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Experimental airborne colonization of piglets with livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA)

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AMPs	antimicrobial peptides
BfR	Bundesinstitut für Risikobewertung
CA-MRSA	community-aquired methicillin-resistant *Staphylococcus aureus*
CC	clonal complex
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLSI	Clinical and Laboratory Standards Institute
ConA	concanavalin A
DG group	dexamethasone group
e.g.	exempli gratia
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EG group	endotoxin group
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FCS	fetal calf serum
HA-MRSA	hospital-associated methicillin-resistant *Staphylococcus aureus*
HD group	high dose group
IMDM	Iscove´s Modified Dulbecco´s Medium
LA-MRSA	livestock-associated methicillin-resistant *Staphylococcus aureus*
LD group	low dose group
LPS	lipopolysaccharide
MD group	median dose group
MHB	Mueller Hinton broth
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered-saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
RBC	red blood cell
S. aureus	*Staphylococcus aureus*
SCC*mec*	staphylococcal cassette chromosome *mec*

III. List of Abbreviations

spa	staphylococcal protein A
ST	sequence type
TIA group	tiamulin group
TLR	toll-like receptor
TSB	Trypton Soy broth
wbc	white blood cell count

1. General remarks

Antimicrobial resistance is a rising concern in human and veterinary medicine (Dahms et al. 2014b) and is therefore a major issue of public health. Whilst nowadays the number of human deaths caused by resistant bacteria is around 700.000 per year, the proportion might increase to 10 million deaths per year by 2050 (Jim O`Neill 2014). Combatting the rise in antimicrobial resistance is one of the main topics of the One Health approach, which addresses critical public health subjects in human and veterinary medicine (Sellera and Lincopan 2019). Methicillin-resistant *Staphylococcus aureus* (MRSA), one of the first opportunistic microorganism causing healthcare-associated infections, is still an important pathogen and one of the leading causes of bloodstream infections in Europe (ECDC 2018). In the past, the occurrence of MRSA was especially associated to healthcare systems, but findings by Voss et al. (2005) lead to a change in perception: The author described multiple ineffective MRSA decolonization attempts of a six-month old farmers´ girl before surgery. Voss et al. (2005) traced the repeated re-colonization of the girl back to MRSA-positive healthy pigs and was, therefore, the first author who described the connection between asymptomatically MRSA colonized livestock and human MRSA carriage (Dahms et al. 2014b). Triggered by these findings, different countries investigated livestock for the presence of MRSA and found that this newly emerged MRSA is widespread among animal husbandry. The main reservoir was found in pigs with a prevalence of up to 100% (EFSA 2018). While healthcare- associated MRSA isolates continued to decline in 2017, there is an increasing detection of livestock- associated MRSA in humans since 2007, which highlights the importance of LA-MRSA as an One Health issue (Kinross et al. 2017).

Besides the occurrence of MRSA-positive animals, airborne LA-MRSA has been regularly detected in pigsties and air exhausts of these buildings in the past (Friese et al. 2012; Schulz et al. 2012a; Bos et al. 2016; Ferguson et al. 2016; Angen et al. 2017). This finding, in combination with the positive correlation of individual MRSA carriage and the presence of MRSA in the air, suggests a possible airborne transmission route. Additionally, an environmental spread of MRSA through the air cannot be excluded. In a study published in 2018, LA-MRSA was found to survive in settled stable dust for up to several weeks (Feld et al. 2018). This is of major importance since dust is presumed to be the most likely source of airborne LA-MRSA (Friese et al. 2012).

Until now, knowledge about the real impact of LA-MRSA contaminated air on the colonization status of pigs is limited, although investigations are warranted (Bisdorff et al. 2012).

Therefore, the aims of our study were:

- to identify the necessary airborne MRSA concentration for an MRSA colonization of piglets in a newly established animal model

- to evaluate possible predisposing factors for an LA-MRSA colonization in piglets

1.1 *Staphylococcus aureus (S. aureus)*

Staphylococcus aureus (*S. aureus*) is a commensal Gram-positive bacterium that is able to colonize the skin and mucous membranes of humans and animals, without causing clinical signs. In humans, 20% are persistent and 30% are transient MRSA carriers. Half of the human population is never colonized with *S. aureus* (Kluytmans 1997). Although *S. aureus* is detectable at various sampling sites, its primary reservoir and ecological niche is thought to be the vestibulum nasi (Kluytmans 1997). However, clinically inapparent colonization can evolve into serious *S. aureus* infections (Huijsdens et al. 2006) with resistance to antimicrobial agents complicating treatment approaches (Lowy 2003).

The first resistances of *S. aureus* were detected shortly after the launch of penicillin – the first beta-lactam antibiotic – in 1944 (Livermore 2000) and are induced by the production of an enzyme referred to as beta-lactamase (Graveland et al. 2011a). This plasmid-encoded enzyme hydrolyzes the beta-lactam ring of penicillin (Lowy 2003; Graveland et al. 2010) resulting in ineffectiveness of this antibiotic agent. By the late 1960s, 80% of the community and hospital-acquired staphylococcal isolates already showed this kind of resistance (Lowy 2003). Hence, the semisynthetic beta-lactamase stable penicillin derivate methicillin was developed and introduced into the clinical practice in 1960. Soon after (1961), the first methicillin resistance was detected (Jevons et al. 1961)**.** In contrast to penicillin resistance, methicillin resistance is chromosomally mediated by a *mecA* gene, which is located on a mobile genetic element termed staphylococcal cassette chromosome *mec (*SCC*mec)* (Lowy 2003). The *mecA* gene codes for a variant penicillin-binding protein (PBP2a or PBP2b), which mediates the resistance to beta- lactam antibiotics by a decreased affinity for most semisynthetic penicillins (Hartman and Tomasz 1984; Lakhundi and Zhang 2018). Usually, beta-lactam antibiotics target the penicillin-binding protein (PBP) that is necessary for the peptidoglycan synthesis, resulting in the inhibition of the bacterial cell wall synthesis (Tipper 1985). Although more than 99% of the circulating methicillin resistant staphylococcal isolates harbor the *mecA* gene, there are also phenotypic methicillin-resistant *S. aureus,* which contain *mecA* homologous genes termed *mecB* and *mecC* (Becker et al. 2014). These variants of the *mecA* gene also mediate the resistance to beta-lactam antibiotics (García-Álvarez et al. 2011). The *mecC* gene was first described in 2011 in clinical *S. aureus* isolates from humans and bulk milk samples and is located chromosomally, similar to the *mecA gene* (García-Álvarez et al. 2011). The recent detection of the plasmid-borne *mecB* gene in *S. aureus* isolates poses a new threat for the public health due to the risk of facilitated transmission compared to the chromosomally integrated elements (Becker et al. 2014).

Despite epidemiological overlaps between different groups of MRSA, Cuny et al. (2013) propose the maintenance of the MRSA discrimination for practical reasons. MRSA was first detected in a British hospital (Jevons 1961) and was initially expected to be a solely hospital-associated pathogen (HA-MRSA). The clinical picture of infections caused by HA-MRSA is manifold, ranging from minor skin infections to endocarditis or sepsis or even necrotizing pneumonia (Deurenberg et al. 2007; Goerge et al. 2017). The nosocomial nature is suspected when an infection appears at least 48 h after admission to hospital (Graveland et al. 2011a). On the contrary, an infection that occurs 48 h of admission, is defined as community-acquired MRSA (CA-MRSA) (Deurenberg et al. 2007). CA-MRSA was firstly reported in 1993 and is of special concern because it causes infections of skin and soft tissues in otherwise healthy humans, without any known risk factors (Davis et al. 2007; Deurenberg et al. 2007). In the early 21th century, a new linage of MRSA was observed in humans with contact to pigs (Armand-Lefevre et al. 2005; Voss et al. 2005). This MRSA differed from the already known types of MRSA as it was non-typeable by the standard genetic typing method of pulsed-field gel electrophoresis (PFGE) using the restriction endonuclease *SmaI* (Voss et al. 2005). The origin of this so-called livestock-associated MRSA (LA-MRSA) is assumed to be of human nature as human sensitive *S. aureus* of this lineage may spread to livestock with subsequently acquisition of methicillin resistance (Price et al. 2012). To identify *S. aureus* strains and to predict epidemiological and phylogenetic relationships, different genotyping methods are used (Chambers and Deleo 2009). One of them is the multilocus sequence typing (MLST), where a sequence analysis of seven housekeeping genes is conducted in order to provide a discriminatory allelic profile called the sequence type (ST) (Chambers and Deleo 2009). *S. aureus* strains with five identical housekeeping gene sequences are grouped into same clonal complexes (CC) (Deurenberg et al. 2007). Most isolates of LA-MRSA belong to the clonal complex (CC) CC398 and MLST sequence type (ST) ST398 and are associated with a variety of *S. aureus* protein A (*spa*) types (Khanna et al. 2008; Köck et al. 2009). A slightly easier alternative to the MLST method is s*pa* typing: a single-locus sequence typing method for *S. aureus,* based on sequence polymorphisms of the *spa* gene (Deurenberg et al. 2007; Cuny et al. 2016).

1.2 Livestock-associated MRSA (LA-MRSA) in animals

In recent years, a lot of research has been undertaken to investigate the spread of LA-MRSA in animal husbandry. Due to its assumed limited host specificity, LA-MRSA colonize a range of animals, such as pigs, cattle, poultry, companion animals and horses. Thereby, animals are mostly asymptomatically carriers of LA-MRSA and infections are rarely seen (Becker et al. 2017).

Since LA-MRSA was first isolated in persons with regular close contact to pigs (Armand-Lefevre et al. 2005; Voss et al. 2005), subsequent research was initially focused on pig farming.

In Germany, the LA-MRSA prevalence in pigsties varies between 52% (Alt et al. 2011), 70% (Köck et al. 2009) and 96% (Fischer et al. 2017). Particularly high prevalences were detected in fattening herds (Friese et al. 2012) with large pig farms and wean-to-finish herds (Alt et al. 2011). The MRSA prevalence ranges from 23% (van Duijkeren et al. 2008) to 71% (Broens et al. 2011a) in the Netherlands, 86.3% in Belgium (Pletinckx et al. 2011) and 45% in Canada (Khanna et al. 2008). The most common *spa* types occurring in German pig production are t011 and t034 (Alt et al. 2011), with the *spa* types t108, t1451 and t2510 also occurring (Köck et al. 2009). MRSA ST398 harbors different *spa* types with ongoing reports of new spa types (Graveland et al. 2011a).

In general, the occurrence of MRSA-positive pig farms varies hugely with values ranging from 0.1 to 100.0% in different European countries (EFSA 2018). In the case of Norway, the prevalence of 0.1% might reflect the success of eradication programs. In other cases, it might be a result of different sampling methods. For instance, it was shown that, although nasal swabs are the best sampling method for a MRSA detection, the chance of identifying MRSA positive pigs increases with the number of sampling sites (Dewaele et al. 2011). In addition, the timing of sampling might influence the MRSA status of pigs: weaned piglets are often found to carry LA-MRSA, possibly due to the high exposure to inhalable dust (Takai et al. 1998), which is suspected to be an important factor in LA-MRSA transmission (Feld et al. 2018).

LA-MRSA can be detected in isolates from skin and urinary-genital tract infections in pigs as well as from the metritis-mastitis-agalactia syndrome (MMA) syndrome (Schwarz et al. 2008). However, pigs are often LA-MRSA positive without showing any clinical symptoms and the overall prevalence is high. The opposite is true in dairy cattle (Spohr et al. 2011). The prevalence in milk samples lies between 1.4–16.7% in German dairy herds but is often associated with subclinical and clinical mastitis (Spohr et al. 2011). In other European countries, the LA-MRSA prevalence in dairy herds ranges from 0-7.4% in Belgium (Vanderhaeghen et al. 2010b) and 15-60 % in Italy (Luini et al. 2015; Locatelli et al. 2017). This low LA-MRSA prevalence in cattle is in accordance with Verhegghe et al. (2013), who found pigs significantly more likely to be colonized with MRSA than cattle and poultry. The low MRSA prevalence in poultry found by Verhegghe et al. (2013) is supported by the findings by Friese et al. (2013), who detected a MRSA prevalence of 22.5% in broiler fattening farms and 25.9% in turkey fattening farms in Germany. In Belgium and the Netherlands, an MRSA prevalence of 3.3-7% for broiler and laying chickens was detected by Pletinckx et al. (2011), Nemeghaire et al. (2013), and Mulders et al. (2010). In contrast to pigs, where most of the isolates belong to the LA-MRSA category, MRSA isolates in poultry were also assigned to HA-MRSA (Nemeghaire et al. 2013). In companion animals (cats and dogs), MRSA isolates have mostly been identical to the HA-MRSA lineages of human origin. This indicates that contact to human MRSA carriers might be a risk factor for these animals to become colonized by MRSA

(Loeffler and Lloyd 2010). However, a German-wide survey conducted between 2010 and 2012 revealed that 13.5 and 7.7% of *S. aureus* positive wound samples in dogs and cats, respectively, contained LA-MRSA CC398 (Vincze et al. 2014a). This is in accordance with findings of a French study, which isolated MRSA CC398 in 21.4% and 26.5% of clinical cases in dogs and cats, respectively (Haenni et al. 2017). Therefore, the replacement of human MRSA strains by MRSA CC398 in pet animals is considered possible (Haenni et al. 2017). Also, in horses, we can assume that the previously predominant lineage CC8-MRSA has been replaced by LA-MRSA, especially due to a specific sublineage of MRSA CC398, designed as clade c (Abdelbary et al. 2014; Vincze et al. 2014b; Haenni et al. 2017). In Germany, Cuny et al. (2016) found an LA-MRSA proportion of 84.5% in clinical isolates of horses, mostly belonging to the aforementioned clade c.

1.3 Livestock-associated MRSA (LA-MRSA) in humans

Animals colonized by LA-MRSA pose a major threat in human medicine, especially for people regularly exposed to livestock (farmers, veterinarians and slaughterhouse personal) (Springer et al. 2009). To get a better understanding of risk factors for LA-MRSA colonization in humans, many studies have been investigated the MRSA colonization status of persons working with livestock (Graveland et al. 2011b; Khanna et al. 2008; van den Broek et al. 2009). In farmers, LA-MRSA carriage was estimated to range from 14% (van den Broek et al. 2009) to 86% (Cuny et al. 2009). Working on LA-MRSA-positive farms level influences the LA-MRSA colonization in humans: working on MRSA-positive farms is associated with a higher chance of testing MRSA-positive (van Duijkeren et al. 2008), which suggests that the carriage of LA-MRSA in farmers is strongly exposure related (Graveland et al. 2011b). In veterinarians, the colonization with LA-MRSA was found to range between 4.6% (Wulf et al. 2006) and 45% (Verkade et al. 2013; Cuny et al. 2009). Besides working with living animals, the slaughtering process seems to be an additional risk factor for LA-MRSA carriage (Gilbert et al. 2012). For instance, a nasal LA-MRSA prevalence of 5.6% in slaughterhouse workers was detected by van Cleef et al. (2010).

LA-MRSA carriers are a presumable source for the introduction of this resistant bacterium into hospital or other healthcare facilities (Becker et al. 2017). Although there are several studies suggesting that LA-MRSA has a limited ability to colonize humans (Graveland et al. 2011b; Cuny et al. 2013), the number of human LA-MRSA carriers has increased in recent years particularly in areas with high pig production. This LA-MRSA carriage leads to the typical clinical picture usually observed in *S. aureus* infections (Becker et al. 2017; Cuny et al. 2013), when turn into an infection (Bonten and Weinstein 1996). Whereas the general rate of LA-MRSA carriage at hospital admission ranges between 0.08–0.2% in Germany (Cuny et al. 2013), Köck et al. (2013) found a LA-MRSA proportion of all MRSA-positive clinical isolates of up to 29% in a hospital in northwestern Germany - the area with the highest pig density in the

country. Particularly high prevalences of LA-MRSA in patient isolates in this region was also observed by Schaumburg et al. (2012). According to Feingold et al. (2012), the high regional density of livestock is an important risk factor for the LA-MRSA carriage in humans. Infections caused by LA-MRSA have subsequently been reported most frequently for humans living in livestock-dense areas or working with livestock (Becker et al. 2017). It was shown that LA-MRSA CC398 lacks many virulence factors usually harbored by HA- and CA-MRSA strains (Cuny et al. 2013). For instance, exotoxins, such as pyrogenic toxin superantigens and exfoliative toxins responsible for the development of staphylococcal toxin syndromes (staphylococcal toxic shock syndrome, the staphylococcal enterotoxin-caused food poisoning and the staphylococcal scalded skin syndrome) were absent in most MRSA CC398 isolates (Becker et al. 2017). This indicates a low potential of these MRSA clones to cause toxin-mediated diseases (Cuny et al. 2013). Many studies (van Duijkeren et al. 2008; Köck et al. 2009; Vanderhaeghen et al. 2010a) described the absence of the Panton–Valentine leukocidin (PVL) in MRSA CC398, an *S. aureus*-specific exotoxin (Deurenberg et al. 2007), which is assumed to be a major virulence factor of CA-MRSA (Diep and Otto 2008). Nowadays, there are few reports of the sporadic occurrence of PVL-positive MRSA CC398 strains, of which less strains were isolated from clinical human cases (Becker et al. 2017). However, LA-MRSA CC398 seems to be equally capable as the HA-MRSA strains of causing diseases in humans (Cuny et al. 2013) especially in persons exposed to livestock (Goerge et al. 2017).

1.4 Livestock-associated MRSA (LA-MRSA) in the environment

LA-MRSA was found in pig holdings, especially in stable dust, feces and boot swabs, which contain a mixture of both feces and dust (Friese et al. 2012). Pletinckx et al. (2011) detected this resistant bacterium on the floor, the wall and air samples of pigsties. Airborne LA-MRSA in pig houses were also detected by Harper et al. (2010), Dewaele et al. (2011), Friese et al. (2012), and Ferguson et al. (2016). Friese et al. (2012) showed an airborne LA-MRSA prevalence of 85.2% in LA-MRSA positive German pig barns with a median concentration of 2.6×10^2 cfu/m^3. Similar exposure levels of LA-MRSA in the air of pigsties have been observed by Masclaux et al. (2013), Angen et al. (2017), and Madsen et al. (2018). In a recent study, the practicability of different sampling methods for detecting MRSA-positive stables were compared, and air sampling, with a sensitivity of 99%, was recommended for initial screenings (Agersø et al. 2014). This highlights the frequent occurrence and importance of airborne LA-MRSA in MRSA-positive pig farms. Airborne LA-MRSA cannot only be detected inside pig barns, but also in the exhaust air of pigsties. A persistent LA-MRSA contamination of the surrounding areas of pigsties, especially downwind, is assumed due to MRSA emitted by exhaust air (Schulz et al. 2012b). Schulz et al. (2012b) proved airborne LA-MRSA from 50 meters up to distances of 150 meters downwind from pigsties in low concentrations ranging from 2 cfu/m^3 up to 14 cfu/m^3. Airborne MRSA was also detected in 215 meters distance from

the barns by Ferguson et al. (2016). Gibbs et al. (2006) found antibiotic-resistant *S. aureus* at least 150 meters downwind in decreasing concentration with increasing distance to the livestock.

1.5 Transmission of livestock-associated MRSA (LA-MRSA)

It is unquestionable that the main transmission route of LA-MRSA is the direct contact to MRSA-positive individuals and MRSA-contaminated surfaces. Broens et al. (2012a) noticed that keeping pigs with MRSA-positive pen mates massively increases the likelihood of colonization. In humans, the spread of MRSA occurs mainly through hand-to-nose-contact (Wulf et al. 2006) as well as touching MRSA-contaminated surfaces within the farming environment (Bisdorff et al. 2012). Whereas Alt et al. (2011) declare the direct contact to MRSA-positive livestock as the sole relevant route for MRSA transmission from animals to humans, Cuny et al. (2009) and Dahms et al. (2014a) also propose an indirect MRSA transmission via dust. Dust is a mixture of excrement, litter, feed, skin, and feathers and bacteria sloughed from animals (Zhao et al. 2014) and generated for the most part by the animals themselves (Gustafsson 1999). According to Friese et al. (2012) dust is the most likely source of airborne LA- MRSA. Microorganisms can become airborne when dried particles are disturbed by air flow or animal activity (Zhao et al. 2014). MRSA aerosols originate from the skin, feces and nostrils of MRSA-positive pigs (Masclaux et al. 2013) and might disseminate MRSA inside the pig barn. This may lead to MRSA transmission without direct contact to MRSA carriers (Friese et al. 2012). This is also assumed by Harper et al. (2010) and Masclaux et al. (2013), who advocate an indirect MRSA transmission by inhalation of MRSA-contaminated air. Working in a barn has been shown to increases the likelihood of acquiring MRSA. This is primarily due to inhalation of the stirred up MRSA contaminated dust. Furthermore, working in barns might increase the respiratory rates due to accelerated physical efforts, which is followed by higher nasal MRSA deposition (Angen et al. 2017).

According to Dewaele et al. (2011), the occurrence of MRSA-positive air and dust might also be a source for MRSA to spread to other farms in the neighborhood (Bisdorff et al. 2012; Schulz et al. 2012a) or to animals living in the vicinity (Schulz et al. 2012b). Furthermore, a recontamination of disinfected farms might be possible by MRSA re-entrainment of MRSA-positive air or vectors like rodents (Schulz et al. 2012b). Also, people living near to MRSA-positive livestock may be at higher risk to be colonized (Gibbs et al. 2004).

1.6 Possible predisposing factors for colonization with livestock-associated MRSA (LA-MRSA)

We assume that the high prevalence of LA-MRSA colonization in pigs is of multifactorial nature, influenced inter alia by the pre-immune status of piglets. In intense livestock farming, many stress factors affect the immune system of the animals and might decrease the resistance to

pathogens. However, to date, data concerning the influence of the immune status on MRSA colonization of pigs is scarce. In one publication, the level of different *S. aureus* specific antigens was investigated with respect to the pigs´ MRSA status (permanent, intermittent or non-carrier). Since no association between MRSA carriage and humoral response was detected, the influence of the humoral immune response on MRSA carriage was neglected (Espinosa-Gongora et al. 2015a). However, given the pathogenesis of multifactorial diseases, we assumed that stress imitated by dexamethasone treatment would enhance the MRSA colonization of piglets after experimental MRSA exposure.

In addition, air contaminants, especially endotoxins, affect respiratory health. As a part of the outer membrane of Gram-negative bacteria, endotoxins play an indisputable role in the development of respiratory diseases. Therefore, we addressed the relationship between aerogene endotoxin exposure and sensitization of the piglets´ airways for MRSA colonization.

On the other hand, certain microbial flora may have an antagonistic effect on MRSA and thus impede experimental colonization (Moodley et al. 2011). Then, MRSA might compete with other microbes (Edwards et al. 2012) for attachment sides to establish a colonization. Should these attachment sides already be occupied by other microbes, such as methicillin-sensitive *Staphylococcus aureus* (MSSA), colonization fails (Verstappen et al. 2014). In support of this theory, research suggests that MSSA carriage may protect against MRSA colonization (Dorado-García et al. 2013). Consequently, piglets who are natural carriers of MSSA may require higher doses of MRSA for successful establishment. In other pig MRSA colonization models, MSSA carriage was held responsible for the MRSA colonization failure (Broens et al. 2012b and Jouy et al. 2012). SanMiguel et al. (2017) found that topical antibiotic treatment influences the residential bacteria on the skin and decreases the cutaneous host defense against *S. aureus* colonization.

2. Outline of the Study

The study “Experimental airborne colonization of piglets with livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA)” was carried out between 2014 and 2017 and funded in parts by the Federal Ministry of Risk Assessment (BfR, grant no.: 1329-530).

2.1 Airborne LA-MRSA exposure of the piglets in the aerosol chamber and MRSA colonization monitoring

For each group, nine MRSA - negative tested piglets, aged approximately 21 days, were exposed to a defined concentration of airborne MRSA (ST398) (10^2 cfu/m^3 low dose group (LD group), 10^4 cfu/m^3 median dose group (MD group), and 10^6 cfu/m^3 high dose group (HD group)) in the aerosol chamber for 24 hours. An LA-MRSA suspension, containing a defined bacterial concentration of 10^4 cfu/mL (LD group), 10^6 cfu/mL (MD group), and 10^9 cfu/mL (HD group) was prepared, stored in syringes on ice and aerosolized by using a perfusion pump (see Figure 2) with an ultrasonic nebulizer located in the aerosol chamber’s ceiling. A sketch of the aerosol chamber is included (Rosen et al. 2018). As depicted in Figure 3, the piglets were transported from the experimental pig barn to the aerosol chamber using a hygiene lock. In the aerosol chamber, the piglets received water and feed ad libitum (Figure 3, Figure 4). During MRSA exposure, air samples were taken via impingement. This was carried out during three 30-minute intervals at an air flow of 8.5 and 12.5 liters per minute (see Figure 5). Five animal samples (nasal, skin, pharyngeal, conjunctival and rectal swabs) and different environmental samples were taken three times a week for a period of 21 days and subsequently investigated for the presence of MRSA. At the end of the observation period, the animals were euthanized, and different tissues and organs were analyzed to investigate the spread of MRSA.

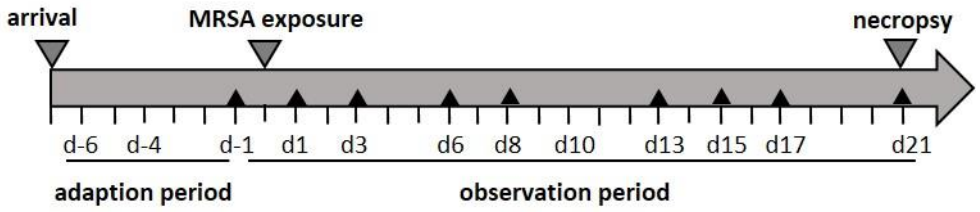

***Figure 1**: Timeline of the animal trials*

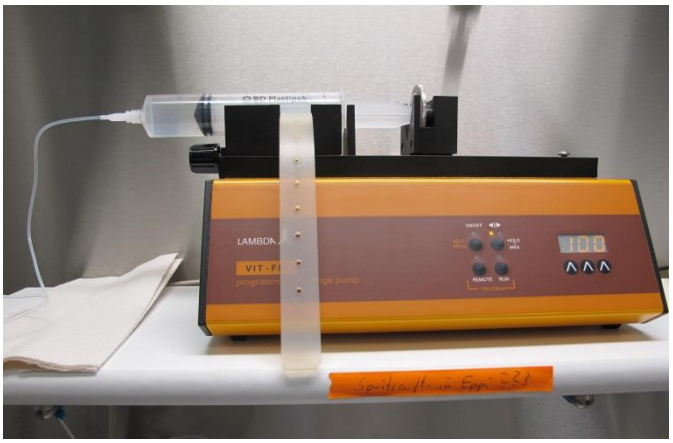

Figure 2: *Syringe containing the MRSA suspension fixed in the perfusion pump*

Figure 3: *Transport of the piglets into the aerosol chamber*

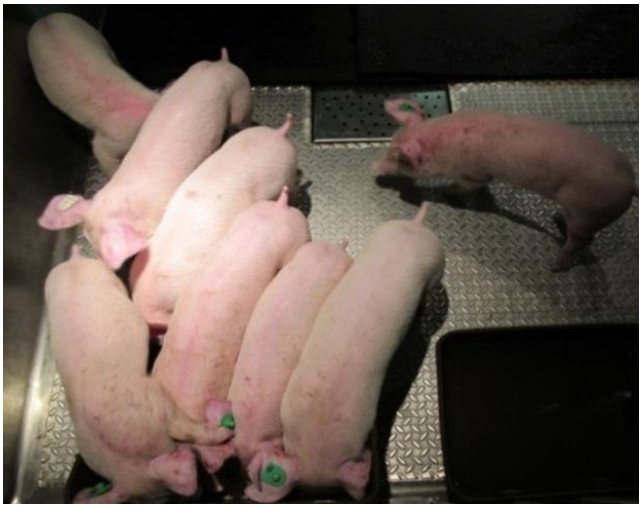

Figure 4*: Piglets in the aerosol chamber during airborne MRSA exposure*

Figure 5: *Air sampling via impingement (AGI-30 Impinger)*

2.2.1 Air samples

The air samples were collected in phosphate-buffered-saline (PBS) using AGI-30 Impingers and proceeded immediately. After the remaining volume of sampling fluid was measured, 100 µl of an appropriate dilution were streaked out threefold onto chromogenic MRSA screen agar (CHROMagar MRSA, MAST Diagnostica GmbH) and incubated aerobically at 37°C. After 24 hours, MRSA was identified phenotypically and confirmed with MALDI TOF mass spectrometry.

2.2.2 Animal and Environmental samples

The swab samples were extracted in PBS and after retaining samples were taken (stored at 4°C), enriched using a two-step enrichment (MHB+ (Mueller Hinton broth) supplemented with 6.5% NaCl) (Oxoid, Wesel, Germany) and Trypton Soy broth (TSB+) (Oxoid, Wesel, Germany) containing 75 mg/L aztreonam (Molekula GmbH, Munich, Germany) and incubated at 37°C for the qualitative analysis of MRSA. After 24 h, the sample fluid was streaked out onto the chromogenic MRSA screen agar. All MRSA-positive samples were identified phenotypically and confirmed with MALDI TOF mass spectrometry. Qualitative MRSA-positive samples were quantified by plating 100 µl of the retained samples threefold onto chromatic MRSA screen agar and identified as described before.

2.2.3 Organs

To decontaminate the outer layer, all organs and tissues were immersed in 96% ethanol and flamed. Then, the specimen was cut into pieces, transferred into MHB+, and homogenized using a stomacher homogenizer (stomacher 400 circulator, Seward Limited, West Sussex, United Kingdom). After retaining samples were taken (stored at 4°C), the samples were

incubated for qualitative analysis at 37°C for 24 h. Then, the incubated MHB+ was transferred into TSB+ and incubated again overnight at 37°C. When MRSA was detected qualitatively, 100 µl of the retained samples were plated threefold onto chromatic MRSA screen agar and identified as described above.

2.2.4 *Spa* Typing of MRSA isolates

S*pa* typing was performed for one isolate of the impinger sample and the last MRSA positive nasal swab isolate of each animal as described in the original research article (Rosen et al. 2018). In addition, one isolate of every MRSA-positive tonsil was *spa* typed. The isolates were confirmed as *spa* type t011 using the PCR method described by Kahl et al. (2005). LGC Genomics GmbH performed the sequencing and the sequences were analyzed using BioNumerics version 6.6.

2.2.5 Blood sample analyses of selected groups

Two groups of nine MRSA-negative piglets, each, were exposed to immunomodulation factors to elucidate the influence of the immune system on colonization success by airborne MRSA. One group was treated with dexamethasone (dexamethasone 4 mg/mL, Vetiquinol) on nine consecutive days (DG group). The other group was challenged with airborne bacterial endotoxin (4 µg/m^3) from *E. coli* O111:B4 (SIGMA ALDRICH, Darmstadt, Germany) (EG group). Both groups were then exposed to an airborne MRSA concentration of 10^4 cfu/m^3 for 24 hours.

Blood samples were taken of the DG and EG group as well as a control group and investigated for specific immunological parameters (in cooperation with Dr. Friederike Ebner and Stefanie Schmidt, Institute of Immunology, Freie Universität Berlin. The proliferation capacity was analyzed to investigate the influence of dexamethasone on the $CD4^+$ T cells in the DG group in comparison to the control group. For this, mononuclear cells from porcine peripheral blood (PBMC) were isolated and stained with the proliferation marker carboxyfluorescein diacetate succinimidyl ester (CFSE, eBiosciences). CFSE-labeled porcine PBMCs were transferred to IMDM supplemented with 10% FCS and 1% Penicillin-Streptomycin (all PAN-222 Biotech, Aidenbach, Germany) and seeded into 96-well round bottom plates. A proliferation was induced by adding concanavalin A (ConA) (2µg/mL, Sigma-Aldrich) and assessed by comparing unstimulated and ConA-stimulated PBMC using flow cytometry. For the flow cytometry, cells were stained with pig specific antibodies. Cells were acquired on BD FACS Canto II with BD FACS Diva software and analyzed using FlowJo v9 software (Tree Star) for proliferative capacity of $CD4^+$ T cells.

To analyze the influence of immunomodulation due to dexamethasone and airborne endotoxin on the total leucocyte counts and differential cell count, the blood was treated with red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA) and white blood

cells were counted using Neubauer chamber (Marienfeld, Germany). To determine the differential cell count, a blood smear was stained using the Romanowsky method (DiffQuick, Labor + Technik, Eberhard Lehmann GmbH, Germany) and two hundred cells were counted and classified. Details are described in Rosen et al. (2020).

3. Publications

3.1 Publication I

Rosen, Kerstin; Roesler, Uwe; Merle, Roswitha; Friese, Anika (2018): **Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model**. In: *Frontiers in microbiology* 9, S. 1542. DOI: 10.3389/fmicb.2018.01542.

