Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* in pigs: (co-) colonization dynamics and clonal diversity

Fetsch, A.[*1], *Roesler, U.*[2], *Kraushaar, B.*[1], *Friese, A.*[2]

Abstract

Methicillin-susceptible *Staphylococcus (S.) aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) are colonizers of skin and mucosa. In humans, MSSA and MRSA compete for colonization space in the anterior nares and one clone can be found rather than differing types of MSSA and MRSA. We investigated the colonization dynamics and clonality of both, MSSA and MRSA in pigs over a longer time period. Eighteen sows were nasally sampled three times every ten weeks. Additionally, environmental samples were taken. Samples were investigated for MSSA and MRSA, respectively. *Spa*-typing was done with up to five MRSA and MSSA isolates found per sample and time point; selected isolates were further investigated by microarray. 38.9 % of sows were infrequently MSSA/MRSA co-colonized and 16.7 % were permanent carriers of MSSA. The majority of sows showed a changing colonization status. CC398 and CC9 associated *spa*-types were exclusively found among MRSA and MSSA, respectively. In 44.4 % of sows at least two different types of MSSA were present at the same time and sample. Strains of the same clonal lineage showed a high genetic identity despite their origin. MSSA of different *spa*-types but 100 % identical microarray profiles were found in sows and their environment. Our results show that pigs may be colonized with MSSA and MRSA at the same time, i.e. that MSSA/MRSA do not exclude each other in the anterior nares of pigs. Highly identic clones are present in sows and their environment, but pigs can be colonized with different clones at the same time.

Introduction

Methicillin-susceptible *Staphylococcus (S.) aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) are colonizers of skin and mucosa and may cause severe infections both, in humans and animals. In the last decade, MRSA were frequently detected in healthy farm animals. Especially MRSA of the clonal complex (CC) 398 are widespread in the pig population in Europe (Graveland *et al.*, 2011) and are also able to cause serious diseases in animals and humans (van der Mee-Marquet *et al.*, 2014). Livestock professionals (farmers, veterinarians etc.) have a higher risk to get colonized with these so called livestock-associated (LA-) MRSA (van Cleef *et al.*, 2011). In this context MSSA carriage in pig farmers as a possible protective effect against the colonization with MRSA was postulated (van Cleef *et al.*, 2014). A multiple strain colonization of *S. aureus* has already been shown in humans (Votintseva *et al.*, 2014) and in pigs (Espinosa-Gongora *et al.*, 2014; Vandendriessche *et al.*, 2014). We aimed to investigate the colonization dynamics of both, MSSA and MRSA in sows over a longer time period (30 weeks). Hence, the clonal diversity of MSSA and MRSA found was investigated as little is known about the clonality of MSSA, neither in pigs nor their environment while it is well described that the majority of MRSA found in pigs belong to CC398 (EFSA, 2009). Special emphasize was also given to the animals´ environment as this might have an important impact as a potential source for colonization (Broens *et al.*, 2012).

Material and Methods

Sampling

Eighteen sows and their environment were investigated. The animals were sampled three times at intervals of about ten weeks. The sows were housed in four groups (A, B, C, and D) within one barn with possible direct contact between group A and B as well as between group C and D. One anterior nostril of every pig was sampled using a dry cotton swab (Sarstedt AG & Co. KG, Nümbrecht, Germany). Additionally, in every group environmental samples from walls, toys, brushes, watering places, and troughs were collected at the 2nd and 3rd sampling time by swabbing five different locations each using the same cotton swab moistened with 1.5 ml phosphate buffered saline (PBS), together with one boot swab sample and a pooled dust sample from the whole barn.

Laboratory analyses

Isolation

Each swab was incubated in 10 ml Mueller Hinton broth with 6.5 % NaCl (MHB+) overnight at 37°C. One loop of the MHB+ was then streaked onto the *S. aureus* selective agar (BBL™ CHROMagar™ Staph aureus, Becton and Dickinson, USA) and incubated under aerobic conditions for 24 hours at 37°C. Another 1 ml of the MHB+ was added to 9 ml tryptone soy broth including 75 mg/l aztreonam and 3.5 mg/l cefoxitin (TSB+) to grow MRSA aerobically at 37°C for 17 hours. After that, a loop-full of TSB+ was streaked onto selective MRSA agar (CHROMagarMRSA™, MAST Diagnostica GmbH, Reinfeld, Germany) and incubated at the same conditions.

