Colonization and transmission of meticillin-susceptible and meticillin-resistant *Staphylococcus aureus* in a murine nasal colonization model

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Nasal colonization by *Staphylococcus aureus* is an important risk factor for the development of a nosocomial infection. Acquisition of nasal colonization by *S. aureus* increases mortality in hospitalized patients, but little is known about the transmission dynamics of *S. aureus*. To study *S. aureus* transmission, colonization and colonization persistence, we developed a murine transmission model. In 20 cages, 2 out of 10 mice were nasally inoculated (at 5×10^8 c.f.u. per mouse) with either meticillin-susceptible *S. aureus* (MSSA) (10 cages) or meticillin-resistant *S. aureus* (MRSA) (10 cages). On days 5, 15, 25 and 40, all mice in a cage were swabbed or sacrificed and nasal colonization and c.f.u. were determined in all 10 mice by nasal dissection or by nasal swab. Spread and subsequent stable colonization by both MSSA and MRSA from colonized to uncolonized mice within a cage was seen. At day 5, an increased number of colonized mice were observed in the MSSA group compared to the MRSA group ($P=0.003$). On day 40, the mean number of c.f.u. per mouse was higher for MRSA than for MSSA ($P=0.06$). Faecal–oral transmission was shown to be a possibly important transmission route in this model. These results suggest a more rapid spread of MSSA compared to MRSA. However, MRSA shows a more stable nasal colonization after a longer period of time.

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INTRODUCTION

Staphylococcus aureus is a major pathogen in nosocomial and community-acquired infections. Approximately 30 % of healthy individuals carry *S. aureus* on the skin and/or mucous membranes. A positive carrier status significantly increases the chance of acquiring a nosocomial infection, predominantly of wounds, catheters and intravascular devices (Wertheim *et al.*, 2005).

In an intensive care unit (ICU) setting, approximately 30 % of acquired infections are caused by *S. aureus* (Ibelings & Bruining, 1998). Mortality rates are significantly increased in patients carrying meticillin-resistant *S. aureus* (MRSA); overall ICU mortality was 18 % in a French study, and mortality increased to 45 % in MRSA-colonized patients and to 58 % in MRSA-infected patients (Girou *et al.*, 1998).

Little is known about the dynamics of transmission of *S. aureus* in an ICU setting. In our ICU, MRSA was shown to be more epidemic than meticillin-susceptible *S. aureus*

(MSSA) (Vriens *et al.*, 2002). In a European study which assessed the spread of *S. aureus* in different ICUs, no difference in epidemicity could be shown between MRSA and MSSA (Bloemendaal *et al.*, 2009).

In the present study, we assess transmission, colonization and colonization persistence of *S. aureus* in a murine nasal colonization model. To answer the question whether MRSA is more contagious than MSSA, the possible differences in transmission and colonization dynamics between a genetically identical strain set of MSSA and MRSA were investigated. The whole genome of these isolates has been sequenced and shown to not have any genetic differences, except for the presence of a type IV staphylococcal cassette chromosome *mec* (SCC*mec*) in the MRSA (Bloemendaal *et al.*, 2010). These strains have previously been described by Wielders *et al.* (2001).

METHODS

Animals. The mice used for this study were 6-week-old female BALB/c mice, obtained from Harlan and Charles River. The animals were kept in a separate room in the Infections Unit of the General Animal Laboratory of the University of Utrecht. The mice were housed in

Abbreviations: ICU, intensive care unit; MLVA, multilocus variable number of tandem repeats analysis; MRSA, meticillin-resistant *S. aureus*; MSSA, meticillin-susceptible *S. aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*.

groups of 10 in filter-top cages and were fed water and food *ad libitum*. The study was performed with permission of the local Animal Ethics Committee (DEC).