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Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model

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Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) was first found in 2005 and is up to date widespread in animal husbandry reservoir – focusing on pig farming. The regular detectability of MRSA in the air of pigsties as well as in exhaust air of pig farms (mean count: 10^2 cfu/m^3) poses the question whether an airborne spread and, therefore, a MRSA colonization of animals *via* the airborne route exists. To answer this question, we exposed three groups of nine MRSA-negative tested piglets each to a defined airborne MRSA concentration (10^2, 10^4, and 10^6 cfu/m^3) in our aerosol chamber for 24 h. In the following observation period of 21 days, the MRSA status of the piglets was monitored by taking different swab samples (nasal, pharyngeal, skin, conjunctival, and rectal swab). At the end of the experiment, we euthanized the piglets and investigated different tissues and organs for the spread of MRSA. The data of our study imply the presence of an airborne MRSA colonization route: the animals exposed to 10^6 cfu/m^3 MRSA in the air were persistent colonized. The piglets exposed to an airborne MRSA concentration of 10^4 cfu/m^3 were transient, and the piglets exposed to an airborne MRSA concentration of 10^2 cfu/m^3 were not colonized. Consequently, a colonization *via* the airborne route was proven.

Keywords: livestock, ST398, pig, aerosol chamber, swine, antibiotic resistance

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INTRODUCTION

For more than a decade, it is known that the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) is no longer restricted to the well-known hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA). Livestock – and of outstanding importance – pig farming is a reservoir where MRSA was first described in 2005 (Voss et al., 2005). To distinguish it from the already known MRSA, these variants were referred as livestock-associated MRSA (LA-MRSA). Most of the LA-MRSA isolates are assigned to the clonal complex (CC) 398 and the sequence type (ST) 398 with the predominant *spa* type t011. LA-MRSA is not limited to farm animals anymore and could also be found in companion animals like cats and dogs as well as horses (Vincze et al., 2014). In the last years, there was an increase of MRSA isolates associated to livestock in hospitals of rural areas (Becker et al., 2017).

The main transmission route of MRSA is direct contact to animals as well as living or non-living vectors. In several studies, these resistant bacteria were also found in the air of pig barns as well as in exhaust air of pig farms (Pletinckx et al., 2011; Friese et al., 2012; Ferguson et al., 2016).

Furthermore, Ferguson et al. (2016) reported deposited MRSA in soil up to a distance of 215 m of the pig farm surrounding. The spread of MRSA into neighboring farms by the airborne way is a more likely scenario. However, the role of an airborne transmission between animal farms is still unclear. To investigate the possibility of a colonization of piglets through MRSA contaminated air, three groups of piglets were experimentally exposed to different MRSA concentrations as defined aerosol in an aerosol chamber. This study aimed to determine the concentration of airborne MRSA needed for a transient or a persistent MRSA carriage of piglets. Until now, MRSA transmission models are quite artificial with regard to MRSA transmission (nasal drop-in, oral inoculums) or taking a long time for obtaining colonized piglets (colonization of piglets at birth by vaginal MRSA-positive sows). Therefore, in the present study, a new model was also established for colonization of MRSA in piglets through airborne route. For the first time, MRSA colonization was conducted with conventional raised non-antibiotic-treated piglets habiting a common bacterial flora such as methicillin-sensitive *Staphylococcus aureus* (MSSA) in a model that imitates the field conditions of a transmission of MRSA *via* the airborne route as far as possible.

MATERIALS AND METHODS

The animal study was permitted by the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales; number 0403/12).

Study Design

In order to determine the specific dose necessary for a successful airborne MRSA colonization of piglets, three groups were exposed once to a defined MRSA concentration in the air for 24 h using an aerosol chamber. The first group was exposed to 10^2 colony forming units (cfu)/m^3 (low dose group: LD), the second group to 10^4 cfu/m^3 (mid dose group: MD), and a third one to 10^6 cfu/m^3 (high dose group: HD). A control group (CT) was treated equally and housed for 24 h in the chamber without any MRSA exposure. The MRSA concentration used for the LD group is equal to the mean MRSA concentration found in the barn air of pigsties (Friese et al., 2012). The MD group was exposed to an airborne MRSA concentration of 10^4 cfu/m^3 MRSA – according to Friese et al. (2012) the highest MRSA concentration that was detected in the air of pig farms. Before the exposure in the aerosol chamber, all groups had a 7-day period of acclimatization. Thereafter, the piglets were sampled three times a week. These swabs were analyzed in order to determine the MRSA colonization (see **Figure 1**). Additionally, the environment of the kept pigs was investigated. After an observation period of 21 days, the piglets were euthanized, and different tissues and organs were analyzed qualitatively and quantitatively for the presence of MRSA.

To verify the airborne MRSA dosage that resulted in a transient MRSA colonization of the piglets, the MD group (10^4 cfu/m^3) was repeated. The animals were treated equally to the other groups.

Aerosol Chamber

In the present study, an aerosol chamber (**Figure 2**) made of stainless steel was used to generate bio aerosols under defined climatic parameters (relative humidity of 70%, temperature of 26°C and an air flow of 100 m^3/h). The chamber has a base area of 3.2 m^2 and a volume of 7 m^3. The MRSA suspension was aerosolized by using a perfusion pump in combination with an ultrasonic nebulizer (Broadband Ultrasonic Generator, Sono-Tek Corporation, Milton, MA, United States) integrated into the ceiling. The perfusion rate of the pump was adapted to the different desired MRSA concentrations in the air. In the ceiling of the aerosol chamber is one port for exhaust air and one entry port for applying fresh air. The aerosol was dispersed by an axial ventilator situated in the center of the ceiling. Air samples were taken using impingement at different levels: 1.6 m (high position: HP), 0.8 m (middle position: MP), and 0.3 m (low position: LP; exposure level of the piglets) above the ground.

The particle size distribution in the air was measured by an aerosol spectrometer monitor (Grimm, model 1.109, GRIMM

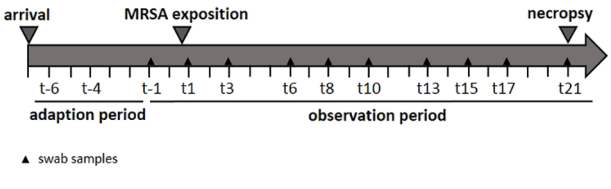

FIGURE 1 | Study design.

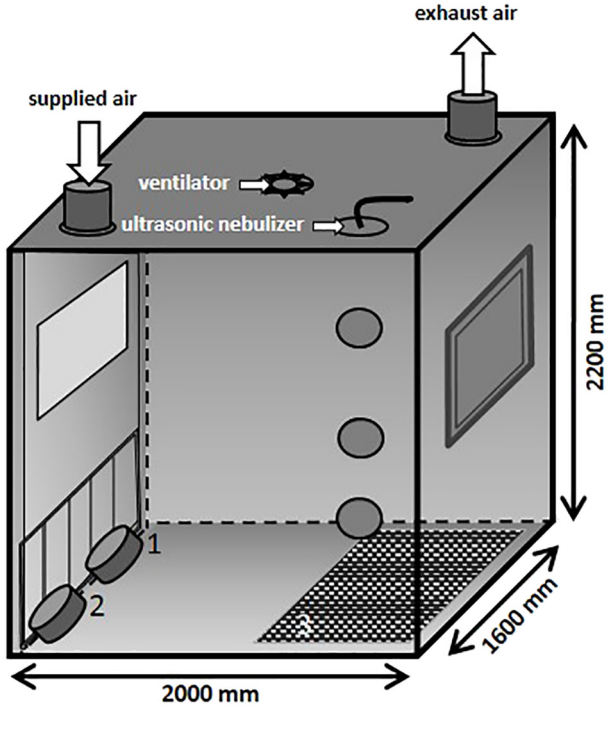

FIGURE 2 | Schematic illustration of the aerosol chamber.

Aerosol Technik Ainring GmbH & Co., KG, Germany). During the animals' exposure, the chamber contained rubber mats (3) that covered half of the ground as well as two troughs for water (1) and feed (2) positioned on the ground and fixed to the chamber door (see **Figure 2**), respectively. The piglets were moving around freely during the whole exposure time.

Air sampling was done using an AGI-30 impinger (Zinsser Analytic, Frankfurt am Main, Germany), filled with 30 mL of phosphate-buffered-saline (PBS; Oxoid, Wesel, Germany). It was connected to the aerosol chamber *via* probes made of steel. The air was sampled for 30 min using an air flow of 12.5 L/min. The flow was verified by using a rotameter. In previous studies, we validated the different MRSA concentrations found in the air of the chamber to reach the required bacterial loads for the animal trials. Therefore, we aerosolized MRSA suspensions under the defined parameters of 26°C, 70% relative humidity, and an airflow of 100 m^3/h. The MRSA suspension was adapted until the targeted concentration in the air was achieved. The final MRSA suspensions were tested at least three times to confirm the reproducibility. To test the aerosol distribution within the chamber, the MRSA concentration was measured *via* impingement in the three different heights: HP, MP, and LP.

Bacterial Strain and Preparation of MRSA Suspension

The MRSA strain of the ST 398 was originally provided by the "Federal Institute for Risk Assessment" (BfR) and was isolated from a healthy pig. This MRSA ST 398 strain (strain ID: BfR 08S00974, ITU 1179) was used by Szabó et al. (2012), thereby establishing a nasal colonization model for LA-MRSA.

The MRSA suspension was prepared as follows: first, 100 µl of the specific MRSA culture, that was aerobically incubated overnight in Mueller Hinton broth (Oxoid, Wesel, Germany) with the addition of 6.5% NaCl (MHB+) in a shaking incubator (Multitron, Infors HT, Germany), was plated onto blood base agar (Blood Agar Base No. 2, Oxoid, Wesel, Germany) and was incubated for 8 h at 37°C to achieve the exponential growth phase. Afterward, all colonies of one plate were suspended in 3 mL PBS and homogenized using glass beads and vortexing for 3 min. The MRSA suspension was adjusted to 0.5 McFarland standard by adding PBS to receive a concentration of approx. 1×10^8 cfu/mL. This bacterial concentration was confirmed by measuring the optical density at 600 nm (OD600; OD600 needed to be a value between 0.073 and 0.11) and counting the bacteria using the Neubauer chamber (C-Chip Neubauer improved, Carl Roth GmbH + Co., KG, Karlsruhe, Germany). The suspension was diluted with PBS to gain the predefined specific concentration for the subsequent aerosolization as defined in the preliminary studies.

The MRSA suspension needed for the experiment was divided into portions of 50 mL and loaded in syringes. For the animal exposure, which lasted 24 h, eight syringes with MRSA suspension was used and they were stored on ice until usage. For the LD and MD groups, a new syringe with suspension was applied every 4 h, for the HD group every 75 min due to a higher necessary perfusion rate.

Animals and Animal Housing

This study included 36 weaned, gender mixed piglets at an age of approximately 21 days. Three groups with nine piglets each were used to determine the MRSA dose needed for a possible airborne colonization, another group of nine animals served as a control group. The animals were housed in the experimental animal facility of the Centre for Infection Medicine of the Department for Veterinary Medicine of Freie Universität Berlin. A strict hygiene regime was performed concerning the entry facility and the used experimental pig barn. The barn was cleaned every day. Manure was removed and the floor was cleaned with water. The staff being in contact with the piglets as well as the pig barn and the aerosol chamber were confirmed MRSA-negative prior to the experiments. Protective clothing was used including snoods and respiratory masks and was changed each time. During the observation period, behavior and health condition were observed daily, and rectal temperature at every sampling time point and the weight of the piglets were monitored weekly.

Samples

Aerosol Chamber

The aerosol chamber was disinfected prior to the animals' exposure. After that, the walls and floor of the aerosol chamber were sampled using PBS-moistened cotton gauze swabs to confirm the negative MRSA status.

The MRSA concentration in the air was measured during the entire aerosol exposure of the piglets at three time points: 1, 9, and 17 h after starting the aerosolization. Therefore, two air samples (HP and MP) were taken simultaneously using impingement as described above. The lowest sampling location (LP) in the height of the animals was not used due to the risk of injuries to the animals and to avoid an influence of results.

Furthermore, a potential presence of MRSA on the walls of the aerosol chamber after the 24 h exposure and after removing the animals was analyzed. For this, an area of 900 cm^2 on two chamber walls at a height of 1.5 m was sampled using PBS-moistened cotton gauze swabs.

Animal Samples

Nasal, pharyngeal, conjunctival, skin, and rectal swabs were collected the day before and after the MRSA exposure and then three times a week for 21 days.

For the skin and the rectal samplings, cotton swabs with a diameter of 5 mm (nerbe plus GmbH, Winsen, Germany) were used. The skin swab was moistened with PBS. All other samples were taken with sterile dry 3 mm cotton swabs (nerbe plus GmbH, Winsen, Germany). The nasal colonization was examined by scrubbing on the nasal mucosa of both nostrils consecutively in a depth of about 1 cm. The pharyngeal swab was taken by opening the piglets' mouth and scrubbing the pharynx. For investigating the fecal shedding of MRSA, a dry cotton rectal swab was taken. To determine the skin's colonization with MRSA, the region behind the ears was swabbed three times on every site. The conjunctival status was investigated applying a single dry cotton swab on both eyes.

At the end of the experiment, the piglets were necropsied to investigate the occurrence of MRSA in the internal organs. The following organs were examined under sterile conditions: ileocaecal, mandibular and lung lymph nodes, palatine tonsils, tracheal bifurcation, lung, and spleen.

Environmental Samples

Environmental MRSA contamination of the experimental pig barn was evaluated using moistened swab samples (diameter 5 mm) of the ground, the walls, the feeding trough, and the water trough as well as the toys. The ground and the wall of the pen were sampled by scrubbing two different locations of approximately 20 cm^2. The feed trough and water trough as well as the toy were tested on approximately 20 cm^2 at only one position. All samples were handled individually and processed in laboratory within 2 h after sampling.

Laboratory Analyses

Air Samples

After the sampling, the remaining PBS in the impinger was quantified for the calculation of the total MRSA concentration.

The air samples were processed subsequent to sampling. One hundred microliters of an appropriate dilution was streaked out threefold onto chromatic MRSA screen agar (CHROMagar MRSA, MAST Diagnostica GmbH) and incubated aerobically at 37°C. MRSA was identified phenotypically after 24 h of culturing. The MRSA concentration in the air could be calculated by counting the colonies.

Swab Samples

For quantification, the swab samples were extracted in 1.5 mL PBS and vortexed gently, and 600 µl were stored at 4°C (retained samples). The remaining sample fluid as well as the swab was mixed with 9 mL MHB+ (Oxoid, Wesel, Germany). After an incubation of 24 h at 37°C, 1 mL of the MHB+ was transferred into 9 mL of Trypton Soy broth (Oxoid, Wesel, Germany) containing 75 mg/L Aztreonam (Molekula GmbH, Munich, Germany) and 3.5 mg/L Cefoxitin (Altmann Analytik GmbH & Co., KG, Munich, Germany; TSB+) and incubated again at 37°C overnight. Every sample fluid was streaked out onto the chromatic MRSA screen agar using a 10 µl inoculation loop. Five MRSA positive subjected colonies per sample were picked and transferred onto Columbia agar with sheep blood plus (Oxoid, Wesel, Germany), incubated at 37°C overnight and confirmed with MALDI TOF mass spectrometry. In case of positive results after the sample enrichment, 100 µl of the retained samples were plated onto chromatic MRSA screen agar in a threefold approach and quantified as described.

The cotton gauze swabs from sampling the aerosol chamber were vortexed and enriched with 180 mL MHB+ at 37°C in the shaking incubator after retained samples were taken. The following day, 20 mL were transferred into 180 mL TSB+ and incubated again for 24 h. The samples were streaked out onto chromatic MRSA screen Agar. MRSA identification was carried out as described before.

Internal Organs

In the laboratory, the samples were processed immediately after the necropsy using the same procedure as previously published by Szabó et al. (2012).

The outer layer of the organs or tissues was decontaminated by flaming with 96% ethanol (except for ileocaecal lymph node and tracheal bifurcation), cut into pieces (excluding tracheal bifurcation), weighed to 10 g, and added with 90 mL MHB+. In case of lower mass, the whole sample was used and diluted 1:10 with MHB+. The specimen was homogenized for 2 min at 200 rpm using a stomacher (stomacher 400 circulator; Seward Limited, West Sussex, United Kingdom). A sample fluid of 1 mL was stored at 4°C and with the remaining sample a two-step enrichment with MHB+ and TSB+ was conducted as described before. Qualitative MRSA-positive samples were quantified and colonies, suspected to be MRSA, were verified as described before.

Spa Typing of MRSA Isolates

For each group, the *spa* typing was conducted for one isolate of every impinger sample and one MRSA isolate origination from the last positive nasal swab of each animal, respectively. In addition, one isolate of every MRSA-positive tonsil of the HD was *spa* typed. All isolates were confirmed as *spa* type t011 using the PCR according to Kahl et al. (2005). LGC Genomics GmbH performed the sequencing. The sequences were analyzed using BioNumerics version 6.6.

Statistical Analysis

The software SPSS, version 24 (SPSS, Inc., Chicago, IL, United States) was used to perform the statistical analysis. We used a generalized regression model to estimate the effect of the MRSA concentration in the air on the prevalence of MRSA-positive individuals in population (logistic regression models) or on the number of log cfu per individual sample (linear regression models). Animal and type of swab sample were considered as random factors, while day of sampling was considered as repeated measurements in all models.

The influence of the type of swab sample, the group, and the sampling day as well as their interactions were investigated in one set of models. The influence of group and the type of sample including necropsy only at day 21 were determined in a second set of models. Third, the influence of environmental samples was analyzed in a set of models including type of sampling, group and investigation day as fixed factors.

p-Values <0.05 were regarded statistically significant. Model diagnosis included normality tests of residuals and visual investigation of homoscedasticity. Results displayed refer to the multivariable models described above.

RESULTS

MRSA Aerosol

Table 1 shows the MRSA concentration in the air measured *via* impingement. The first rows of this table show the results of the evaluation tests concerning the validation of the three targeted

MRSA concentrations for the subsequent animal exposures. The results of the air samplings during the animal exposure of all three groups are included. It shows that the targeted MRSA concentrations in the air were reproducible and that all three groups were exposed to the defined MRSA concentration in the air during the different experiments. The results of impingement, especially the values close to minimum and maximum, indicate a very good reproducibility of the aerosol generation. Furthermore, we achieved a very good distribution of airborne MRSA within the aerosol chamber since there was no difference in MRSA concentration between the different located impingers from the chamber's top to the ground.

The particle size counted by the Grimm aerosol spectrometer was between 3.1 μm (minimum) and 3.7 μm (maximum) for all groups.

The MRSA-negative status of the aerosol chamber was confirmed before starting every animal exposure by sampling the wall using cotton gauze swabs. After the exposure, MRSA was qualitatively detectable on the chamber wall for the LD and MD groups and quantifiable for the HD group with a concentration of 2 and 0.7 cfu/cm^2, respectively.

MRSA in the Animals' Environment

Methicillin-resistant *Staphylococcus aureus* in the pig barn can act as a source for MRSA re-colonization of the animals. Five different swab samples were taken to determine the MRSA status of different surfaces. The concentration of airborne MRSA during the exposure ($p < 0.001$) as well as the sampling day ($p = 0.035$) had a significant influence on the percentage of MRSA-positive environmental swabs. For the LD group, all environmental samples taken from the barn were negative during the whole observation period after the MRSA aerosol exposure. However, 27% (12/45) of the environmental swabs originating from the MD and 98% (44/45) from the HD group taken after the exposure were MRSA positive during the whole observation period. The type of swab sample did not significantly influence the likelihood of an environmental swab being MRSA positive ($p = 0.282$). In the MD group, most MRSA-positive samples were found the first day after the exposure (t1; $n = 3/5$) with 10 cfu/swab and at the end of the observation period (t21; $n = 4/5$). Thus, positive samples were the ground, the feeding and the water trough and, additionally at day 21, the wall. For the other sampling points, one environmental swab was found to be positive only (mostly the ground) with exception of days 15 and 17 where all samples were negative. A quantification was possible at the beginning of the observation period (t1 and t3) only. Except for the toy at day 18, all environmental samples were MRSA positive within the HD group. There, more than half of the samples were quantifiable. The highest number of MRSA/swab was found in ground samples of the pig barn after the exposure (10^2 cfu/swab) and decreased to 0.1 cfu/swab at the end of the observation period (t21) – similar to the decrease of the MRSA concentration of all other environmental swabs over time. The MRSA concentration found in the ground samples differs significant from the other environmental swabs (from $p < 0.0001$ to $p = 0.011$).

Clinical Symptoms

No clinical signs were observed in any group during the whole observation period. The body weight development of the exposed animals was comparable to the animals of the control group.

Animal Colonization

Piglets of the HD group exposed to 10^6 cfu/m^3 airborne MRSA were persistently colonized over the whole observation time. By contrast, piglets of the MD group exposed to 10^4 cfu/m^3 airborne MRSA were transiently colonized and piglets of the LD group exposed to 10^2 cfu/m^3 airborne MRSA were not colonized. The control group remained MRSA negative for the whole observation period. In general, the sampling day ($p < 0.001$) had a significant influence on the MRSA status of a piglet. Also, the MRSA dosage in the air significantly influenced the MRSA status of the piglets: the probability of the LD group's animals being MRSA positive at respective sampling points of the observation period was significant lower ($p < 0.001$) compared to the MD group. By contrast, animals of the HD group were at significant higher ($p < 0.001$) risk being MRSA positive during the course of time. Interestingly, the type of sample had no significant effect on the likelihood having a MRSA-positive status within the whole observation period ($p = 0.414$). On the other hand, there was a significant influence on the MRSA status of the pigs ($p = 0.011$) when considering the type of swab sample in the course of time. For example, the likelihood of the skin swab being MRSA positive

TABLE 1 | MRSA concentration in the air in cfu/m^3 for preliminary tests and the animal exposure for the low dose group (LD), median dose group (MD), and high dose group (HD).

		LD (3×10^2 cfu/m^3)	MD (3×10^4 cfu/m^3)	HD (3×10^6 cfu/m^3)
		MRSA in air (cfu/m^3)	MRSA in air (cfu/m^3)	MRSA in air (cfu/m^3)
Validation	Mean	6.4×10^2	3.0×10^4	5.0×10^6
Tests ($n = 3$)	Minimum	2.3×10^2	1.6×10^4	2.8×10^6
	Maximum	1.3×10^3	6.3×10^4	7.5×10^6
Animal	Mean	4.2×10^2	3.6×10^4	5.2×10^6
Exposure ($n = 3$)	Minimum	1.3×10^2	1.6×10^4	3.9×10^6
	Maximum	7.1×10^2	6.3×10^4	6.9×10^6

The data shown here for the preliminary tests based on three repeated measurements using three impingers in three positions (HP, MP, and LP) for each of the three target dosages. Data on the animal exposure are based on three measurements using two impingers (HP and MP) during the 24 h animal exposure.

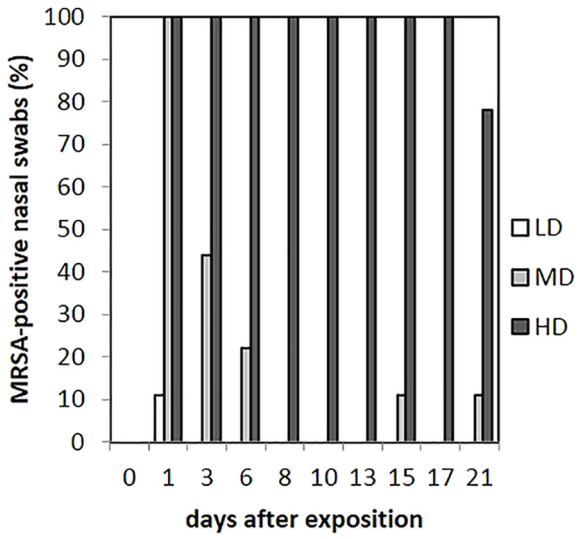

FIGURE 3 | Percentages of MRSA-positive nasal swabs from piglets of the low (LD), mid (MD), and high (HD) dose group over the observation period.

increased at the end of the observation period compared to the pharyngeal swab ($p = 0.009$).

Nasel Swabs

As presented in **Figure 3**, in the LD group, one animal ($n = 1/9$) showed one MRSA-positive nasal swab directly after the exposure (day 1) only. Then, all nasal swabs were MRSA negative during the whole observation period. For the MD group, all nasal swabs ($n = 9/9$) were MRSA positive directly after the exposure and decreased continuously until day 6 ($n = 2/9$; **Figure 3**). At days 15 and 21, one nasal swab ($n = 1/9$) was MRSA positive, respectively, and isolated from piglet no. 61 (t21) and no. 62 (t15). These animals had MRSA-positive nasal swabs before at days 1 and 3. Animal no. 59 was the only one being MRSA positive for all first three sampling points after exposure. During the entire observation period, 17 out of 81 nasal swabs of the MD group were qualitative MRSA positive, whereas 6 swabs were quantifiable with about 10 cfu/swab sample at day 1. For the HD group, all nasal swabs ($n = 9/9$) were MRSA positive at all sampling times except the last one (**Figure 3**). For that group, **Figure 4** shows the MRSA concentration in the nasal swabs over the observation period. At day 1, all nasal swabs were quantifiable with a mean count of 10^4 cfu/swab. During the time, the MRSA concentration per swab sample as well as the number of quantifiable samples decreased close to the detectable concentration limit of 5 cfu/swab. At the end of the observation period, quantification was possible sporadically only.

There was a significant difference in the occurrence of MRSA-positive nasal and skin swabs ($p = 0.011$) including the samples from all groups.

Statistical analysis showed that the MRSA concentration of positive nasal swabs over time was twofold higher compared to the pharyngeal swab {$p < 0.0001$; odds ratios (OR) = 2, 141 [95% confidence interval (CI)]}.

Skin Swabs

As seen in **Figure 5A**, no animal of the LD group had MRSA-positive skin swabs during the whole observation period. 89% ($n = 8/9$) of the skin was tested MRSA positive directly after the exposure for animals of the MD group with an increase to 100% (9/9) at day 3. Then, the MRSA status of the skin varied over the time. Positive skin swabs were detectable until the end of the observation period. In animal no. 61, MRSA could be monitored on the skin for the first four time points as well as day 21. Piglet no. 58 showed positive skin swabs at day 1 as well as day 3. After a period of MRSA-negative skin samples, this piglet became MRSA positive again at the end of the observation period. Quantification of the MD groups' skin swabs was possible for all qualitative MRSA-positive swabs of day 1 and day 3 with a mean MRSA concentration of 10^2 cfu/swab. For the HD group, all skin swabs were tested MRSA positive during the whole observation period (see **Figure 5A**). **Figure 4** shows *inter alia* the MRSA concentration of quantifiable skin swabs of the HD group. Quantification was possible for the majority of samples for all sampling points. The mean count decreased from 10^4 cfu/swab (day 1) to 3.2×10 cfu/swab (day 21). Furthermore, comparable to the nasal swabs, the skin swabs' MRSA concentration was significant higher compared to the MRSA concentration of the pharyngeal swab ($p < 0.0001$; OR = 5.33; 95% CI).

Pharyngeal Swabs

Within the LD group, all pharyngeal swabs were MRSA negative during the whole observation period. As presented in **Figure 5B** for the MD group, all pharyngeal swabs ($n = 9/9$) were MRSA positive directly after the exposure (day 1) and decreased to 11% ($n = 1/9$) at day 6. Then, MRSA was detected sporadically only. The last positive sample on day 17 originated from piglet no. 63, which was also positive for this kind of swab sample at days 1 and 3. In animal no. 59, MRSA was found in four pharyngeal swabs at days 1, 3, 8, and 10 after the exposure.

All animals of the HD group had MRSA-positive pharyngeal swabs for all sampling points (see **Figure 5B**). Quantification was possible for 67% ($n = 54/81$) of these samples, whereas the mean count was about 10^2 cfu/swab ($n = 9/9$) directly after the exposure (day 1) and decreased to approximately 10 cfu/swab for the last sampling points (see **Figure 4**).

The presence of MRSA-positive pharyngeal swabs was only significantly lower compared to the skin swabs ($p = 0.015$). The MRSA concentration of the pharyngeal swabs was significantly lower compared to the nasal as well as the skin swabs' [$p < 0.0001$; OR = 2.141 (nasal swab) and 5.224 (skin swab), 95% CI] MRSA concentration during the time. The MRSA concentration of the pharyngeal swabs was significantly higher ($p = 0.001$; OR = 0.657; 95% CI) compared to the MRSA concentration of the conjunctival swabs.

Conjunctival Swabs

Animals of the LD group showed MRSA-negative conjunctival swabs. As shown in **Figure 6A**, MRSA-positive conjunctival swabs of the MD group could be proven at day 1 for 55% ($n = 5/9$) and day 8 for 44% ($n = 4/9$) of the animals.

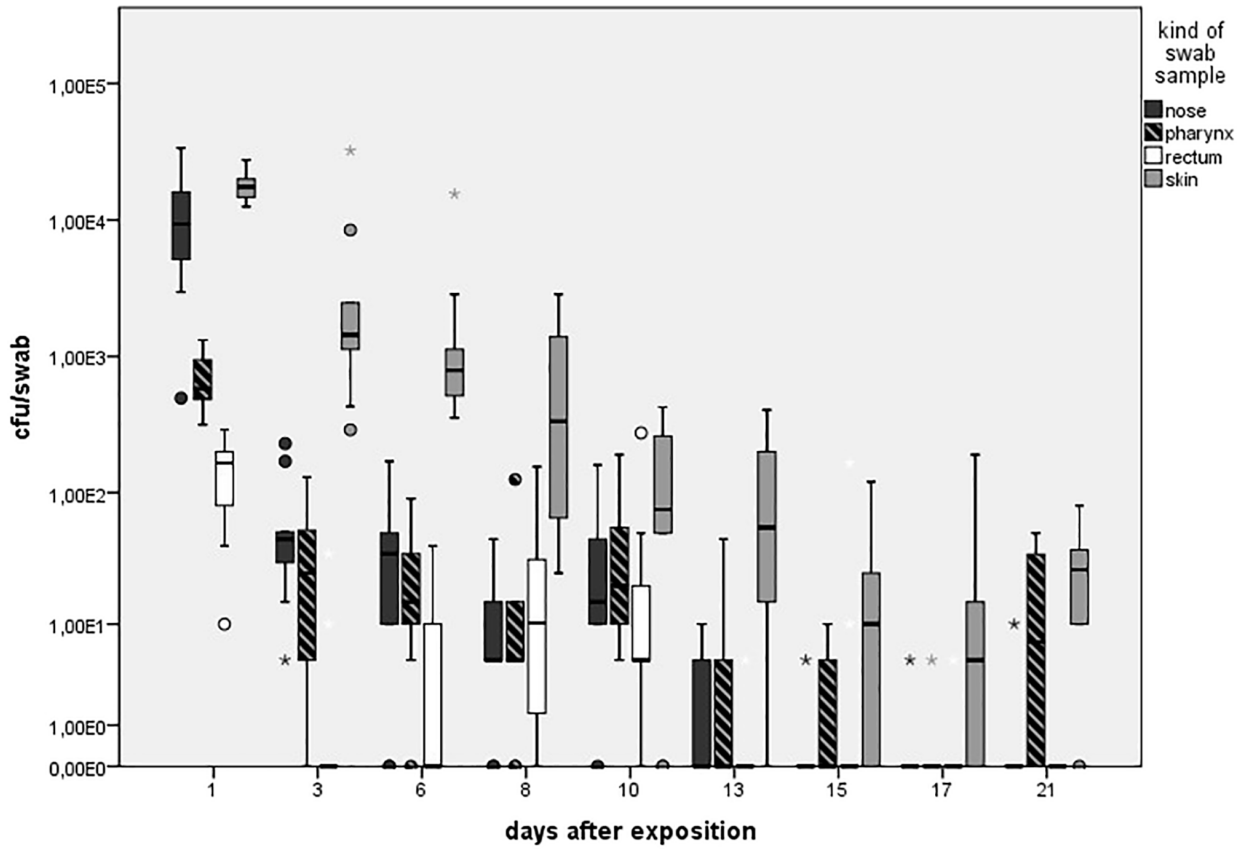

FIGURE 4 | Mean count of quantifiable MRSA-positive swabs (cfu/swab sample) colored in different shades for the high dose group (HD) over the observation period of 21 days. Boxes show the lower quartile, median, and the upper quartile. The ends of the whiskers show the lowest datum within 1.5 interquartile range of the lower quartile and the highest datum within 1.5 interquartile range of the lower quartile and the highest datum within 1.5 interquartile range of the upper quartile. Dots represents outliners. Asterisk represents extreme values. Number of quantifiable swabs for nose/skin/pharyngeal/rectal swabs at day 1 (9/9/9/9), day 3 (9/9/8/3), day 6 (7/9/8/3), day 8 (8/9/7/6), day 10 (8/7/9/7), day 13 (4/8/4/1), day 15 (0/6/3/0), day 17 (1/5/8/1), and day 21 (1/7/5/2).

