Screening of Genes Encoding Adhesion Factors and Biofilm Formation in *Staphylococcus aureus* Isolates from Poultry

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Screening of genes encoding adhesion factors and biofilm formation in *Staphylococcus aureus* isolates from poultry

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Abstract

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Staphylococcus aureus isolates recovered from poultry between 1970 and 1972 (90 old isolates) and in 2006 (81 recent isolates) were screened for the presence of *bap*, *icaA* and *icaD* genes, associated with biofilm formation and for *bbp*, *cna*, *ebpS*, *eno*, *fib*, *fnbA*, *fnbB*, *clfA* and *clfB* genes that encode microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Most of the old isolates were collected from broiler breeders affected by staphylococcal tenosynovitis and arthritis, whilst nearly all recent isolates were derived from the nose and cloaca of healthy broilers. Ten recent isolates belonged to the animal-associated MRSA ST398. All isolates were positive for *clfA*, *clfB*, *eno*, *fnbA*, and negative for *bap* and *bbp*. All recent isolates were also positive for *ebpS* and *cna*, and all old isolates were positive for *fib*. The average number of genes encoding adhesins and biofilm-associated proteins in both groups was eight per isolate. All MRSA ST398 isolates in this study were positive for *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA* and *clfB*. No relevant differences were found between the presence of adhesin and biofilm formation genes in old and recent *S. aureus* isolates or in isolates from healthy compared with diseased chickens. From this study, there is no indication that the presence of these genes has changed over time and no specific association between the presence of certain MSCRAMM or biofilm genes in poultry *S. aureus* isolates and the isolate's capacity to cause disease could be found.

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Introduction

Staphylococcus aureus is an important facultative pathogen in both human and veterinary medicine. In poultry it may, as in other animal species and in humans, belong to the normal skin microflora (Devriese, 1980). Furthermore, it can be involved in problems such as osteomyelitis, bumble foot and arthritis and it is most commonly isolated from the joints, tendon sheaths and bone of affected animals (Andreasen *et al.*, 2003). The virulence of a particular *S. aureus* strain is attributed to the combined effect of extracellular factors and toxins, together with the adhesive and invasive properties of the strain, due to the presence of adhesins, biofilm formation and resistance to phagocytosis (Projan & Novick, 1997). The definition of a biofilm is "a microbial derived sessile community characterized by cells that are irreversibly attached to a substratum of interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibiting an altered phenotype with respect to growth rate and gene transcription" (Donlan & Costerton, 2002). Two steps appear to be involved in the molecular mechanisms implicated in host colonization by *S. aureus*. The first step is bacterial adhesion to host extracellular matrix and plasma proteins mediated by different proteins of the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). The second step is growth-dependent accumulation of bacteria in multilayered cell clusters (intercellular adhesion) where genes involved in biofilm formation come into play (Heilmann *et al.*, 1996; Cucarella *et al.*, 2001; Clarke &

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Foster, 2006). In human *S. aureus* strains, several of these virulence factors play a role in life-threatening infections such as endocarditis and osteomyelitis (Foster & McDevitt, 1994; Peacock *et al.*, 2000; Pragman & Schlievert, 2004). As the lesions of *S. aureus* in poultry very often consist of arthritis, tenosynovitis, osteomyelitis, bacterial chondronecrosis or femoral head necrosis and endocarditis (Butterworth, 1999), it is likely that MSCRAMMs also play a significant role in the pathogenesis of these diseases. However, there is no literature with data concerning the presence of MSCRAMM genes in poultry *S. aureus* isolates. *S. aureus* strains possessing MSCRAMM genes can be detected by means of binding assays with purified matrix molecules or by means of PCR technology (Tristan *et al.*, 2003).