S. aureus strains and growth conditions. The *S. aureus* strains used for the experiment were the MSSA WKZ-1 and MRSA WKZ-2 (type IV SCCmec), previously described by Wielders *et al.* (2001) and Jansen *et al.* (2006). Recent whole genome sequencing has shown that, apart from the type IV SCCmec in WKZ-2, no differences including single nucleotide polymorphisms were present. Therefore, WKZ-1 and WKZ-2 are a fully isogenic MRSA–MSSA pair (Bloemendaal *et al.*, 2010). The strains were stored at -80°C . Strains were grown on 5% sheep blood agar for 24 h at 37°C . Bacteria were suspended in saline and quantified by optical density measurement set at $\text{OD}_{660}=1$ (1.3×10^9 cells ml^{-1}). To confirm a comparable c.f.u. count, WKZ-1 and WKZ-2 were plated by a serial dilution on 5% sheep blood agar plates.

Transmission from live mice. The study was performed in two different experimental set-ups. In the first experiment, the same mice were swabbed nasally on sample days to assess colonization (four cages total). In the second setup, the mice were euthanized and the nasal area was dissected to assess colonization (16 cages total).

Ten cages with 10 mice each were assigned to each bacterial strain. In each of the 10 MSSA cages, two mice were inoculated with 5×10^8 c.f.u. MSSA WKZ-1 in 20 μl saline. In each of the 10 MRSA cages, two mice were inoculated with 5×10^8 c.f.u. MRSA WKZ-2 in 20 μl saline. Inoculation was performed deep in the nose using a sterile pipette. The inoculated mice were marked by an ear tag. They were replaced in the cage after approximately 2 min and no sneezing was observed in these 2 min.

On sample days (days 5, 15, 25 and 40 post-inoculation), all mice in two MSSA cages and all mice in two MRSA cages were swabbed nasally using a sterile loop. The tips of the loops were cut off with sterile scissors and transferred into 200 μl saline. After rigorous vortexing, 10 μl was plated on mannitol salt agar (MSA) plates and incubated for 48 h at 37°C . No c.f.u. count was obtained using this method.

On the same sample days, all mice in two MSSA and all mice in two MRSA cages were euthanized by cervical dislocation. The external nasal region was disinfected with 80% alcohol. Nasal tissue was excised with sterile scissors and dissected in two equal parts. One part was grown for 24 h in 4 ml trypsin soy broth with 5 mg aztreonam l^{-1} (Squibb) at 37°C . After rigorous vortexing, 10 μl supernatant was plated on MSA plates and incubated for 48 h at 37°C . The other nasal part was ground in 100 μl saline. The tissue suspension was centrifuged at 3000 g for 10 min. The supernatant was plated on 5% sheep blood agar plates in a dilution series to obtain a nasal c.f.u. count.

Faecal–oral transmission. To assess the relevance of intestinal colonization in the transmission of *S. aureus* in a murine model, faeces was obtained from all mice at sacrifice and from the floor of the MSSA and MRSA cages. All obtained droppings were dissected. One part was used to assess *S. aureus* colonization of faeces. The other part was introduced in ‘clean cages’ holding six non-inoculated mice. One cage for MSSA and one cage for MRSA was used. In total, 20–30 droppings were introduced per ‘clean cage’. The mice were euthanized on day 15 after introduction of the droppings and nasal colonization was assessed by nasal dissection.

Intestinal colonization. Faeces samples were taken from all sacrificed mice in order to assess *S. aureus* colonization of the digestive tract. Faeces samples were suspended in 4 ml saline and 100 μl suspension was pipetted into 4 ml trypsin soy broth with 5 mg

aztreonam l^{-1} and incubated for 24 h at 37°C . After vortexing, 10 μl broth was plated on MSA plates and incubated for 48 h at 37°C .

Strain verification. The presence of *S. aureus* was confirmed by DNase test and coagulase test in all experiments. To confirm strain identity, isolates were typed by multilocus variable number of tandem repeats analysis (MLVA), as described by Ikawaty *et al.* (2008).

Statistical analyses. Data were analysed using the Statistical Package for Social Sciences (SPSS) version 12.0.1. Categorical variables were compared using the chi-square test and continuous variables were compared using the Mann–Whitney *U*-test. Continuous variables were calculated by paired *t*-test. Significance was set at $P < 0.05$.