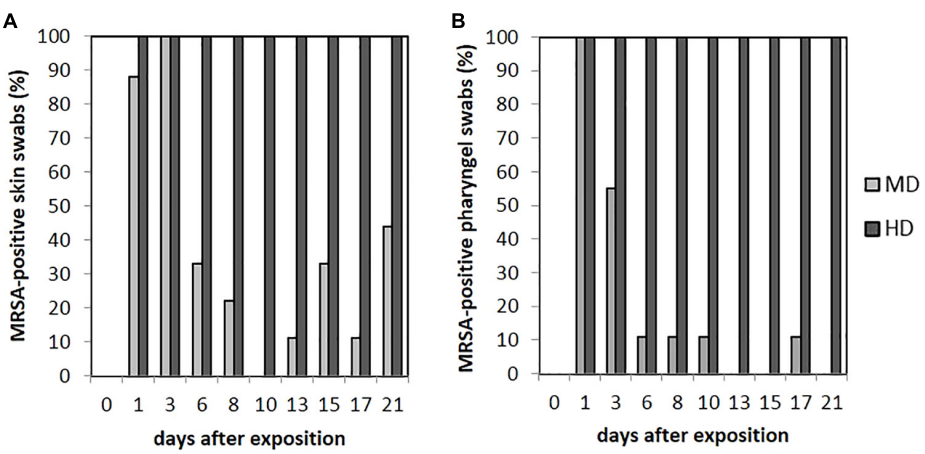

FIGURE 5 | Percentages of MRSA-positive skin **(A)** and pharyngeal **(B)** swabs from piglets of the mid (MD) and high dose (HD) group over the observation period.

In addition, MRSA-positive conjunctival swabs were seen sporadically. A quantification of these kinds of swab samples was not possible. For the HD group, most of the conjunctival swabs were MRSA positive during the completely observation period (see **Figure 6A**). Here, a quantification was possible until day 9 and, additionally for one sample at day 21. The MRSA load per

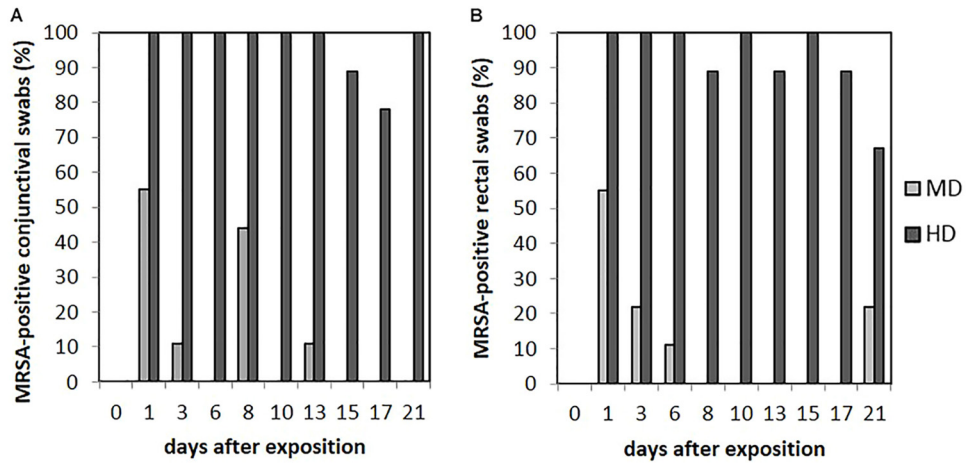

FIGURE 6 | Percentages of MRSA-positive conjunctival **(A)** and rectal **(B)** swabs from piglets of the mid (MD) and high dose (HD) group over the observation period.

swab sample was between 5 and 10^2 cfu/swab. The probability of the conjunctival swabs being MRSA positive was significant lower compared to the skin swabs ($p = 0.006$).

Fecal Excretion

We did not observe any MRSA-positive rectal swabs within the LD group. As illustrated in **Figure 6B**, the MD group showed MRSA-positive swabs of the rectum the first three sampling points. The detection rate of positive swabs decreased from 55% ($n = 5/9$) at the beginning of the observation period to 11% ($n = 1/9$) at day 6. Apart from day 21 where two piglets had MRSA-positive rectal swabs, the remaining samples were MRSA negative. One out of the two positive samples at day 21 derived from piglet no. 61 that was also positive at day 1; the other positive rectal swab originated from piglet no. 56 that had shown no MRSA-positive swab of this region before. In the HD group, the percentage of MRSA-positive rectal swabs ranged from 89% ($n = 8/9$) to 100% ($n = 9/9$; see **Figure 6B**). All MRSA-negative swabs are attributable to two animals. According to **Figure 4**, the mean MRSA concentration of the rectal swabs was 10^2 cfu/swab directly after the exposure and decreased over the time. The number of quantifiable samples decreased from 100% directly after exposure (day 1) to sporadic quantification for the end of the observation period. A significant difference between the occurrence of MRSA-positive rectal and skin swabs was found ($p = 0.006$). Considering the other types of swab samples, there was no significant difference (from $p = 0.073$ to $p = 0.494$). In addition, the MRSA load of the rectal swabs was significant lower compared to the nasal, skin, and pharyngeal swabs (from $p < 0.0001$ to $p = 0.030$).

Internal Organs

In contrast to the HD group, the investigated internal organs of the LD and MD group did not show any MRSA colonization. In animals exposed to the highest MRSA concentration in the air, MRSA was detected in tonsils only. There the bacterial count was between 8.7×10^1 and 2.8×10^4 cfu/tonsil. The MRSA concentration in tonsils was significantly higher compared to all types of animal swab samples taken at day 21 [$p < 0.0001$; from OR = 0.140 (conjunctival swab) to OR = 0.237 (rectal swab); 95% CI].

Spa Typing

The selected isolates were confirmed as *spa* type t011.

Second Evaluation of the Mid Dose Group

The MD group was repeated to confirm the transient piglets' MRSA colonization when exposed to the airborne MRSA concentration of 10^4 cfu/m^3. For the second MD group, the mean concentration of MRSA in the air during the animals' exposure was 4.32×10^4 cfu/m^3. The number of MRSA-positive swab samples at the respective time points was – for each kind of swab sample – comparable to the MD group previously performed (data not shown). In the same manner as for the first MD group, all investigated internal organs were MRSA negative. The statistical analysis revealed no significant difference ($p = 0.776$) in the probability of the animals being MRSA positive at respective sampling points of the observation period between the two MD groups.

DISCUSSION

Our study aimed to identify the required dose for a successful (experimental) MRSA colonization of piglets *via* the airborne transmission route. The MRSA concentration in the air required for a long-term colonization was 10^6 cfu/m^3 for an exposure time of 24 h. Furthermore, an exposure to 10^4 cfu/m^3 resulted in a transient colonization of the animals. The exposure to the lowest used MRSA dose of 10^2 cfu/m^3 did not lead to a colonization. Statistical analysis underlines these differences in the MRSA detection when comparing the three animal groups in the course of time. Therefore, it is very likely that an airborne

colonization route exists depending on the MRSA concentration in the air.

In the past, several models for an experimental MRSA colonization were conducted. However, all studies used more or less artificial methods for MRSA exposure. Most of them utilized nasal drop-in only (Broens et al., 2012; Jouy et al., 2012; Szabó et al., 2012; Verstappen et al., 2014) or the combination with skin (Crombé et al., 2012) or gastrointestinal (Moodley et al., 2011) inoculation. The dosage used here was between 10^7 cfu/mL (Verstappen et al., 2014) and 10^8 cfu/mL (Moodley et al., 2011; Broens et al., 2012; Crombé et al., 2012; Szabó et al., 2012), whereas this dosage did not always result in a successful colonization (Moodley et al., 2011; Broens et al., 2012). Jouy et al. (2012) used a MRSA concentration of 10^4 cfu/mL for the nasal drop-in without resulting in persistent nasal colonization. Oral inoculation of 50 mL containing 10^9 cfu/mL resulted in a colonization but also in death of most of the pigs induced by pneumonia (Broens et al., 2012). In our model, as expected for a colonization in contrast to an infection, no clinical signs occurred. The usage of a high dosage in combination with one specific inoculation method to reach a stable colonization probably falsifies the transfer of results to field conditions. According to Crombé et al. (2012), the need of high dosages of MRSA could result in a greater transmission between animals due to a higher amount of MRSA inoculated animals compared to animals in the field. Although we used a lower airborne MRSA concentration compared to Szabó et al. (2012) using the nasal drop-in method with 5×10^8 cfu/animal, our animals showed a higher colonization status at all time points. A model developed by Moodley et al. (2011) simulated a natural colonization by an experimental vaginal colonization of sows leading to stable MRSA colonized piglets over 4 weeks.

Furthermore, some studies used piglets with absent nasal microbiota (Verstappen et al., 2014) or by antibiotic treatment (Moodley et al., 2011) influenced natural nares microflora to enhance the ability for MRSA to colonize pigs. By contrast, our model used conventional raised, non-antibiotic treated animals. There is some evidence that a higher MRSA dosage for colonization is required in the presence of MSSA due to occupied attachment sides. Several authors make MSSA carriage responsible for MRSA colonization failure (Broens et al., 2012; Jouy et al., 2012). Co-colonization experiments with MRSA and MSSA conducted by Verstappen et al. resulted in a statistically higher MSSA than MRSA colonization. However, the piglets used in our study harbored MSSA naturally. Nevertheless, in comparison with other colonization models, our dosage is lower compared to the other models described despite the natural MSSA carriage of our animals.

Evaluation of the Airborne Colonization Model

The airborne way of exposure seems to be less artificial than the nasal drop-in method used in the other studies and imitates field conditions in a reasonable manner. In conventional pig farms, MRSA occurs in dust and was found in air samples regularly (Friese et al., 2012). According to Ferguson et al. (2016), the size of airborne particles depends on the collection points: In the barn air, MRSA was bound on particles larger than 5 μm originating from feces. By contrast, MRSA found in the surrounding of pig barns was bound on particles originating from feces or epithelial cells with less than 5 μm. These reports indicate an early deposition of large particles and a prolonged stay and, therefore, wider spread of smaller particles in the air. The particle size measured in our aerosol chamber was around less than 5 μm and, therefore, imitated the entry of MRSA in the barn *via* the airborne route very well. The mean concentration found in barn air according to Friese et al. (2012) was about 10^2 cfu/m^3. Within our study, in contrast, the identified dosage for a persistent colonization is much higher with 10^6 cfu/m^3 over 24 h. This could be due to various reasons: the duration of exposure for 24 h in the aerosol chamber is not comparable to the exposure duration of animals in conventional pigsties. There, piglets are exposed often to MRSA for the completely fattening period of 6 months. An increase of the exposure time could be a next step to investigate the temporal influence for MRSA colonization. All animals exposed to the MRSA concentration of 10^4 cfu/m^3 were MRSA positive directly after the exposure with a decrease of MRSA colonization over time. It is very likely that the MRSA colonization success would be bigger when exposed longer or repeatedly – similar to field conditions where often a continual MRSA load in the air exists. It is also important to note that farm animals are challenged by other factors that could influence the MRSA colonization success. Possible factors are antibiotic treatment, immunosuppression induced by stress, bacterial endotoxins, and harmful (ammonia, hydrogen sulfide, and hydrocarbons) gases in barn air. Therefore, more research on these topics needs to be done. In addition, the aerosolized MRSA suspension itself could be a potential reason for a lower colonization capacity. Crombé et al. (2013) postulated before that the preparation process of MRSA suspensions could influence the physiological state of MRSA and, therefore, the ability to colonize animals. Aside from that, the aerosolization procedure could influence the bacteria negatively or, the time of particle distribution through the whole aerosol chamber. However, in our study, there was no difference of MRSA viability between different measure points within the chamber since the concentration of airborne MRSA measured in the different heights *via* impingement was similar.

Airborne MRSA Colonization as the Initial Transmission Route in Our Animal Model

A weakness of our animal model is the inability to differentiate between an exclusive airborne colonization and a colonization additionally caused by contact to MRSA-contaminated chamber surfaces or animals' skin due to bacterial deposition. However, there are several indications for the assumption that the main and initial colonization way of the piglets was dominated *via* the airborne route rather than by contact to contaminated surfaces. Almost all animals of groups MD and all of HD had MRSA-positive skin swabs on days 1 and 3 after exposure. If direct contact to contaminated surfaces such as skin would be the main

transmission way, the animals of the MD group should be MRSA positive for a longer time – especially on skin. However, at day 6 after the exposure, only one-third of the skin swabs were MRSA positive, whereas the animals' skin of the HD group remain positive until the end of the experiment. Obviously, the presence of MRSA on skin within the MD group was not sufficient to act as a source for MRSA re-colonization by direct contact, e.g., nose-skin contact. In addition, nose swab samples were negative within the MD group at day 8 after the exposure. Thus, the initial MRSA exposure dosage in the air was most likely responsible for the stable MRSA detection on the animals' skin within the HD group. A colonization *via* contaminated walls or ground floor inside the aerosol chamber seem rather unlikely as the MRSA load on the wall surfaces after exposure was very low. Whereas quantification of MRSA in both swabs taken of aerosol chamber walls was not possible for MD group, the sampled walls of HD group were quantifiable but in a low MRSA concentrations of 2 and 0.7 cfu/cm^2. This surface concentration seems to be too low to act as a dominant source for MRSA transmission by direct contact. A further indication for an initial colonization *via* the airborne route is that our airborne MRSA concentration was lower compared to the necessary MRSA concentration for a successful colonization of pigs used in the nasal drop-in model. Therefore, it is very unlikely that deposited airborne MRSA on the animals' skin was a sufficient source for MRSA colonization.

MRSA Colonization and Contamination of the Animals

Another interesting point to discuss is the differentiation between a true colonization and a transient contamination of the animals.

We strongly assume that in the LD group, no colonization occurred: One piglet of this group showed one MRSA-positive nasal swab directly after the exposure. MRSA detection in the anterior nose at one time point is no evidence for a true colonization. The following sampling, this piglet became MRSA negative and remained negative. This underlines the finding (Angen et al., 2017) where nasal swabs were taken from persons after staying 1 h in a pig barn. Almost all persons were sampled MRSA positive; 48 h later, most of the persons were MRSA negative again. Becoming MRSA negative after a short time indicates a transient contamination, not a colonization.

We assume that the animals of the HD group were stable colonized. All skin and pharyngeal swabs were MRSA positive the whole time. MRSA was detectable over the observation period in 97.5% of the nasal, 96.3% of the conjunctival, and 92.5% of the rectal swabs. The quantification was possible for all kinds of swab samples the first days after exposure and decreased over time. Our data suggest a stable colonization of piglets occurred due to a high number of MRSA-positive swab samples per animal at the different sampling points until the day of necropsy.

For the MD group, we assume that the animals were transiently colonized. Eight out of nine animals (89%) were MRSA positive on the skin directly after the exposure. The following decrease of MRSA-positive skin swabs over the time suggests a contamination rather than a true colonization of the animals' skin. For the skin, nasal as well as conjunctival swabs, it is difficult to distinguish between a true colonization and a transient contamination of the animals immediately after the aerosol exposure due to a possible direct deposition of aerosolized MRSA from air on these sampling sites. The steep decline of MRSA-positive conjunctival swabs from day 1 to day 3 after exposure as well as the sporadic detection of MRSA during the observation period suggests a contamination of the piglets' conjunctiva. Not only the detection rate, also the MRSA load of the skin decreased during the following sampling time points, since quantification was possible for the first two observation points after exposure only. This could be an indicator for the absence of proliferation and, therefore, for a true colonization (Jouy et al., 2012). On the other hand, there was the recurrence of MRSA-positive skin swabs within the MD group from day 13 until the end of the observation period. The recurrence of MRSA-positive swab samples at the last sampling points after their absence at the samplings before was also seen for the nasal and pharyngeal swabs. This might be caused by recontamination due to other persistently MRSA-positive animals of the study group associated with MRSA contamination of the animals' surrounding.

Methicillin-resistant *Staphylococcus aureus*-positive rectal swabs are a result of swallowed bacteria and, therefore, assigned to true colonization. The decrease of MRSA-positive rectal swabs within the first week after exposure indicates a temporary colonization. Two positive swabs of the rectum reoccurred at day 21 simultaneously to positive nasal and skin swabs. However, a quantification was not possible for rectal swabs, which indicates a low MRSA load there. The presence of MRSA in the pharynx can be attributed to true colonization rather than contamination. The number of positive samples decreased over time, which underlines a transient colonization. We suspect that the presence of MRSA-positive pharyngeal swabs at the end of the observation period is a consequence of recolonization, especially in context of the recurrence of other positive swab samples. However, due to the intensive cleaning of our experimental pig barn once a day, the MRSA load in the environment was reduced and, therefore, also its capacity to act as a source for MRSA spreading. It seems more likely that MRSA-positive animals contaminated the environment, since the number of positive tested environmental swabs increased similar to the number of positive animals. The animals with the numbers 63 and 58 of the MD group were MRSA positive on six out of nine sampling points and could therefore act as permanent carriers. Animal number 61 showed MRSA-positive nasal, rectal, and skin swabs at day 21. This may indicate that one animal was colonized stable in the MD group.

Statistical analysis show that the kind of swab sample does not influence the animals' MRSA status when considering the whole observation period for determining their status. This is because almost all swab samples were MRSA positive directly after the exposure and, thus, all animals had a positive status. However, the MRSA status is significantly influenced by the different swab sample types when considering the sampling time point. This result underlines the distinction between true

colonization and transient contamination. Directly after the MRSA exposure, almost all swabs of every animal were MRSA positive, but the number of positive swabs decreased over time since the sampling sites were probably only contaminated. However, specific sampling sites remained MRSA positive for a longer time. Those were favored MRSA colonization sites like the head's mucosa. The nasal mucosa was the most preferred location for a MRSA colonization. This is also shown by the significant twofold higher detection rate of MRSA in nasal swabs compared to pharyngeal swabs within the HD group.

The results of the second MD group show the strong reproducibility of the transient experimental MRSA colonization of the piglets by exposing these animals to an airborne MRSA concentration of 10^4 cfu/m^3. This shows that our airborne colonization model gives reproducible results and is, therefore, a valid colonization model for further investigations.

Spread of MRSA Into Organs and Tissues

As Crombé et al. (2012) already pointed out, the inability to distinguish between true colonization and transient contamination of animals is a well-known problem given the fact, that there are no defined criteria for colonization. They assumed true colonization when *post-mortem* isolation of MRSA in the animals' throats was possible. This matches the findings of our persistent colonized HD group, where MRSA was present in the tonsils of all animals in rather high concentrations. In addition, in the HD group, MRSA could be found during the completely observation period while the bacterial load of MRSA decreased. Tonsils are the first line of defense targeting bacteria after nasal or oral uptake. Szabó et al. (2012) used nasal drop-in with a dosage of 10^8 cfu/mL and had similar results concerning the tonsils. However, they found MRSA also in other investigated organs. The probable reason for limited spread of MRSA in organs of our animals is the usage of a lower MRSA dosage and the different exposure route.

The experimental exposure of the piglets to MRSA *via* the airborne route within our study also imitate a possible entry of airborne MRSA in pig barns. Sources could be neighbored MRSA-positive barns within the same farm or maybe other farms nearby. With our model, we were able to expose piglets to defined MRSA concentrations in the air in order to investigate the effect of specific airborne bacteria dosages. We achieved a stable, reproducible colonization of conventional raised, non-pretreated piglets *via* a natural like way of airborne exposure with a MRSA dosage that is lower compared to the already existing MRSA colonization models. In conclusion, the animal model reported in this study, is a useful tool to investigate the colonization kinetic in dependence of various factors influencing the MRSA colonization in future.

ETHICS STATEMENT

This study was approved in accordance with the Directive 2010/63/EU and with the German Animal Welfare Act by the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin, LAGeSo) under the registration number G 0403/12. The study was carried according to the institutional guideline for animal welfare of the Freie Universität Berlin.

AUTHOR CONTRIBUTIONS

UR and AF performed the study design. UR and AF designed the animal experiments. KR performed the laboratory work. AF and KR performed the sampling. RM performed the statistical analysis. KR evaluated the final data and wrote the manuscript. All authors have read and approved the final draft of the article.

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REFERENCES

Angen, Ø., Feld, L., Larsen, J., Rostgaard, K., Skov, R., Madsen, A. M., et al. (2017). Transmission of methicillin-resistant *Staphylococcus aureus* to human volunteers visiting a swine farm. *Appl. Environ. Microbiol.* 83:e01489-17. doi: 10.1128/AEM.01489-17

Becker, K., Ballhausen, B., Kahl, B. C., and Köck, R. (2017). The clinical impact of livestock-associated methicillin-resistant *Staphylococcus aureus* of the clonal complex 398 for humans. *Vet. Microbiol.* 200, 33–38. doi: 10.1016/j.vetmic.2015.11.013

Broens, E. M., Graat, E. A. M., van de Giessen, A. W., Broekhuizen-Stins, M. J., and de Jong, M. C. (2012). Quantification of transmission of livestock-associated methicillin resistant *Staphylococcus aureus* in pigs. *Vet. Microbiol.* 155, 381–388. doi: 10.1016/j.vetmic.2011.09.010

Crombé, F., Argudín, M. A., Vanderhaeghen, W., Hermans, K., Haesebrouck, F., and Butaye, P. (2013). Transmission dynamics of methicillin-resistant *Staphylococcus aureus* in pigs. *Front. Microbiol.* 4:57. doi: 10.3389/fmicb.2013.00057

Crombé, F., Vanderhaeghen, W., Dewulf, J., Hermans, K., Haesebrouck, F., and Butaye, P. (2012). Colonization and transmission of methicillin-resistant *Staphylococcus aureus* ST398 in nursery piglets. *Appl. Environ. Microbiol.* 78, 1631–1634. doi: 10.1128/AEM.07356-11

Ferguson, D. D., Smith, T. C., Hanson, B. M., Wardyn, S. E., and Donham, K. J. (2016). Detection of airborne methicillin-resistant *Staphylococcus aureus* inside

and downwind of a swine building, and in animal feed: potential occupational, animal health, and environmental implications. *J. Agromed.* 21, 149–153. doi: 10.1080/1059924X.2016.1142917

Friese, A., Schulz, J., Hoehle, L., Fetsch, A., Tenhagen, B. A., Hartung, J., et al. (2012). Occurrence of MRSA in air and housing environment of pig barns. *Vet. Microbiol.* 158, 129–135. doi: 10.1016/j.vetmic.2012.01.019

Jouy, E., Le Roux, A., Kéranflec'h, A., Granier, S. A., Laurent, F., Kempf, I., et al. (2012). Methicillin-resistant *Staphylococcus aureus* ST398 contamination and transmission in pigs after a low dose inoculation. *Lett. Appl. Microbiol.* 54, 518–523. doi: 10.1111/j.1472-765X.2012.03239.x

Kahl, B. C., Mellmann, A., Deiwick, S., Peters, G., and Harmsen, D. (2005). Variation of the polymorphic region X of the protein a gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in *Staphylococcus aureus*. *J. Clin. Microbiol.* 43, 502–505. doi: 10.1128/JCM.43.1.502-505.2005

Moodley, A., Latronico, F., and Guardabassi, L. (2011). Experimental colonization of pigs with methicillin-resistant *Staphylococcus aureus* (MRSA): insights into the colonization and transmission of livestock-associated MRSA. *Epidemiol. Infect.* 139, 1594–1600. doi: 10.1017/S0950268810002888

Pletinckx, L. J., Verhegghe, M., Dewulf, J., Crombé, F., De Bleecker, Y., Rasschaert, G., et al. (2011). Screening of poultry-pig farms for methicillin-resistant *Staphylococcus aureus*: sampling methodology and within herd prevalence in broiler flocks and pigs. *Infect. Genet. Evol.* 11, 2133–2137. doi: 10.1016/j.meegid.2011.07.008

Szabó, I., Beck, B., Friese, A., Fetsch, A., Tenhagen, B. A., and Roesler, U. (2012). Colonization kinetics of different methicillin-resistant *Staphylococcus aureus* sequence types in pigs and host susceptibilities. *Appl. Environ. Microbiol.* 78, 541–548. doi: 10.1128/AEM.05327-11

Verstappen, K. M., Duim, B., van Nes, A., Snijders, S., van Wamel, W. J. B., and Wagenaar, J. A. (2014). Experimental nasal colonization of piglets with methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. *Vet. Microbiol.* 174, 483–488. doi: 10.1016/j.vetmic.2014.09.019

Vincze, S., Stamm, I., Kopp, P. A., Hermes, J., Adlhoch, C., SemmLer, T., et al. (2014). Alarming proportions of methicillin-resistant *Staphylococcus aureus* (MRSA) in wound samples from companion animals, Germany 2010-2012. *PLoS One* 9:e85656. doi: 10.1371/journal.pone.0085656

Voss, A., Loeffen, F., Bakker, J., Klaassen, C., and Wulf, M. (2005). Methicillinresistant *Staphylococcus aureus* in pig farming. *Emerg. Infect. Dis.* 11, 1965–1966. doi: 10.3201/eid1112.050428

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Publication II

Kerstin Rosen, Friederike Ebner, Stefanie Schmidt, Susanne Hartmann, Roswitha Merle, Anika Friese, Uwe Roesler (2018): **Influence of immune status on the airborne colonization of piglets with methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC) 398**. In: *European Journal of Microbiology and Immunology* 10, S. 1-10. DOI: 10.1556/1886.2019.00024.

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Influence of Immune Status on the Airborne Colonization of Piglets with Methicillin-Resistant Staphylococcus aureus (MRSA) Clonal Complex (CC) 398

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Colonized vertebrates including humans and pigs are to date the main reservoirs of livestock-associated Methicillin-resistant *Staphylococcus aureus* (LA-MRSA). Currently, the mechanisms underlying colonization of pigs are not fully understood.

We investigated the influence of piglet pre-immune status on airborne MRSA colonization. Three groups of MRSA-negative piglets were primed and exposed to airborne LA-MRSA (10^4 colony forming units (cfu)/m^3) in an aerosol chamber for 24 h. One group was treated intramuscularly with dexamethasone (1 mg/kg body weight) to imitate weaning stress. The second group was exposed to bacterial endotoxin containing MRSA aerosol. Both conditions play a role in the development of multifactorial diseases and may promote MRSA colonization success. The third group served as control.

The piglets' MRSA status was monitored for 21 days via swab samples. At necropsy, specific tissues and organs were analyzed. Blood was collected to examine specific immunological parameters.

The duration of MRSA colonization was not extended in both treated groups compared to the control group, indicating the two immune-status influencing factors do not promote MRSA colonization. Blood sample analysis confirmed a mild dexamethasone-induced immune suppression and typical endotoxin-related changes in peripheral blood. Of note, the low-dose dexamethasone treatment showed a trend of increased MRSA clearance.

Keywords: livestock, ST398, aerosol chamber, swine, antibiotic resistance

Introduction

More than 10 years after the rise of livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in 2005, LA-MRSA is still a major public health concern. Although its primary reservoir is pig farming, the so-called LA-MRSA has been repeatedly isolated from dogs, cats, and horses [1, 2]. Given the high prevalence of LA-MRSA in pig farming, possible spread within and between farms, and its zoonotic potential, detailed knowledge about transmission is essential. In Germany, an MRSA prevalence between 52% and 96% is reported in pig farming [3]. We recently identified the airborne MRSA concentration of 10^6 colony forming units (cfu)/m^3 as an effective dose for airborne MRSA colonization of piglets [4]. Piglets exposed to this MRSA concentration for 24 h in an aerosol chamber were persistently colonized with MRSA. An airborne MRSA concentration of 10^4 cfu/m^3 resulted in transiently colonized animals. In contrast to the mean concentration of 10^2 cfu/m^3 found in the barn air of pigsties [5], the experimental dose required for a permanent MRSA colonization is relatively high. We assume that successful colonization and the high MRSA prevalence detected in pigsties are multifactorial. In this work, we focused on the immunological state of piglets and its impact on MRSA colonization. In intensive livestock farming, many stress factors influence immunity and contribute to decreased resistance to pathogens. According to Amadori et al., common sources of chronic stress include poor or harmful climate conditions, pain, and increased infection pressure [6]. These result in an increased glucocorticoid secretion with decreased immunity, predisposing for the onset of diseases with opportunistic pathogenic micro-organisms [7]. Previous work has demonstrated that stress, especially after changing the environment often coincides with the exposure to new micro-organisms resulting in increased susceptibility to infection [8]. Following the pathogenesis of multifactorial diseases, we assumed that stress imitated by dexamethasone treatment would enhance MRSA colonization of piglets after experimental exposure. In addition, diseases of the respiratory tract are among the most common diseases of pigs in intensive pig farming. Air contaminants, especially endotoxins, are also potential underlying causes affecting the respiratory tract. As a part of the outer membrane of Gram-negative bacteria, endotoxin plays an indisputable role in the development of respiratory diseases. Holst et al. reported that airborne endotoxin affects the respiratory health of pigs due to inflammation [9]. According to Urbain et al., endotoxin facilitates the respiratory diseases in pigs due to its proinflammatory nature [10]. In guinea pigs, endothelial cell damage was observed when these animals were exposed for several weeks to airborne endotoxin [11].

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We, therefore, addressed the relationship between airborne endotoxin exposure and sensitization of the piglets' airways for MRSA colonization. The overall aim of our study was to investigate if stress induced by dexamethasone and the presence of airborne endotoxin might act as contributory factors for airborne MRSA colonization of pigs and, therefore, contribute to the high MRSA prevalence observed in pigsties.

Material and Methods

Study Design. In our study, we investigated the effect of immunomodulating factors on MRSA colonization of piglets. We pre-treated groups of piglets (n = 9) with dexamethasone (dexamethasone treated group, DG) or included bacterial lipopolysaccharides (LPS) from *E. coli* O111:B4 (endotoxin treated group, EG) during MRSA exposure. Both groups were exposed to aerosolized MRSA for 24 h using an aerosol chamber. A control group (CG) of 9 piglets (n = 9) was exposed to airborne MRSA alone as described previously [4]. All 3 groups were exposed to an airborne MRSA concentration of 10^4 cfu/m^3 as we previously demonstrated that this dose results in transient MRSA colonization of piglets [4]. Blood samples were taken to investigate the total leucocytes, including differential blood counts and the proliferation capacity of CD 4^+ T cells. To monitor the MRSA status of the piglets, 5 different swab samples (nasal, skin, pharyngeal, conjunctival, and rectal swab) were taken from each piglet 3 times a week for 21 days (observation period). At the end of the experiment, piglets were sacrificed by euthanasia to investigate the spread of MRSA into different tissues and organs. All samples were analyzed qualitatively and quantitatively for the presence of MRSA.

Aerosol Chamber. To expose piglets to MRSA, an aerosol chamber with a base area of 3.2 m^2 and a volume of 7 m^3 was used as described previously [4]. The MRSA suspension was prepared and transported by a perfusion pump with a rate of 9 mL/h to an ultrasonic nebulizer (Broadband Ultrasonic Generator, Sono-Tek) that generated the aerosol. A ceiling ventilator dispersed the aerosol whose particle size distribution was measured by a spectrometer monitor (Grimm, model 1.109, GRIMM Aerosol Technik Ainring GmbH & Co. KG, Germany). During exposure, the aerosol chamber was equipped with rubber mats covering half of the ground. The piglets were provided with feeding and water troughs and were allowed to move freely in the aerosol chamber.

During MRSA exposure, air samples were taken using an AGI-30 Impinger (Zinsser Analytic, Germany) to confirm the targeted MRSA concentration in the air and to investigate the aerosol distribution within the aerosol chamber. The impinger sampling positions were at 2 different heights (middle position – 0.8 m (MP) and high position – 1.6 m (HP)) and filled with 30 mL of phosphate-buffered saline (PBS) serving as sampling fluid.

Bacterial Strain and Preparation of MRSA Suspension. For our experiments, a MRSA strain (strain ID: BfR 08S00974, ITU 1179) of the sequence type (ST) 398 and the *spa* type t011 was obtained from the "Federal Institute for Risk Assessment" (BfR) and used as previously reported [4, 12]. Briefly, MRSA was streaked out on blood base agar plates (Blood Agar Base No. 2, Oxoid, Germany) and incubated for 8 h. Then, the plates were suspended with PBS, adjusted to McFarland 0.5 and diluted to the defined MRSA concentration. The suspension was split into portions of 50 mL and stored on ice until aerosolization.

Animals and Animal Housing. The piglets arrived at our facilities at the age of approximately 3 weeks and were exposed to airborne MRSA (10^4 cfu/m^3) 7 days later (day −1; Figure 1). For this study, 27 weaned, gender-mixed piglets were used. Two groups (n = 9, each) were used to investigate the effects of immunomodulatory factors on the MRSA colonization success. A third group (n = 9) served as control and was exposed to airborne MRSA without additional immunomodulating factors.

The piglets were housed at the research facility of the Centre for Infection Medicine of Freie Universitaet Berlin under a strict hygiene management. During the handling of the animals, protective clothes, snoods, and masks were used. The pig barn was cleaned daily with water. All persons in contact with the piglets, the pig barn itself, and the aerosol chamber were confirmed to be MRSA-negative before arrival of the piglets. During the experiment, the piglets' behavior and general condition were monitored daily. Internal temperature was measured 3 times per week and before taking the swab samples. Body weight development was monitored at least once weekly.

