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GENETIC ANALYSIS OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM RABBITS. PRELIMINARY RESULTS.

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ABSTRACT

Colonization by livestock-associated Methicillin-Resistant *Staphylococcus aureus* (LA-MRSA) has been increasingly reported on rabbit farms in Europe. The aim of this study was to sequence the MRSA strains isolated from rabbit lesions of different lineages from farms in the Iberian Peninsula. Thirteen isolates of four different lineages (ST2855, ST146, ST398, ST4774) were sequenced and used in comparative genomics. The genetic characteristics of these strains were similar in the same ST. The most important findings were the presence of the CRISPR-cas system in the strains belonging to ST2855, the presence of phages with Sa7int and Sa3int, and the presence of SaPI with integrase type II in the ST398 lineage. These findings required further investigation to assess if there was any effect on the pathogenesis of staphylococcal infections.

Key words: *S. aureus*, MRSA, rabbit lesions.

INTRODUCTION

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have become a growing problem in human medicine since the 1960s, and more recently in veterinary medicine with the appearance of livestock-associated MRSA (LA-MRSA). *S. aureus* frequently infects commercial rabbits. The first case of MRSA on a rabbit farm was described in Italy (Agnoletti et al., 2014). Recently, the analysis of 240 *S. aureus* isolates obtained from commercial rabbitries from the Iberian Peninsula identified 12.5% as MRSA (Moreno-Grúa et al., 2018). This resistance is given by genes *mec*A or *mec*C within a chromosomal cassette called SCC*mec* (Boyce et al., 2005). In addition to resistance to methicillin, different SCC*mec* carry other genes involved in resistance to other antibiotics (Partridge et al., 2018). After finding many MRSA strains from rabbit farms in the Iberian Peninsula in 2018, an in-depth analysis of these strains was necessary. In this work, these strains were genetically analyzed to describe resistance genes, and the presence or absence of mobile genetic elements carrying virulence genes.

MATERIALS AND METHODS

Bacterial isolates

MRSA strains were isolated from different rabbit lesions during the 2014-2017 period (Moreno-Grua et al., 2018). Thirteen strains (2 ST146 strains, 2 ST398 strains, 7 ST2855 strains and 2 ST4774 strains), isolated from rabbit farms in the Iberian Peninsula, were sequenced and used in comparative genomics. Jwt, a highly virulent MSSA strain of the ST121 lineage, the genome of which has been reported (Viana et al., 2015a) and DL9, a low-virulence MSSA strain of the ST96 lineage, were used in comparative genomics.

Complete genome sequencing

Strains were streaked in TSA plates and incubated at 37ºC for 24 h. Bacteria were collected with a loop and homogenized in specific tubes previously sent by the sequencing laboratory. Information on the strains was provided on the laboratory's website (http://microbesng.com/).

After obtaining the sequences, the genetic characteristics of the mobile genetic elements were studied, such as the presence of different virulence factors, toxins and host adaptation genes, using different informatics tools: RAST (Rapid Annotation using Subsystem Technology, http://rast.theseed.org/), the NCBI's BLAST tool (https://blast.ncbi.nlm.nih.gov/) and computer programs to visualize genomes. Genome information was divided into different fragments called contigs. Then some PCRs were performed to join a few of them. These PCR products were sent to Eurofins Genomics for sequencing.

RESULTS AND DISCUSSION

Whole genome sequencing is becoming a frequent and cheap technique to study different organisms. After sequencing however, information needs to be processed by specific programs handled by experts. Finally, it is needed to interpret the results for optimal analyses. Here the complete genomes of 13 strains were sequenced in an external laboratory and the annotation of contigs was done with the RAST server. Afterward, basic tools were used to look for different genes and to compare them between distinct strains.

Several differences were found among all the strains (Table 1); for instance, only the strains belonging to ST146 carried the enterotoxin gene cluster called *egc* (*seg*, *sei*, *sem*, *seo*, *seu*). These enterotoxins seem to be involved in infection and pathogenicity in rabbits as Viana et al. (2015b) described how ST121 strains (high-virulent MSSA strains) had more enterotoxins than ST96 (low-virulent MSSA strains).

Table 1: Relation between types of lesions and genotypic characteristics in the sequenced MRSA isolates.