Identification and characterization of MSSA and MRSA isolates

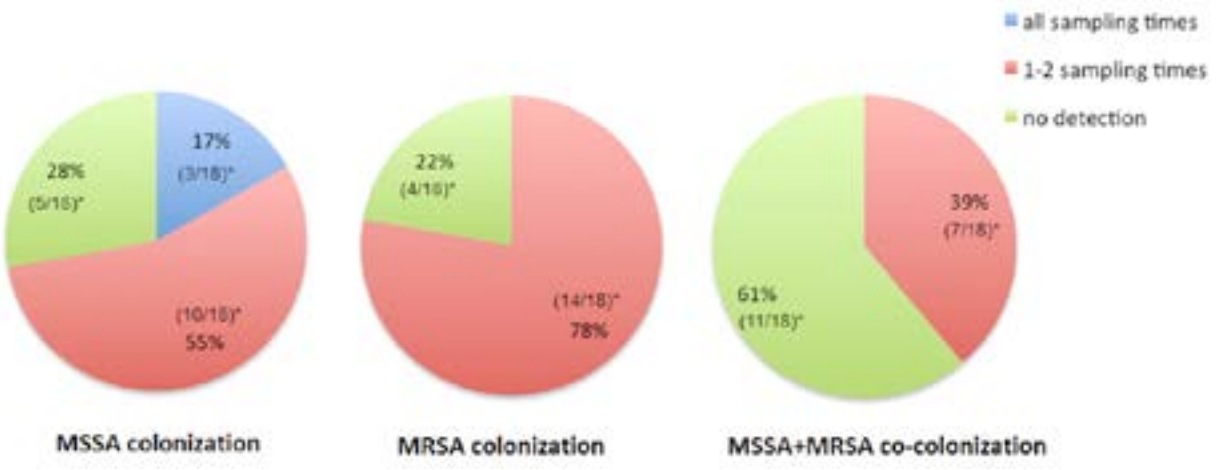
Up to five MRSA and MSSA isolates per sample were further characterized. All susceptive isolates were phenotypically examined. Subsequently DNA of the isolates was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Isolates were confirmed as MSSA and MRSA, resp., by means of an in-house multiplex-Real-Time PCR (adapted to (Fosheim *et al.*, 2011; Kilic *et al.*, 2010)). *Spa*- (Shopsin *et al.*, 1999) and in case of MRSA, SCC*mec*- (Zhang *et al.*, 2005) typing were also conducted.

To investigate the clonal identity of strains isolated from the animals and the environment, a subset of 62 MSSA and MRSA isolates was selected and investigated by means of a highly discriminatory microarray analysis (Identibac *S. aureus* Genotyping, Alere Technologies GmbH, Jena, Germany) according to the manufacturer's instructions.

Results

Overall, 38.9 % of pigs were infrequently MSSA/MRSA co-colonized; 16.7 % were permanent car-riers of MSSA while the majority of pigs (66.7 %) showed a changing colonization status (Figure 1).

Figure 1 Frequency of detection of MSSA, MRSA, and MSSA/MRSA co-colonization in 18 sows



* Numbers in brackets mean the number of animals where MSSA, MRSA or MSSA/MRSA co-colonization was detected in relation to the total number of animals tested mbers in brackets mean the number of animals where MSSA, MRSA or MSSA/MRSA co-colonization was detected in relation to the total number of animals tested

MSSA and MRSA were also detected in environmental samples: In total, 16 out of 54 (29.6 %) environmental swabs were MSSA positive and 11 (20.4 %) were MRSA positive; both, MSSA and MRSA were also detected in the same environment and at the same sampling time.

CC398 (t034, t011) and CC9 (t337, t1430, and t13816) associated *spa*-types were exclusively found among MRSA and MSSA, respectively. All of the 120 MRSA isolated from animals and the environment harbored a SCC-*mec* cassette of type V. In 44.4 % of pigs MSSA of up to two different *spa*-types were present at the same time and sample. MSSA strains were highly identical (96.5 % homogeneity) and clearly distinct from the selected MRSA strains, irrespective of their origin (animal vs. environment). Strains of the same clonal lineage showed a high genetic identity despite their origin.