Dexamethasone Treatment. As presented in Figure 1, the piglets were treated with dexamethasone (dexamethasone 4 mg/mL, Vetiquinol) on 9 consecutive days. The treatment started the day after arrival (day −6) and ended 2 days after airborne MRSA (10^4 cfu/m^3) exposure in the aerosol chamber (day 2), aiming an MRSA exposure under dexamethasone treatment. Dexamethasone was applied intramuscularly (i.m.) with a dosage of 1 mg/kg body weight. Prior to the dexamethasone application, each DG piglet was weighed daily to calculate the exact amount of dexamethasone applied.

Airborne Bacterial Endotoxin Treatment during MRSA Exposure. The second group (EG) was challenged simultaneously with bacterial endotoxin and airborne MRSA (10^4 cfu/m^3) for 24 h. LPS from *E. coli* O111:B4 (Sigma Aldrich, Darmstadt, Germany) was added to the MRSA suspension used for aerosolization in the aerosol chamber. We chose an endotoxin concentration of 4 μg/m^3 as this was the maximum concentration found in the air of pig barns by Zejda et al. [13]. To achieve the targeted endotoxin concentration in air, the required amount of the LPS-powder in the MRSA suspension for aerosolization was calculated. We dissolved 0.9 mg of LPS per mL of bacterial suspension and vortexed the suspension gently.

Blood Samples. To investigate specific immunological parameters, blood samples of at least 5 mL were taken at different days prior to and after aerosol exposure, at day −6, day −4, day −1, day 1, day 3, day 6, day 13, and day 21 from all piglets (Figure 1). The blood was taken from the vena cava cranialis with single-use needles (∅/*L* 0.90 × 40, Sterican®, B. Braun Melsungen AG, Germany) and VACUETTE blood tubes with lithium heparin (Greiner Bio-One, Germany). The first blood sample of the DG was taken prior to the first application of dexamethasone. Blood samples were examined by the Institute of Immunology (Freie Universitaet Berlin).

Samples

Aerosol Chamber. We used PBS-moistened cotton gauze to confirm the absence of MRSA on the floor (1 sample) and the chamber walls (2 different sampling locations) at a height of 1.5 m before starting the animal exposure in the aerosol chamber. For this purpose, an area of 900 cm^2 was scrubbed.

During aerosolization, the airborne MRSA concentration was measured via impingement for 30 min 1 h, 9 h, and 17 h after starting the MRSA exposure of the piglets to confirm the targeted bacterial load in the air.

After 24 h of MRSA exposure, the MRSA status of the aerosol chamber was investigated by sampling the same areas (wall and floor), using a PBS-moistened cotton gauze as described above.

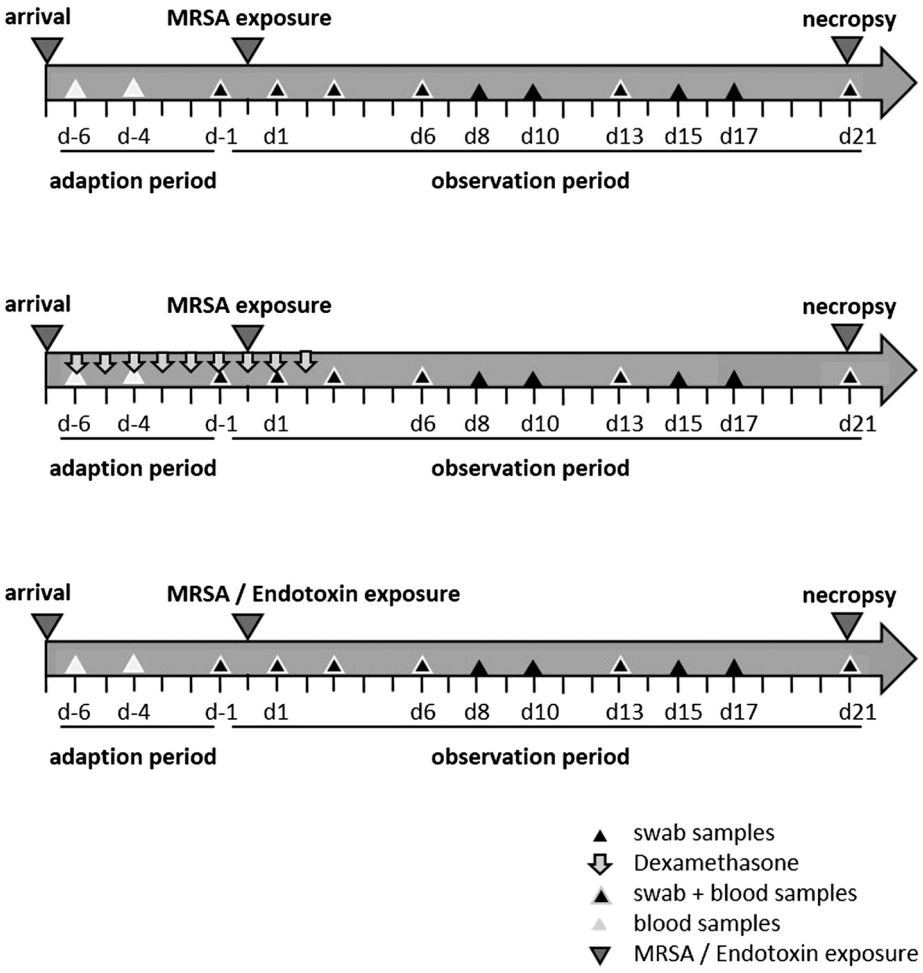

Figure 1. Study design

Animal Samples. To monitor the MRSA status of the piglets, nasal, pharyngeal, conjunctival, skin, and rectal swabs were taken directly before and after exposure (day −1 and day 1; Figure 1). As seen in Figure 1, the sampling was performed 3 times weekly during the ensuing observation period of 21 days as described previously [4]. After the observation period, necropsy was performed according to Szabó et al. and Rosen et al. to investigate the spread of MRSA into the tissues and organs [4, 12]. We examined palatine tonsils, tracheal bifurcation, lung, spleen, and lymph nodes at various sites (ileocaecal, mandibular, and lung) qualitatively and quantitatively for the presence of MRSA.

Environmental Samples. To monitor environmental MRSA contamination, we sampled 5 different locations in the barn. Approximately 20 cm^2 of the wall and the ground of the pig barn at 2 different locations were sampled. In addition, the feeding and water troughs, as well as the enrichment toy, were scrubbed at 1 position. Sampling proceeded within 2 h in the laboratory using PBS-moistened swabs with a diameter of 5 mm (Nerbe Plus GmbH, Winsen [Luhe], Germany).

Laboratory Analyses

Air Samples. After taking the air samples using impingement, the remaining PBS was measured, and a volume of 100 µL of the sampling fluid was streaked out 3 times directly onto chromatic MRSA screen agar (CHROMagar MRSA; MAST Diagnostica GmbH) and incubated aerobically at 37 °C. After 24 h, the MRSA concentration was determined by counting the typical MRSA colonies on the plate.

Swab Samples. All swabs were analyzed qualitatively for the presence of MRSA as described previously [4]. In brief, the swabs were extracted with PBS and vortexed. The fluid including the swab was transferred to a two-step-enrichment with Müller Hinton Broth (Oxoid, Germany) supplemented with 6.5% NaCl (MHB+) and Tryptone Soy Broth (Oxoid, Wesel, Germany) containing 75 mg/L aztreonam (Molekula GmbH, Germany) and 3.5 mg/L cefoxitin (Fluka Analytical, Germany) (TSB+). TSB+ was streaked out onto chromatic MRSA screen agar and incubated overnight at 37 °C. Suspicious colonies were confirmed using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI–TOF MS; MALDI Microflex LT and Biotyper database, Bruker Daltonics, Bremen, Germany).

Internal Organs. The tissues and organs of the dissected piglets were handled as described previously [4]. Briefly, the samples were decontaminated, cut into pieces, homogenized using a stomacher (stomacher 400 circulator; Seward Limited, West Sussex, United Kingdom) and enriched in the two-step-enrichment as mentioned above for the swab samples. A retained sample was stored at 4 °C and quantified in the case of positive results of the enrichment of the associated sample. Confirmation of MRSA suspected colonies was performed as described.

***Spa* Typing of MRSA Isolates.** One isolate of every impinger sample for each group and 1 MRSA isolate of the last positive nasal swab of each animal were *spa* typed. The isolates were confirmed as *spa* type t011 by performing the PCR according to Kahl et al. [14]. LGC Genomics GmbH (Location) performed the sequencing. The sequences were analyzed using BioNumerics version 6.6.

PBMC Isolation and Proliferation Assay. Mononuclear cells from porcine peripheral blood (PBMC) were isolated by density centrifugation of whole blood diluted 1:2 in 0.9%

NaCl using Pancoll solution (density 1.077 g/mL, PAN-Biotech). PBMC were stained with the proliferation marker carboxyfluorescein diacetate succinimidyl ester (CFSE, eBiosciences) at a concentration of 5 mM for 5 min in the dark. CFSE-labeled porcine PBMCs were transferred to IMDM supplemented with 10% FCS and 1% penicillin–streptomycin (all PAN-Biotech, Aidenbach, Germany) and seeded into 96-well round bottom plates (2 Mio cells/200 μL). Proliferation was induced by adding Concanavalin A (ConA, 2 μg/mL; Sigma-Aldrich) and assessed after 5 days by comparing unstimulated and ConA-stimulated PBMC using flow cytometry.

For flow cytometry, cells were stained with the following antibodies specific to pig species: anti-CD4a-Pe-Cy7 (clone 4–12-4, IgG2b, BD Biosciences), anti-CD3ε-PerCP-Cy5.5 (clone BB23-8E6-8C8, IgG2a, BD Biosciences) and anti-CD8α-AlexaFluor® 647 (clone 76–2-11, IgG2a, BD Biosciences). For dead cell exclusion, a fixable viability dye was used in eFluor® 780 (eBiosciences). Cells were acquired on BD FACS Canto II with BD FACS Diva software and analyzed using FlowJo v9 software (Tree Star) for proliferative capacity of CD4+ T cells identified as live$CD3^+CD4^+CFSE^{low}$.

Total Leucocytes and Differential Cell Counts. For total leucocyte counts, blood was treated with a red blood cell (RBC) lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA), and white blood cells (WBC) were counted using a Neubauer chamber (Marienfeld, Germany). To determine differential WBC counts, a blood smear was Romanowsky stained (DiffQuick, Labor + Technik, Eberhard Lehmann GmbH, Germany) and 200 cells were counted and classified. Percentages of lymphocytes, neutrophils, eosinophils, basophils, and monocytes were calculated. Absolute values were calculated by multiplying the total number of leucocytes with the percentage of each cell type.

Statistical Analysis. The software SPSS, version 24 (SPSS, Inc., Chicago, IL) was used to perform the statistical analysis. We used generalized regression models to estimate the effect of possible predisposing factors on the prevalence of MRSA-positive individuals in population (logistic regression models) or on the number of log cfu per individual sample (linear regression models). Animal and type of swab sample were considered as random factors, while day of sampling was considered as repeated measurements in all models. The same analyses were also carried out, stratified in terms of the selected types of swab samples and the differences between 2 groups.

P values < 0.05 were regarded statistically significant. Model diagnostics included normality tests of residuals and visual inspection of homoscedasticity. The displayed results refer to the multivariable models described above.

For analyzing the blood data, statistical analyses were performed using ANOVA with repeated measurements. It was investigated if the percentage of the different cell types in blood differed between the groups "Dexamethasone", "Endotoxin", and "Control" in the course of time. Depending on the results of Mauchly's test of sphericity the parametric, the Greenhouse–Geisser or the Huynh–Feldt estimate was used to test differences between time. The Tukey test was used for multiple comparisons between the groups.

The proliferation data of T cells were analyzed by performing the *t*-test for paired samples, because we compared the animals' data of the "Dexamethasone" and "Control" group at 2 different time points.

Ethics. The animal study was approved by the German Animal Ethics Committee for the protection of animals of the Regional Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin; approval number 0403/12). Piglets were cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and in the German Animal Welfare Law.

Results

Airborne MRSA Concentration Measured during Piglets' Aerosol Exposure. The data for airborne MRSA concentration during exposure indicated the reliable reproducibility of the defined airborne MRSA concentration in the aerosol chamber (Table 1). The close values of minimal and maximal MRSA concentration in the air within each group demonstrated that MRSA was well distributed in the aerosol chamber air.

The Grimm aerosol spectrometer measured a particle size between 3.1 μm and 3.7 μm for all groups.

Prior to MRSA exposure, the aerosol chamber was tested MRSA-negative via sampling the floor and walls for all groups. After exposure, MRSA was detectable qualitatively on the floor and the walls of the aerosol chamber for all groups.

Clinical Symptoms. None of the animals showed any clinical signs during the experiment and the complete observation period. The body weight of the housed animals developed similarly to piglets of the same age.

General MRSA Colonization of the Pigs. Neither the DG nor the EG showed an extended MRSA colonization compared to the CG. The likelihood of animals being MRSA-positive over the course of time was significantly higher ($p \leq 0.001$, multivariable mixed logistic regression) for animals of the CG compared to the DG. Statistical analyses revealed no significant difference ($p = 0.103$, linear regression model) in the MRSA concentration between quantifiable swab samples of the DG and the CG over time. The probability of the animals to be MRSA-positive during the observation period was investigated using ANOVA with repeated measurements. No significant difference between the CG and EG ($p = 0.145$) could be shown. Similarly, the MRSA concentration in swab samples of the CG and EG did not differ statistically significantly ($p = 0.130$, linear regression model).

Total MRSA-Positive Swab Samples over the Entire Observation Period. Figure 2 shows the number of MRSA-positive swab samples per animal of all 3 groups for the respective sampling points during the entire observation period. In all groups, the number of MRSA-positive swab samples per animal was highest directly after exposure (day 1) and decreased over time. Like the CG and EG, all piglets ($n = 9/9$) of the DG showed MRSA-positive swabs directly after exposure (day 1) with a median of 3 positive swab

Table 1. MRSA concentration in the air in cfu/m^3 of the exposure of the control group (CG), dexamethasone-treated group (DG), and endotoxin-challenged group (EG). The data shown here are based on 3 measurements using 2 impingers (HP and MP) during the 24-h animal exposure

		Control group (3×10^4 cfu/m^3)	Dexamethasone group (3×10^4 cfu/m^3)	Endotoxin group (3×10^4 cfu/m^3)
		MRSA in air (cfu/m^3)	MRSA in air (cfu/m^3)	MRSA in air (cfu/m^3)
Animal Exposure	Mean	3.6E+04	2.1E+04	3.2E+04
	Min.	1.6E+04	1.5E+04	2.5E+04
	Max.	6.3E+04	2.7E+04	4.0E+04

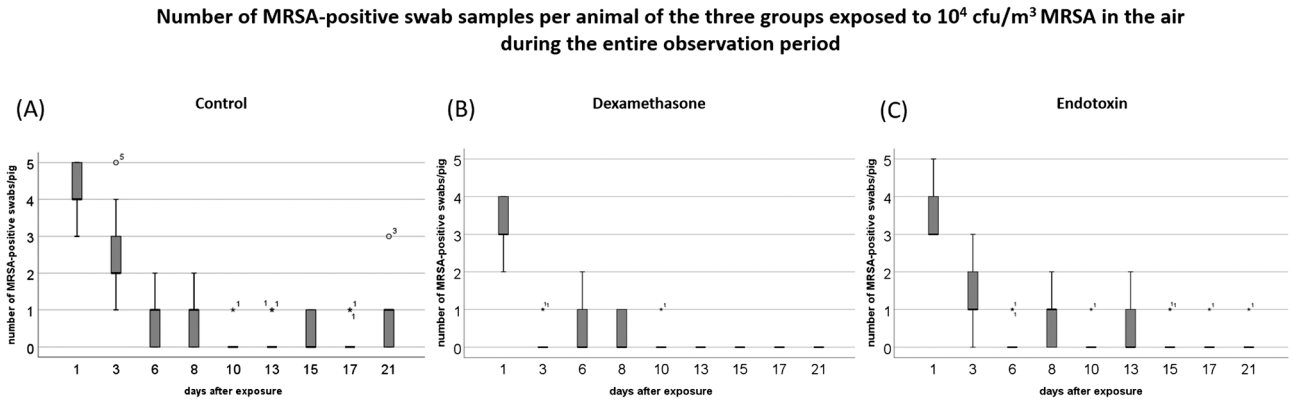

Figure 2. Mean count of MRSA-positive swabs per piglet of the control group (A), dexamethasone group (B), and endotoxin group (C) exposed to 10^4 cfu/m^3 MRSA in the air during the entire observation period over the observation period of 21 days. Boxes show the lower quartile, median, and the upper quartile. The ends of the whiskers show the lowest datum within 1.5 interquartile range of the lower quartile and the highest datum within 1.5 interquartile range of the lower quartile and the highest datum within 1.5 interquartile range of the upper quartile. Dots represent the outliners. Asterisk represents extreme values

samples per animal (Figure 2B). Most of them were nasal (n = 9/9), skin (n = 9/9), and rectal swabs (n = 7/9). For the further sampling points, 80% of all detected MRSA-positive samples of the DG were derived from skin swabs, whereas the last MRSA-positive swab sample was obtained at day 10 from 1 animal. In the DG, from day 3 to day 10, 1 (n = 1/9) to 3 (n = 3/9) animals showed at least 1 MRSA-positive swab sample. In the EG, the number of MRSA-positive samples per animal decreased from a median of 3 positive swab samples per animal at day 1 to less than 1 MRSA-positive swab sample at the end of the observation period (Figure 2C). The number of MRSA-positive animals decreased from all piglets (n = 9/9) of the EG directly after exposure to 1 (n = 1/9) for the last sampling points (day 17 and day 21). According to Figure 2A, a median of 4 swab samples per piglet of the CG at day 1 was observed and decreased to less than 1 MRSA-positive swab at day 21, whereas 55% of the animals (n = 5/9) of this CG were still MRSA-positive at this time point.

The MRSA status of each animal over the course of time is depicted in the Supplementary Material (S1).

Nasal, Skin, and Pharyngeal Swabs. The stratified multivariable mixed logistic regression model for nasal swabs revealed a significantly higher probability for animals of the CG to have MRSA-positive nasal swabs ($p \leq 0.001$) and skin swabs ($p \leq 0.001$) during the experiment compared to the those of the DG. In contrast, there were no significant differences in the probability of having MRSA-positive nasal (p = 0.695) and skin swabs (p = 0.081) between the CG and EG group (stratified multivariable mixed logistic regression model for nasal and skin swabs). According to the pharyngeal swabs, there were no significant differences in the probability of the animals having MRSA-positive samples between all the 3 groups (p = 0,550 DG versus CG and p = 0,787 EG versus CG, stratified multivariable mixed logistic regression model for pharyngeal swabs).

As presented in Figure 3A and 3B, all animals of the DG showed MRSA-positive nasal and skin swabs directly the exposure (day 1). Although the nasal swabs remained MRSA-negative in the DG, MRSA-positive skin swabs were detectable until day 10 with a percentage between 11% (n = 1/9) at day 3 and day 10 as well as 33% (n = 3/9) at day 3 and day 6. As shown in Figure 3C, 2 animals (n = 2/9) of the DG showed MRSA-positive pharyngeal swabs directly after exposure (day 1). The last MRSA-positive swab of the pharynx was detected (n = 1/9) at day 3.

All animals (n = 9/9) of the EG had MRSA-positive nasal and skin swabs at day 1. Then, in both types of swab samples, the number of MRSA-positive swabs decreased, and MRSA was found only sporadically at different sampling points (Figure 3A and 3B). As seen in Figure 3C, 7 out of 9 pharyngeal swabs (n = 7/9) were MRSA-positive at day 1 for the EG. The MRSA detectability decreased at day 3, and MRSA was found again at day 15 only.

The results of CG were previously published [4]. In summary, almost all nasal, skin, and pharyngeal swabs of the CG were MRSA-positive directly after exposure (Figure 3A–3C). For all types of samples, the number of MRSA-positive swabs decreased over time.

Conjunctival and Rectal Swab. As presented in Figure 3D, the animals of the DG showed MRSA-positive conjunctivas on day 1 (n = 2/9) and day 6 (n = 1/9) only. The MRSA-positive rectal swabs were observed exclusively in 77% (n = 7/9) of the DG's piglets directly after exposure (Figure 3E).

The MRSA-positive conjunctival swabs of the EG were noted at day 1 and day 21 only (Figure 3D). There were only 2 animals in the EG showing MRSA-positive rectal swabs at day 1 (n = 2/9) and 1 (n = 1/9) animal at day 3 (Figure 3E).

More than half of the animals (n = 5/9) from the CG cohort showed MRSA-positive conjunctival swabs at day 1 (Figure 3D). Then, MRSA was detected sporadically. The MRSA-positive rectal swabs of the CG were observed in more than half of the animals (n = 5/9) at day 1. Afterwards, MRSA was detectable sporadically only.

MRSA in the Experimental Environment of the Kept Animals. During the observation period, 5 different swab samples of the pig animal facility were taken to observe the MRSA status of the piglets' environment as a possible source for recolonization. The environmental swabs of the DG and EG groups were MRSA-positive directly after exposure (day 1) only. For the DG, MRSA was found on the wall, water, and feeding trough. For the EG, MRSA was also detectable on the ground floor. For the CG, with the exception of days 15 and 17, at least 1 environmental swab was MRSA-positive over the entire observation period.

Internal Organs. MRSA was not found in tissues or organs of any group 21 days after MRSA exposure.

***Spa* Typing.** The selected isolates were confirmed as *spa* type t011.

Immunological Parameters of the Blood Samples. Figure 4A shows the total leucocyte counts and differential white blood cell counts of the dexamethasone-treated animals prior to the first administration of dexamethasone (day −6) and 48 h after (day −4) compared to the CG. We observed a significant decrease in the total leucocyte count (p = 0.003,

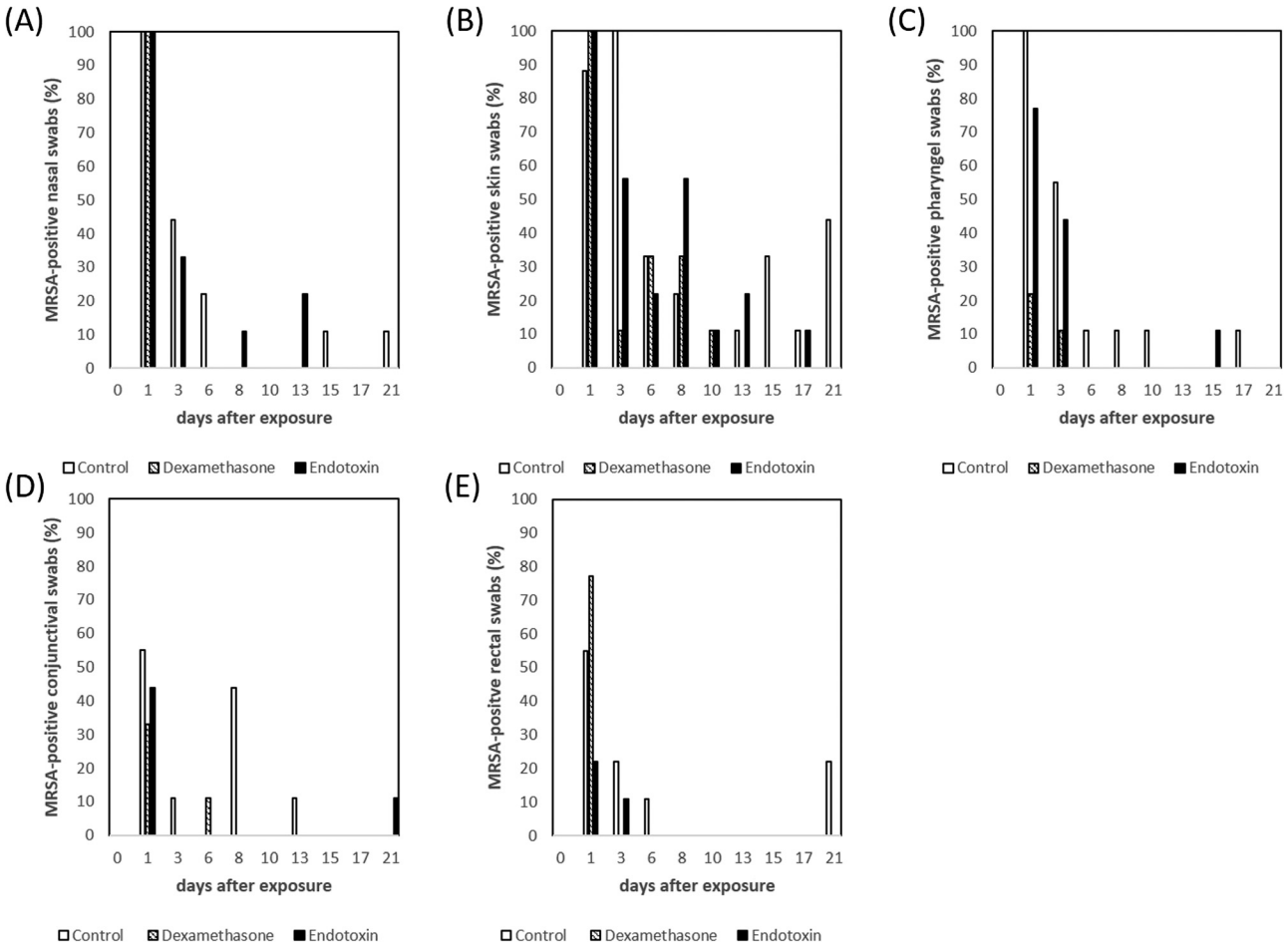

Figure 3. Percentages of MRSA-positive nasal (A), skin (B), pharyngeal (C), conjunctival (D), and rectal (E) swabs from the piglets of the control group (CG), dexamethasone group (DG) and endotoxin group (EG) group over the observation period

paired *t*-test) that was specific to the dexamethasone-treated group, while animals of the CG had unchanged total cell counts ($p = 0.779$). To assess immunosuppression in more detail, we performed differential cell counts and found that the decrease of total leucocytes was due to a specific decrease ($p = 0.001$, paired *t*-test) in numbers of lymphocytes, whereas the decrease in neutrophils was not significant ($p = 0.392$). However, the CG showed a significant increase ($p = 0.002$) in neutrophils and a significant decrease ($p = 0.017$) in lymphocyte counts. Therefore, we decided to more closely assess immunosuppression by dexamethasone treatment and evaluated the suppressive effects of the corticosteroid dexamethasone on lymphocytes in vitro. For that purpose, we stimulated PBMC labeled with the proliferation marker ConA, and assessed the frequencies of proliferated $CD4^+$ T cell after 5 days by flow cytometry (Figure 4B). As shown in Figure 4C and 4D, glucocorticoid treatment significantly reduced the capacity of $CD4^+$ T cells to proliferate ($p = 0.017$, paired *t*-test). In contrast, no significant effect on the proliferative capacity of $CD4^+$ T cells was detected in the control group ($p = 0.186$).

Peripheral Blood Cell Counts. Figure 5A illustrates the mean total leukocyte counts over the entire experimental period for all groups. ANOVA analysis for repeated measurements revealed no significant differences in the leucocyte count over time between all groups ($p = 0.387$ DG versus CG and $p = 0.466$ EG versus CG).

Figure 5B depicts the neutrophil counts for all 3 groups during the observation period. In contrast to the DG ($p = 0.867$), statistical analysis revealed significant differences between the EG and the CG ($p = 0.002$) over time. The neutrophil counts of the EG were significantly increased compared to the CG from day −1 until the end of the observation period ($p = \leq 0.001$ to $p = 0.024$). The increase in neutrophil counts in the DG from day 1 to day 3 is notable, despite being not statistically significant compared to the other groups.

Figure 5C shows the lymphocyte counts for all 3 groups. The statistical analysis showed significant differences between all groups ($p = 0.004$ DG versus CG and $p = 0.003$ EG versus CG) over time.

Discussion

Our study aimed to investigate possible predisposing factors for MRSA colonization success focusing on the immunological state of the piglets. In addition to the stress induced by weaning and its effects on the adaptive immune system [15], piglets are exposed to new environmental conditions including climatic changes, different microorganisms, and dust containing bacterial endotoxins. Here, we imitated a weakened immunological state of weaned pigs via dexamethasone treatment prior to exposing them to MRSA, investigating the question if immune stress at this age promotes colonization by MRSA. Indeed, we hypothesized an extended MRSA colonization of dexamethasone-treated animals in contrast to animals exposed to airborne MRSA alone. However, our data show a significantly shorter MRSA colonization of dexamethasone-treated animals after an airborne exposure over time. Remarkably, most of the MRSA-positive samples of the DG were skin swabs showing a significant reduced detection compared to the CG indicating contamination rather than colonization. The poor ability of MRSA to colonize the piglets is

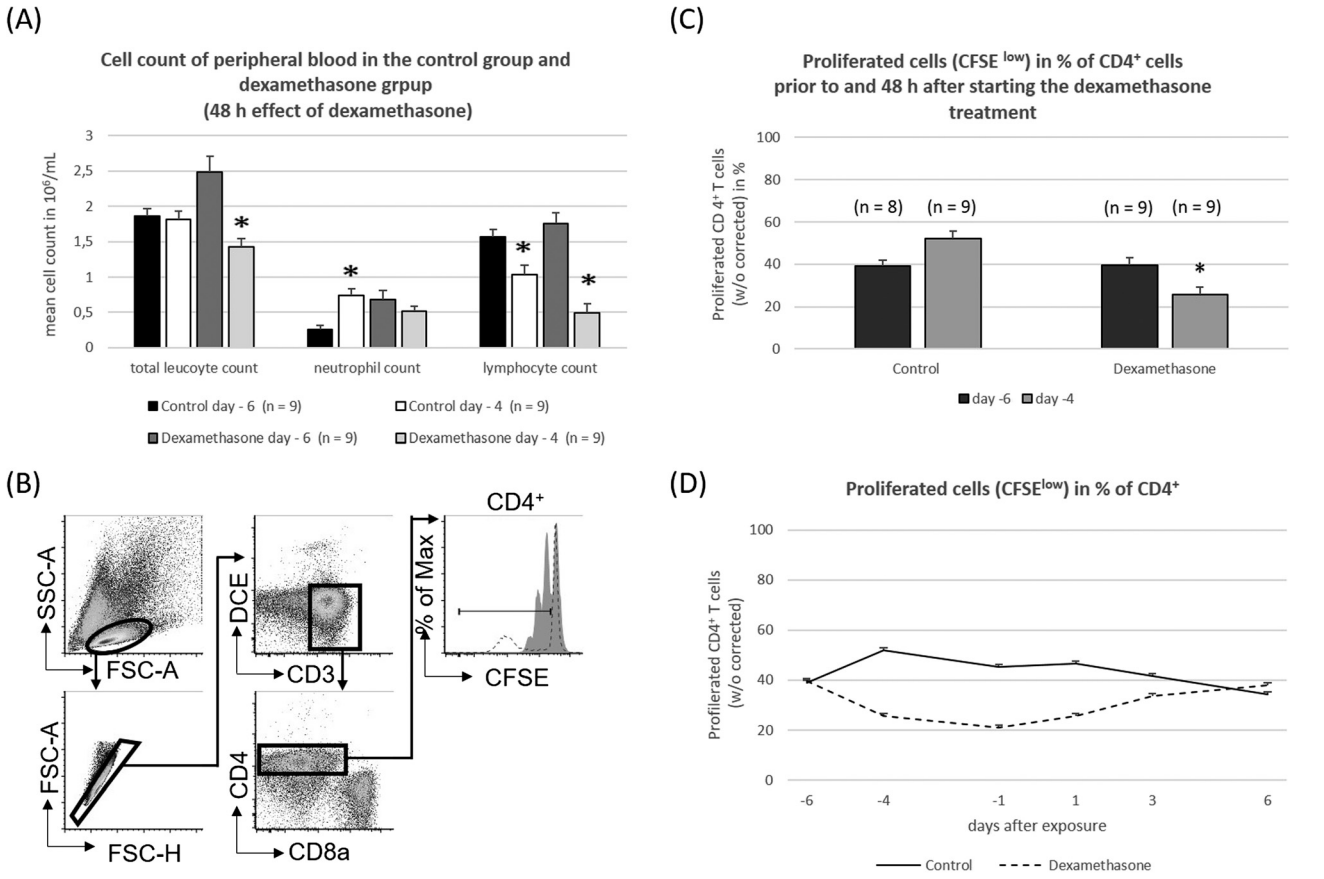

Figure 4. Dexamethasone treatment effects. A) The effect of dexamethasone administration (1 mg/kg body weight/day) on total leucocyte counts, neutrophil, and lymphocyte numbers at the beginning (day −6) and 48 h after the first application of Dexamethasone (day −4). Asterisks indicate significantly different values comparing *t*-6 and *t*-4: $p \leq 0.005$. PBMCs were CFSE-labeled and stimulated with ConA (2 μg/mL) to assess their proliferative capacity. B) Flow cytometric gating strategy to analyze frequency of proliferated cells by CFSE-dilution ($CD3^+CD4^+CFSE^{low}$). C) Mean values of proliferated $CD4^+$ T cells corrected for $CFSE^{low}$ frequencies of unstimulated (w/o) controls. Asterisks indicate significant differences ($p = 0.001$) between *t*-6 (before treatment) and *t*-4 (after onset of Dexamethasone treatment). D) Mean values of proliferated $CD4^+$ T cells corrected for $CFSE^{low}$ frequencies of unstimulated (w/o) controls over prolonged observation time

presumably due to dexamethasone-induced higher MRSA clearance—an effect, which has not yet been investigated in piglets before. This is confirmed by the absence of MRSA in the nasal and rectal swabs from day 3 and the pharyngeal swabs from day 6 after exposure with a significantly lower number of MRSA-positive nasal swabs in the dexamethasone-treated animals compared to the CG. Although the sampling procedure was carried out with the greatest of care, the MRSA-positive conjunctival swab of 1 animal at day 3 might be a contamination due to its MRSA-positive skin at this sampling point.

