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Summarizing discussion

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SUMMARIZING DISCUSSION

In this thesis, colonization, carriage and transmission of *S. aureus* were studied and the most important findings and conclusions are explicated in this chapter. In spite of all the research that has been performed on nasal *S. aureus* (de)colonization in animals, probably the best way to study this is by using the *S. aureus* human inoculation model (1-4). Unfortunately, we encounter ethical problems regarding the use of this human model, and a proper human-like animal model is still missing. Furthermore, in order to prevent carriage of and infection with *S. aureus*, especially in risk groups, understanding carriage and detecting reservoirs in hospitals is essential.

The first aim of this thesis was to gain more insights into the colonizing capacity of *S. aureus*. Therefore, a novel experimental decolonization and carriage model in rhesus macaques (*Macaca mulatta*) was set up, and a study with the human inoculation model was performed. A secondary aim was to investigate, using whole-genome sequencing (WGS), whether nosocomial acquisition of *S. aureus* via healthcare workers (HCWs) occurred in neonates admitted to a neonatal intensive care unit (NICU). We also studied whether transmission and the presence of specific virulence genes support the occurrence of neonatal bloodstream infections.

MAJOR FINDINGS AND CONCLUSIONS

In **Chapter 2** *S. aureus* colonization and carriage were investigated to enhance existing knowledge about the colonizing capacity of emerging, livestock-associated *S. aureus* strains in humans. In addition, the ethical restrictions of the human inoculation model challenged us to an experiment, to develop a new animal model in rhesus macaques. In **Chapter 2.1** we developed an experimental decolonization and inoculation procedure in rhesus macaques. Rhesus macaques are natural hosts of *S. aureus* (5). They were therefore considered to be an excellent model, compared to other animal models where sometimes the importance of the animal being a natural host seems to be ignored. Twenty rhesus macaques, all nasal *S. aureus* carriers, were split up into two groups for a decolonizing treatment. To check for nasal *S. aureus* carriage, animals were swabbed 4–5 times with one-week intervals. We defined carriage as animals in which at least 80% of the nasal cultures were *S. aureus* positive. For decolonization, the nose was approached with either a topical treatment with mupirocin nasal ointment only (treatment A), or a combination treatment of mupirocin nasal ointment with systemic trimethoprim/sulfadiazine (treatment B). **In this novel rhesus macaque model, we showed that we could decolonize the nose of *S. aureus* carriers, using standardized protocols, for a period of 10 weeks or more. There was no significant difference between the two treatments, as all animals became negative for *S. aureus* in the nose**

between days 7-21. After 63 days, 60% of all animals in both treatment groups persistently cultured negative in the nose. Furthermore, with respect to the time of first recurrence of *S. aureus*, there was no significant difference between the two treatment groups when only the nose was analyzed. However, analysis of recurrence of *S. aureus* at any site (throat or rectum) showed a trend, suggesting that treatment B was more effective than treatment A. Following decolonization, the anterior nares were inoculated with a human *S. aureus* strain 8325–4, to investigate whether carriage could be induced. **We were able to inoculate the noses of the rhesus macaques with human *S. aureus* strain 8325–4, but we did not observe stable, long-term colonization with this strain.** Contrary to this observation, in previous studies, *S. aureus* 8325–4 was shown to persist for at least four weeks in the human nose, despite the presence of a defect in the sigma factor (SigB) locus (3, 4, 6). By choosing strain 8325-4, we had the benefit of being able to use our previous experience in human inoculation experiments in which this strain was also used. In addition, this strain is suitable for genetic modification and can be applied for both knockout (3, 4) and knock-in studies.

We conclude that we developed in rhesus macaques, a natural host for *S. aureus*, an experimental decolonization and inoculation procedure, which can be used for *S. aureus* decolonization and inoculation studies in a properly controlled fashion.

In contrast to other *S. aureus* animal models, the major future benefit of this model is that rhesus macaques allow long-term follow-up when using them for (de)colonization studies, as these animals do not need to be sacrificed. Also, the pathogen-host interplay could very well be studied in this animal because of its natural *S. aureus* carrier state and its genetically close relatedness to humans.

In **Chapter 2.2** the intrinsic capacity of a non-human *S. aureus* strain (ST398) to colonize the human nose was studied. This strain emerges in livestock and people in close contact with livestock are presumed to be at risk for carriage, but data on its colonizing capacity in humans is lacking. An artificial human nasal inoculation experiment was performed with a mixed inoculum (10*7 bacteria per strain per nostril) of a bovine methicillin-susceptible *S. aureus* (MSSA) ST398 (CC398) strain and a human MSSA ST931 (CC8) strain. Subsequently, their ability to survive in the anterior nares was determined. The driving force to perform this study was, as earlier mentioned, that methicillin-resistant *S. aureus* (MRSA) strains of lineage ST398 are described in literature as predominantly occurring in livestock, while they have the ability to also colonize humans. Furthermore, an increase of livestock-associated (LA)-MRSA ST398 infections was documented in hospitalized patients without a link to livestock. **We demonstrate in our human inoculation model, that *S. aureus* ST398 of bovine origin is capable of surviving in the nose of 10 healthy volunteers for at least 21 days, when inoculated 7 weeks after an eradication treatment with mupirocin and chlorhexidine-containing soap.** In the first days after inoculation, we observed a rapid decrease in bacterial load of both strains in the nares of all volunteers, resulting in the elimination of both strains within 21 days in 4 volunteers. Interestingly, in the remaining 10 volunteers, ST398 could