Discussion

From the sows of our study, 38.9 % were co-colonized with MSSA and MRSA at the same time and sample, resp. at least once during the study period. These findings do not support the hypothesis that MSSA and MRSA compete for colonization space. Others too concluded that MRSA seem to add to the burden of *S. aureus* rather than replacing it (Vandendriessche *et al.*, 2014), also in humans (Cespedes *et al.*, 2005).

Spa-typing and microarray analyses showed a clear discrimination between MSSA and MRSA originating from the sows of our study. On the one hand MSSA found in sows mainly belonged to CC9 associated *spa*-types. Other authors confirm these findings but did also detect CC398 associated *spa*-types (Hasman *et al.*, 2010; Vandendriessche *et al.*, 2014). On the other hand, the majority of MRSA found in pigs belong to CC398 associated *spa*-types (EFSA, 2009; Smith and Pearson, 2011), also in the present study. Interestingly, none of the study pigs harbored MRSA of more than one differing *spa*-type at the same time and over the study period, resp. In contrast, two different *spa*-types of MSSA were present at the same time and sample in the majority of pigs. A multiple-strain colonization in human nasal carriers of *S. aureus* been shown recently (Votintseva *et al.*, 2014). These findings call for a critical review of the number of isolates that need to be analyzed, particularly in case of in-depth epidemiological investigations.

Molecular analysis of MSSA and MRSA isolated within our study revealed that strains detected both, at animal level and in the environment were highly identical or, in the latter case, did even show 100 % homogeneity in the microarray cluster analysis. This may allow for the conclusion that clones are exchanged between pigs and their environment.

Conclusion

Pigs may be colonized with MSSA and MRSA at the same time, i.e. MSSA/MRSA do not exclude each other in the anterior nares of pigs;

Pigs can be colonized with different clones at the same time;

Highly identic MSSA and MRSA clones are present in pigs and their environment.

Acknowledgements
This study was supported by the Federal Institute for Risk Assessment (internal BfR-grant 44-001). Parts of this work were carried out in the framework of the project 'MedVet-Staph' funded by the German Federal Ministry of Education and Science (BMBF), research grant: 01KI1014C. The authors gratefully acknowledge excellent technical assistance of H. Jansen, M. Thieck, Y. Kelner-Burgos, K. Steege, U. Kämpe, D. Leese, F. Leonhard, and S. Scheunemann.
A manuscript about this study (Fetsch *et al.*) has been submitted to the journal Veterinary Microbiology.