The blood samples of our dexamethasone-treated piglets revealed a decrease of the lymphocyte count in peripheral blood in combination with decreased proliferation capacity of the $CD4^+$ T cells, indicating mild immunomodulation of the adaptive immune system under dexamethasone application. A decline of lymphocytes after weaning was also observed in the study of Kick et al. and is a result of increased blood cortisol concentration induced by stress [15]. In prior studies, dexamethasone treatment also resulted in lymphocytopenia in pigs [8, 16–18]. Therefore, we assume that the dexamethasone treatment was partly effective to imitate the moderate stress occurring after weaning in the field; however, it is likely that there are other, uncharacterized effects of dexamethasone contributing to the defense of MRSA colonization. Additionally, other studies show that the success of immunosuppression by glucocorticoids in pigs is inconsistent, and Flaming et al. described pigs to be remarkably resistant to dexamethasone treatment with similar doses [18]. Our findings might be explained by different studies that investigated the effect of glucocorticoid treatment at low doses. In a recent review, Cain and Cidlowski summarized the effect of glucocorticoids on the immune system and proposed a biphasic, dose-dependent influence on rat models and human macrophages [19]. According to Lim et al. [20], a low-dose treatment of glucocorticoids resulted in an elevated expression of innate immune genes (for instance, cytokines and chemokines), sensitizing the organism towards pathogens. Therefore, a possible explanation for the decreased MRSA colonization in dexamethasone-treated animals is the stimulation of innate immunity by low-dose dexamethasone resulting in more rapid MRSA elimination. According to Kulkarni et al., dexamethasone treatment decreases the gene expression of antimicrobial peptides (AMPs)—secreted by innate immune cells (monocytes, macrophages, neutrophils and epithelial cells)—in THP-1 monocytes [21]. AMPs are involved in the early defense against pathogens and play, inter alia, a key role in the host cutaneous defense against *S. aureus* [22]. We hypothesize that AMP levels in the nasal epithelium of the piglets were reduced due to dexamethasone resulting in an enhanced adhesion ability of MRSA during the airborne MRSA exposure and its following microinvasion. The lower MRSA concentration (of free, non-attached or microinvaded MRSA) in the nasal swabs (DG) compared to the control directly after exposure might confirm this hypothesis. We assume a subsequent MRSA eradication by a strong local immune response and local inflammation attributed to the end of dexamethasone treatment at day 2 and its fast degradation [25]. This is

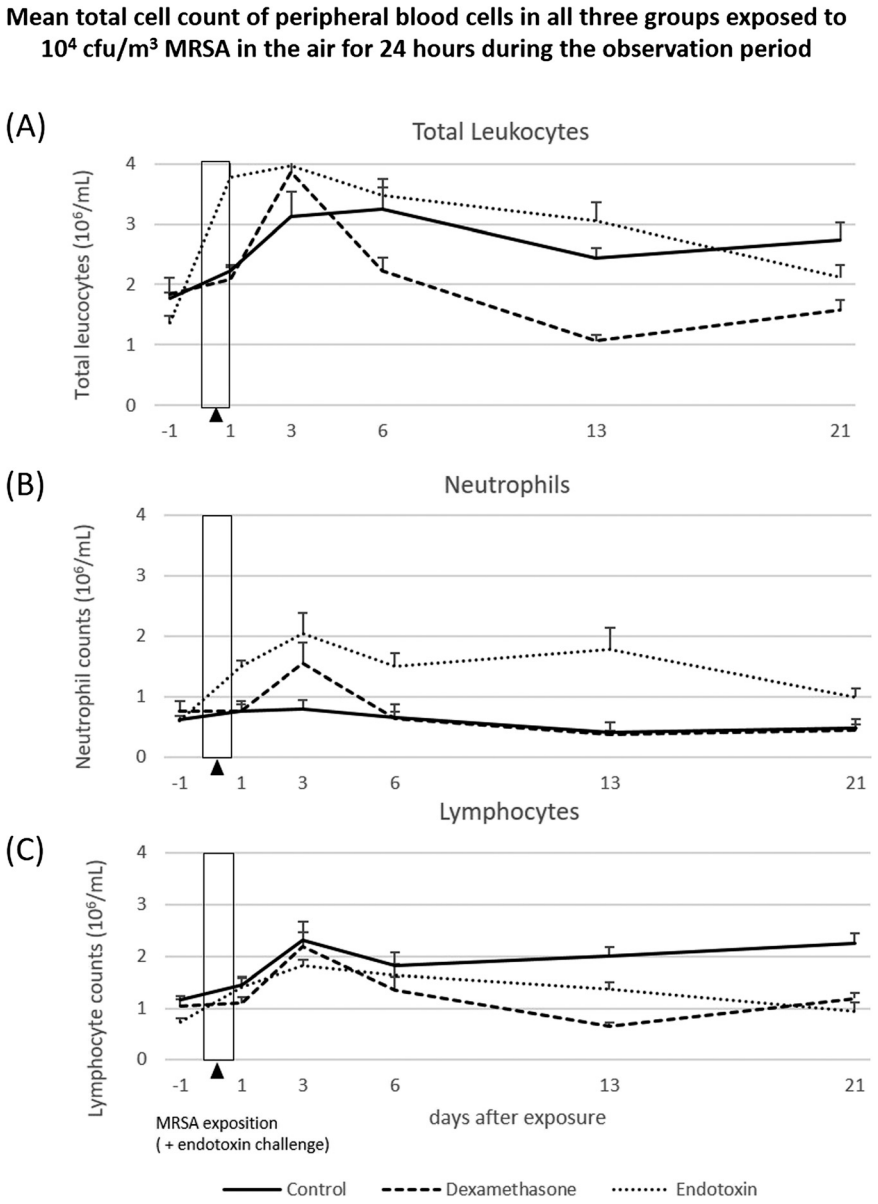

Figure 5. Mean total cell count of peripheral blood cells in the dexamethasone (DG), endotoxin (EG), and control group (CG) exposed to 10^4 cfu/m^3 MRSA in the air for 24 h during the observation period

supported by increased neutrophil counts in the peripheral blood at day 3, 1 day after stopping dexamethasone treatment. This hypothesis provides a further possible explanation for the significantly shorter MRSA colonization of the DG compared to the CG, indicating low susceptibility to MRSA due to low-dose dexamethasone treatment combined with the specific time of stopping the treatment. According to our data, this treatment regimen does not promote MRSA colonization of piglets when exposed via the airborne transmission route.

We also examined the hypothesis that the presence of airborne endotoxin promotes airborne MRSA colonization by acting as a respiratory hazard. In our study, the piglets were exposed to an endotoxin-containing MRSA aerosol resulting in an airborne endotoxin concentration of 4 μg/m^3, corresponding to the maximum endotoxin concentration found in pig barns [26]. However, the particle size of endotoxin is also a crucial factor influencing the place of deposition in the respiratory tract. In our study, the aerodynamic diameter of the aerosolized particles was between 3.2 and 3.7 μm and endotoxin was, therefore, able to penetrate the alveolar region of the lung [27]. The statistical analyses demonstrate that there is no relationship between airborne endotoxin and MRSA colonization success in piglets, although our data reveal an endotoxin-associated effect on peripheral blood cells after endotoxin exposure. In humans, endotoxin is shown to damage respiratory endothelial cells [28] facilitating the ability of MRSA to colonize the respiratory mucosa. In light of possible injury to the upper respiratory tract epithelium, we expected a longer nasal and pharyngeal MRSA colonization acting as a source for spread and subsequent recolonization. Here again, the statistical analyses revealed no prolonged MRSA colonization in the EG compared to the CG, neither for the nose nor for the pharynx. The comparable nasal MRSA colonization of both groups suggests an intact nasal mucosa of the piglets despite airborne endotoxin exposure. Our data are supported by a study by Urbain et al. where no changes in the cell composition of nasal fluid after endotoxin nebulization were found, and therefore, the influence of airborne endotoxin on intact nasal mucosa was denied [10]. The cell counts of blood taken after exposure in the aerosol chamber (day 1) mirror the exposure to an endotoxin-containing MRSA aerosol and are not related to endotoxin alone. Since no changes in total leucocyte and neutrophil count were found after the exposure to airborne

MRSA alone (CG), the increase of the total leukocytes due to a significant increase of neutrophils detected in the EG is interpreted as being induced by endotoxin. Alterations in the peripheral blood of endotoxin-exposed pigs were also found by other groups [10, 29]. Thereby, our data imply that the effect of airborne endotoxin in our aerosol chamber on the immune system is comparable to those in animals continuously exposed to high levels of endotoxin in pig barns. Although airborne endotoxin exposure in the aerosol chamber resulted in a typical immune response, the effect on MRSA colonization success of piglets could not be found. One possible explanation might be the duration of endotoxin exposure. Short-term exposure (i.e., 6 h) had no effects on the composition of nasal lavage, bronchoalveolar lavage (BAL), and blood components attributed to the endotoxin exposure alone [31]. Jolie et al. found a significantly higher neutrophil and alveolar macrophage concentration in the BAL after long-term endotoxin exposure (i.e., 15 weeks), indicating a systemic inflammatory response, though in the absence of clinical signs [30]. Furthermore, in a field study, discrepancies were found in the immune response between pigs and broilers despite the equal endotoxin concentration in the environment of investigated barns [29]. Roque et al. explained their findings with the longer endotoxin exposure of pigs (5-month fattening period) compared to broilers (1-month fattening period) [29]. The authors concluded that endotoxin weakens the piglets' immune defense against pathogens underlining that a possible role of airborne endotoxin favoring MRSA colonization in field cannot be neglected. In livestock, pigs are constantly exposed to endotoxin during the complete fattening period and, therefore, a MRSA colonization promoting effect of endotoxin-containing air might be possible despite no effects being observed when exposing the animals for 24 h in an aerosol chamber.

Additional pollutants in the pigsties' environment (e.g., ammonia, carbon dioxide, and hydrogen sulfide) may also affect the airways and could influence, together with endotoxin, MRSA colonization as discussed by Urbain et al. [10]. The authors could show that exposing pigs to airborne endotoxin resulted in damaged nasal mucosa only when challenging the animals with ammonia beforehand. In another study by Folgemark et al., guinea pigs were exposed to endotoxin with and without β(1,3)-D-glucans—a cell wall component of fungi—and found stronger inflammatory airway responses in the animals exposed to both agents compared to the animals challenged with endotoxin alone [32]. The findings of Urbain et al. and Folgemark et al. support the assumption that airborne endotoxin might be a contributing factor for developing respiratory diseases when combined with other common factors occurring in pig barn air [10, 32]. Our results indicate that airborne endotoxin alone—at least when exposed for 24 h only—does not promote MRSA colonization in piglets.

The discrepancy between the airborne MRSA dose regularly found in pigsties (10^2 cfu/m^3) by Friese et al. [5] associated with the high MRSA prevalence in pigs and the effective dose for successful permanent colonization found in our animal trial (10^6 cfu/m^3 [4]) is presumably due to the multifactorial nature of the pigsties' environment. Pigs are naturally exposed to numerous other factors that may influence the ability of MRSA to colonize pigs. Apart from the diverse pollutants in the pigs' environment, the MRSA colonization might also be driven by treatment with antibiotics and metallic oxide. In our study, dexamethasone decreased the number of lymphocytes 48 h after treatment same as that of weaned piglets in the field [15]. Due to the understudied effects of dexamethasone, this treatment regime resulted in a higher clearance of MRSA and was, therefore, potentially not adequate to imitate chronic weaning stress in pigs occurring in intensive pig production. Airborne endotoxin exposure in the aerosol chamber did not lead to a different MRSA colonization. Hence, future studies should use combinations of possible predisposing factors promoting MRSA colonization in order to study successful MRSA colonization in pigs.

In conclusion, we have shown that the airborne LA-MRSA colonization is independent of the piglets' immune status. MRSA in the pigs' environment is considered one of the main sources for the animals' colonization [33, 34]. To prevent a reentry of dust-borne MRSA from particles deposited in the environment, reducing the MRSA burden by a stringent cleaning and disinfection regime is crucial.

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Authors' Contribution

UR performed the study design. UR and AF designed the animal experiments. SH, FE, and SS performed the sampling and investigation protocol for the analysis of immunological parameters. FE and SS performed the immunological laboratory work and evaluated the dataset of the immunological parameters. RM performed the statistical analysis. KR performed the microbial laboratory work, performed the sampling, evaluated the final data, and wrote the manuscript. All authors have read and approved the final draft of the article.

Conflict of Interest

Nothing to declare.

Supporting Information

Number of MRSA-positive swab samples per animal of the 3 groups exposed to 10^4 cfu/m^3 MRSA in the air during the entire observation period. Each color represents 1 animal, which was sampled with 5 different swab samples for investigating the presence of MRSA, respectively.

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References

1. Vincze S, Stamm I, Kopp PA, Hermes J, Adlhoch C, Semmler T, Wieler LH, et al. Alarming proportions of methicillin-resistant Staphylococcus aureus (MRSA) in wound samples from companion animals, Germany 2010-2012. PloS one. 2014;9:e85656.

2. Cuny C, Witte W. MRSA in equine hospitals and its significance for infections in humans. Vet Microbiol. 2017;200:59–64.

3. Alt K, Fetsch A, Schroeter A, Guerra B, Hammerl JA, Hertwig S, Senkov N, et al. Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. BMC Vet Res. 2011;7:69.

4. Rosen K, Roesler U, Merle R, Friese A. Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model. Front Microbiol. 2018;9:1542.

5. Friese A, Schulz J, Hoehle L, Fetsch A, Tenhagen B-A, Hartung J, Roesler U. Occurrence of MRSA in air and housing environment of pig barns. Vet Microbiol. 2012;158:129–35.

6. Amadori M, Stefanon B, Sgorlon S, Farinacci M. Immune system response to stress factors. Ital J Anim Sci. 2009;8:287–99.

7. Amadori M, Zanotti C. Immunoprophylaxis in intensive farming systems: The way forward. Vet Immunol Immunopathol. 2016;181:2–9.

8. Wallgren P, Wilén I-L, Fossum C. Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. Vet Immunol Immunopathol. 1994;42:301–16.

9. Holst H, Edqvist L-E, Kindahl H, Rylander R. Hematological, Blood Biochemical, and Cytological Bronchoalveolar Lavage Studies in Prepubertal Gilts after Endotoxin Inhalation and Ingestion. J Vet Med A. 1994;41:159–66.

10. Urbain B, Prouvost J-F, Beerens D, Ansay M, Gustin P. Acute Effects of Endotoxin Inhalation on the Respiratory Tract in Pigs: Interaction with Ammonia. Inhalation Toxicol. 1996;8:947–68.

11. Fogelmark B, Sjösstrand M, Rylander R. Pulmonary inflammation induced by repeated inhalations of f(1,3)-D-glucan and endotoxin. 1994:85–90.

12. Szabó I, Beck B, Friese A, Fetsch A, Tenhagen B-A, Roesler U. Colonization kinetics of different methicillin-resistant Staphylococcus aureus sequence types in pigs and host susceptibilities. Appl Environ Microbiol. 2012;78:541–48.

13. Zejda JE, Barber E, Dosman JA, Olenchock SA, Mc Duffie HH, et al. Respiratory Health Status in Swine Producers Relates to Endotoxin Exposure in the Presence of Low Dust Levels. JOM. 1994;1994:49–56.

14. Kahl BC, Mellmann A, Deiwick S, Peters G, Harmsen D. Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in Staphylococcus aureus. J Clin Microbiol. 2005;43:502–5.

15. Kick AR, Tompkins MB, Flowers WL, Whisnant CS, Almond GW. Effects of stress associated with weaning on the adaptive immune system in pigs. J Anim Sci. 2012;90:649–56.

16. Harada K, Muramatsu M, Suzuki S, Tamura Y, Sawada T, Takahashi T. Evaluation on the pathogenicity of Erysipelothrix tonsillarum for pigs by immunosuppression with cyclophosphamide or dexamethasone. Res Vet Sci. 2011;90:20–2.

17. Salak-Johnson JL, McGlone JJ, Norman RL. In vivo glucocorticoid effects on porcine natural killer cell activity and circulating leucocytes. J Anim Sci. 1996;584–92.

18. Flaming KP, Goff BL, Frank DE, Roth JA. Pigs are Relatively Resistant to Dexamrthasone Induced Immunosuppression. Comp Haematol Int. 1994;218–25.

19. Cain DW, Cidlowski JA. Immune regulation by glucocorticoids. Nat Rev Immunol. 2017;17:233–47.

20. Lim H-Y, Müller N, Herold MJ, van den Brandt J, Reichardt HM. Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. Immunology. 2007;122:47–53.

21. Kulkarni NN, Gunnarsson HI, Yi Z, Gudmundsdottir S, Sigurjonsson OE, Agerberth B, Gudmundsson GH. Glucocorticoid dexamethasone down-regulates basal and vitamin D3 induced cathelicidin expression in human monocytes and bronchial epithelial cell line. Immunobiology. 2016;221:245–52.

22. Ryu S, Song PI, Seo CH, Cheong H, Park Y. Colonization and infection of the skin by *S. aureus*: immune system evasion and the response to cationic antimicrobial peptides. Int J Mol Sci. 2014;15:8753–72.

23. Brown AF, Leech JM, Rogers TR, McLoughlin RM. Staphylococcus aureus Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. Front Immunol. 2014;4:507.

24. Im S-A, Gerelchuluun T, Lee C-K. Evidence for Direct Inhibition of MHC-Restricted Antigen Processing by Dexamethasone. Immune network. 2014;14:328–32.

25. Wyns H, Meyer E, Watteyn A, Plessers E, de Baere S, de Backer P, Croubels S. Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs. Vet J. 2013;198:286–88.

26. Zejda JE, Barber E, Dosman JA, Olenchock SA, McDuffie HH, Rhodes C, Hurst T. Respiratory Health Status in Swine Producers Relates to Endotoxin Exposure in the Presence of Low Dust Levels. 1994;38:49–56.

27. Basinas I, Sigsgaard T, Kromhout H, Heederik D, Wouters IM, Schlünssen V. A comprehensive review of levels and determinants of personal exposure to dust and endotoxin in livestock farming. J Exposure Sci Environ Epidemiol. 2015;25:123–37.

28. Thorn J. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. Inflammation research official journal of the European Histamine Research Society. 2001;50:254–61.

29. Roque K, Shin KM, Jo JH, Lim GD, Song ES, Shin SJ, Gautam R, et al. Association between endotoxin levels in dust from indoor swine housing environments and the immune responses of pigs. J Vet Sci. 2018;19:331–38.

30. Jolie R, Bäckström L, Olsen L, Chase C. A 15-Week Eperimental Exposure of Pigs to Airborne Dust with Added Endotoxin in a Coontinious Flow Exposure Chamber. Can J Vet Res. 1999;129–37.

31. Urbain B, Mast J, Beerens D, N'guyen T, Goddeeris B, Ansay M, Gustin P. Effect of inhalation of dust and endotoxin on respiratory tracts of pigs. Am J Vet Res. 1999;60:1055–60.

32. Folgemark B, Sjöstrand M, Rylander R. Pulmonary inflammation induced by repeated inhalations of f(1,3)-D-glucan and endotoxin. J Exp Path. 1994;85–90.

33. Pletinckx LJ, Verhegghe M, Dewulf J, Crombé F, de Bleecker Y, Rasschaert G, Goddeeris BM, de Man I. Screening of poultry-pig farms for methicillin-resistant Staphylococcus aureus: sampling methodology and within herd prevalence in broiler flocks and pigs. Infect Genet Evol. 2011;11:2133–37.

34. Broens EM, Espinosa-Gongora C, Graat EAM, Vendrig N, van der Wolf PJ, Guardabassi L, Butaye P, et al. Longitudinal study on transmission of MRSA CC398 within pig herds. BMC Vet Res. 2012;8:58.

4. Unpublished Data

4.1 Preliminary trials

We conducted preliminary tests to evaluate a potential reduction of the concentration of detected airborne MRSA:

- during storage of the syringe, containing the MRSA suspension for aerosolization
- during the sampling procedure using impingement (influence on the biological collection efficiency)

4.1.1 Storage trials

Possible changes of the suspensions' MRSA concentrations during storage in 50 mL syringes, prior to aerosolization in the aerosol chamber, were investigated in this trial.

Triplicates of syringes containing a defined MRSA concentration in PBS were stored for up to 24 h. The MRSA concentration of the suspension was determined at 0 h, 4 h, and 24 h after preparation. This was done by streaking out 100 microliters of an appropriate dilution threefold onto sheep blood agar (Oxoid, Wesel, Germany), counting and phenotypically identifying MRSA colonies.

The samples were stored and analyzed after:

- 0 h
- 4 h, stored at room temperature
- 24 h, stored on ice

In the animal trial, syringes in the perfusion pump were replaced every 4 hours during MRSA aerosol generation. To imitate the conditions during the animal trial, the suspension analyzed after 4 h was stored at room temperature (see Figure 7). Samples analyzed after 24 h were stored on ice just like the syringes used in the animal trial before nebulizing the MRSA suspension. These storage trials were conducted for the three different MRSA concentrations in the suspension.

Figure 6: *Storage of MRSA suspension containing at room temperature (left figure) and syringes on ice (right figure)*

To expose the piglets to the defined airborne MRSA concentration of 3×10^2 cfu/m^3 (low dose group - LD group), 3×10^4 cfu/m^3 (median dose group - MD group), and 3×10^6 cfu/m^3 (high dose group - HD group), each MRSA suspension had to contain a defined concentration of MRSA (determined in preliminary tests; data not shown).

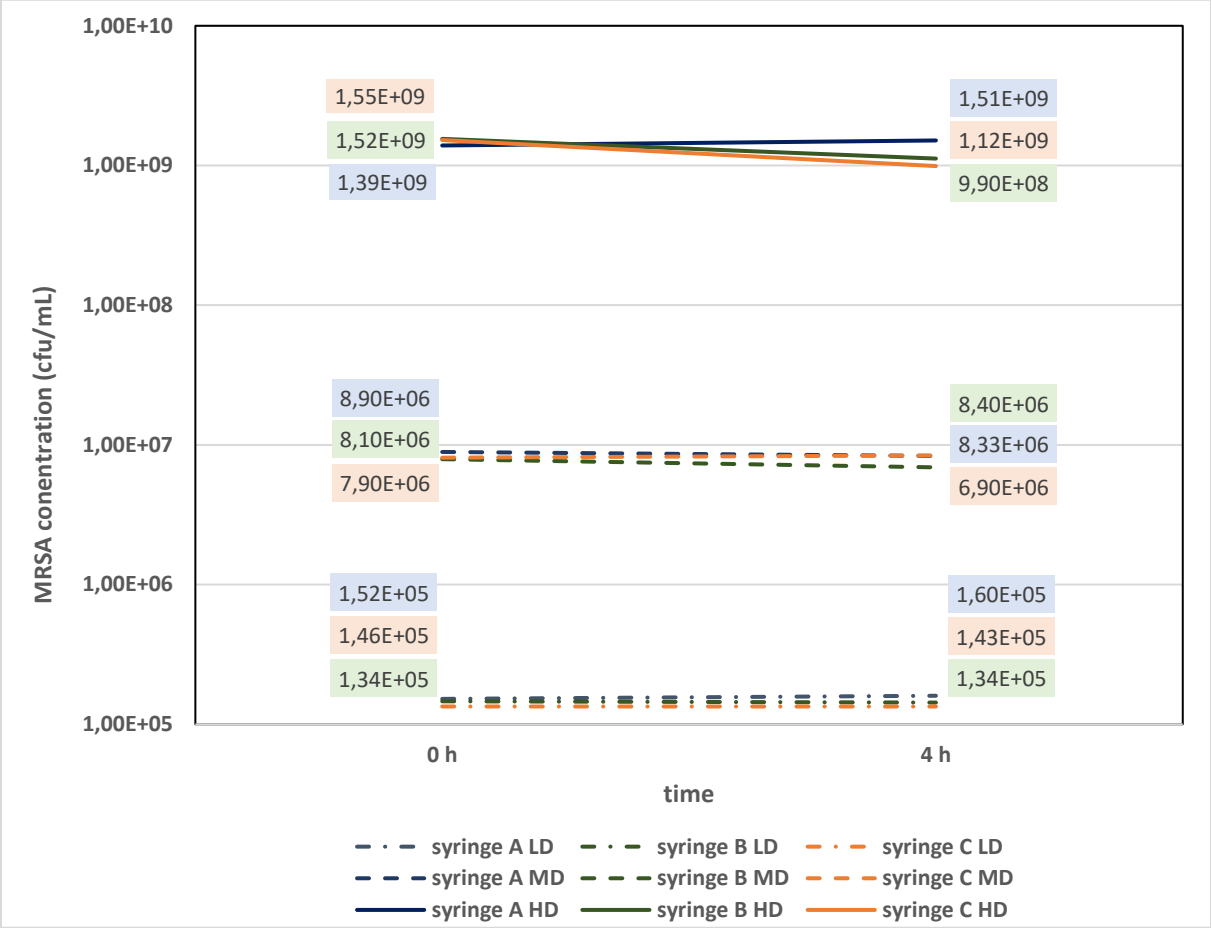

***Figure 7**: Changes in MRSA concentrations in suspensions containing different initial MRSA concentrations (LD, MD, and HD) in triplicate (syringe A-C) after 4 h of storage at room temperature*

Figure 7 shows the necessary MRSA concentrations of the suspensions for airborne MRSA exposure in the LD, MD, and HD group during 4 h of storage at room temperature. Each line represents one MRSA suspension (syringe A-C). The MRSA concentration was determined immediately after preparation (0 h) and after 4 h of storage at room temperature. This figure shows that storage at room temperature over a period of 4 h does not relevantly influence the MRSA concentration in the suspensions.

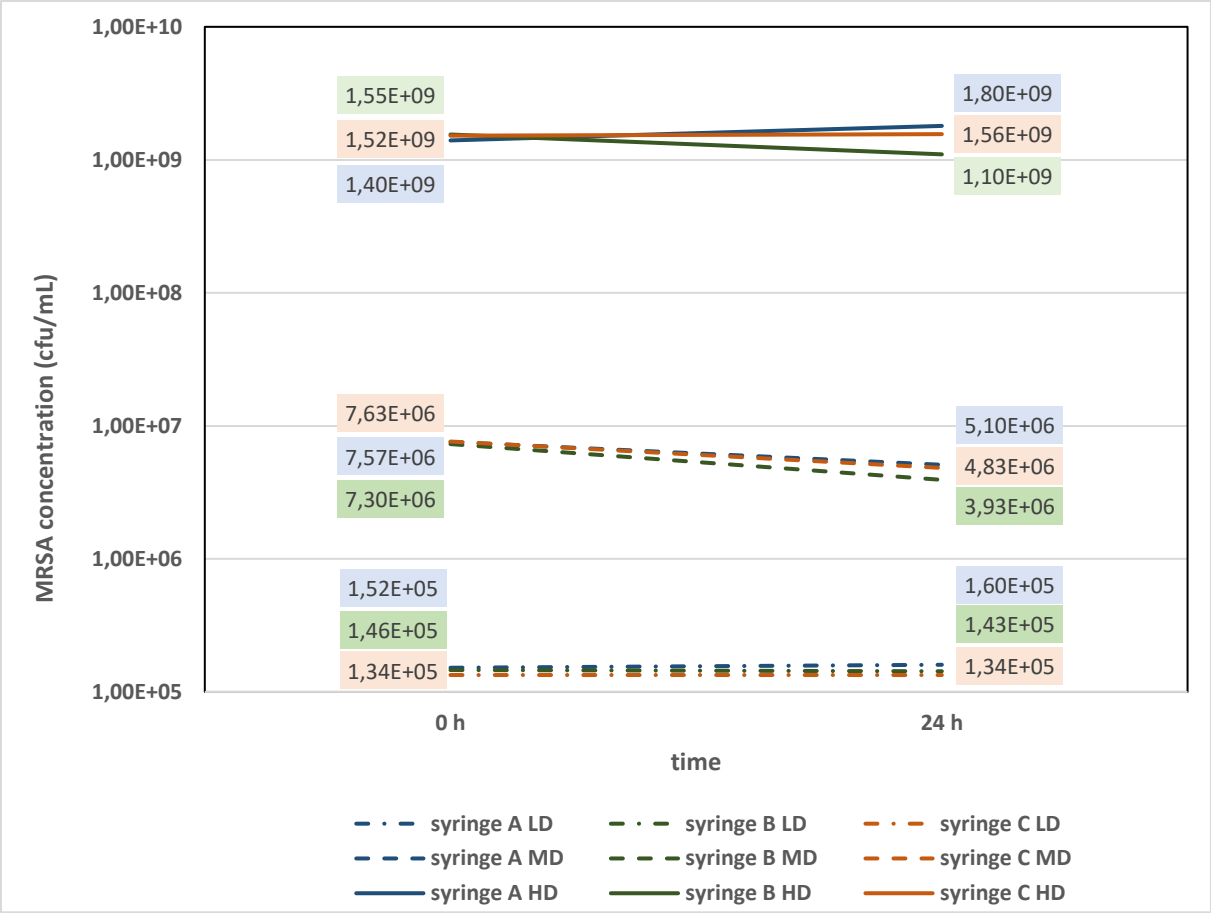

***Figure 8**: Changes in MRSA concentrations in suspensions containing different initial MRSA concentrations (LD, MD, and HD) in triplicate (syringe A-C) after 24 h of storage on ice*

Figure 8 depicts the necessary MRSA concentrations of the suspensions for airborne MRSA exposure in the LD, MD, and HD group during 24 h of storage on ice.

Each line represents one MRSA suspension (syringe A-C). The MRSA concentration was determined immediately after preparation (0 h) and after 24 hours of storage on ice. There was no relevant reduction of the MRSA concentration during storage.

In conclusion, storage of the MRSA suspensions did not influence the MRSA concentrations and is therefore possible under the conditions defined above.

4.1.2 Biological collection efficiency using the AGI- 30 Impinger

Bursting of bubbles and passage of air through the collection fluid during sampling may have a negative effect on the detectability of airborne MRSA (Terzieva et al. 1996). In this trial, we set out to identify whether a change in MRSA concentration took place in the impinger fluid (PBS) when impingement was used for air sampling. Three impingers were artificially spiked with a defined concentration of LA-MRSA. To imitate the conditions of the air sampling in our animal trials, the impingers were connected to vacuum pumps and operated using room air. The concentration of MRSA in the sampling fluid was determined directly before and after sampling, by streaking out 100 microliters of an appropriate dilution of each sampling fluid threefold onto sheep blood screen agar and incubating aerobically at 37°C for 24 h.

We calculated the concentration of MRSA in 30 mL of PBS when impingement is conducted for 30 minutes (12,5 L/min) with an airborne MRSA concentration of 3×10^2 cfu/m^3 (LD group exposure), 3×10^4 cfu/m^3 (MD group exposure), and 3×10^6 cfu/m^3 (HD group exposure). These concentrations were then used for artificial enhancement of the three impingers.

