotypes (A1/II1/δ, A1/II1/η and C1/I1/β). The same occurred in enterotoxins genes (Table 4), with some exceptions: *sed, seh, selk, sell* and *selu* were variable in seven 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 of enterotoxins (three of them belonging to the genotype B1/IV2/β and one isolated belonging to the genotype D1/IV2/α).
Respect MLST typing, ST96 strains lacked *sdr*E and *bbp*, and belonged only to *agr*III group unlike ST121 strains.

Table 3: Percentage of rabbit *S. aureus* isolates positive to adhesins, type of capsular polysaccharide and *agr* subgroup among prevalent genotypes.

Genotype	ST	Isolates	*cna*	*sdr*D	*sdr*E	*bbp*	*fnb*B	*cap*5	*cap*8	*agr*I	*agr*II	*agr*III	*agr*IV
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	121	4	100%	100%	100%	100%	100%	-	100%	-	-	100%	-
C1/I1/β	121	6	100%	83%	83%	100%	-	-	100%	-	-	100%	-
D1/IV2/α	121	2	-	100%	100%	-	100%	100%	-	100%	-	-	-
B1/IV1/α	96	6	100%	100%	-	-	100%	-	100%	-	-	100%	-
B1/IV2/β	96	3	100%	-	-	-	100%	-	100%	-	-	100%	-

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

Table 4: Percentage of rabbit *S. aureus* isolates positive to enterotoxins among prevalent 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	121	4	100%	-	-	-	-	-	-	-	-	50%
B1/IV1/α	96	6	67%	-	17%	-	-	50%	-	-	-	50%
B1/IV2/β	96	3	-	-	-	-	-	-	-	-	-	-
C1/I1/β	121	6	33%	-	100%	-	-	-	-	-	-	33%
D1/IV2/α	121	2	-	-	-	-	-	50%	-	-	-	-

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

The more prevalent lesions: mastitis, pododermatitis, abscesses, conjunctivitis and otitis, were associated with different profiles of virulence factors. However, the type of infection could not be related to any combination of virulence factors.

CONCLUSION

In conclusion, the type of infection could not be related to any combination of virulence factors, although it is well known that the presence of combinations of virulence factors plays an important role in the host or even in tissue specificity in *S. aureus* infections. However, the great majority of isolates belonging to the same genotype were related to the same virulence factors, even though certain virulence factors were variable inside a genotype. This variability could be due to variants or

new types of MGEs, as mentioned above, insertion of transposons or insertion sequences. These insertions would cause mutations in the genes for its integration which would promote genetic diversity and sometimes adaptation to a new environment. In agreement with Jarraud *et al.* (2002) we postulate that the basic unit of bacterial pathogenicity could be the clone or lineage, which expands because it possesses particular combinations of virulence and regulatory genes in the appropriate genetic background.

ACKNOWLEDGEMENTS

This study was supported by grants from the CEU Cardenal Herrera University (PRCEU-UCH01/08, 23/08, 33/09 and 01/11) Banco Santander/CEU-UCH: Copernicus Program (PRCEU-UCH/COOP 01/08), Generalitat Valenciana (GV05/202, ACOMP/2009/207, ACOMP/2010/062) and the Comisión Interministerial de Ciencia y Tecnología (AGL2008-00273/GAN and AGL2011-30170-CO2-02).

REFERENCES

Cucarella C., Solano C., Valle J., Amorena B., Lasa I., Penades, J.R. 2001. *Bap*, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol 183: 2888-2896*.

Fitzgerald J.R., Hartigan P.J., Meaney W.J., Smyth, C.J. 2000. Molecular population and virulence factor analysis of *Staphylococcus aureus* from bovine intramammary infection. *J Appl Microbiol 88: 1028-1037*.

Haveri M., Hovinen M., Roslof A., Pyorala S. 2008. Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. *J Clin Microbiol 46: 3728-3735*.

Jarraud S., Mougel C., Thioulouse J., Lina G., Meugnier H., Forey F., Nesme X., Etienne J., Vandenesch, F. 2002. Relationships between *S. aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. *Infect Immun 70: 631-641.*

Vancraeynest D., Hermans K., Haesebrouck F. 2004. Genotypic and phenotypic screening of high and low virulence *Staphylococcus aureus* isolates from rabbits for biofilm formation and MSCRAMMs. *Vet Microbiol 103: 241-247.*

Viana D., Selva L., Segura P., Penades J.R., Corpa J.M. 2007. Genotypic characterization of *Staphylococcus aureus* strains isolated from rabbit lesions. *Vet Microbiol 121: 288-298.*

Zhu Y. 2010. *Staphylococcus aureus* virulence factors synthesis is controlled by central metabolism. *Dissertations & Theses in Veterinary and Biomedical Science. University of Nebraska – Lincoln.*