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The first purpose of the present study was to determine if the presence of MSCRAMM genes and genes associated with biofilm formation in poultry-associated *S. aureus* isolates has changed over time. Therefore, the presence of these genes was compared in *S. aureus* isolates from poultry that were collected between 1970 and 1972 (old isolates) and in 2006 (recent isolates) by using PCR technology. Since most of the old isolates were collected from broiler breeders affected by tenosynovitis, whilst nearly all recent isolates were derived from the nose and cloaca of healthy broilers, the study secondly also provided indications about the possible association of certain genes with the capacity of the strain to cause disease in poultry.

Material and Methods

Bacterial isolates. One hundred and seventy one *S. aureus* isolates from poultry were tested. Ninety of these isolates were collected between 1970 and 1972 (old isolates) and 81 in 2006 (recent isolates). Eighty-three old isolates were isolated from broiler breeders affected by tenosynovitis, four were obtained from nares and skin of healthy chickens. The origin of three old isolates could not be traced. The strains had been lyophilized and stored at -20°C (Devriese *et al.*, 1972).

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For collecting the recent isolates, 150 different randomly selected Belgian industrial broiler farms were sampled between May and December 2006. In total, 81 *S. aureus* isolates were obtained, from 39 different farms. Two of these isolates came from lesions of swollen head. Seventy-nine isolates were derived from nares and cloaca of healthy broilers. For obtaining the latter isolates, samples were taken from the nose and cloaca of five broilers per farm with a sterile cotton swab. Isolates were characterized as *S. aureus* by colony morphology, standard biochemical methods and growth on modified Baird-Parker medium (Devriese, 1981). Amplification of the *femA* gene, which has been reported to be specific for *S. aureus* (Vannuffel *et al.*, 1995; Mehrotra *et al.*, 2000), was used as a control to confirm the diagnosis of *S. aureus*.

In a previous study it was shown that ten out of the 81 recent isolates are methicillin-resistant *S. aureus* (MRSA), all belonging to the animal associated sequence type ST398 (Nemati *et al.*, 2008).

DNA extraction and PCR assay. DNA extraction was carried out as described before (Vancraeynest *et al.*, 2004). Amplification of *icaA* and *icaD* (intercellular adhesion gene A and D), *bap* (encoding biofilm associated protein), *bbp* (encoding bone sialoprotein binding protein), *clfA* and *clfB* (encoding clumping factors A and B), *cna* (encoding collagen binding protein), *ebpS* (encoding elastin binding protein), *eno* (encoding laminin binding protein), *fmbA* and *fmbB* (encoding fibronectin binding proteins A and B) and *fib* (encoding fibrinogen binding protein) were performed by 12 PCR reactions in a thermal cycler (Mastercycler, Eppendorf AG, Hamburg, Germany). The composition of the PCR mixture and the thermal cycling conditions used for amplification of the genes were as described earlier by Vancraeynest *et al.* (2004). The reference strain *S. aureus* V329 kindly provided by J.R. Penadés was used as a positive control for amplification of *bap* (Cucarella *et al.*, 2001). For amplification of *clfA*, *clfB*, *cna*, *ebpS*, *eno*, *fmbA*, *fmbB*, *icaA* and *icaD*, *S. aureus* strain KH1 and for *bbp* and *fib* *S. aureus* strain KH 103 were used as a positive control respectively (Vancraeynest *et al.*, 2004). Amplification of the *femA* gene was used as a control to confirm the quality of the DNA extraction in each PCR reaction. Primers used in the PCR assays, as well as the expected amplicon sizes and the references, are shown in Table 1.

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Statistical analysis. Comparison of the presence of the different genes was performed firstly between isolates derived from diseased versus isolates from healthy chickens, and secondly between old and recent isolates. Chi-square tests were done wherever possible. When frequencies of five or less were present, Fisher's exact test was used. One-sided testing was performed. Possible differences in the number of MSCRAMM and biofilm genes were investigated using Kruskal Wallis ANOVA for isolates originating from sick or healthy animals, and also from recent and old isolates. Differences were significant when P-values were <0.05. Statistical analyses were performed using SPSS version 17.00.