References

- *Broens, E.M., Espinosa-Gongora, C., Graat, E.A., Vendrig, N., Van Der Wolf, P.J., Guardabassi, L., Butaye, P., Nielsen, J.P., De Jong, M.C., Van De Giessen, A.W., 2012. Longitudinal study on transmission of MRSA CC398 within pig herds. BMC veterinary research 8, 58.*
- *Cespedes, C., Said-Salim, B., Miller, M., Lo, S.H., Kreiswirth, B.N., Gordon, R.J., Vavagiakis, P., Klein, R.S., Lowy, F.D., 2005. The clonality of Staphylococcus aureus nasal carriage. The Journal of infectious diseases 191, 444-452.*
- *EFSA, 2009. Scientific Opinion of the Panel on Biological Hazards on a request from the European Commission on Assessment of the Public Health significance of meticillin resistant Staphylococcus aureus (MRSA) in animals and foods. The EFSA Journal 993, 1-73.*
- *Espinosa-Gongora, C., Dahl, J., Elvstrom, A., van Wamel, W.J., Guardabassi, L., 2014. Individual predisposition to Staphylococcus aureus colonization in pigs based on quantification, carriage dynamics and serological profiles. Applied and environmental microbiology.*
- *Fosheim, G.E., Nicholson, A.C., Albrecht, V.S., Limbago, B.M., 2011. Multiplex real-time PCR assay for detection of methicillin-resistant Staphylococcus aureus and associated toxin genes. Journal of clinical microbiology 49, 3071-3073.*
- *Graveland, H., Duim, B., van Duijkeren, E., Heederik, D., Wagenaar, J.A., 2011. Livestock-associated methicillin-resistant Staphylococcus aureus in animals and humans. International journal of medical microbiology : IJMM 301, 630-634.*
- *Hasman, H., Moodley, A., Guardabassi, L., Stegger, M., Skov, R.L., Aarestrup, F.M., 2010. Spa type distribution in Staphylococcus aureus originating from pigs, cattle and poultry. Veterinary microbiology 141, 326-331.*
- *Kilic, A., Muldrew, K.L., Tang, Y.W., Basustaoglu, A.C., 2010. Triplex real-time polymerase chain reaction assay for simultaneous detection of Staphylococcus aureus and coagulase-negative staphylococci and determination of methicillin resistance directly from positive blood culture bottles. Diagnostic microbiology and infectious disease 66, 349-355.*
- *Shopsin, B., Gomez, M., Montgomery, S.O., Smith, D.H., Waddington, M., Dodge, D.E., Bost, D.A., Riehman, M., Naidich, S., Kreiswirth, B.N., 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of Staphylococcus aureus strains. Journal of clinical microbiology 37, 3556-3563.*
- *Smith, T.C., Pearson, N., 2011. The emergence of Staphylococcus aureus ST398. Vector borne and zoonotic diseases (Larchmont, N.Y.) 11, 327-339.*
- *van Cleef, B.A., Monnet, D.L., Voss, A., Krziwanek, K., Allerberger, F., Struelens, M., Zemlickova, H., Skov, R.L., Vuopio-Varkila, J., Cuny, C., Friedrich, A.W., Spiliopoulou, I., Paszti, J., Hardardottir, H., Rossney, A., Pan, A., Pantosti, A., Borg, M., Grundmann, H., Mueller-Premru, M., Olsson-Liljequist, B., Widmer, A., Harbarth, S., Schweiger, A., Unal, S., Kluytmans, J.A., 2011. Livestock-associated methicillin-resistant Staphylococcus aureus in humans,*

Europe. Emerging infectious diseases 17, 502-505.

- *van Cleef, B.A., van Benthem, B.H., Verkade, E.J., van Rijen, M., Kluytmans-van den Bergh, M.F., Schouls, L.M., Duim, B., Wagenaar, J.A., Graveland, H., Bos, M.E., Heederik, D., Kluytmans, J.A., 2014. Dynamics of methicillin-resistant Staphylococcus aureus and methicillin-susceptible Staphylococcus aureus carriage in pig farmers: a prospective cohort study. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 20, O764-771.*
- *van der Mee-Marquet, N.L., Corvaglia, A., Haenni, M., Bertrand, X., Franck, J.B., Kluytmans, J., Girard, M., Quentin, R., Francois, P., 2014. Emergence of a novel subpopulation of CC398 Staphylococcus aureus infecting animals is a serious hazard for humans. Frontiers in microbiology 5, 652.*
- *Vandendriessche, S., Vanderhaeghen, W., Larsen, J., de Mendonca, R., Hallin, M., Butaye, P., Hermans, K., Haesebrouck, F., Denis, O., 2014. High genetic diversity of methicillin-susceptible Staphylococcus aureus (MSSA) from humans and animals on livestock farms and presence of SCCmec remnant DNA in MSSA CC398. The Journal of antimicrobial chemotherapy 69, 355-362.*
- *Votintseva, A.A., Miller, R.R., Fung, R., Knox, K., Godwin, H., Peto, T.E., Crook, D.W., Bowden, R., Walker, A.S., 2014. Multiple-strain colonization in nasal carriers of Staphylococcus aureus. Journal of clinical microbiology 52, 1192-1200.*
- *Zhang, K., McClure, J.A., Elsayed, S., Louie, T., Conly, J.M., 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant Staphylococcus aureus. Journal of clinical microbiology 43, 5026-5033.*

[1] National Reference Laboratory for coagulase-positive *staphylococci* incl. S*taphylococcus aureus*, Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 12277 Berlin, Germany
[2] Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin, Robert-von Ostertag-Straße 7-13, 14163 Berlin, Germany

**corresponding author:* alexandra.fetsch@bfr.bund.de