RESULTS

Inoculation and spread

Using two different sampling methods to assess the nasal carriage of MSSA and MRSA in mice, transmission of both bacterial strains from inoculated mice to non-inoculated mice was observed (Table 1).

In the combined results from the two sampling methods (Fig. 1), the mean number of mice colonized by MSSA was 7.8 per cage on day 5, whereas MRSA had colonized a mean of 4.3 mice ($P=0.001$). On day 15, the mean number of mice colonized by MSSA and MRSA was 9.8 and 8.5, respectively ($P=0.02$); on day 25, it was 7.3 and 9.0 mice, respectively ($P=0.01$); on day 40, 4.0 and 5.3 mice were colonized, respectively (not significant).

The number of c.f.u. per strain on the days post-inoculation can be seen in Fig. 2. A near significant difference ($P=0.06$) was seen on day 40.

All but one of the inoculated mice remained positive for *S. aureus* colonization throughout the duration of the experiments. The negative mouse was found on day 40. All animals survived until the day of sacrifice. No complications were observed.

Intestinal colonization

Simultaneous colonization of nares and faeces was seen in all but six cases. On day 5, two (non-inoculated) MRSA mice were found to be negative for nasal colonization but positive for faecal colonization. In the other four cases, a mouse was found positive for nasal colonization but negative for faecal colonization. This was the case in one MSSA mouse on day 5. Two MSSA mice and one MRSA mouse showed this colonization pattern on day 15.

Faecal–oral transmission

All freshly obtained droppings from nasally colonized mice which were introduced in the clean cages were positive for *S. aureus*. All mice were colonized by *S. aureus* in the case of MSSA and MRSA introduction.

Table 1. Number of mice colonized by MSSA and MRSA on different days for both experimental methods separately and cumulatively

Days*	MSSA (n)			MRSA (n)		
	Swab (20)	Dissection (20)	Total (40)	Swab (20)	Dissection (20)	Total (40)
5	16	15	31	10	7	17
15	20	19	39	20	14	34
25	16	13	29	20	16	36
40	4	12	16	10	11	21

*Days post-inoculation.

Strain verification

All collected *S. aureus* strains had identical MLVA types.

DISCUSSION

Many studies have been undertaken to assess virulence and resistance mechanisms in *S. aureus*. There is no conclusive evidence for an increased or decreased virulence of MRSA compared to MSSA (Huesca *et al.*, 2002; Mizobuchi *et al.*, 1994), although a meta-analysis does show an increase in mortality in MRSA bacteraemia compared to MSSA bacteraemia (Cosgrove *et al.*, 2003). No definite explanation for this increase can be provided. A delay in appropriate treatment due to underestimation of MRSA in a non-outbreak situation is a likely reason.

One may presume that the epidemicity (transmission, colonization and colonization persistence) of MRSA should not differ from that of MSSA, as both strains are essentially identical, apart from SCCmec. A number of

studies have been performed on fitness cost of resistance acquisition by different strains. Ender *et al.* (2004) showed a reduced growth rate after integration of a type I SCCmec in an MSSA strain in a direct competition growth experiment. Lee *et al.* (2007) showed a fitness cost after type I SCCmec incorporation, but not after type IV SCCmec incorporation. This was assessed by a glucose uptake and a growth rate experiment. Karauzum *et al.* (2008) showed a decreased fitness in an ST8 MRSA compared to an ST8 MSSA, but not in an ST5 MRSA and MSSA (both type IV SCCmec), in an adhesion model and a murine sepsis model. A decrease in fitness was also seen after acquisition of resistance to other antimicrobials (McCallum *et al.*, 2006; Nagaev *et al.*, 2001; O'Neill *et al.*, 2006). In theory, the presence of the SCCmec cassette may result in an increased or decreased epidemicity. This contrary effect may be due to polar effects due to the insertion of SCCmec into *orfX* on the staphylococcal chromosome. Genes on the SCCmec may influence regulatory peptides through an unknown mechanism as shown by Kaito *et al.* (2008). They describe the role of the *fudoh* gene in the ability of *S. aureus* to spread on agar and to infect mice. The presence of *fudoh* decreased the spread rate of both MSSA and MRSA. In addition, in a murine infection model, *fudoh*-positive *S. aureus* isolates were less virulent. A microarray showed that this gene also affects the expression of a number of other genes which are not located on SCCmec. The function and location of these genes are not stated. The *fudoh* gene has been found on type II and III SCCmec. It was not shown to be present on type I, IV and V SCCmec.