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Results

All poultry *S. aureus* isolates included in this study were positive for *clfA*, *clfB*, *eno* and *fmbA*. None of the isolates harboured *bap* or *bbp*. Furthermore, all recent isolates were positive for *ebpS* and *icaD* and all old isolates carried *fib*. None of the old *S. aureus* isolates harboured the *fmbB* gene. All isolates from healthy birds also carried *ebpS* and *icaD* and none of the isolates from affected chickens were positive for *fmbB*. Statistically significant differences between old and recent isolates as well as between isolates from healthy and affected poultry were only found for the genes *ebpS*, *fib*, *fmbB* and *icaD*. Table 2 is an overview of these results, as well as the results of the statistical comparison per gene. Twelve MSCRAMM and biofilm genes were screened in this study. In the old isolates, 23 isolates were positive for nine, 26 isolates were positive for eight, 31 isolates were positive for seven, and 10 isolates were positive for six genes (median: 8 genes per isolate). In the recent isolates, eight isolates carried nine, 52 isolates carried eight, and 21 isolates carried seven of the investigated genes (median 8 genes per isolate). In isolates derived from healthy animals, 22 isolates carried seven, 52 carried eight and nine isolates were positive for nine of the screened genes. *S. aureus* isolates from sick birds harboured

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in ten cases six, in 29 cases seven, in 26 cases eight and in 20 cases nine of the investigated genes. No significant differences were found when comparing these numbers between old and recent isolates ($p = 0.279$) nor between isolates originating from healthy and sick animals ($p = 0.183$).

All MRSA isolates in this study were positive for *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA*, *clfB* and negative for *icaA*, *bap*, *bbp* and *fib*.

Discussion

S. aureus is an opportunistic bacterium in poultry. Up until now, no data were available on the presence of virulence factors belonging to the MSCRAMM family or responsible for biofilm formation in poultry *S. aureus*. The present study investigated the presence of genes encoding these virulence factors in poultry *S. aureus* isolates, as they may contribute to host colonization and to the capacity to cause *S. aureus* lesions in poultry, such as arthritis and tenosynovitis.

The presence of *icaD*, *ebpS* and *fnbB* in the recent isolates was significantly higher than in the old isolates and the result for *fib* was opposite. The differences between both groups for *fib* and *fnbB* were associated with the MRSA isolates included in this study. From all isolates in this study, only the MRSA isolates were positive for *fnbB* and were negative for *fib*. In a previous study, it has been shown that these MRSA isolates were all belonging to the animal associated ST398 (Nemati *et al.*, 2008). As they are clonal, it is possible that they possess the same adhesin- and biofilm-encoding genes because of preservation of these genes during evolution of the clone. In this case, this may suggest that the presence of these MSCRAMM and biofilm formation genes has given the strain a selective advantage, such as better host colonization. However, horizontal transfer of these genes cannot be excluded, nor can genetic hitchhiking, which would mean that the MSCRAMM and biofilm genes are present due to selection operating upon linked genes. The results also showed a significant difference for *ebpS* and *icaD*. Although this result is significant, it is probably irrelevant, since the percentage of positive strains was very high in both recent and old isolates: 89 % positive for *ebpS* and 93% for *icaD* in the old strains versus 100% positive for both genes in the recent strains (Table 2).

The MRSA ST398 isolates in this study were all positive for *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA* and *clfB*. Thus, these isolates contain one of the genes associated with biofilm formation and seven known MSCRAMM genes, meaning that they have a good genetic capacity for adherence. This might be of importance not only as a first step in poultry colonization, but also zoonotically. Indeed, the MRSA strains belonging to ST398 have since 2005 been described to be easily transferred from animals, in casu pigs, to humans (Voss *et al.*, 2005).