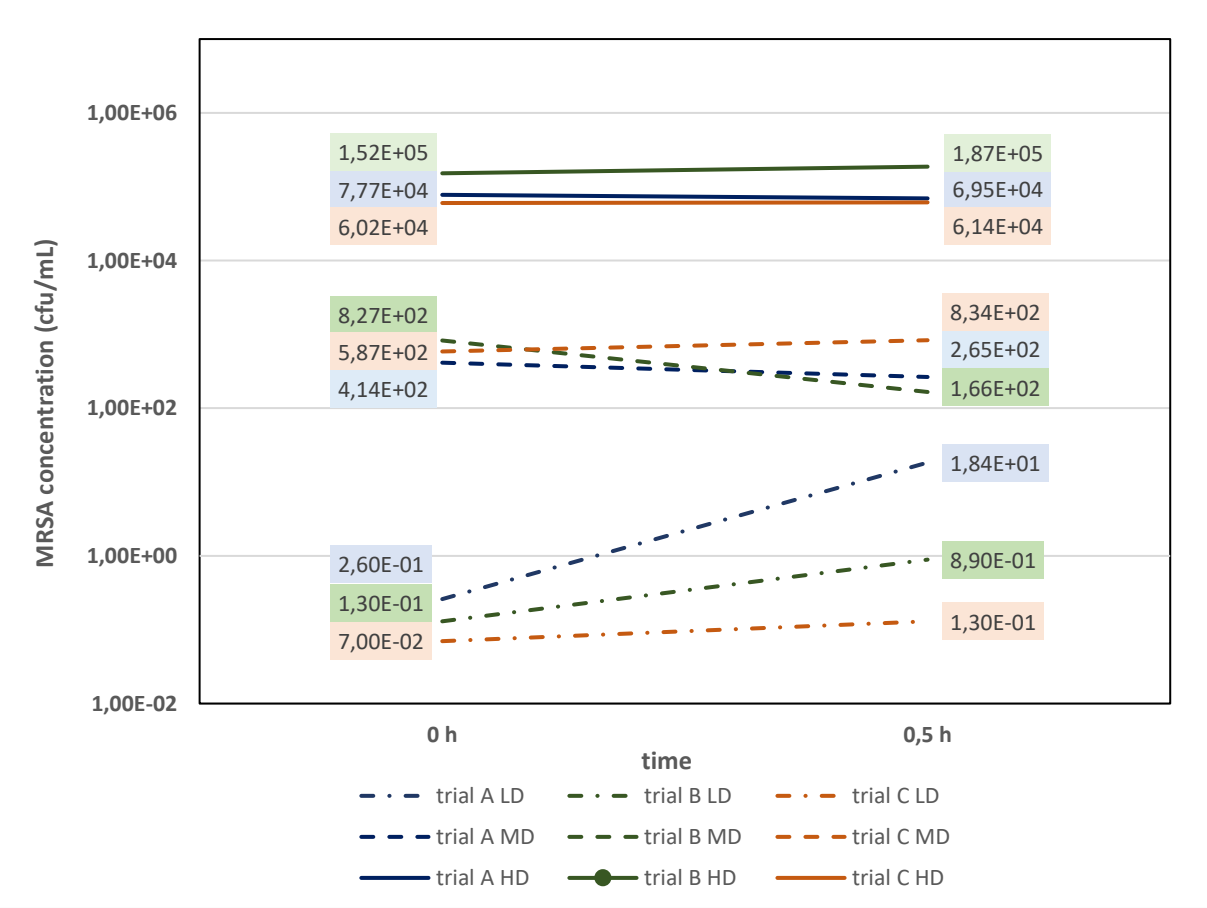

***Figure 9**: MRSA concentration of the impinger fluid, spiked with MRSA concentrations expected during low-dose, medium-dose and high-dose exposure to airborne MRSA, before and after sampling.*

Figure 9 shows the MRSA concentrations of the sampling fluids before and after 30 minutes of air sampling. Each line represents one trial. This figure shows that the sampling procedure has limited influence on the MRSA concentration in the impinger fluid.

To sum up, this method of air sampling does not have a noteworthy effect on the MRSA concentration in the sampling fluid.

4.2. Tiamulin treatment of pigs prior to exposure to airborne MRSA

4.2.1 Background

In our study, one piglet showed respiratory symptoms, lethargy and an increased rectal temperature during the adaption period. This animal was treated with an antibiotic (Florfenicol) and a non-steroidal anti-inflammatory drug (Meloxicam) and was excluded from the trial. Since the chosen treatment did not result in recovery, the piglet was euthanized. Due to increased body temperature of the remaining animals, a metaphylactic treatment with tiamulin was conducted. Animals were observed as usual.

4.2.2 Material and Methods

Upon arrival, the piglets were aged 21 to 24 days (Rosen et al, 2018). All animals in the tiamulin group (TIA group) were treated with tiamulin (Denagard, Novartis Tiergesundheit GmbH, Munich, Germany) supplied by the Ruminant and Swine Clinic of the Freie Universität Berlin. Following the instructions for use, a dose of 2.5 ml per animal was applied on three consecutive days (see Figure 10). The treatment was stopped 24 h before exposure to 10^4 cfu/m^3 of airborne MRSA in the aerosol chamber over a period of 24 hours. This airborne MRSA concentration resulted in animals showing a shorter period of MRSA colonization when compared to untreated animals (control group) exposed to a similar airborne MRSA concentration.

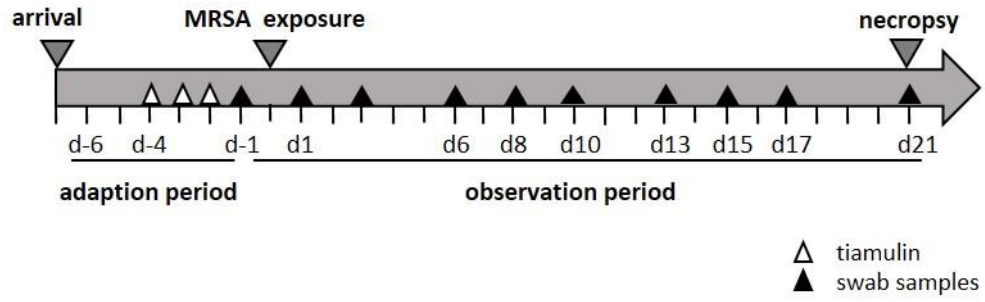

Figure 10: *Study design of the tiamulin group*

Five different animal swab samples (nasal, skin, pharyngeal, conjunctival and rectal swab) and five environmental samples (ground, wall, water, feeding trough, and toy) were taken three times a week during an observation period of 21 days. At the end of this period, the animals were euthanized and dissected. Different organs and tissues were qualitatively and

quantitatively investigated for the presence of MRSA. The laboratory proceedings of sample analyses is described in detail in Rosen et al. (2018).

Statistical analysis was performed using SPSS, version 24 (SPSS, Inc., Chicago, IL). We used generalized regression models to estimate the effect of the tiamulin treatment on the prevalence of MRSA positive individuals in the population (logistic regression models). The animal was considered a random factor, while sampling days were considered repeated measurements in all models. P-Values <0.05 were regarded as statistically significant. Model diagnostics included normality tests of residuals and visual inspection of homoscedasticity. Results displayed refer to the multivariable models described above. The same analysis was carried out stratified for selected types of swab samples and differences between two groups.

4.2.3 Results

4.2.3.1 Airborne MRSA exposure

		Control Group	**Tiamulin Group**
		MRSA conc. in air (cfu/m^3)	MRSA conc. in air (cfu/m^3)
animal	mean concentration	3.6×10^4	3.4×10^4
exposure	min. concentration	1.6×10^4	2.9×10^4
	max. concentration	6.3×10^4	3.8×10^4

Table 1: *MRSA concentration in the air in cfu/m^3 during the exposure of the control and tiamulin group. The data shown here are based on three measurements using two impingers (high position (HP) and middle position (MP)) during the 24 hours animal exposure as described in Rosen et al. (2018).*

Tab. 1 shows the MRSA concentration of the TIA group and the control group during exposure to airborne MRSA for 24 hours. The data demonstrate that both groups experienced comparable exposure. The fact that maximum and minimum MRSA concentrations are close between groups implies a well-distributed MRSA aerosol in the aerosol chamber.

Prior to MRSA exposure of the piglets, two aerosol chamber walls as well as the floor was swabbed and tested MRSA-negative. After 24 hours of exposure, both walls tested MRSA-positive. A quantification was not possible.

The mean particle size in the TIA group measured by the Grimm counter (Grimm, model 1.109, GRIMMAerosol Technik Ainring GmbH & Co., KG, Germany) was 4.3 µm.

4.2.3.2 Environmental samples

In the TIA group, MRSA concentrations of 10 to 30 cfu/swab were detected directly after exposure (day 1) in the sampled water and feeding trough. At the following samplings, all

environmental swabs tested MRSA-negative. In the control group, MRSA was found in the environmental swabs throughout the entire observation period.

4.2.3.3 Clinical Symptoms

In the TIA group, one animal was euthanized due to respiratory symptoms. The other piglets of this group developed an increased rectal body temperature of up to 40.3°C, which returned to the physiological level after tiamulin treatment. No clinical signs occurred in animals of the control group.

4.2.3.4 Animal colonization

Statistical analysis revealed that animals in the TIA group were significantly less likely ($p \leq 0.001$) to test MRSA-positive over the course of the observation period, when compared to the control group.

Nasal swabs

All animals of the TIA group had MRSA- positive nasal swabs directly after exposure (day 1). Two out of eight MRSA- positive nasal swabs (n = 2/8) were quantifiable at day 1 with an MRSA concentration of 5 cfu/swab. In contrast to the control group (previously published by Rosen et al. (2018)), the nasal swabs of animals of this group remained MRSA negative throughout the entire observation period. In the control group, all animals showed MRSA-positive nasal swabs directly after exposure (day 1) and MRSA-positive nasal swabs were sporadically detected until the end of the observation period (see Figure 14A). There is a significantly decreased probability ($p \leq 0.001$) of animals showing MRSA-positive nasal swabs in the TIA group compared to the control group over time.

Skin swabs

Directly after exposure, MRSA was detected in all skin swab samples (day 1) (n = 8/8) with a decrease to 25% (n = 2/8) on day 3. Throughout the rest of the observation period, the skin swabs of the TIA group remained MRSA-negative. In the control group, almost all animals showed MRSA –positive skin swabs at the first two sampling points (n = 8/9 on day 1 and n = 9/9 on day 3) after exposure. Over time, the number of MRSA-positive skin swabs decreased, with individual ones continuing to test positive until the end of the observation period (day 21). Statistical analyses of the skin swabs revealed that animals were significantly less likely ($p \leq 0.001$) to show MRSA-positive skin swabs over the course of the observation period if they were treated with tiamulin.

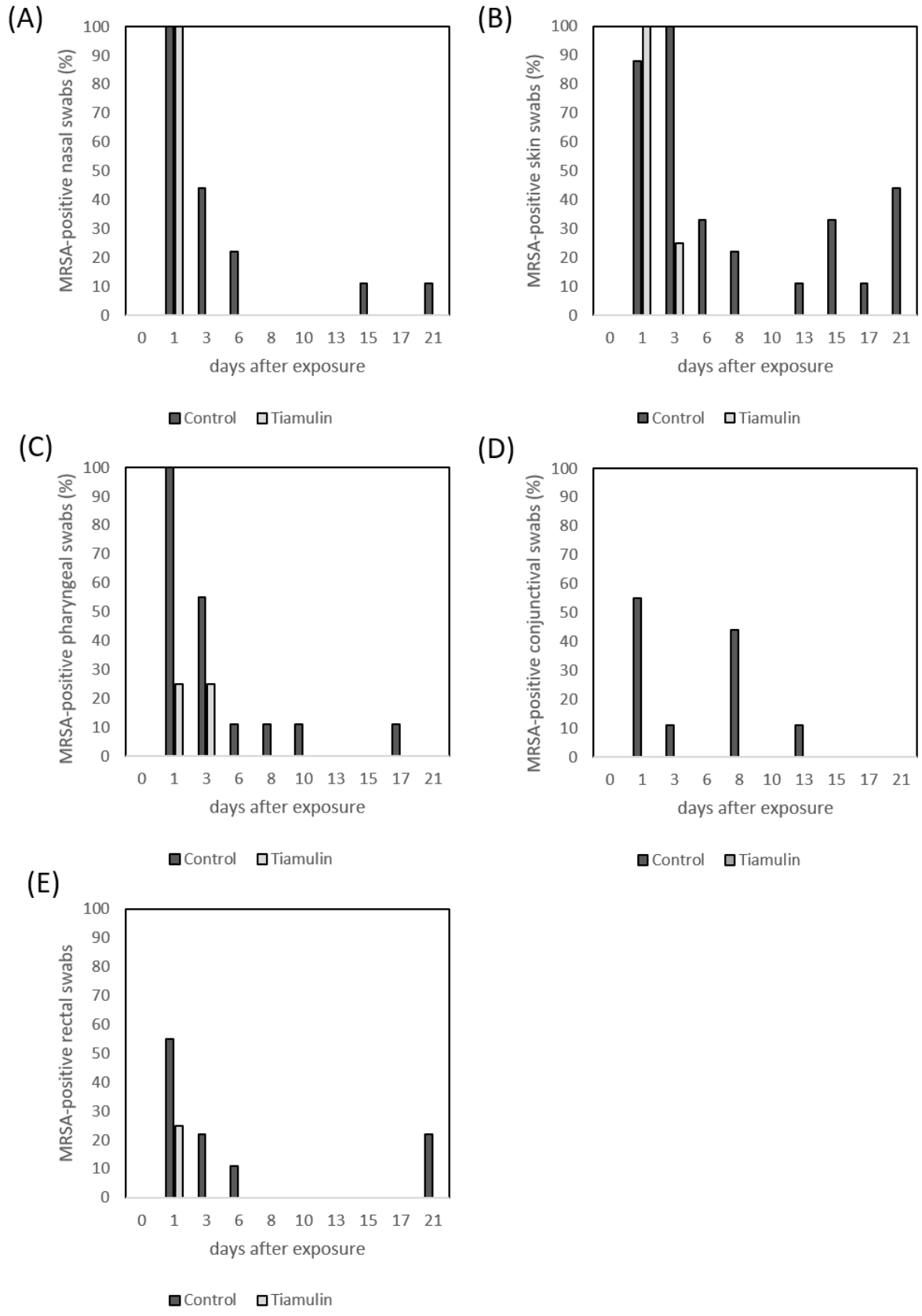

Figure 11 A - E: *Percentages of MRSA-positive nasal (A), skin (B), pharyngeal (C), conjunctival (D), and rectal (E) swabs from piglets of the TIA group over the observation period*

Pharyngeal swabs

In the TIA group, 25% (n = 2/8) of the animals showed MRSA-positive pharyngeal swabs only on day 1 and day 3 after exposure. For both sampling points, the quantification of MRSA was not possible. In the control group, the detectability of MRSA in the pharyngeal swabs decreased from 100% on day 1 (n = 9/9) to 11% (n = 1/9) on day 17. Day 17 also represented the last day where MRSA was found in pharyngeal swabs. Statistical analysis shows no significant differences (p = 0.773) between the TIA group and control group regarding pharyngeal samples harboring MRSA over the course of the observation period.

Conjunctival swabs

In the TIA group, all conjunctival swabs were MRSA-negative throughout the entire observation period. In the control group, five out of nine conjunctival swabs (n = 5/9) were MRSA-positive directly after exposure (day 1). In this group, MRSA-positive conjunctival swabs were found sporadically throughout the observation period.

Rectal swabs

Directly after exposure (day 1), MRSA was only found in two (n = 2/8) rectal swabs of TIA group animals. On day 1, five out of nine animals (n = 5/9) had MRSA-positive rectal swabs in the control group. At the following sampling points, the number of MRSA positive swabs decreased until day 6 (n = 2/9). Two animals showed MRSA positive rectal swabs again at day 21.

Internal Organs

All investigated organs of the TIA and the control group were MRSA negative.

5. Discussion

Besides the wide spread of LA-MRSA in livestock, human infections associated with this type of MRSA are worsening rapidly. Therefore, it is necessary to estimate the impact of airborne MRSA transmission pathways on the MRSA status of individuals. In this context, we established a new airborne MRSA colonization animal model using an aerosol chamber. Additionally, different possible predisposing factors for MRSA colonization of pigs were investigated to elucidate the mechanism behind airborne MRSA colonization. We found that an airborne exposure to 10^6 cfu/m^3 and 10^4 cfu/m^3 MRSA for 24 hours resulted in persistent and transient MRSA colonization in pigs, respectively. We can conclude that exposure to airborne MRSA exclusively, leads to persistently colonized pigs. Furthermore, we detected that the assumed predisposing factors for MRSA colonization did not result in a longer MRSA colonization of the piglets. In contrast, it was found that dexamethasone treatment significantly reduces the MRSA colonization in pigs.

5.1 Animal MRSA colonization models

MRSA colonization models in pigs are a useful tool to investigate the colonization kinetics, transmission between animals and different colonization properties of several LA-MRSA sequence types. To the best of our knowledge, airborne transmission was never used before for MRSA colonization of piglets. In previous studies, MRSA suspensions were used for oral or nasal transmission, whereas some studies practiced more than one of these application pathways (Crombé et al. 2012a; Moodley et al. 2011). In one colonization model, MRSA colonized piglets were obtained due to birth given by experimental MRSA colonized sows (Moodley et al. 2011). MRSA colonization models can be used to gather information concerning transmission pathways and possible factors influencing MRSA colonization. This information can then be utilized to explore the mechanisms behind high MRSA prevalence on farm level. Detailed understanding of the MRSA transmission ways is required to establish measures to reduce MRSA prevalence in livestock. The airborne MRSA colonization model imitates the field conditions in a more natural way than other already established MRSA colonization models. It additionally enables risk assessment for the occurrence of airborne MRSA in the farm environment as possible source for colonization of humans and livestock in proximity of the farm.

In our study, piglets were exposed to an MRSA aerosol. However, it remains questionable whether transmission by direct contact can be completely ruled out in this model. We assume that MRSA transmission by direct contact to MRSA contaminated surfaces of the aerosol chamber or to deposited MRSA on the animals ‘skin play a negligible role and initial animal colonization in our model was primarily airborne. Almost all animals of the median dose group (10^4 cfu/ m^3 MRSA - MD) and the high dose group (10^6 cfu/ m^3 MRSA - HD) showed MRSA-

positive skin swabs at day 1 and day 3 after exposure. Piglets from the high dose group, that showed permanent MRSA colonization, all had MRSA-positive skin swabs until the end of the observation period. One third of the transiently MRSA colonized piglets of the median dose group showed MRSA- positive skin swabs only until day 6 after exposure. This may be explained by the lower initial doses of MRSA during exposure. It appears that the presence of MRSA on the animals´ skin, which might be a result of MRSA aerosol deposition, was not sufficient for MRSA re-colonization via direct contact, e.g. nose-skin contact. The prolonged detectability of MRSA in skin swabs of the HD group could be explained by the higher initial airborne MRSA exposure. In addition, in the MD group, MRSA concentrations on the aerosol chamber surfaces (wall and floor) was too low for quantification. In the HD group, MRSA concentrations were near to the detection limit (2 and 0.7 cfu/cm^2). For us, the MRSA load on the surfaces and on the piglets‘ skin is therefore rather unlikely to act as relevant source for animals to become MRSA-positive. This is underlined by the fact that the nasal MRSA inoculation dose needed for a successful MRSA colonization in the nasal drop- in model is 10^8 cfu/ml.

5.1.1 MRSA colonization versus contamination of the piglets

One weakness of our animal model is the inability to distinguish clearly between true MRSA colonization and MRSA contamination. This is a common problem, which was already discussed by Goerge et al. (2017). The author came to the conclusion that the distinction between persistent colonization and repeated contamination is challenging or even technically impossible. However, our study shows a statistically significant relationship between the airborne MRSA concentration, which were piglets exposed to, and the likelihood of becoming MRSA carriers. Therefore, in our study we assume a MRSA contamination of LD groups´ animals, a transient MRSA colonization occurring in the MD group and a true persistent MRSA colonization of the animals of the HD group. This is in line with Angen et al. (2017) and Bos et al. (2016), who were able to show a positive correlation between airborne MRSA exposure level and the nasal MRSA colonization status of humans.

The airborne MRSA exposure for 24 h of the LD group to the mean MRSA concentration found in the barn air of pigs (10^2 cfu/m^3) by Friese et al. (2012) failed to result in MRSA colonization. There, MRSA was detectable in the nose of only one animal directly after the exposure (day 1) only. This animal remained MRSA-negative until the end of the observation period. Similar observations were made by Angen et al. (2017). There, nasal swabs were taken from persons exposed to airborne MRSA (10^2 cfu/m^3) for one hour in pigsties and were almost uniformly positive directly after exposure. After 48 hours, all samples were MRSA-negative. This short MRSA carriage was regarded as a transient MRSA contamination (Angen et al. 2017). Thus, we conclude that becoming MRSA-negative after a short period of time, as was the case for the one piglet in the LD group, suggests transient contamination rather than colonization.

5. Discussion

A stable MRSA colonization was found in the animals of the HD group. There, MRSA was detectable in all skin and pharyngeal swabs until the end of the observation period. In the nasal, conjunctival and rectal swabs, MRSA was detectable in 97.5%, 96.3%, and 92.5% of cases, respectively. MRSA quantification was possible for all swab samples upon the first sampling after exposure and decreased over time. Despite the fact that the animals were only exposed to MRSA once for 24 hours and immediately transferred to a clean, MRSA-negative barn, the number of MRSA-positive swab samples per animal at different sampling occasions until the day of necropsy was high.

The MRSA colonization dynamics found in MD group animals suggest a transient MRSA colonization of the piglets. In the MD group, MRSA-positive swabs decreased from time of exposure to the end of the observation period. This indicates a transient MRSA contamination rather than a true colonization of animals. Additionally, according to Sakwinska et al. (2010) individuals colonized with low levels of *S. aureus* are more likely to eliminate the bacteria than individuals colonized with high bacterial loads. This is because theses bacteria are eliminated by nasal epithelial cell shedding and mucus flow (Edwards et al. 2012).

It is particularly difficult to differentiate between true colonization and a transient contamination in skin, nasal and conjunctival swabs. It becomes even more difficult if swabs are taken immediately after bacterial aerosol exposure since direct deposition of aerosolized MRSA is possible at these sampling sites.

In our study, there was steep decline in MRSA detectability between first and following samplings of the conjunctiva, where MRSA was detected sporadically only. This suggests an MRSA contamination. Similarly, the skin swabs showed a decrease in MRSA detection as well as MRSA concentration decreased over time. This might indicate the absence of proliferation and, therefore, the absence of true colonization (Jouy et al., 2012) and may just reflect a contamination due to exposure to *S. aureus* (Espinosa-Gongora et al. 2015b). Presence of MRSA in rectal swabs is a result of swallowed bacteria and, therefore, associated with true colonization. MRSA detected in pharyngeal swabs can also be attributed to true colonization rather than contamination. The decrease of MRSA-positive rectal and pharyngeal swabs within the first week after exposure indicates a temporary colonization.

With the exception of conjunctival swabs, all types of swab samples showed a reoccurrence of MRSA at the end of the observation period within the MD group. Here, it can be assumed that direct contact to MRSA carriers or MRSA-contaminated environment lead to recolonization of so far MRSA-negative piglets (Broens et al. 2012b). The experimental pigpen of our animals was cleaned thoroughly once a day to reduce the environmental load of MRSA and its capacity to act as a source for spreading. However, it seems more likely that MRSA-positive animals contaminated the environment, since the number of positive tested environmental swabs

increased similarly to the number of positive animals. Crombé et al. (2013) summarized that the spread of MRSA on herd level can be a result of just a few MRSA-positive carriers. In our study, two animals in the MD group were MRSA positive at six out of nine sampling points and could, therefore, have acted as permanent carriers. Additionally, only one animal of the MD group showed MRSA-positive nasal, rectal, and skin swabs at day 21, indicating stable colonization. This is in accordance with findings of Espinosa-Gongora et al. (2015b), who found that the minority of pigs in animal houses were truly colonized and contributed to the maintenance of *S. aureus*. Moreover, Crombé et al. (2012a) noted the general absence of well-defined criteria to describe true colonization in animals. In our study groups were defined as colonized when 70% of the animals showed at least one MRSA-positive swab sample out of five sampling in the space of two weeks. Furthermore, the presence of MRSA positive organs is assumed to indicate a stable MRSA colonization (Crombé et al. 2012a). MRSA was found in all tonsils of all animals in the HD group, additionally supporting the assumption that true colonization took place in this group. According to Jouy et al. (2012), MRSA- positive tonsils, possibly acting as reservoir for further dissemination, indicate the efficiency of a MRSA inoculation and are useful for detecting silent MRSA carriers.

Statistical analysis in our study shows that the type of swab sample does not influence the likelihood of animals testing positive for MRSA when the whole observation period is considered. This might be surprising as some swab samples are at higher risk of being contaminated with MRSA than others. However, this statistical outcome could be explained by the fact that almost all swab samples were MRSA positive directly after the exposure and thus, all animals had a positive MRSA status at this time point. If the specific timing of sampling is taken into consideration, however, MRSA status is significantly influenced by the swab sample type. Directly after exposure to MRSA, nearly all swabs samples in all animals tested positive for MRSA. Since the sampling sites were most probably only contaminated, not truly colonized, the number of positive swab samples decreased over time. For instance, in the MD group, only one animal showed an MRSA-positive conjunctival swab at day 13 after exposure. In the HD group, all animals showed MRSA-positive conjunctivas at this point in time. When considering the sampling time point, the statistical analysis showed significant differences between both groups and underline rather a temporary contamination than a true colonization of conjunctival swabs the in the MD group. This difference was statistically significant and underlines the likelihood of contamination having taken place in the MD group as opposed to true colonization in the HD group.

Certain colonization sites, such as mucosal sites in the head, remained MRSA positive for a longer time. MRSA were twice as likely to be detected in nasal swabs than in pharyngeal swabs in the HD group. This highlights the fact that the nasal mucosa is the preferred site of MRSA

colonization. Precisely this nasal colonization was also shown in field studies and other animal trials.

5.1.2 Modes of MRSA administration

The mode of bacterial administration in an animal model influences the deposition site (Zhao et al. 2014). Therefore, different bacterial doses may be required for successful bacterial colonization. In addition, the mode of administration should imitate the field conditions as closely as possible, in order to allow the transfer of results to the natural conditions in livestock. Artificial MRSA inoculation is most frequently performed using nasal drop-in (Broens et al. 2012b; Jouy et al. 2012; Szabó et al. 2012), in combination with application on the skin (Crombé et al. 2012a) or gastrointestinal inoculation (Moodley et al. 2011) represent a considerable artificial way of MRSA colonization. The use of aerosols, as for the initial transmission, represents a much more natural mode of colonization. Stable MRSA colonization of piglets was also achieved by Moodley et al. (2011), using intravaginal MRSA inoculation of sows before farrowing. This transmission pathway also reflects natural transmission conditions but is a time-consuming procedure.

In experimental colonization models, exposure doses typically range from 10^7 and 10^8 cfu MRSA/mL and do not always result in stable MRSA colonization (Moodley et al. 2011; Broens et al. 2012b). In a study by Jouy et al. (2012), a dose of 10^4 cfu/mL, administered via nasal drop-in, led to transiently MRSA contaminated animals. This underlines the need for MRSA inoculation doses between 10^7 and 10^8 cfu/mL for a persistent MRSA colonization in pigs. In contrast to findings by Broens et al. (2012b), where most of the MRSA colonized animals died of pneumonia after oral inoculation with 50 mL of 10^9 cfu/mL, in our model, no clinical signs occurred. This is to be expected for a colonization in contrast to an infection. Results gained from studies using artificial application of high doses of MRSA, may not be fully transferable to field conditions. Transmission of MRSA between animals is expected to be greater in experimentally colonized animals than in natural livestock conditions (Crombé et al. 2012a) Szabó et al. (2012) used a MRSA concentration of 10^8 cfu/animal in their nasal drop-in model. The same strain of MRSA, at a concentration of two log units lower, led to a higher MRSA prevalence when aerosolized from in our study.

The airborne route imitates the natural conditions in livestock in a realistic way. Nevertheless, in order to transfer the results found in our study to field conditions, it is important to compare the size of the airborne MRSA particles found in the aerosol chamber with the airborne MRSA particles detected in the field. The size of MRSA-bound particles is important since this determines on the one hand, the deposition depth in the airways (Clauß 2015) and on the other hand, the ability and duration of staying airborne (Zhao et al. 2014). The particle size of the MRSA aerosol generated in the aerosol chamber was approximately 3.6 µm and therefore able

to penetrate the respiratory tract (Green et al. 2006). Small particles might be particularly important for airborne colonization (Ferguson et al. 2016) as larger particles do not remain airborne for a long time. However, also large MRSA-carrying particles constitute a source for MRSA colonization, depositing in the upper respiratory tract (Madsen et al. 2018; Graveland et al. 2011). In the field, airborne bacteria were often found as large aggregates or attached to other particles (Clauß et al. 2011). According to Donham et al. (1986), inside of pig barns *S. aureus* and MRSA are bound to particles lager than 5 µm whereas outside, MRSA is found on particles less than 5 µm in diameter. This indicates an early deposition of large particles and a prolonged stay and therefore wider spread of smaller particles in the air (Schulz et al. 2011). For the animal exposure in our study, we used the airborne MRSA concentration found by Friese et al. (2012) using the AGI 30 impinger, which imitates the bacterial exposure of the upper human respiratory tract (Springorum et al. 2011). This airborne MRSA concentration was also found in the recent past by Madsen et al. (2018), although impaction was used as an alternative sampling method.

A further point requiring discussion is the duration of MRSA exposure in the aerosol chamber. An exposure of 24 hours is much shorter than the natural exposure of fattened pigs. Bos et al. (2016) found an exposure-response-relationship between airborne MRSA in pig barns and the nasal MRSA colonization in humans working there for at least 20 hours. MRSA-exposure is defined by the airborne MRSA concentration and the duration of exposure. Prolonged MRSA exposure in our study could have altered the results of the MRSA colonization statuses.

In the field, the number of transiently colonized pigs was found to outweigh the number of permanently colonized pigs, possibly due to repeated MRSA contamination (Bangerter et al. 2016).This has also been suggested for humans: Van Cleef et al. (2011) and Köck et al. (2012) assumed that in farmers, who were carriers of MRSA, continuous MRSA exposure and subsequent contamination was more likely source than permanent colonization. Hence, repeated MRSA exposure might mirror the field conditions more precisely and might have resulted in other doses for successful MRSA colonization.

5.2 Possible predisposing factors for MRSA colonization

The high LA-MRSA prevalence of pigs in animal housing in the field (Köck et al. 2009; Alt et al. 2011; Fischer et al. 2017) suggests that additional factors may account for the high LA-MRSA colonization. Several individual factors may influence the ability of MRSA to colonize the pigs. Verstappen et al. (2017) found that despite general exposure to MRSA-positive dust, not all the pigs in their study tested positive for MRSA. They hypothesized that certain preventive factors may be to blame for this. However, until now, predisposing factors for MRSA colonization are not defined. A suppressed immune status is often made responsible for the development of MRSA infections (Anker et al. 2018). However, little is known about the impact

of the immunological state of the animal on the MRSA status. In our study, we also investigated the influence of the immunological condition of piglets on MRSA colonization.

5.2.1 Post weaning stress imitated by dexamethasone treatment

In piglets, increased rates of MRSA carriage are often observed around weaning (Broens et al. 2011a; Dewaele et al. 2011; Broens et al. 2012a). Hence, the time around weaning is of special interest when study MRSA colonization. According to Campbell et al. (2013), weaning is one of the most stressful periods in the life of pigs, increasing the risk for diseases. Stress - especially changing the environment - result in a decreased immune function in pigs (Wallgren et al. 1994) and coincides in time with the exposure to new micro-organisms. Therefore, we assumed that stressful conditions might enhance MRSA colonization in piglets. To imitate the stress at weaning, piglets were treated with dexamethasone according to the protocol of Harada et al. (2011) to induce a mild immune suppression. In contrast to our expectations, animals treated with dexamethasone showed significantly shorter periods of MRSA colonization than the untreated control group. The low detectability of MRSA in the skin swabs in combination with the absence of MRSA in the other types of swab samples shortly after the exposure suggests that animals were contaminated rather than colonized. This indicates poor MRSA colonization ability under dexamethasone treatment. We assume that the decreased MRSA colonization may be a result of an increase MRSA clearance. The dexamethasone application might have triggered the immune response showing evidence that there are not well-known side effects of dexamethasone at pigs. In our study, the blood data of the dexamethasone treated animals shows a mild immunomodulation, as we detected a decreased lymphocyte count and proliferation capacity of the $CD4^+$ T-cells. Dexamethasone-induced lymphocytopenia in pigs has been observed in other studies in the past (Flaming et al. 1994; Wallgren et al. 1994; J. L. Salak-Johnson et al. 1996; Harada et al. 2011) but while we detected a slight decrease in neutrophils within the first 48 h of dexamethasone treatment, Harada et al. (2011) found a neutrophilia at this time. In general, immunosuppression due to glucocorticoids in pigs is not consistent and questionable. Flaming et al. (1994) observed that the immune system of pigs is notable resistant to a dexamethasone treatment using similar doses. In a recent study, the immunosuppressive effect of dexamethasone in pigs is doubted as the exacerbation of a Seneca Valley virus infection in dexamethasone treated and untreated pigs was of comparable severity (Buckley et al. 2018). This underlines that an immunosuppressive effect of dexamethasone in piglets needs to be discussed and investigated in the future. However, we assume that the dexamethasone treatment was at least effective to imitate the weaning stress: in the study of Kick et al. (2012) weaning was found to be followed by a decline of lymphocytes- probably due to the stress-increased blood cortisol concentration -an effect which was also observed in our study after dexamethasone treatment.

5. Discussion

There are different hypotheses to explain the decreased MRSA carriage of piglets under dexamethasone treatment observed in our study.