Isolate ID	**Lesion**	**ST (MLST)**	**SCC*mec* type**	***agr* types**	**IEC types**	***egc* cluster**
Sp-795	Otitis	146	IV	II	E	GIMNOU
Sp-986	Dermatitis	146	IV	II	E	GIMNOU
Sp-1006	Conjunctivitis	398	V	I	-	-
Sp-1032	Mastitis	398	V	I	-	-
P-991	Metritis	2855	III	III	-	-
Sp-993	Mastitis	2855	III	III	-	-
Sp-1000	Metritis	2855	III	III	-	-
Sp-1001	Hepatitis	2855	III	III	-	-
Sp-1002	Metritis	2855	III	III	-	-
P-1009	Pneumonia	2855	III	III	-	-
P-985	Metritis	2855	*mec*C	III	-	-
Sp-987	Metritis	4774	*mec*C	III	-	-
Sp-999	Mastitis	4774	*mec*C	III	-	-

P, Portugal; Sp, Spain

No sequenced strain carried genes lukS/F-PV, *tst*, *eta* or *bap*. This finding could indicate that these strains do not need these genes to colonize and develop infections in rabbits, as observed before in ST121 strains in rabbits vs. humans (Viana et al., 2015a). An ST121 strain carrying the *pvl* gene has been recently detected and affected a rabbit farm in China (Wang et al., 2019). This strain caused severe lung infections, which may indicate adaptation of ST121 to lung conditions as it has been reported that PVL produces necrotizing pneumonia (Diep et al. 2010). All the strains harbored both leukocidins *luk*AB and *luk*ED, except for strains ST398 that had only *luk*AB leukocidin. The strains belonging to ST2855 were the only ones with the CRISPR-cas system. CRISPR is widespread in both bacteria and archaea, and protects from viral infections. When a bacterium detects the presence of viral DNA, it integrates short fragments of viral DNA into the CRISPR locus. The recombination between

phages and CRISPR-cas loci facilitates horizontal gene transfer in staphylococci (Varble et al. 2019). This finding in strains ST2855 needed further investigation to assess if there was any effect on pathogenesis. Moreover, all the strains contained the three subunits of the *hlg*ABC locus.
Interestingly, lack of virulence factors and enterotoxins of ST96 and ST2855 could allow them to survive in the host and to produce infection later, as demonstrated recently by Tuchscherr et al., 2019. These authors observed that more low-cytotoxicity strains survived and were less efficiently cleared by the host than highly cytotoxic strains, which makes them a source of chronic infections.
Another important gene related with host adaptation and infection is *dlt*B. This gene encodes for a membrane protein involved in transporting positive charges outside bacteria (Collins et al. 2002). Variations in the *dlt*B gene were studied. It was observed that *dlt*B remained invariable within the same ST, except for strains ST398, which presented changes in amino acids 2, 227 and 405.
Regarding mobile genetic elements (MGE), it was observed that all the sequenced strains carried at least one bacteriophage in the genome (Table 2). MGE are an important source of genetic variability and transmission of virulence factors. The first studies about MGE in rabbit strains hypothesized that rabbit ST121 strain had lost MGE because they do not need them to develop infections (Viana et al., 2015). The strains carrying prophage with Sa3int had a truncated β-hemolysin sequence. Interestingly, strain DL9 (ST96) had this phage, while strains ST2855, which belong to CC96, did not carry this phage and, therefore, the β-hemolysin sequence was intact.

Table 2: Presence of prophages, *hlb* and virulence factors in the phages in the sequenced strains and controls.

Isolate ID	ST (MLST)	Phage int	*hlb*	*sak*	*chp*	*scn*
Jwt	121	12	+	-	-	-
DL9	96	3,7	-	+	+	+
Sp-795	146	3,5	-	+	-	+
Sp-986	146	1,3	-	+	-	+
Sp-1006	398	2,9	+	-	-	-
Sp-1032	398	2	+	-	-	-
P-991	2855	7	+	+	-	-
Sp-993	2855	7	+	+	-	-
Sp-1000	2855	2,6,7	+	+	-	-
Sp-1001	2855	2,7	+	+	-	-
Sp-1002	2855	2,7	+	+	-	-
P-1009	2855	2,7	+	+	-	-
P-985	2855	2,7	+	+	-	-
Sp-987	4774	New	+	-	-	-
Sp-999	4774	New	+	-	-	-

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The presence of virulence factors in prophages was also studied. No virulence factors were detected in the phage of the ST121 control strain. On the contrary, all the phages with Sa7int carried the *sak* gene. Sa3int from the ST96 control strain carried genes *sak*, *chp* and *scn*, while Sa3int from ST146 carried only *sak* and *scn* (Table 2). SaPIs were found only in strains ST398, which carried integrase type II SaPI.

CONCLUSIONS

In this work, MRSA strains were genetically analyzed to describe resistance genes and the presence or absence of MGE carrying virulence genes. The genetic characteristics of strains were similar in the same ST. The most important findings were the presence of the CRISPR-cas system in the strains belonging to ST2855, the presence of phages with Sa7int and Sa3int, and the presence of SaPI with integrase type II (ST398). These findings needed to be further investigated to assess if there was any effect on the pathogenesis of staphylococcal infections.

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