SCREENING OF HIGH AND LOW VIRULENCE *STAPHYLOCOCCUS AUREUS* ISOLATES FROM RABBITS FOR MSCRAMM GENES

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ABSTRACT

At rabbit flock level, two types of *Staphylococcus aureus* infections can be distinguished. In the first type, caused by low virulence strains, the infection remains limited to a small number of animals. The second type of infection is caused by high virulence strains, which spread throughout the rabbitry. The pathogenetic capacity of a particular *S. aureus* strain is attributed to a combination of extracellular factors and properties such as adherence. Twentyeight high virulence and 34 low virulence *S. aureus* isolates recovered between 1998 and 2003 were used to study the prevalence of genes encoding for microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). PCR assays were performed to detect *bbp* (encoding bone sialoprotein binding protein), *clf*A and *clf*B (encoding clumping factors A and B), *cna* (encoding collagen binding protein), *ebp*S (encoding elastin binding protein), *eno* (encoding laminin binding protein), *fnb*A (encoding fibronectin binding protein A), *fnb*B (encoding fibronectin binding protein B) and *fib* (encoding fibrinogen binding protein). All rabbit *S. aureus* strains harboured *clf*A and *clf*B. The prevalences of *ebp*S, *eno*, *fnb*A and *fib* did not reveal striking differences between high and low virulence strains. *Fnb*B prevalence in high virulence isolates was significantly lower than in low virulence isolates and *cna* was absent in high virulence strains. It was remarkable that only high virulence strains were positive for *bbp*. This could lead to the development of a diagnostic PCR test to screen asymptomatic rabbits for the presence of these strains, in order to prevent the entry of these rabbits in unaffected rabbitries.

Key words: *Staphylococcus aureus*, rabbit, adhesion.

INTRODUCTION

In individual rabbits, all *S. aureus* infections have a similar clinical appearance, with lesions of pododermatitis, subcutaneous abscesses and mastitis (OKERMAN *et al*., 1984; HOLLIMAN and GIRVAN, 1986; ROSSI *et al*., 1995; DEVRIESE *et al*., 1996). Sporadically, internal organ abscesses are observed as well, predominantly in lungs, liver and uterus. This leads to poor production results, infertility and death. Suckling young may die as a result of agalactia in the doe. At rabbit flock level, two clinical types of *S. aureus* infections can be distinguished. In the first type, caused by low virulence strains, the

infection remains limited to a small number of animals. This type only has a minor economic importance. The second type of infection is caused by the high virulence strains, which spread throughout the rabbitry. This leads to chronic problems. Highly virulent strains causing an epidemic spread of disease in rabbitries typically belong to the biotype-phage type combination "mixed CV-C"-3A/3C/55/71 (OKERMAN *et al*., 1984; HERMANS *et al*., 1999). At one occasion however, a biotype – phage type combination "mixed CV-C" - 29/79/42E/92/D11/HK2 caused an epidemic spread as well (DEVRIESE *et al*., 1996).

The pathogenesis of a particular *S. aureus* strain is attributed to a combination of extracellular factors, such as the secretion of toxins, and properties such as the capability to adhere to matrix molecules. Different proteins of the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are involved in *S. aureus* adhesion (FOSTER and HOOK, 1998). It has been shown that high virulence *S. aureus* strains from rabbits have a better capacity to colonize the host than low virulence *S. aureus* strains (HERMANS *et al*., 2000). This may be caused by a difference in adhesive capabilities between high and low virulence strains. In addition, MSCRAMMs may mediate *S. aureus* attachment to different cell types (DZIEWANOWSKA *et al*., 1999). Staphylococcal MSCRAMMs can be detected with binding assays using purified matrix molecules. However, these assays are expensive. Moreover, some adhesins are redundant: there are two fibronectin binding proteins and three receptors for fibrinogen (Clumping factors A and B and fibrinogen binding protein). Another complicating factor in the use of binding assays is the fact that some MSCRAMMS bind to more than one matrix molecule. A more practical approach is to use PCR technology for the detection of adhesion genes (TRISTAN *et al*., 2003).The goal of the present study was to perform a genotypic screening for a set of MSCRAMM genes among high and low virulence *S. aureus* strains from rabbits.