In the recent past, glucocorticoids were found to increase the innate immune system pathway despite decreasing its function. The mode of action - enhancing or suppressing the immune system – is likely to be influenced amongst by factors such as the applied dose and the temporal relationship between the glucocorticoid treatment and noxious stimuli (Cain and Cidlowski 2017). Cain and Cidlowski (2017) reviewed the functions of glucocorticoids and suggest that the immune system is regulated by glucocorticoids in a biphasic manner: low doses promote the expression of innate immune genes resulting in a rapid immune response to noxious stimuli whereas high concentrations lead to an immune suppression (Cain and Cidlowski 2017). This is underlined by the research of Lim et al. (2007) who investigated the effects of glucocorticoids at different doses on rats macrophages. They found an increased expression of immune defense genes (for instance cytokines and chemokines) when treated with low doses and its reduced expression when using high doses of glucocorticoids. Therefore, the low dose administration of dexamethasone in our study might have alerted the piglets' immune system resulting in a more rapid MRSA clearance.

A further explanation for the shortened MRSA carriage of the dexamethasone treated piglets might be a possible decrease of the antimicrobial peptides (AMPs) gene expression in THP-1 monocytes under dexamethasone treatment (Kulkarni et al. 2016). AMPs secreted by innate immune cells (monocytes, macrophages, neutrophils and epithelial cells), are involved in the early defense against pathogens and play a key factor in the cutaneous defense against *S. aureus* (Ryu et al. 2014). Low levels of AMPs occurring in *S. aureus*-carriers seem to facilitate the adhesion of these bacteria to the nasal mucosa when compared to non-carriers (Brown et al. 2014). Therefore, we assume that a reduced level of AMPs in the epithelium of the piglets' nose due to dexamethasone treatment results in an enhanced adhesion followed by a microinvasion of the aerosolized MRSA. This hypothesis is supported by the lower MRSA concentration in the nasal swabs of the dexamethasone treated group compared to the control group directly after exposure. In MRSA carriers, the presentation of MRSA by antigen presenting cells after microinvasion results in immune tolerance (Brown et al. 2014). We assumed, however, that a strong local immune response and local inflammation with subsequent MRSA elimination took place shortly after stopping the dexamethasone treatment at day 2. This is underlined by the neutrophils´ increase in the peripheral blood at day 3, which is probably a result of taking off dexamethasone since the degradation of dexamethasone in pigs is notably fast (Wyns et al. 2013). The interplay between dexamethasone administration and point in time of stopping dexamethasone treatment possibly results in an increased defense against MRSA.

Furthermore, glucocorticoid treatment seems to induce the upregulation of the Toll-like receptors 2 (TLR-2) (Frank et al. 2010). This is of importance since Toll-like receptors activate the innate immune responses to bacteria (Skerrett et al. 2017) and were found to be important for the elimination of invading bacteria (Takeuchi et al. 2000). More precisely, TLR-2 is likely to be a key factor in the innate immune system response to staphylococcal infections, The absence of TLR-2 has been associated with increased susceptibility to infections with *S. aureus* (Fournier and Philpott 2005). TLR-2 plays an important role in the recognition of *S. aureus* (Skerrett et al. 2017) and is involved in the in the early immune response against nasal *S. aureus* colonization (González-Zorn et al. 2005). A study of Quinn and Cole (2007) compared the initial *S. aureus* colonization process of two *S. aureus* strains with different colonization ability. They found that the *S. aureus* strain, which was able to colonize the nasal mucosa, caused a delay about 4 hours in TLR-2 expression. During these 4 hours, the *S. aureus* strain was able to evade the host's innate immune mechanism and therefore able to colonize the epithelium successfully (Quinn and Cole 2007). The inhibitory role of TLR-2 during nasal colonization of *S. aureus* and its upregulation under glucocorticoid treatment conditions, leads us to the hypothesis that in our study, the dexamethasone group experienced an increase in TLR-2 and was able to clear the MRSA rapidly.

The temporal relationship between the glucocorticoid administration and the noxious stimulus might also has an impact on the immune response (Cain and Cidlowski 2017) and, therefore, MRSA colonization. Frank et al. (2010) investigated the correlation between time of corticosterone administration and LPS challenge in the immune system of rats. They found an increased inflammatory response when corticosterone was administered before LPS exposure. Anti-inflammatory effects were observed when corticosterone was given after the LPS challenge. These results are in line with our study in that the animals were first treated with dexamethasone and then exposed to airborne MRSA. In summary, our data indicate that the administration of low dose dexamethasone results in a lower susceptibility of the piglets to MRSA colonization. Probably, the timing of application additionally plays and important role.

5.2.2 Airborne bacterial endotoxin as respiratory hazard

In intensive pig farming, respiratory diseases account for a significant amount of animal morbidity and mortality and are responsible for considerable economic losses (Maes et al. 2001; Knetter et al. 2014). Respiratory disorders are of complex nature and the animals` susceptibility to respiratory pathogens might be increased by different environmental factors (Knetter et al. 2014). Van Duijkeren et al. (2008) presume that respiratory diseases predispose pigs to MRSA colonization. In a previously study of Knetter et al. (2014), an increased susceptibility of pigs to respiratory infections due to increased lung inflammation and tissue damage was observed after organic swine dust extract exposure. A key component of organic dust in barns are endotoxins - cell-wall components of dead Gram-negative bacteria (Seedorf

et al. 1998) - which are ubiquitously present in the environment. These are considered capable of provoking alteration in the human respiratory system resulting in various respiratory diseases (Schierl et al. 2007). Since inhalation of airborne endotoxins causes respiratory health damage in humans, it might also contribute to respiratory disorders in pigs - especially since pigs are continuously exposed to organic dust containing endotoxin (Holst et al. 1994). Moreover, as highly potent proinflammatory substance (Michel et al. 1997) stimulating the immune system, endotoxin exposure might contribute to the elimination of commensal nasal bacteria and, therefore, promote the settlement of immune evading bacteria such as MRSA (Masclaux et al. 2013). To elucidate the role of endotoxins in the MRSA colonization process of piglets, we exposed pigs to an MRSA aerosol containing endotoxins at an airborne lipopolysaccharide (LPS) (purified endotoxin) concentration of 4 µg/m^3. This corresponds with the maximum endotoxin concentration found in pig barns investigated by Zejda et al. (1994). In our survey, the aerodynamic diameter of the aerosolized particles was found to range from 3.2 and 3.7 µg/m^3 and the LPS was, therefore, predicted to be deposited in the alveolar region of the lungs (Basinas et al. 2015).

Statistical analysis reveals that piglets exposed to airborne endotoxins showed no enhanced MRSA colonization despite typical endotoxin-related effects on the peripheral blood cells. Nasal epithelial cells represent the entrance to the respiratory tract and play a key role in the initial defense against microorganism invasion (Yang et al. 2017). The adhesion of MRSA to nasal epithelial cells is the initial step for a successful colonization (Weidenmaier et al. 2012). Since epithelial cell damage leave it more susceptible to bacterial action (Souza Xavier Costa et al. 2017) we hypothesized that endotoxin-induced epithelial cell damage, as primary target for endotoxins (Thorn 2001), would facilitate MRSA adhesion and result in prolonged MRSA colonization. This epithelial cell damage after endotoxin exposure was previously documented in guinea pigs (Fogelmark et al. 1994). Nasal and pharyngeal swabs were expected to test positive for MRSA over a prolonged period of time and act as a source for subsequent spread of MRSA to other tissues. However, the statistical analyses reveal that no prolonged MRSA colonization of nose and pharynx was detected in LPS groups compared to the control group. This indicates an intact nasal mucosa of the piglets despite airborne endotoxin exposure. This assumption is supported by the findings of Urbain et al. (1996b) who did not find effects of nebulized endotoxin on the pigs' nasal mucosa. Even a direct nasal installation of endotoxin solution showed no modification of the nasal fluid composition (Urbain et al. 1996b). In their study, while endotoxin nebulization did not result in a change in the white blood cell count (wbc), pigs challenged with similar endotoxin doses applied intrabronchially or intravenously showed these changes in the white blood cell count. Based on this findings, Urbain et al. (1996b) suggested a weak systemic response of the pigs to nebulized endotoxin. In our study, the piglets exposed to the airborne endotoxin in the aerosol chamber showed an increase in

neutrophils directly after exposure, which implies a systemic reaction. Although the animals were exposed to combined MRSA and LPS aerosol, this blood neutrophilia can be attributed to the LPS exposure alone, since no changes in the total leucocyte and neutrophil count were found after the sole exposition to airborne MRSA (control group). Similar changes in peripheral blood parameters after endotoxin exposure have been documented previously. Roque et al. (2018) investigated the relationship between airborne endotoxin exposure and the immunological profiles of fattening pigs, which represent a more sensitive marker for response to acute LPS exposure than the change in lung function or respiratory symptoms (Michel et al. 1997). Since our blood data are in correspondence with those found in the study of Roque et al. (2018), we assume that the airborne LPS in our aerosol chamber had an effect on the immune system of the piglets and is comparable to pigs continuously exposed to high endotoxin levels in pig barns. Although the LPS exposure in the aerosol chamber resulted in a typical immune response, an effect on the MRSA colonization of piglets could not be detected.

In our study, the aerosolized endotoxin showed a small particle size and was therefore able to penetrate deep into the lungs. Under field conditions, endotoxins are usually found bound on dust, resulting in larger particle sizes. This discrepancy might have affected the lung dose (Jolie et al. 1999) causing an increased endotoxin deposition in the lower respiratory tract, which would have facilitated the MRSA colonization of piglets due to induced epithelial cell damage. However, clearly distinguishing between endotoxin effects and dust-related effects on the respiratory health under field conditions is not possible (Urbain et al. 1999). Urbain et al. (1999) showed that after short-term aerogenous endotoxin exposure of pigs, pulmonary airway inflammation was positively correlated with the dust concentration only. Different endotoxin concentrations showed no additional effect on the respiratory health (Urbain et al. 1999). Jolie et al. (1999) found a significantly higher neutrophil and alveolar macrophage concentration in the bronchoalveolar lavage fluid after a long-term endotoxin exposure (15 weeks) indicating a subclinical problem of the respiratory tract - but also in this study LPS was bound on dust. On the contrary, chronic exposure to defined dust concentrations in combination with different concentrations of ammonia, but without endotoxin, did not result in an increased incidence of respiratory diseases in pigs (Done et al. 2005). In another study by Urbain et al. (1996b) endotoxin nebulization alone resulted in only a moderate respiratory reaction of pigs. The author attributed this finding to the dilution of endotoxin in the respiratory system and the barrier function of the epithelial cells (Urbain et al. 1996b). It is likely that a combination of endotoxins and other airborne components are involved in the aetiology of pigs´ respiratory diseases (Romberger et al. 2002). In our study, we observed a systemic endotoxin-related reaction in piglets, characterized by changes of white blood cell count after airborne endotoxin exposure. Of course, the duration of the airborne endotoxin exposure might influence the effect on the respiratory health and, therefore, MRSA colonization: A dose-

dependent increase of inflammatory mediators in the airways was observed by Cleave et al. (2010). Moreover, the different responses of the immune systems of pigs and broilers after equal endotoxin exposure in a field study by Roque et al. (2018) was attributed to the longer exposure time of pigs (five-month fattening period) compared to broilers (one-month fattening period). The persistent natural exposure to endotoxins in the field throughout the complete fattening period, in contrast to the 24 h exposure in our study, together with the harmful effects of endotoxins on the immune system found by Roque et al. (2018) indicates that endotoxins might facilitate the MRSA colonization in pigs when exposed for an extended time.

5.2.3 Other factors influencing the airborne MRSA colonization

5.2.3.1 Antibiotic treatment

Standard antimicrobial medication is used frequently around the time of weaning (Slifierz et al. 2015) and may be a factor in the spread of MRSA in pigs (van Duijkeren et al. 2008). Tacconelli et al. (2008) reported a 2-fold higher chance for MRSA carriage in humans when previously exposed to antibiotics. This might also hold true for animals. Van Duijkeren et al. (2008) identified antibiotic group medication as risk factor for MRSA carriage in pigs. Similarly, in a study of Graveland et al. (2010), the use of antibiotics was found to be associated with MRSA-positive veal calves. Although not statistically significant, a similar trend was observed in studies on pigs (Alt et al. 2011, Broens et al. 2011a and Broens et al. 2011b). Besides treatment with antibiotics, indirect exposure to antibiotics in dust might result in the selection of resistant bacteria. Hamscher et al. (2003) found residues of up to five different antibiotics in pig-house dust sampled between 1981 to 2000. Continuous inhalation of subtherapeutic concentrations of antibiotics might result in development of antibiotic resistance (Hamscher et al. 2003). In the past, MRSA was found particularly resistant to antimicrobial agents that were frequently used in the respective pig farms (Neeling et al. 2007; Mutters et al. 2016). It was shown that the exposure to subinhibitory antibiotic concentrations increase the cell adhesion of *S. aureus* (Bisognano et al. 1997), which might contribute to a higher MRSA colonization in pigs. Furthermore, antibiotic contact might eradicate the antibiotic- sensitive bacteria in the nasal cavity and allow the subsequent recolonization with antibiotic-resistant bacteria such as MRSA (Noble et al. 1964). These factors must be taken into consideration when assessing the dose of airborne MRSA required for pigs in our study to become persistent MRSA carriers. In our study, all piglets were naturally colonized with MSSA, at least on the nasal and pharyngeal mucosa.

We hypothesized that piglets treated with an antibiotic before exposure to airborne MRSA would show enhanced colonization. The antibiotic used was the pleuromutilin tiamulin because of its regularly and exclusive use in food producing animals (pigs and poultry), particularly for the treatment of respiratory and diarrheal diseases occurring after weaning.

5. Discussion

In our study the tiamulin treatment was conducted before exposure the airborne MRSA in order to reduce the commensal bacterial concentration and pave the way for successful colonization of MRSA. Exposure to MRSA took place 24 hours after the last antibiotic treatment, at which point we expected a subtherapeutic residual concentration of tiamulin and its metabolites on the piglets´ skin and mucosa. We expected this to additionally provided the aerosolized MRSA with a survival advantage over commensal flora and encourage their selection. A further reason for using tiamulin and not the also in porcine medicine commonly used tetracycline was, that the majority of previously isolated strains of MRSA and MSSA have shown tetracycline resistance (Price et al. 2012). Tetracycline treatment might therefore have led to selection for both *S. aureus* lineages, rather than only the methicillin resistant variant (Price et al. 2012). Furthermore, due to the presence of this resistance in MRSA as well as in MSSA isolates, tetracycline is not considered the main driving force for MRSA selection (Guardabassi et al. 2013), rendering it unsuitable for our investigation. Considering the tiamulin antibiotic, Rubin et al. (2011) detected significantly higher minimal inhibitory concentration (MIC) for porcine MRSA ST398 compared to MSSA of porcine origin. In the past, the emergence of MRSA isolates with increased MICs to tiamulin has been observed more and more frequently (Rubin et al. 2011). This is most likely due to the frequent use of pleuromutilin in porcine medicine resulting in selection of pleuromutilin-resistant staphylococci (van Duijkeren et al. 2014).

Contrary to our hypothesis, our data imply that pigs who received treatment with tiamulin before exposure to airborne MRSA show shorter periods of MRSA carriage than the untreated control group. Statistical analysis reveals that animals in the TIA group are significantly less likely to test positive for MRSA throughout the observation period than animals in the control group. Tiamulin metabolites have been detected, inter alia, in the muscle, fat and skin of pigs after an oral tiamulin treatment (Nicholas 2017). The absence of MRSA in nasal swabs from day 3 after exposure as well as the fast decline of MRSA on the skin might therefore be explained by the presence of tiamulin metabolites ´on the skin and mucosa. From day 6 after the airborne MRSA exposure, all swab samples tested negative for MRSA. This suggests a selection disadvantage of our MRSA strain compared to the commensal flora. It was shown that the antimicrobial activity of excreted metabolites exhibits 67% of the activity of the parent drug (Nicholas 2017). Active antimicrobial tiamulin metabolites in feces and urine of the piglets may therefore have led to an additional contamination and might have contributed to the decreased MRSA colonization compared to the control group. The possible presence of tiamulin residues in combination with exposure to a strain of MRSA with a low level of resistance to it may have resulted in elimination of the aerosolized MRSA. Our aerosolized MRSA strain was isolated from a healthy pig showing a MIC of 8 mg/L. Due to the fact that no cut-off values of *S. aureus* and tiamulin are available from the CLSI), guidelines, different MIC breakpoints are used in

studies to determine resistance of *S. aureus* to tiamulin. According to Fessler et al. (2010), MICs ≥ 16 mg/L indicate a tiamulin resistance of *S. aureus*. Other publications used a breakpoint of 2 mg/L (Overesch et al. 2011; Meyer et al. 2012). Since the epidemiological cut-off value for *S. aureus* wild types and tiamulin is 2 mg/L (EUCAST 2019), we assumed that the MRSA strain used in our study was at least less susceptible to tiamulin than the commensal flora of the piglets. However, the quick elimination of the aerosolized MRSA found in our study might be explained by the usage of an MRSA strain showing a slightly increased MIC only. Using a more resistant MRSA to tiamulin could have been resulted in an enhanced MRSA colonization of the antibiotic treated group compared to the untreated control group.

For the investigation of the effect of antibiotic treatment on MRSA colonization, our animal model does come with some limitations. It has been shown that a stepwise exposure of *S. aureus* to tiamulin results in an increased MIC, which underlines the ability of *S. aureus* to acquire tiamulin resistance (Gentry et al. 2007). In contrast to the restricted time in our experimental study, pigs in animal houses are permanently exposed to antibiotic residues in dust (Hamscher et al. 2003). This causes a continuous selective pressure on the nasal microbiota, possibly resulting in selection for MRSA. Therefore, the single tiamulin treatment used in our study did not accurately imitate the situation in the field. Furthermore, in our model, the piglets were first treated with tiamulin and then exposed to airborne MRSA. Under field conditions, antibiotic treatment commonly occurs when MRSA colonization is already established. This change in order might be a further explanation of our findings: once established, MRSA might have a selective advantage over the commensal flora when antimicrobial agents are used (Broens et al. 2012). In contrast to our experiment, larger frequencies of antibiotic therapy occur in pigsties and the MRSA exposure takes commonly place during the entire fattening period and is not limited to 24 hours. An array of previous MRSA colonization studies has shown that the role of antibiotic treatment is generally difficult to evaluate in animal models. For instance, in order to facilitate colonization with a tetracycline resistant MRSA, Moodley et al. (2011) treated pigs with a standard therapeutic tetracycline regime before and after exposing them to the strain. Despite this procedure, MRSA colonization was not successful. This highlights the difficulties associated with imitating the selective antibiotic pressure occurring under field conditions in experimental studies. Nevertheless, the use of antibiotics is associated with a selective advantage of MRSA when compared to susceptible nasal strains (Broens et al. 2012a). The use of antibiotics may therefore promote the acquisition of pig-associated MRSA (van Duijkeren et al. 2008). However, MRSA can also be found and transmitted in pigs without any antimicrobial treatment (Weese et al. 2011; Crombé et al. 2012b; Sørensen et al. 2017). Hence, Broens et al. (2012b) assume that antibiotics are not required for the MRSA colonization and transmission, but that selective antibiotic pressure might influence the success of MRSA colonization. Therefore, the

necessary airborne MRSA concentration for a successful MRSA colonization in field might be lower than figured out in our study.

Finally, the ability of MRSA to colonize individuals could also be influenced by the presence of other commensal bacteria: it has been shown that the colonization with *Staphylococcus epidermidis, Corynebacterium* spp., pneumococci, and other staphylococcal species correlated negatively with the colonization of *S. aureus* (Uehara et al. 2000; Iwase et al. 2010; Lijek et al. 2012; Verstappen et al. 2017).

5.2.3.3 Environmental factors

Under field conditions, pigs are exposed to many potentially harmful environmental agents, such as dust, composed of a myriad of components (Knetter et al. 2014) and pollutants. These environmental agents may influence the susceptibility towards MRSA.

In our study, we investigated the effect of exposure to airborne agents on airborne colonization in piglets using airborne endotoxins as the only example. However, endotoxins may interact with other pollutants or agents in the dust, possibly promoting the exacerbation of respiratory diseases (Urbain et al. 1996b and Thorn (2001). In a study of Urbain et al. (1996b), pigs only showed nasal epithelial cell damage after endotoxin inhalation if they were exposed to ammonia beforehand. This indicates an increased responsiveness of the nasal mucosa to endotoxins due to ammonia (Urbain et al. 1996b).

The administration of *S. aureus* to nasal epithelial cells damaged by environmental toxicants in a rat model, resulted in a tissue infiltration. This was not the case in the control group, where the bacteria were sequestered within mucous clumps (Harris et al. 2009). These findings favor the hypothesis the presence of harmful agents in the environment results in increased susceptibility of the pigs´ nasal cavity towards MRSA colonization.

Furthermore, ammonia was found to decrease the ciliated mucosa of pigs (Urbain et al. 1996a), which might facilitate attachment of MRSA to the mucosal surface (Narita et al. 1995). Since MRSA contaminated dust deposited in the nasal cavities is normally removed by the ciliary transport and subsequent swallowing (Graveland et al. 2011b), a reduction in the ciliary function might also facilitate MRSA colonization.

In addition, Folgemark et al. (1994) observed a synergistic effect of β(1,3)-D-glucan, a cell wall component of fungi, and endotoxin inhalation on the respiratory inflammation in guinea-pigs. This, in combination with the study of Urbain et al. (1996b), supports the relevance of endotoxins as contributory factor for the development of respiratory diseases, when combined with other factors commonly occurring in pig barn air. The combined exposure of pigs to airborne endotoxin and other potentially pathogenic agents commonly occurring in the air of pigsties e.g.: ammonia, β(1,3)-D- glucan, and hydrogen sulfide is closer to the natural situation

(Holst et al. 1994) and may interfere with the MRSA colonization in piglets. Airborne endotoxin exposure alone – at least when exposed for 24 hours only - does not promote the MRSA colonization in piglets. Further studies with extended endotoxin exposure times and additional airborne components, regularly occurring in pigsties, may lead to more information.

6. Conclusion

In our study, we established a newly experimental airborne MRSA colonization model of piglets and attempted to imitate the natural conditions occurring in the field as closely as possible. Colonization of airborne MRSA seems to be a function of i) bacterial concentration in the air, ii) duration of exposure, iii) detrimental effects on the airways, iv) the interplay of MRSA with the commensal microbiota including selective pressure due to antibiotics and v) the immune status of weanling pigs. Conventionally raised, non-antibiotic treated, MRSA- negative piglets, harboring the sensitive variant of *S. aureus* (MSSA) in the nasal cavity and pharynx were used to investigate the necessary dose of airborne MRSA for transient and persistent MRSA colonization. The detected MRSA concentration in the air necessary for MRSA carriage in pigs might be affected by the duration of exposure in the aerosol chamber and different an array of other factors occurring under field conditions. Nonetheless, this animal model is a useful tool to investigate possible predisposing factors for MRSA colonization under controlled conditions in the future. The strong reproducibility of the transient experimental MRSA colonization of the piglets exposed to an airborne MRSA concentration of 10^4 cfu/m^3 shows that our airborne colonization model is a valid colonization model for further investigations. Although the main transmission route is indisputably via direct contact, the occurrence of an airborne mode of MRSA transmission cannot be neglected. This study underlines the very complex nature of the MRSA colonization influenced inter alia by different environmental and host factors. Further research on this topic is warranted to provide more insights into the multifaceted mechanism of MRSA colonization in piglets.

7. Summary

The airborne transmission of LA-MRSA sequence type ST398, firstly detected in 2005, has received a lot of research attention since their occurrence has become a regularity on pig farms, the main reservoir.

Controlling the transmission and spread of LA-MRSA is a major issue regarding “One Health” due to its ability to colonize not only the entire livestock, but also humans. An increasing prevalence in regions with high livestock density highlights this importance. Understanding the various mechanisms behind MRSA colonization of piglets is of utmost necessity in order to establish measures for the prevention of further spreading of LA-MRSA.

In our study, we established a newly experimental airborne MRSA colonization model of piglets, which can be used for reliable and reproducible MRSA colonization of piglets under less artificial conditions than found in other MRSA colonization models of pigs.

A transient MRSA colonization of the piglets was detected when exposing the animals to an airborne MRSA concentration of 10^4 cfu/m^3 for 24 hours in the aerosol chamber. Exposure to an airborne MRSA concentration of 10^6 cfu/m^3 resulted in persistently MRSA colonized piglets. In addition, we investigated possible predisposing factors for successful MRSA colonization.

We explored the role of weaning stress in MRSA colonization by imitating the stressed immunological state using a dexamethasone treatment. In addition, we investigated the effect of presence of airborne bacterial endotoxins on the MRSA colonization status of pigs. Our results indicate that despite their systemic effects on the organism, these factors do not promote the MRSA colonization in piglets.

The limited exposure time of the piglets in the aerosol chamber contradicts the time of airborne MRSA exposure during the complete fattening period in field conditions and must be mentioned as a limitation of the study.

A variety of environment factors may interact with MRSA colonization in the field. This underlines the difficulties in imitating field conditions in experimental colonization models. Moreover, antibiotic treatment of the piglets with the frequently used antibiotic tiamulin did not promote MRSA colonization, additionally demonstrating the difficulties in imitating field conditions in an animal model.

Further research is warranted for deeper insights into the possible mechanisms influencing the MRSA colonization of piglets. This knowledge is essential to limit the further spreading of LA-MRSA in the livestock and thus the transmission to humans.

8. Zusammenfassung

Experimentelle aerogene Kolonisierung von Schweinen mit livestock-assoziiertem Methicillin-resistenten*Staphylococcus aureus* (LA-MRSA)

Im Jahr 2005 wurde der Livestock-assoziierte Methicillin-resistente *Staphylococcus aureus* (LA-MRSA) Sequenztyp ST398 in landwirtschaftlichen Nutztierhaltungen erstmalig nachgewiesen, wobei bis heute die Schweinehaltung das Hauptreservoir für diesen Sequenztyp darstellt. Seitdem dort LA-MRSA auch regelmäßig in der Stall- sowie in der Abluft nachgewiesen wird, herrscht rege Diskussion über eine mögliche aerogene Übertragung von Tier zu Tier, Tier zu Mensch oder vice versa.

Die Verhinderung einer aerogenen Übertragung sowie weiteren Verbreitung von LA-MRSA, der aufgrund seiner geringen Wirtsspezifität in der Lage ist nicht nur alle landwirtschaftlichen Nutztiere, sondern auch den Menschen zu kolonisieren, stellt eines der Hauptthemen des „One Health"- Gedankens dar, der die Interaktion zwischen der Veterinär- und Humanmedizin sowie Umwelt zum Gegenstand hat. Die zunehmende Prävalenz von LA-MRSA in Gegenden mit hoher Nutztierdichte unterstreicht zudem die Notwendigkeit der Eindämmung von LA-MRSA. Dabei stellt unter anderem das Verständnis der verschiedenen Kolonisationsmechanismen bei Schweinen, inklusive der aerogenen Kolonisierung, eine herausragende Grundlage bei der Etablierung von Präventionsmaßnahmen gegen die weitere Verbreitung dar.

In der vorliegenden Arbeit wurde daher ein neues, experimentelles aerogenes LA-MRSA-Kolonisationsmodel für Schweine etabliert, welches zu einer zuverlässigen und reproduzierbaren LA-MRSA-Kolonisierung dieser Tiere führt. Dabei ist die LA-MRSA-Exposition in diesem Modell im Vergleich zu anderen bereits publizierten LA-MRSA-Kolonisationmodellen weniger artifiziell und imitiert die natürlichen Bedingungen im Feld so gut wie möglich.

Eine aerogene LA-MRSA Exposition von Schweinen mit einer Konzentration von 10^4 cfu/m^3 für 24 Stunden führte dabei zu einer temporären, transienten LA-MRSA-Kolonisierung der Tiere, wohingegen die Schweine, die für 24 Stunden mit einer aerogenen LA-MRSA Konzentration von 10^6 cfu/m^3 exponiert wurden, eine dauerhafte Kolonisierung zeigten. Darüber hinaus wurden mit Hilfe dieses neu etablierten Kolonisierungsmodelles weitere präsumtive prädisponierende Faktoren für eine aerogene LA-MRSA Kolonisierung untersucht: So wurde der Einfluss von Stress bei frisch abgesetzter Ferkel auf die aerogene LA-MRSA Kolonisierung untersucht, indem das Immunsystem durch die Gabe von Dexamethason moduliert wurde. Zusätzlich wurde der Einfluss von in der Luft vorhandenem bakteriellen Endotoxin auf die aerogenen LA-MRSA Kolonisierung untersucht. Unsere Ergebnisse implizieren, dass diese beiden untersuchten Faktoren trotz nachgewiesener systemischer

Effekte einer Immunsuppression, die aerogene LA-MRSA Kolonisierung beim Schwein nicht fördern.

Auch wenn das aerogene LA-MRSA Kolonisierungsmodell den natürlichen Kolonisationsweg so gut wie möglich imitiert, gibt es doch limitierende Faktoren, die in Bezug auf die Übertragung der Ergebnisse *in praxi* beachtet werden müssen:

Zum einen ist die aerogene LA-MRSA-Expositionsdauer der Schweine in der Aerosolkammer mit 24 Stunden im Gegensatz zu der tatsächlich vorkommenden aeorgenen LA-MRSA Exposition der Schweine während der kompletten Mastperiode begrenzt. Zum anderen können eine Reihe weiterer Umweltfaktoren, die unter natürlichen Bedingungen vorkommen, mit der LA-MRSA Kolonisierung interagieren. Dies hebt die generelle Schwierigkeit von experimentellen Kolonisationsmodellen hervor, die Feldbedingungen zu imitieren.

Somit besteht auch in Zukunft weiterer Forschungsbedarf, um tiefergehende Einblicke in die Mechanismen zu erhalten, die eine aerogene MRSA-Kolonisierung des Schweines beeinflussen könnten. Derartiges Wissen ist essentiell, um die weitere Verbreitung von LA-MRSA bei landwirtschaftlichen Nutztieren, aber auch beim Menschen zu reduzieren oder gar zu verhindern.

9. References

Abdelbary, Mohamed M. H.; Wittenberg, Anne; Cuny, Christiane; Layer, Franziska; Kurt, Kevin; Wieler, Lothar H. et al. (2014): Phylogenetic analysis of Staphylococcus aureus CC398 reveals a sub-lineage epidemiologically associated with infections in horses. In: *PloS one* 9 (2), e88083. DOI: 10.1371/journal.pone.0088083.

Agersø, Y.; Vigre, H.; Cavaco, L. M.; Josefsen, M. H. (2014): Comparison of air samples, nasal swabs, ear-skin swabs and environmental dust samples for detection of methicillin-resistant Staphylococcus aureus (MRSA) in pig herds. In: *Epidemiology and infection* 142 (8), S. 1727–1736. DOI: 10.1017/S095026881300280X.

Alt, Katja; Fetsch, Alexandra; Schroeter, Andreas; Guerra, Beatriz; Hammerl, Jens A.; Hertwig, Stefan et al. (2011): Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. In: *BMC veterinary research* 7, S. 69. DOI: 10.1186/1746-6148-7-69.

Angen, Øystein; Feld, Louise; Larsen, Jesper; Rostgaard, Klaus; Skov, Robert; Madsen, Anne Mette; Larsen, Anders Rhod (2017): Transmission of Methicillin-Resistant Staphylococcus aureus to Human Volunteers Visiting a Swine Farm. In: *Applied and environmental microbiology* 83 (23). DOI: 10.1128/AEM.01489-17.

Anker, J. C. H.; Koch, A.; Ethelberg, S.; Mølbak, K.; Larsen, J.; Jepsen, M. R. (2018): Distance to pig farms as risk factor for community-onset livestock-associated MRSA CC398 infection in persons without known contact to pig farms-A nationwide study. In: *Zoonoses and public health* 65 (3), S. 352–360. DOI: 10.1111/zph.12441.

Armand-Lefevre, Laurence; Ruimy, Raymond; Andremont, Antoine (2005): Clonal comparison of Staphylococcus aureus isolates from healthy pig farmers, human controls, and pigs. In: *Emerging infectious diseases* 11 (5), S. 711–714. DOI: 10.3201/eid1105.040866.

Bangerter, Patrick Daniel; Sidler, Xaver; Perreten, Vincent; Overesch, Gudrun (2016): Longitudinal study on the colonisation and transmission of methicillin-resistant Staphylococcus aureus in pig farms. In: *Veterinary microbiology* 183, S. 125–134. DOI: 10.1016/j.vetmic.2015.12.007.

Basinas, Ioannis; Sigsgaard, Torben; Kromhout, Hans; Heederik, Dick; Wouters, Inge M.; Schlünssen, Vivi (2015): A comprehensive review of levels and determinants of personal exposure to dust and endotoxin in livestock farming. In: *Journal of exposure science & environmental epidemiology* 25 (2), S. 123–137. DOI: 10.1038/jes.2013.83.

9. References

Becker, Karsten; Ballhausen, Britta; Kahl, Barbara C.; Köck, Robin (2017): The clinical impact of livestock-associated methicillin-resistant Staphylococcus aureus of the clonal complex 398 for humans. In: *Veterinary microbiology* 200, S. 33–38. DOI: 10.1016/j.vetmic.2015.11.013.