still be detected after 21 days. In half of this latter group we observed, after a decrease in bacterial loads of both strains, that the loads stabilized after 21 days. In the remaining five individuals, cell counts for strain ST398 increased at the end of the follow-up period, where in most of these cases the human strain was already eliminated. Our data clearly indicate that in 28.6% of the volunteers *S. aureus* is rapidly eradicated, even when exposed to significant numbers of bacteria. Yet, 71.4% of the volunteers were unable to eradicate any of the inoculated *S. aureus* strains. **Based on microarray analysis, no evidence was found that survival of ST398 in the human host was due to the acquisition of mobile genetic elements (MGEs).** This suggests that animal ST398 is able to survive for several weeks in the human nares without gaining or losing MGEs.

In conclusion, MSSA strain 5062 of bovine origin (ST398, CC398) is capable of surviving in the human nose for at least 21 days and can successfully outcompete a human strain 1036 (ST931, CC8).

It is a matter of debate whether we should fear exposure to *S. aureus* ST398, in relation to the risk of becoming a *S. aureus* carrier. As we studied the colonizing capacity of this strain in a controlled fashion, exposing the volunteers to a significant amount of bacteria, we need to be cautious about drawing conclusions. We think that people in contact with livestock may be exposed to lower numbers of bacteria than in our experimental setting. This may explain the fact that people exposed to LA-MRSA in the community, may lose the strain within 24 hours (7), in contrast to what we have observed. Van Cleef et al. discuss that it could very well be that people who are temporarily in contact with livestock, are just being contaminated instead of being colonized with LA-MRSA (7). Among infection prevention control professionals, it is well-known that the success of a transmission of MRSA should be checked only about eight hours after exposure. The reason for this approach is that there is a high chance of culturing positive for *S. aureus* immediately after exposure. When culturing the day after, the positive results spontaneously turn negative, for instance because often, contamination does not lead to successful colonization. Further research is needed to investigate if colonization with *S. aureus*, whether or not due to inoculation in experimental settings, is dependent on bacterial loads in the way we assume. As we pretreated the volunteers with mupirocin nasal ointment before inoculation, we could only speculate about the possible positive effect of this treatment on the development of *S. aureus* nasal colonization. The treatment could have eradicated more bacteria from the nasal cavity than only *S. aureus*, which may be beneficial for *S. aureus* regrowth and recolonization after a period of time. For example, we know that *Staphylococcus epidermidis* (*S. epidermidis*) has a negative influence on *S. aureus* nasal colonization (8, 9), and by eradicating this bacterium, the chances of *S. aureus* may improve.

In **Chapter 3,** nosocomial transmission of *S. aureus* was studied in the highest-risk population for the development of hospital-acquired infections (HAIs): neonates on a NICU. Neonates are regularly confronted with invasive *S. aureus* infections, in particular bacteremia,

which makes this bacterial species one of the most important pathogens in this high-risk group (10-12). All-cause mortality among neonates suffering from *S. aureus* bacteremia varies between 10 and 20% (13, 14). So how, and from whom or where, do these neonates become contaminated, leading to colonization and infections with this bacterium? It is relatively unknown what the contribution of the parents is in the transmission of *S. aureus* to their newborns. Meanwhile, literature describes a variety of *S. aureus* sources in the hospital that might influence the neonatal *S. aureus* carriage state, from non-carriage to carriage. NICU outbreaks are frequently described with respect to *S. aureus* (15-22), and because of the currently available advanced typing techniques, transmission via HCWs has already been shown (21, 22). By demonstrating possible sources of *S. aureus* and transmission routes, awareness will rise, allowing us to avoid, or at least minimalize, the transmission of *S. aureus* to neonates to prevent HAIs. In **Chapter 3.1,** we performed a retrospective analysis in which *S. aureus* isolates of neonates and HCWs were analyzed by WGS to detect relationships. The isolates of neonates were derived from different patient materials, including blood cultures, but we also included screening cultures. The main goal in this study was to explore whether HCWs could possibly be involved as a source in *S. aureus* transmission to neonates, by showing indistinguishable genomes in both groups in the same time period. **By using WGS, we showed that HCWs might serve as a reservoir for the transmission of methicillin-susceptible *S. aureus* (MSSA) to neonates on a level IV NICU. In 4 WGS clusters, a total number of 4 HCWs and 35 neonates were involved, of which 10 neonates suffered from neonatal bacteremia.** Indistinguishable MSSA isolates, causing 10 of the total number of 20 episodes of neonatal MSSA bacteremia, were found in both neonates and HCWs, and were already present in HCWs or neonates admitted earlier. Data concerning MSSA transmission from HCW to neonate are scarce since, normally, no contact investigation is performed when a neonate is positive for MSSA, in contrast with MRSA findings. In three studies, a MSSA transmission from HCW to patient was described. Gomez-Gonzalez et al. showed, -albeit by use of Pulse Field Gel Electrophoresis, a technique with less discriminatory power -, that HCWs acted as a reservoir for neonates who later on developed a MSSA sepsis (16). In another study, using Raman spectroscopy typing, a HCW proved to be the source of an outbreak in neonates with MSSA bullous impetigo (18). In addition, Price et al., applying WGS, showed indications for the transmission from patients, the environment and HCWs, to other patients on an adult intensive care unit, leading to colonization and infections with genetically identical *S. aureus* isolates (23).