The average number of genes was similar between both groups, namely eight genes per isolate for both groups. This is a high number compared to strains from other species that have been screened before: Vancraeynest *et al.* (2004) showed that rabbit *S. aureus* harboured seven MSCRAMM genes per isolate and human clinical isolates even harboured only six (Tristan *et al.*, 2003). This might indicate that poultry *S. aureus* isolates have a very good capacity of host colonization.

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The poultry *S. aureus* strains used in this study had been isolated with an interval of about 36 years. From the data obtained, there are no indications that *S. aureus* strains from poultry have acquired MSCRAMM or biofilm encoding genes over time. However, conclusions cannot be drawn on evolution over a longer period. As it is virtually impossible to obtain older poultry *S. aureus* collections than the one used here, examining long-term evolution is currently not feasible.

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Several authors have compared the frequency of presence of certain MSCRAMM genes between isolates from diseased and healthy individuals and have drawn conclusions about the involvement of certain MSCRAMM genes in the pathogenesis of *S. aureus*. For instance, Peacock *et al.* (2002) compared the presence of virulence genes in *S. aureus* isolates from healthy human blood donors and from patients with invasive disease. They noticed that amongst others *fnbA*, *cna* and *ica* were significantly more common in invasive isolates. When the frequency of MSCRAMM and biofilm genes is compared between the groups of *S. aureus* isolates from diseased and healthy chickens, no relevant significant differences could be found. This is similar to the results of the comparison of old and recent isolates, which can be explained by the fact that most old isolates were obtained from sick and most recent isolates from healthy birds. From this study, no conclusion can be drawn about the involvement of specific MSCRAMM and biofilm formation genes in the pathogenesis of *S. aureus* infections in poultry. As *S. aureus* is an opportunistic pathogen, it should be remembered here that it only causes disease under certain predisposing conditions and that the virulence factors will only come into play at that time.

As stated above, 10/81 recent isolates were MRSA, belonging to the animal associated sequence type ST398. These isolates were positive for *icaD*, *cna*, *ebpS*, *eno*, *fnbA*, *fnbB*, *clfA*, *clfB* and were negative for *icaA*, *bap*, *bbp* and *fib*. The result for *ebpS*, *eno*, *fnbA*, *clfA* and *clfB* is in agreement with what Otsuka *et al.* (2006) found for MRSA strains with various ST types and originating from various countries. Taneike *et al.* (2006) described that all MRSA strains derived from nosocomial outbreaks in Japan were negative for *fnbB*, and nearly all of them harboured *bbp*. This is opposite to the results from our study. Furthermore, Otsuka *et al.* (2006) showed that for *icaA*, only pandemic ST30 strains from Japan were negative and for *cna* only these strains and PVL-negative Japanese region-specific strains (ST89) were positive while all MRSA isolates in our study were negative for *icaA* and positive for *cna*.

In conclusion, no relevant differences could be found between the presence of MSCRAMM and biofilm genes in *S. aureus* isolates from affected chickens (old isolates) and from nose and cloaca of healthy chickens (recent isolates). From this study, there is no indication that the presence of these genes has changed over time. Furthermore, no specific association between the presence of certain MSCRAMM or biofilm genes in poultry *S. aureus* isolates and the isolate's capacity to cause disease could be found. Further studies should focus on different collections of isolates, such as recent collections from healthy broiler breeders or from broilers affected by *S. aureus*, to check the validity of this conclusion.

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Table 1. *Primers used in this study*