MATERIALS AND METHODS

Bacterial isolates

The present studies focused on 62 rabbit *S. aureus* isolates which were chosen to be epidemiologically unrelated. Twenty eight of these strains were high virulence strains, all isolated from commercial rabbitries with chronic problems of staphylococcosis. One of these high virulence strains was atypical because it was not sensitive to phages 3A, 3C, 55 or 71 (DEVRIESE *et al*., 1996). The other strains were low virulence strains. The strains were isolated in Belgium (39), France (17), the Netherlands (3), Spain (2) and the United Kingdom (1).

PCR assay on MSCRAMM genes

A PCR assay was performed to detect the presence of *bbp*, *clf*A and *clf*B, *cna*, *ebp*S, *eno*, *fnb*A, *fnb*B and *fib* in all isolates.

To prepare DNA, one colony of bacterial cells was suspended in 20 µl lysis buffer (0.25% SDS, 0.05 N NaOH) and heated at 95°C for 5 minutes. The cell lysate was spinned down by short centrifugation, and then diluted by adding 180 µl distilled water. Another centrifugation for 5 minutes at 16000 g was performed to remove the cell debris. Supernatants were frozen at -20°C until further use.
The detection of *ebp*S and *fnb*A was done by means of simplex PCR tests. For the detection of *bbp, clf*A, *clf*B *cna, eno, fnb*B and *fib* two multiplex PCR assays were performed.

For the detection of *ebp*S and *fnb*A each 50 µl PCR mixture contained 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Invitrogen, Merelbeke, Belgium), 200 µM of each dNTP, 100 pmol of both primers and 5 µl DNA sample. For the two multiplex PCRs (*bbp*, *cna*, *eno* and *clf*A, *clf*B, *fib*, *fnb*B respectively) each 50 µl PCR mixture contained 3 mM $MgCl_2$, 1.5 U Taq DNA polymerase, 40 µM of each dNTP, 100 pmol of both primers and 5 µl DNA sample. DNA amplification was performed with a DNA thermal cycler (T1 Thermocycler, Biometra, Göttingen, Germany). The thermal cycling conditions for the two multiplex PCRs were those described by Tristan *et al*. (2003). *Ebp*S and *fnb*A were amplified using an initial denaturation step of 1 min at 94°C, followed by 30 cycles of amplification (denaturation for 45 s at 94°C, annealing for 45 s at 50°C and elongation for 1 min at 72°C) and a termination step of 7 min at 72°C.

After amplification, 4 µl amplicon was mixed with 2 µl sample buffer (50% glycerol, 1 mM cresolred) and electrophoresis was performed on 1.5% agarose gels. After electrophoresis, gels were visualized under U.V. light and photographed. The Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, St. Leon-Rot, Germany) was used as a DNA size marker.

Sequencing

As no control strains were available for *bbp*, *clf*A, *clf*B, *cna*, *ebp*S, *eno*, *fnb*A, *fnb*B and *fib*, the amplicons obtained in the respective PCR assays were subjected to DNA sequencing to confirm their specificity. This was done using the BigDye ® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a ABI Prism™ 3100 Genetic Analyzer. The electropherograms were exported and converted to Kodon (Applied Maths, Sint-Martens-Latem, Belgium). Sequences were compared to published sequences obtained from GenBank (accession numbers Y18653, Z18852, AJ224764, M81736 U48826, AF065394, X95848, X62992 and X72014 respectively) by using Blast software.