In the present study, we have developed a murine epidemicity model to study the transmission and colonization persistence of *S. aureus*. To this end, we used a MSSA and a MRSA strain (WKZ-1 and WKZ-2, respectively), which are genetically identical, except for a type IV SCCmec cassette in the WKZ-2 strain. As a basis for our method, we used the murine nasal colonization model as described by Kiser *et al.* (1999), which showed a stable colonization of the murine nares with *S. aureus* after inoculation with at least 10^8 c.f.u.

The results of our study also show a stable colonization of all inoculated mice for 40 days after inoculation with 5×10^8 c.f.u. in accordance with the finding of Kiser *et al.*

Fig. 1. Mean number of mice colonized per 10 mouse cage by MSSA and MRSA. Mice were inoculated with 5×10^8 c.f.u. MSSA (black bars) and MRSA (grey bars). Samples were taken on days 5, 15, 25 and 40 post-inoculation.

Fig. 2. Number of c.f.u. for MSSA (black bars) and MRSA (grey bars) groups on days 5, 15, 25 and 40 in a box plot (median and range) as obtained by culturing after nasal tissue dissection.

(1999). Furthermore, our results show a successful spread of both MSSA and MRSA from inoculated mice to non-inoculated mice within a cage. This result was found in two different experimental set-ups and can therefore be considered reproducible. The newly colonized mice showed a stable colonization for at least 15 days and almost half of the mice were still colonized by *S. aureus* on day 40. The inoculated mice showed a more stable colonization than the non-inoculated mice. This could be explained by the idea that the number of c.f.u. inoculated is far larger than the number of c.f.u. transmitted by natural spread. Kiser *et al.* (1999) showed that to achieve stable colonization at least 10^8 c.f.u. was necessary. The natural transmission will not achieve these numbers.

A difference was found in the rate of transmission between MSSA and MRSA. MRSA was seen to spread less rapidly than MSSA. However, there was no significant difference in the number of colonized mice after 40 days. In contrast to the number of mice colonized, the number of c.f.u. was found to be higher in MRSA than MSSA on day 40, suggesting a more stable colonization after a longer period of time. This may indicate an intrinsic factor on the type IV SCCmec on the WKZ-2 chromosome influencing colonization persistence, but further experiments should be undertaken to assess this suggestion. Sequence analysis of the SCCmec element did not yield an obvious gene candidate able to explain the difference.

To assess the relevance of digestive tract colonization and coprophagia in a murine bacterial epidemicity model, we studied intestinal colonization and faecal–oral transmission by introducing colonized faeces in cages holding non-*S. aureus* colonized mice. A major limitation in this experiment was the impossibility of excluding the role of

environmental contamination by the faecal *S. aureus*, due to ethical restrictions (i.e. cages without ground covering). Therefore, it is not possible to prove that the positive colonization of all clean mice was attributable to coprophagia. It does, however, show the relevance of digestive tract colonization in the transmission of *S. aureus* in a murine model. Furthermore, the experiment shows a clear correlation between nasal colonization and digestive tract colonization by *S. aureus*. The few cases of negative nasal carriage and positive digestive tract *S. aureus* carriage could be due to non-detectable colonization rates in the murine nares in the early stages of colonization.

We believe we have demonstrated the possibility of using a murine nasal colonization–transmission model for the assessment of epidemicity of *S. aureus* strains. We have shown a stable colonization and spread of both MSSA and MRSA. The differences seen in the epidemicity of the genetically highly related WKZ-1 and WKZ-2 strains should be further investigated in order to determine the influence on transmission by the introduction of the SCCmec in *S. aureus*.

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