Gene targeted	Primer sequence PCR	Annealing temperature(°C)	Amplicon size (bp)	Reference
<i>icaA</i>	5'CCTAACTAACGAAAGGTAG-30, 5'AAGATATAGCGATAAGTGC-3	49	1315	Vasudevan <i>et al.</i> (2003)
<i>ica D</i>	5'AAACGTAAGAGAGGTGG-30, 5'GGCAATATGATCAAGATAC-30	49	381	Vasudevan <i>et al.</i> (2003)
<i>bap</i>	5'CCCTATATCGAAGGTGTAGAATTG-30, 5'GCTGTTGAAGTTAATACTGTACCTGC-30	62	971	Cucarella <i>et al.</i> (2001)
<i>bbp</i>	5'AACTACATCTAGTACTCAACAACAG-30, 5'ATGTGCTTGAATAACACCATCATCT-30	55	575	Tristan <i>et al.</i> (2003)
<i>cna</i>	5'GTCAAGCAGTTATTAACACCAGAC-30, 5'AATCAGTAATTGCACTTTGTCCACTG-30	55	423	Tristan <i>et al.</i> (2003)
<i>ebpS</i>	5'AGAATGCTTTTGCAATGGAT-30, 5'AATATCGCTAATGCACCGAT-30	50	652	Vancraeynest <i>et al.</i> (2004)
<i>eno</i>	5'ACGTGCAGCAGCTGACT-30, 5'CAACAGCATYCTTCAGTACCTTC-30	55	302	Tristan <i>et al.</i> (2003)
<i>fnbA</i>	5'CATAAATTGGGAGCAGCATCA-30, 5'ATCAGCAGCTGAATTCCCAT-30	50	127	Vancraeynest <i>et al.</i> (2004)
<i>fnbB</i>	5'GTAACAGCTAATGGTCGAATTGATACT-30, 5'CAAGTTCGATAGGAGTACTATGTTC-30	55	524	Tristan <i>et al.</i> (2003)
<i>fib</i>	5'CTACAACTACAATTGCCGTCAACAG-30, 5'GCTCTTGTAAGACCATTTTCTTCAC-30	55	404	Tristan <i>et al.</i> (2003)
<i>clfA</i>	5'ATTGGCGTGGCTTCAGTGCT-30, 5'CGTTTCTTCCGTAGTTGCATTTG-30	55	292	Tristan <i>et al.</i> (2003)
<i>clfB</i>	5'ACATCAGTAATAGTAGGGGGCAAC-3', 5'TTCGCACTGTTTGTGTTTGCAC-3'	55	205	Tristan <i>et al.</i> (2003)

Table 2. Comparison of the distribution of MSCRAMM genes and genes associated with biofilm formation between recent and old *S. aureus* isolates from poultry and between *S. aureus* isolates from healthy and diseased chickens. [‘]pppppppppppppppppppp;

Gene	Number (%) of positive recent isolates	Number (%) of positive old isolates	P value	Number (%) of positive isolates from healthy chickens	Number (%) of positive isolates from diseased chickens	P value
	n=81	n=90		n=83	n=85	
<i>bap</i>	0 (0)	0 (0)	0.9999	0 (0)	0 (0)	0.9999
<i>bbp</i>	0 (0)	0 (0)	0.9999	0 (0)	0 (0)	0.9999
<i>clfA</i>	81 (100)	90 (100)	0.9999	83 (100)	85 (100)	0.9999
<i>clfB</i>	81 (100)	90 (100)	0.9999	83 (100)	85 (100)	0.9999
<i>cna</i>	37 (45.7)	50 (55.6)	0.1967	39 (47.0)	45 (52.9)	0.4402
<i>ebpS</i>	81 (100)	80 (88.9)	0.0013*	83 (100)	76 (89.4)	0.0017*
<i>eno</i>	81 (100)	90 (100)	0.9999	83 (100)	85 (100)	0.9999
<i>fib</i>	71 (87.7)	90 (100)	0.0004*	73 (87.9)	85 (100)	0.0006*
<i>fnbA</i>	81 (100)	90 (100)	0.9999	83 (100)	85 (100)	0.9999
<i>fnbB</i>	10 (12.3)	0 (0)	0.0004*	10 (12.1)	0 (0)	0.0006*
<i>icaA</i>	31 (38.3)	28 (31.1)	0.3009	31 (37.4)	26 (30.6)	0.3545
<i>icaD</i>	81 (100)	84 (93.3)	0.0196*	83 (100)	79 (93.0)	0.0153*

* indicates a significant difference (p<0.05)