With our data, we can confirm that HCWs might be an important reservoir and link in the transmission of MSSA to neonates.

These data show that more insight is needed into the reservoirs, driving forces and transmission routes of *S. aureus* in the NICU. As long as the reservoirs and routes are unclear, possible intervention measures might focus on the use of personal protective equipment,

the improvement of compliance with hand hygiene and extra (daily) cleaning or disinfection, to prevent transmission from HCWs to patients.

In **Chapter 3.2**, all neonatal *S. aureus* bloodstream isolates, collected over a 7-year period, were analyzed by WGS to detect whether the transmission and genetic makeup of the isolates support the occurrence of neonatal *S. aureus* bacteremia. **By using WGS, among the 104 *S. aureus* isolates studied, 12 different core genome multilocus sequence-typing (cgMLST) clusters of MSSA isolates were found. Seven of these twelve cgMLST clusters included at least two MSSA isolates, cultured from blood of neonates within one month, indicative of transmission.** Transmission should therefore be considered a contributing factor to the frequent occurrence of neonatal *S. aureus* bacteremia, as was recently described by Rouard et al. (24). Although it seems reasonable to assume that transmission, irrespective of the source, can only occur through the hands of HCWs, we cannot prove this, as no culture data of the environment, the HCWs or the parents are present. Besides transmission, it was determined whether the presence of certain virulence factors is associated with neonatal *S. aureus* bacteremia. Since it was difficult to define a suitable control population of neonates, we chose to compare neonatal *S. aureus* bacteremia isolates to all available *S. aureus* genomes from the Refseq Genome Database (N = 10.288 at the time of analysis). **The genes staphylococcal enterotoxin A (*sea*) and toxic shock syndrome toxin 1 (*tsst-1*) were found a factor 2.6 and 3.2 times more often in the MSSA bloodstream isolates, compared to the reference genomes in the Refseq Genome Database.** The overrepresentation of *tsst-1* could not be explained by the frequent presence of MLST ST5 and ST45 in our isolates collection, since *tsst-1* was not associated with these sequence types. On the other hand, 11 of the 25 isolates carrying *sea* were found in ST5 isolates. Still, this cannot be the full explanation for finding an association between *sea* and neonatal MSSA bacteremia. Many studies have been executed on *S. aureus* toxins and their pathogenic roles, particularly on *sea* and *tsst-1*. Previously, it was described that antibody responses to these two specific toxins were higher in patients with *S. aureus* bacteremia, compared to control patients (25). In addition, in a recent publication about a MSSA outbreak on a NICU, *tsst-1* and especially *sea* were found in bloodstream isolates, compared to colonization isolates (24).

The main conclusion from this study is that the occurrence of *S. aureus* bacteremia in neonates could partly be explained by the transmission of MSSA.

The toxins *sea* and *tsst-1* might play a role in neonatal bacteremia. This finding warrants further investigation.

FUTURE PERSPECTIVES

The studies described in this thesis have all contributed to the existing knowledge about *S. aureus* colonization, carriage and transmission in a high-risk population. In the future, the

newly developed animal model might act as model to study the colonizing capacity of different (emerging) *S. aureus* strains such as ST398, and to test the strains' characteristics, for instance. Also, new decolonization strategies can be tested using this model. As mupirocin resistance is increasing, in particular in MRSA strains (26), there is an urgent need to find another effective and easy-to-apply, topical antibacterial drug to eradicate *S. aureus* from the nasal cavity.

From the transmission studies, we have learned that improvements can be made in the field of infection prevention. In future studies in neonatal wards, it would be interesting to prospectively follow up different sources of MSSA in HCWs, the environment and maybe also parents, to detect carriage and to monitor routes of possible transmission, with the main goal to prevent future transmission and infections in neonates. Furthermore, in the future, strains originating from neonates who are colonized by or suffer from *S. aureus* infections, could be compared by using WGS, to determine the number of virulence factors present and to determine whether there are differences between colonization and infection strains. Ideally, it would be interesting to be able to predict whether a strain has more potential to become invasive based on the genetic package, including the toxin genes. The toxins *sea* and *tsst-1* that were found to be more present in neonatal bacteremia isolates, are interesting targets to begin with and figure out what their role is in the pathogenesis of *S. aureus*.

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