RESULTS AND DISCUSSION

PCR assay on MSCRAMM genes

In spite of the fact that PCR technology is an easy technique to detect the presence of adhesion genes, it always carries a risk with it. Inter strain variation in the binding sequence of the used primers could lead to false negative results. However, the primers

used in these studies, designed using known sequences of human *S. aureus* strains, still resulted in amplification in rabbit *S. aureus* strains. Therefore, we can assume that the primer binding sites are conserved areas of the *S. aureus* genome.
All 62 strains tested were positive for *clf*A and *clf*B. All isolates were positive for *fib* and *eno*, except for one low virulence isolate. Ninety-three percent of the high virulence and 94% of the low virulence isolates were positive for *fnb*A. The prevalence of the other MSCRAMM genes among the low virulence strains was as follows: 83% were positive for *fnb*B, 71% were positive for *ebp*S and 56% were positive for *cna*. The prevalence of *fnb*B and *ebp*S in high virulence rabbit *S. aureus* strains was 37% and 76%, respectively. High virulence strains did not contain *cna*. All the "typical" high virulence strains, with phagetype 3A/3C/55/71 (43.5% of the strains tested in this assay) were positive for *bbp*. The "atypical" high – virulence strain belonging to biotype – phage type combination "mixed CV-C" – 29/79/42E/92/D11/HK2 (DEVRIESE *et al*., 1996) and the low virulence strains did not harbour the *bbp* gene.

All rabbit *S. aureus* isolates, except for one high virulence isolate, tested positive for at least one of the fibronectin binding protein genes. All three fibrinogen receptor genes (*clf*A, *clf*B and *fib*) were present in all isolates tested, except for one low virulence isolate.

The prevalences of *clf*A, *clf*B, *cna* and *eno* in the tested strains were comparable with those found in *S. aureus* strains involved in human hematogenous infections. The prevalence of *fnb*A and *fnb*B however was much higher in the rabbit isolates than in human clinical *S. aureus* isolates (TRISTAN *et al*., 2003). PEACOCK and COWORKERS (2002), however, found a prevalence of *fnb*A in human *S. aureus* isolates from healthy blood donors and patients with invasive disease that was comparable with the percentage found in rabbit *S. aureus* isolates. *Clf*A, *clf*B, *ebp*S, *eno*, *fnb*A, *fnb*B and *fib* occurred in both high and low virulence rabbit *S. aureus* strains. However, *fnb*B prevalence in high virulence isolates was lower than in low virulence isolates (37% and 83%, respectively). The *cna* gene was absent in high virulence strains. This is surprising as one would expect the high virulence strains to harbour more MSCRAMM genes, for MSCRAMMs are important virulence determinants. Chi square analysis showed that the differences between the low and the high virulence *S. aureus* strains from rabbits were significant for *bbp*, *cna* and *fnb*B, but not for *clf*A, *clf*B, *ebp*S, *eno*, *fnb*A and *fib*.

The most striking result was the fact that only the typical high virulence *S. aureus* strains from rabbits harboured the *bbp*-gene, encoding for bone sialoprotein binding protein (Bbp). Bbp is associated with osteomyelitis and arthritis in humans (TUNG *et al*., 2000; RYDEN *et al*., 1989; YACOUB *et al*., 1994). As bone and joint infections are not considered typical symptoms of rabbit *S. aureus* infections, a direct link of Bbp to the pathogenesis in rabbits is unlikely. However, as shown by TUNG *et al*. (2000), Bbp displays similarity to a number of cell surface proteins with unknown function (the staphylococcal Sdr family) and to Fbe, a fibrinogen binding protein from *S. epidermidis*. As a consequence, a specificity of Bbp for a host-factor other than bone sialoprotein can not be excluded. The fact that the atypical high virulence strain belonging to biotype - phage type combination "mixed CV-C" – 29/79/42E/92/D11/HK2 (DEVRIESE *et al*., 1996) did not contain the *bbp* gene could either mean that Bbp is not a virulence determinant in high virulence rabbit

S. aureus infections, or that this atypical strain uses another strategy to cause an epidemic spread of disease. Thus, further research is necessary to elucidate the significance of Bbp in the pathogenesis of high virulence rabbit *S. aureus* strains.

A possible application of the fact that the *bbp* gene only occurs in typical high virulence strains is the development of a fast, sensitive and specific PCR assay which can be used as a diagnostic tool to screen asymptomatic rabbits for the presence of these strains. In this way, the entry of high virulence *S. aureus* strains in unaffected rabbitries through asymptomatic carrier rabbits could be avoided. However, further investigations are needed to point out whether this assay would be usable.

Sequencing

The sequences obtained for *bbp, clf*A, *clf*B, *cna, ebp*S , *eno*, *fnb*A, *fnb*B and *fib* amplicons obtained were 97 to 99% identical to the corresponding GenBank sequences (accession numbers Y18653, Z18852, AJ224764, M81736 U48826, AF065394, X95848, X62992 and X72014 respectively).

CONCLUSIONS

The distribution of MSCRAMM genes in high and low virulence *S. aureus* strains is comparable, except for *fnb*B, *cna* and *bbp*. The *bbp* gene is only present in the typical high virulence strains. A possible application is the development of a fast, sensitive and specific PCR assay which can be used as a diagnostic tool to screen asymptomatic rabbits for the presence of these strains to avoid their entrance in healthy rabbitries. Further research is necessary to elucidate the significance of Bbp in the pathogenesis of high virulence rabbit *S. aureus* strains.

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REFERENCES

DEVRIESE L.A., HENDRICKX W., GODARD C., OKERMAN L., HAESEBROUCK F., 1996. A new pathogenic *Staphylococcus aureus* type in commercial rabbits. *J. Vet. Med. B.* **43**: 313-315.

DZIEWANOWSKA K., PATTI J. M., DEOBALD C. F., BAYLES K. W., TRUMBLE W. R., BOHACH G. A., 1999. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect. Immun.* **67**: 4673-4678.

FOSTER T.J., HOOK M., 1998. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* **12**: 484-488.

HERMANS K., DE HERDT P., DEVRIESE L.A., GODARD C., HAESEBROUCK F., 2000. Colonisation of rabbits with *Staphylococcus aureus* after experimental infection with high and low virulence strains. *Vet. Microbiol.* **72**: 277-284.

HERMANS K., DE HERDT P., DEVRIESE L. A., HENDRICKX W., GODARD C., HAESEBROUCK F., 1999. Colonisation of rabbits with *Staphylococcus aureus* in flocks with and without chronic staphylococcosis. *Vet. Microbiol.* **67**: 37-46.

HOLLIMAN A., GIRVAN G.A., 1986. Staphylococcosis in a commercial rabbitry. *Vet. Rec.* **119**: 187-187.

OKERMAN L., DEVRIESE L.A., MAERTENS L., OKERMAN F., GODARD C., 1984. Cutaneous staphylococcosis in rabbits. *Vet. Rec.* **114**: 313-315.

PEACOCK S. J., MOORE C. E., JUSTICE A., KANTZANOU M., STORY L., MACKIE K., O'NEILL G., DAY N. P. J., 2002. Virulent combinations of adhesion and toxin genes in natural populations of *Staphylococcus aureus*. *Infect. Immun.* **70**: 4987-4996.

ROSSI G., STANZEL C., WITTE W., 1995. *Staphylococcus aureus* infections in the rabbit and the transmission of the pathogens with the sperma. 9. Arbeitstagung über Haltung und Krankheiten der Kaninchen, Pelztiere und Heimtiere, 251-257.

RYDEN C., YACOUB A., MAXE I., HEINEGARD D., OLDBERG A., FRANZEN A., LJUNGH A., RUBIN, K., 1989. Specific binding of bone sialoprotein to *Staphylococcus aureus* isolated from patients with osteomyelitis. *Eur. J. Biochem.* **184**: 331-336.

TRISTAN A., YING L., BES M., ETIENNE J., VANDENESCH F., LINA G., 2003. Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infection. *J. Clin. Microbiol.* **41**: 4465-4467.

TUNG H. S., GUSS B., HELLMAN U., PERSSON L., RUBIN K., RYDEN C., 2000. A bone sialoprotein-binding protein from *Staphylococcus aureus*: a member of the staphylococcal Sdr family. *Biochem. J.* **345**: 611-619.

YACOUB A., LINDAHL P., RUBIN K., WENDEL M., HEINEGARD D., RYDEN C. 1994. Purification of a bone sialoprotein-binding protein from *Staphylococcus aureus*. *Eur. J. Biochem.* **222**: 919-925.