Becker, Karsten; Ballhausen, Britta; Köck, Robin; Kriegeskorte, André (2014): Methicillin resistance in Staphylococcus isolates: the "mec alphabet" with specific consideration of mecC, a mec homolog associated with zoonotic S. aureus lineages. In: *International journal of medical microbiology : IJMM* 304 (7), S. 794–804. DOI: 10.1016/j.ijmm.2014.06.007.

Bisdorff, B.; Scholhölter, J. L.; Claußen, K.; Pulz, M.; Nowak, D.; Radon, K. (2012): MRSA-ST398 in livestock farmers and neighbouring residents in a rural area in Germany. In: *Epidemiology and infection* 140 (10), S. 1800–1808. DOI: 10.1017/S0950268811002378.

Bisognano, C.; Vaudaux, P. E.; Lew, D. P.; Ng, E. Y.; Hooper, D. C. (1997): Increased expression of fibronectin-binding proteins by fluoroquinolone-resistant Staphylococcus aureus exposed to subinhibitory levels of ciprofloxacin. In: *Antimicrob. Agents Chemother.* 41 (5), S. 906–913. DOI: 10.1128/AAC.41.5.906.

Bonten, J. M.; Weinstein, Robert A. (1996): The Role of Colonization in the Pathogenesis of Nosocomial Infections 1996, S. 193–200, zuletzt geprüft am 01.10.2018.

Bos, Marian E.H.; Verstappen, Koen M.; van Cleef, Brigitte A.G.L; Dohmen, Wietske; Dorado-Gracia, Alejandro; Graveland, Haitske et al. (2016): Transmission through air as a possible route of exposure for MRSA . In: *Journal of Exposure Science and Envitonmental Epidemiology* 2016 (26), S. 263–269, zuletzt geprüft am 14.08.2018.

Broens, E. M.; Graat, E. A. M.; van der Wolf, P. J.; van de Giessen, A. W.; Jong, M. C. M. de (2011a): Prevalence and risk factor analysis of livestock associated MRSA-positive pig herds in The Netherlands. In: *Preventive veterinary medicine* 102 (1), S. 41–49. DOI: 10.1016/j.prevetmed.2011.06.005.

Broens, E. M.; Graat, E. A. M.; van der Wolf, P. J.; van de Giessen, A. W.; van Duijkeren, E.; Wagenaar, J. A. et al. (2011b): MRSA CC398 in the pig production chain. In: *Preventive veterinary medicine* 98 (2-3), S. 182–189. DOI: 10.1016/j.prevetmed.2010.10.010.

Broens, Els M.; Espinosa-Gongora, Carmen; Graat, Elisabeth A. M.; Vendrig, Nadia; van der Wolf, Peter J.; Guardabassi, Luca et al. (2012a): Longitudinal study on transmission of MRSA CC398 within pig herds. In: *BMC veterinary research* 8, S. 58. DOI: 10.1186/1746-6148-8-58.

Broens, Els M.; Graat, Elisabeth A. M.; van de Giessen, Arjen W.; Broekhuizen-Stins, Marian J.; Jong, Mart C. M. de (2012b): Quantification of transmission of livestock-associated

methicillin resistant Staphylococcus aureus in pigs. In: *Veterinary microbiology* 155 (2-4), S. 381–388. DOI: 10.1016/j.vetmic.2011.09.010.

Brown, Aisling F.; Leech, John M.; Rogers, Thomas R.; McLoughlin, Rachel M. (2014): Staphylococcus aureus Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design. In: *Frontiers in immunology* 4, S. 507. DOI: 10.3389/fimmu.2013.00507.

Buckley, Alexandra; Montiel, Nestor; Guo, Baoqing; Kulshreshtha, Vikas; van Geelen, Albert; Hoang, Hai et al. (2018): Dexamethasone treatment did not exacerbate Seneca Valley virus infection in nursery-age pigs. In: *BMC veterinary research* 14 (1), S. 352. DOI: 10.1186/s12917-018-1693-8.

Cain, Derek W.; Cidlowski, John A. (2017): Immune regulation by glucocorticoids. In: *Nature reviews. Immunology* 17 (4), S. 233–247. DOI: 10.1038/nri.2017.1.

Campbell, Joy M.; Crenshaw, Joe D.; Polo, Javier (2013): The biological stress of early weaned piglets. In: *Journal of animal science and biotechnology* 4 (1), S. 19. DOI: 10.1186/2049-1891-4-19.

Chambers, Henry F.; Deleo, Frank R. (2009): Waves of resistance: Staphylococcus aureus in the antibiotic era. In: *Nature reviews. Microbiology* 7 (9), S. 629–641. DOI: 10.1038/nrmicro2200.

Clauß, M.; Springorum, A. C.; Hartung J. (2011): Size and Composition of Airborne Bacteria Aggregates Collected in Animal Hous Air by a Novel Impactor System, zuletzt geprüft am 16.11.2018.

Clauß, Marcus (2015): Particle size distribution of airborne microorganisms in the environment - a review. In: *Appl Agric Forestry Res,* zuletzt geprüft am 14.11.2018.

Cleave, Jayda; Willson, Philip J.; Town, Jennifer; Gordon, John R. (2010): Fractionation of swine barn dust and assessment of its impact on the respiratory tract following repeated airway exposure. In: *Journal of toxicology and environmental health. Part A* 73 (16), S. 1090–1101. DOI: 10.1080/15287394.2010.482916.

Crombé, Florence; Argudín, M. Angeles; Vanderhaeghen, Wannes; Hermans, Katleen; Haesebrouck, Freddy; Butaye, Patrick (2013): Transmission Dynamics of Methicillin-Resistant Staphylococcus aureus in Pigs. In: *Frontiers in microbiology* 4, S. 57. DOI: 10.3389/fmicb.2013.00057.

Crombé, Florence; Vanderhaeghen, Wannes; Dewulf, Jeroen; Hermans, Katleen; Haesebrouck, Freddy; Butaye, Patrick (2012a): Colonization and transmission of methicillin-

resistant Staphylococcus aureus ST398 in nursery piglets. In: *Applied and environmental microbiology* 78 (5), S. 1631–1634. DOI: 10.1128/AEM.07356-11.

Crombé, Florence; Vanderhaeghen, Wannes; Dewulf, Jeroen; Hermans, Katleen; Haesebrouck, Freddy; Butaye, Patrick (2012b): Colonization and transmission of methicillin-resistant Staphylococcus aureus ST398 in nursery piglets. In: *Applied and environmental microbiology* 78 (5), S. 1631–1634. DOI: 10.1128/AEM.07356-11.

Cuny, Christiane; Abdelbary, Mohamed M. H.; Köck, Robin; Layer, Franziska; Scheidemann, Wolfgang; Werner, Guido; Witte, Wolfgang (2016): Methicillin-resistant Staphylococcus aureus from infections in horses in Germany are frequent colonizers of veterinarians but rare among MRSA from infections in humans. In: *One health (Amsterdam, Netherlands)* 2, S. 11–17. DOI: 10.1016/j.onehlt.2015.11.004.

Cuny, Christiane; Köck, Robin; Witte, Wolfgang (2013): Livestock associated MRSA (LA-MRSA) and its relevance for humans in Germany. In: *International journal of medical microbiology : IJMM* 303 (6-7), S. 331–337. DOI: 10.1016/j.ijmm.2013.02.010.

Cuny, Christiane; Nathaus, Rolf; Layer, Franziska; Strommenger, Birgit; Altmann, Doris; Witte, Wolfgang (2009): Nasal colonization of humans with methicillin-resistant Staphylococcus aureus (MRSA) CC398 with and without exposure to pigs. In: *PloS one* 4 (8), e6800. DOI: 10.1371/journal.pone.0006800.

Dahms, Carmen; Hübner, Nils-Olaf; Cuny, Christiane; Kramer, Axel (2014a): Occurrence of methicillin-resistant Staphylococcus aureus in farm workers and the livestock environment in Mecklenburg-Western Pomerania, Germany. In: *Acta veterinaria Scandinavica* 56, S. 53. DOI: 10.1186/s13028-014-0053-3.

Dahms, Carmen; Hübner, Nils-Olaf; Wilke, Florian; Kramer, Axel (2014b): Mini-review: Epidemiology and zoonotic potential of multiresistant bacteria and Clostridium difficile in livestock and food. In: *GMS Hygiene and Infection Control* (9), zuletzt geprüft am 05.05.2019.

Davis, S. L.; Perri, M. B.; Donabedian, S. M.; Manierski, C.; Singh, A.; Vager, D. et al. (2007): Epidemiology and outcomes of community-associated methicillin-resistant Staphylococcus aureus infection. In: *Journal of clinical microbiology* 45 (6), S. 1705–1711. DOI: 10.1128/JCM.02311-06.

Deurenberg, R. H.; Vink, C.; Kalenic, S.; Friedrich, A. W.; Bruggeman, C. A.; Stobberingh, E. E. (2007): The molecular evolution of methicillin-resistant Staphylococcus aureus. In: *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 13 (3), S. 222–235. DOI: 10.1111/j.1469-0691.2006.01573.x.

9. References

Dewaele, Isabelle; Messens, Winy; Man, Ingrid de; Delputte, Pierre; Herman, Lieve; Butaye, Patrick et al. (2011): Sampling, prevalence and characterization of methicillin-resistant Staphylococcus aureus on two Belgian pig farms. In: *Vet Sci Develop* 1 (1), S. 1. DOI: 10.4081/vsd.2011.e1.

Diep, Binh An; Otto, Michael (2008): The role of virulence determinants in community-associated MRSA pathogenesis. In: *Trends in microbiology* 16 (8), S. 361–369. DOI: 10.1016/j.tim.2008.05.002.

Done, S. H.; Chennells, D. J.; Gresham, A. C. J.; Williamson, S.; Hunt, B.; Taylor, L. L. et al. (2005): Clinical and pathological responses of weaned pigs to atmospheric ammonia and dust. In: *Veterinary Record* 157 (3), S. 71–80. DOI: 10.1136/vr.157.3.71.

Dorado-García, Alejandro; Bos, Marian Eh; Graveland, Haitske; van Cleef, Brigitte Agl; Verstappen, Koen M.; Kluytmans, Jan Ajw et al. (2013): Risk factors for persistence of livestock-associated MRSA and environmental exposure in veal calf farmers and their family members: an observational longitudinal study. In: *BMJ open* 3 (9), e003272. DOI: 10.1136/bmjopen-2013-003272.

Edwards, A. M.; Massey, R. C.; Clarke, S. R. (2012): Molecular mechanisms of Staphylococcus aureus nasopharyngeal colonization. In: *Molecular oral microbiology* 27 (1), S. 1–10. DOI: 10.1111/j.2041-1014.2011.00628.x.

EFSA (2018): The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. In: *EFS2* 16 (2), e0136052. DOI: 10.2903/j.efsa.2018.5182.

Els M Broens1, 2*; Carmen Espinosa-Gongora3; Elisabeth AM Graat1; Nadia Vendrig1; Peter J Van Der Wolf4 (2012): Longitudinal study on transmission of MRSA CC398 within pig herds. In: *BMC veterinary research* 2012, zuletzt geprüft am 14.05.2019.

Espinosa-Gongora, Carmen; Dahl, Jan; Elvstrøm, Anders; van Wamel, Willem J.; Guardabassi, Luca (2015a): Individual predisposition to Staphylococcus aureus colonization in pigs on the basis of quantification, carriage dynamics, and serological profiles. In: *Applied and environmental microbiology* 81 (4), S. 1251–1256. DOI: 10.1128/AEM.03392-14.

Espinosa-Gongora, Carmen; Dahl, Jan; Elvstrøm, Anders; van Wamel, Willem J.; Guardabassi, Luca (2015b): Individual predisposition to Staphylococcus aureus colonization in pigs on the basis of quantification, carriage dynamics, and serological profiles. In: *Applied and environmental microbiology* 81 (4), S. 1251–1256. DOI: 10.1128/AEM.03392-14.

EUCAST (2019): Tiamulin / Staphylococcus aureus International MIC Distribution.

9. References

European Centre for Disease Prevention and Control (2018): SURVEILLANCE REPORT. Surveillance of antimicrobial resistance in Europe 2016, zuletzt geprüft am 06.05.2019.

Feingold, Beth J.; Silbergeld, Ellen K.; Curriero, Frank C.; van Cleef, Brigite A. G. L.; Heck, Max E. O. C.; Kluytmans, Jan A. J. W. (2012): Livestock density as risk factor for livestock-associated methicillin-resistant Staphylococcus aureus, the Netherlands. In: *Emerging infectious diseases* 18 (11), S. 1841–1849. DOI: 10.3201/eid1811.111850.

Feld, Louise; Bay, Hans; Angen, Øystein; Larsen, Anders Rhod; Madsen, Anne Mette (2018): Survival of LA-MRSA in Dust from Swine Farms. In: *Annals of work exposures and health* 62 (2), S. 147–156. DOI: 10.1093/annweh/wxx108.

Ferguson, Dwight D.; Smith, Tara C.; Hanson, Blake M.; Wardyn, Shylo E.; Donham, Kelley J. (2016): Detection of Airborne Methicillin-Resistant Staphylococcus aureus Inside and Downwind of a Swine Building, and in Animal Feed: Potential Occupational, Animal Health, and Environmental Implications. In: *Journal of agromedicine* 21 (2), S. 149–153. DOI: 10.1080/1059924X.2016.1142917.

Fessler, Andrea; Scott, Carly; Kadlec, Kristina; Ehricht, Ralf; Monecke, Stefan; Schwarz, Stefan (2010): Characterization of methicillin-resistant Staphylococcus aureus ST398 from cases of bovine mastitis. In: *The Journal of antimicrobial chemotherapy* 65 (4), S. 619–625. DOI: 10.1093/jac/dkq021.

Fischer, Julia; Hille, Katja; Ruddat, Inga; Mellmann, Alexander; Köck, Robin; Kreienbrock, Lothar (2017): Simultaneous occurrence of MRSA and ESBL-producing Enterobacteriaceae on pig farms and in nasal and stool samples from farmers. In: *Veterinary microbiology* 200, S. 107–113. DOI: 10.1016/j.vetmic.2016.05.021.

Fogelmark, Brigitta; Sjösstrand, Margareta; Rylander, Ragnar (1994): Pulmonary inflammation induced by repeated inhalations of f(1,3)-D-glucan and endotoxin, S. 85–90, zuletzt geprüft am 09.10.2018.

Folgemark, Britta; Sjösstrand, Margareta; Rylander, R. (1994): Pulmonary inflammation induced by repeated inhalations of f(1,3)-D-glucan and endotoxin. In: *Int. J. Exp. Path.* (75), S. 85–90, zuletzt geprüft am 17.12.2018.

Fournier, Bénédicte; Philpott, Dana J. (2005): Recognition of Staphylococcus aureus by the innate immune system. In: *Clinical microbiology reviews* 18 (3), S. 521–540. DOI: 10.1128/CMR.18.3.521-540.2005.

Frank, Matthew G.; Miguel, Zurine D.; Watkins, Linda R.; Maier, Steven F. (2010): Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to E. coli lipopolysaccharide. In: *Brain, behavior, and immunity* 24 (1), S. 19–30. DOI: 10.1016/j.bbi.2009.07.008.

9. References

Friese, A.; Schulz, J.; Zimmermann, K.; Tenhagen, B-A; Fetsch, A.; Hartung, J.; Rösler, U. (2013): Occurrence of livestock-associated methicillin-resistant Staphylococcus aureus in Turkey and broiler barns and contamination of air and soil surfaces in their vicinity. In: *Applied and environmental microbiology* 79 (8), S. 2759–2766. DOI: 10.1128/AEM.03939-12.

Friese, Anika; Schulz, Jochen; Hoehle, Laura; Fetsch, Alexandra; Tenhagen, Bernd-Alois; Hartung, Joerg; Roesler, Uwe (2012): Occurrence of MRSA in air and housing environment of pig barns. In: *Veterinary microbiology* 158 (1-2), S. 129–135. DOI: 10.1016/j.vetmic.2012.01.019.

García-Álvarez, Laura; Holden, Matthew T. G.; Lindsay, Heather; Webb, Cerian R.; Brown, Derek F. J.; Curran, Martin D. et al. (2011): Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. In: *The Lancet Infectious Diseases* 11 (8), S. 595–603. DOI: 10.1016/S1473-3099(11)70126-8.

Gentry, Daniel R.; Rittenhouse, Stephen F.; McCloskey, Lynn; Holmes, David J. (2007): Stepwise exposure of Staphylococcus aureus to pleuromutilins is associated with stepwise acquisition of mutations in rplC and minimally affects susceptibility to retapamulin. In: *Antimicrobial agents and chemotherapy* 51 (6), S. 2048–2052. DOI: 10.1128/AAC.01066-06.

Gibbs, Shawn G.; Green, Christopher F.; Tarwater, Patrick M.; Mota, Linda C.; Mena, Kristina D.; Scarpino, Pasquale V. (2006): Isolation of Antibiotic-Resistant Bacteria from the Air Plume Downwind of a Swine Confined or Concentrated Animal Feeding Operation. In: *Environ Health Perspect* 114 (7), S. 1032–1037. DOI: 10.1289/ehp.8910.

Gibbs, Shawn G.; Green, Christopher F.; Tarwater, Patrick M.; Scarpino, Pasquale V. (2004): Airborne antibiotic resistant and nonresistant bacteria and fungi recovered from two swine herd confined animal feeding operations. In: *Journal of Occupational and Environmental Hygiene* 1 (11), S. 699–706. DOI: 10.1080/15459620490515824.

Gilbert, Maarten J.; Bos, Marian E. H.; Duim, Birgitta; Urlings, Bert A. P.; Heres, Lourens; Wagenaar, Jaap A.; Heederik, Dick J. J. (2012): Livestock-associated MRSA ST398 carriage in pig slaughterhouse workers related to quantitative environmental exposure. In: *Occupational and environmental medicine* 69 (7), S. 472–478. DOI: 10.1136/oemed-2011-100069.

Goerge, Tobias; Lorenz, Marthe Barbara; van Alen, Sarah; Hübner, Nils-Olaf; Becker, Karsten; Köck, Robin (2017): MRSA colonization and infection among persons with occupational livestock exposure in Europe: Prevalence, preventive options and evidence. In: *Veterinary microbiology* 200, S. 6–12. DOI: 10.1016/j.vetmic.2015.10.027.

9. References

González-Zorn, Bruno; Senna, Jose P. M.; Fiette, Laurence; Shorte, Spencer; Testard, Aurélie; Chignard, Michel et al. (2005): Bacterial and host factors implicated in nasal carriage of methicillin-resistant Staphylococcus aureus in mice. In: *Infection and immunity* 73 (3), S. 1847–1851. DOI: 10.1128/IAI.73.3.1847-1851.2005.

Graveland, Haitske; Duim, Birgitta; van Duijkeren, Engeline; Heederik, Dick; Wagenaar, Jaap A. (2011a): Livestock-associated methicillin-resistant Staphylococcus aureus in animals and humans. In: *International journal of medical microbiology : IJMM* 301 (8), S. 630–634. DOI: 10.1016/j.ijmm.2011.09.004.

Graveland, Haitske; Wagenaar, Jaap A.; Bergs, Kelly; Heesterbeek, Hans; Heederik, Dick (2011b): Persistence of livestock associated MRSA CC398 in humans is dependent on intensity of animal contact. In: *PloS one* 6 (2), e16830. DOI: 10.1371/journal.pone.0016830.

Graveland, Haitske; Wagenaar, Jaap A.; Heesterbeek, Hans; Mevius, Dik; van Duijkeren, Engeline; Heederik, Dick (2010): Methicillin resistant Staphylococcus aureus ST398 in veal calf farming: human MRSA carriage related with animal antimicrobial usage and farm hygiene. In: *PloS one* 5 (6), e10990. DOI: 10.1371/journal.pone.0010990.

Green, Christopher F.; Gibbs, Shawn G.; Tarwater, Patrick M.; Mota, Linda C.; Scarpino, Pasquale V. (2006): Bacterial Plume Emanating from the Air Surrounding Swine Confinement Operations. In: *Journal of Occupational and Environmental Hygiene* 3 (1), S. 9–15. DOI: 10.1080/15459620500430615.

Gustafsson, G. (1999): Factors affecting the Release and Concentration of Dust in Pig Houses. In: *Journal of Agricultural Engineering Research* 74 (4), S. 379–390. DOI: 10.1006/jaer.1999.0476.

Haenni, Marisa; Châtre, Pierre; Dupieux-Chabert, Céline; Métayer, Véronique; Bes, Michèle; Madec, Jean-Yves; Laurent, Frédéric (2017): Molecular Epidemiology of Methicillin-Resistant Staphylococcus aureus in Horses, Cats, and Dogs Over a 5-Year Period in France. In: *Frontiers in microbiology* 8, S. 2493. DOI: 10.3389/fmicb.2017.02493.

Hamscher, Gerd; Pawelzick, Heike Theresia; Sczesny, Silke; Nau, Heinz; Hartung, Jörg (2003): Antibiotics in dust originating from a pig-fattening farm: a new source of health hazard for farmers? In: *Environmental health perspectives* 111 (13), S. 1590–1594. DOI: 10.1289/ehp.6288.

Harada, Kazuki; Muramatsu, Masatake; Suzuki, Shoko; Tamura, Yutaka; Sawada, Takuo; Takahashi, Toshio (2011): Evaluation on the pathogenicity of Erysipelothrix tonsillarum for pigs by immunosuppression with cyclophosphamide or dexamethasone. In: *Research in veterinary science* 90 (1), S. 20–22. DOI: 10.1016/j.rvsc.2010.05.009.

9. References

Harper, Abby L.; Ferguson, Dwight D.; Leedom Larson, Kerry R.; Hanson, Blake M.; Male, Michael J.; Donham, Kelley J.; Smith, Tara C. (2010): An overview of livestock-associated MRSA in agriculture. In: *Journal of agromedicine* 15 (2), S. 101–104. DOI: 10.1080/10599241003627110.

Harris, Julie A.; West, Adrian K.; Chuah, Meng Inn (2009): Olfactory ensheathing cells: nitric oxide production and innate immunity. In: *Glia* 57 (16), S. 1848–1857. DOI: 10.1002/glia.20899.

Hartman, Barry J.; Tomasz, Alexander (1984): Low-Affinity Penicillin-Binding Protein Associated with ß-Lactam Resistance in Staphylococcus aures. In: *Division of Infectious Diseases* (2), S. 513–516. Online verfügbar unter https://www.ncbi.nlm.nih.gov/pmc/articles/PMC215458/pdf/jbacter00234-0125.pdf, zuletzt geprüft am 26.09.2018.

Holst, H.; Edqvist, L.-E.; Kindahl, H.; Rylander, R. (1994): Hematological, Blood Biochemical, and Cytological Bronchoalveolar Lavage Studies in Prepubertal Gilts after Endotoxin Inhalation and Ingestion. In: *Journal of Veterinary Medicine Series A* 41 (1-10), S. 159–166. DOI: 10.1111/j.1439-0442.1994.tb00079.x.

Huijsdens, Xander W.; van Dijke, Beatrix J.; Spalburg, Emile; van Santen-Verheuvel, Marga G.; Heck, Max E. O. C.; Pluister, Gerlinde N. et al. (2006): Community-acquired MRSA and pig-farming. In: *Annals of clinical microbiology and antimicrobials* 5, S. 26. DOI: 10.1186/1476-0711-5-26.

Iwase, Tadayuki; Uehara, Yoshio; Shinji, Hitomi; Tajima, Akiko; Seo, Hiromi; Takada, Koji et al. (2010): Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. In: *Nature* 465 (7296), S. 346–349. DOI: 10.1038/nature09074.

J. L. Salak-Johnson; J. J. McGlone; R. L. Norman (1996): In vivo glucocorticoid effects on porcine natural killer cell activity and circulating leukocytes. In: *J ANIM SCI*, S. 584–592, zuletzt geprüft am 09.10.2018.

Jevons, Patricia M. (1961): To-day's Drugs. In: *BMJ* 2 (5251), S. 579. DOI: 10.1136/bmj.2.5251.579.

Jim O`Neill (2014): Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations, zuletzt geprüft am 10.11.2018.

Jolie, Rika; Bäckström, Lennart; Olsen, Linda; Chase, Chris (1999): A 15-Week Experimental Exposure of Pigs to Airborne Dust with Added Endotoxin in a Continuous Flow Exposure Chamber. In: *Can J Vet Res*, S. 129–137, zuletzt geprüft am 09.10.2018.

9. References

Joseph E. Rubin; Katherine R. Ball; Manuel Chirino-Trejo (2011): Antimicrobial susceptibility of Staphylococcus aureus and Staphylococcus pseudintermedius isolated from various animals. In: *Can Vet J* (52), zuletzt geprüft am 14.05.2019.

Jouy, E.; Le Roux, A.; Kéranflec'h, A.; Granier, S. A.; Laurent, F.; Kempf, I. et al. (2012): Methicillin-resistant Staphylococcus aureus ST398 contamination and transmission in pigs after a low dose inoculation. In: *Letters in applied microbiology* 54 (6), S. 518–523. DOI: 10.1111/j.1472-765X.2012.03239.x.

K. P. Flaming; B. L. Goff; D. E. Frank and J. A. Roth (1994): Pigs are Relatively Resistant to Dexamethasone Induced Immunosuppression. In: *Comparative Haematology International*, S. 218–225, zuletzt geprüft am 09.10.2018.

Kahl, Barbara C.; Mellmann, Alexander; Deiwick, Susanne; Peters, Georg; Harmsen, Dag (2005): Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in Staphylococcus aureus. In: *Journal of clinical microbiology* 43 (1), S. 502–505. DOI: 10.1128/JCM.43.1.502-505.2005.

Kelley J. Donham, MS, DVM, William Popendorf, PhD, Urban Palmgren, PhD (1986): Characterization of dusts collected from swine confinement buildings. In: *American Journal of Industrial Medicine* (10), S. 294–297.

Khanna, T.; Friendship, R.; Dewey, C.; Weese, J. S. (2008): Methicillin resistant Staphylococcus aureus colonization in pigs and pig farmers. In: *Veterinary microbiology* 128 (3-4), S. 298–303. DOI: 10.1016/j.vetmic.2007.10.006.

Kick, A. R.; Tompkins, M. B.; Flowers, W. L.; Whisnant, C. S.; Almond, G. W. (2012): Effects of stress associated with weaning on the adaptive immune system in pigs. In: *Journal of animal science* 90 (2), S. 649–656. DOI: 10.2527/jas.2010-3470.

Kluytmans, J. A. J. W. (1997): Nasal Carriage of Staphylococcus aureus: Epidemiology, Underlying Mechanism, and Associated Risks. In: *Clin. Microbiol. REV.* 1997 (Vol. 10), S. 505–520. Online verfügbar unter https://www.ncbi.nlm.nih.gov/pmc/articles/PMC172932/pdf/100505.pdf, zuletzt geprüft am 24.08.2018.

Knetter, Susan M.; Tuggle, Christopher K.; Wannemuehler, Michael J.; Ramer-Tait, Amanda E. (2014): Organic barn dust extract exposure impairs porcine macrophage function in vitro: implications for respiratory health. In: *Veterinary immunology and immunopathology* 157 (1-2), S. 20–30. DOI: 10.1016/j.vetimm.2013.10.019.

Köck, R.; Harlizius, J.; Bressan, N.; Laerberg, R.; Wieler, L. H.; Witte, W. et al. (2009): Prevalence and molecular characteristics of methicillin-resistant Staphylococcus aureus

(MRSA) among pigs on German farms and import of livestock-related MRSA into hospitals. In: *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 28 (11), S. 1375–1382. DOI: 10.1007/s10096-009-0795-4.

Köck, Robin; Loth, Bea; Köksal, Mahir; Schulte-Wülwer, Josef; Harlizius, Jürgen; Friedrich, Alexander W. (2012): Persistence of nasal colonization with livestock-associated methicillin-resistant Staphylococcus aureus in pig farmers after holidays from pig exposure. In: *Applied and environmental microbiology* 78 (11), S. 4046–4047. DOI: 10.1128/AEM.00212-12.

Köck, Robin; Schaumburg, Frieder; Mellmann, Alexander; Köksal, Mahir; Jurke, Annette; Becker, Karsten; Friedrich, Alexander W. (2013): Livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) as causes of human infection and colonization in Germany. In: *PloS one* 8 (2), e55040. DOI: 10.1371/journal.pone.0055040.

Kulkarni, Nikhil Nitin; Gunnarsson, Hörður Ingi; Yi, Zhiqian; Gudmundsdottir, Steinunn; Sigurjonsson, Olafur E.; Agerberth, Birgitta; Gudmundsson, Gudmundur H. (2016): Glucocorticoid dexamethasone down-regulates basal and vitamin D3 induced cathelicidin expression in human monocytes and bronchial epithelial cell line. In: *Immunobiology* 221 (2), S. 245–252. DOI: 10.1016/j.imbio.2015.09.001.

L. Guardabassi; J. Larsen; J.S. Weese; P. Butaye, A. Battisti; J. Kluytmans; D.H. Lloyd; R.L. Skov (2013): Public health impact and antimicrobial selection of meticillin-resistant staphylococci in animals, Journal of Global Antimicrobial Resistance,. In: *Journal of Global Antimicrobial Resistance,* (1), S. 55–62.

Lakhundi, Sahreena; Zhang, Kunyan (2018): Methicillin-Resistant Staphylococcus aureus: Molecular Characterization, Evolution, and Epidemiology. In: *Clinical microbiology reviews* 31 (4). DOI: 10.1128/CMR.00020-18.

Lijek, Rebeccah S.; Luque, Santiago L.; Liu, Qian; Parker, Dane; Bae, Taeok; Weiser, Jeffrey N. (2012): Protection from the acquisition of Staphylococcus aureus nasal carriage by cross-reactive antibody to a pneumococcal dehydrogenase. In: *Proceedings of the National Academy of Sciences of the United States of America* 109 (34), S. 13823–13828. DOI: 10.1073/pnas.1208075109.

Lim, Hee-Young; Müller, Nora; Herold, Marco J.; van den Brandt, Jens; Reichardt, Holger M. (2007): Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. In: *Immunology* 122 (1), S. 47–53. DOI: 10.1111/j.1365-2567.2007.02611.x.

Livermore, David M. (2000): Antibiotic resistance in staphylococci. In: *International Journal of Antimicrobial Agents* 16, S. 3–10. DOI: 10.1016/S0924-8579(00)00299-5.

Locatelli, C.; Cremonesi, P.; Caprioli, A.; Carfora, V.; Ianzano, A.; Barberio, A. et al. (2017): Occurrence of methicillin-resistant Staphylococcus aureus in dairy cattle herds, related swine farms, and humans in contact with herds. In: *Journal of dairy science* 100 (1), S. 608–619. DOI: 10.3168/jds.2016-11797.

Loeffler, A.; Lloyd, D. H. (2010): Companion animals: a reservoir for methicillin-resistant Staphylococcus aureus in the community? In: *Epidemiology and infection* 138 (5), S. 595–605. DOI: 10.1017/S0950268809991476.

Lowy, Franklin D. (2003): Antimicrobial resistance: the example of Staphylococcus aureus. In: *J. Clin. Invest.* 111 (9), S. 1265–1273. DOI: 10.1172/JCI200318535.

Luini, M.; Cremonesi, P.; Magro, G.; Bianchini, V.; Minozzi, G.; Castiglioni, B.; Piccinini, R. (2015): Methicillin-resistant Staphylococcus aureus (MRSA) is associated with low within-herd prevalence of intra-mammary infections in dairy cows: Genotyping of isolates. In: *Veterinary microbiology* 178 (3-4), S. 270–274. DOI: 10.1016/j.vetmic.2015.05.010.

Madsen, Anne Mette; Kurdi, Iman; Feld, Louise; Tendal, Kira (2018): Airborne MRSA and Total Staphylococcus aureus as Associated With Particles of Different Sizes on Pig Farms. In: *Annals of work exposures and health*. DOI: 10.1093/annweh/wxy065.

Maes, D. G.; Deluyker, H.; Verdonck, M.; Kruif, A. de; Ducatelle, R.; Castryck, F. et al. (2001): Non-infectious factors associated with macroscopic and microscopic lung lesions in slaughter pigs from farrow-to-finish herds. In: *Veterinary Record* 148 (2), S. 41–46. DOI: 10.1136/vr.148.2.41.

Masclaux, Frederic G.; Sakwinska, Olga; Charrière, Nicole; Semaani, Eulalia; Oppliger, Anne (2013): Concentration of airborne Staphylococcus aureus (MRSA and MSSA), total bacteria, and endotoxins in pig farms. In: *The Annals of occupational hygiene* 57 (5), S. 550–557. DOI: 10.1093/annhyg/mes098.

Meyer, Cornelia; Fredriksson-Ahomaa, Maria; Stüber, Elisabeth; Thiel, Susanne; Märtlbauer, Erwin (2012): High frequency of multiresistant coagulase-positive Staphylococcus aureus found in slaughter pigs in Uruguay. In: *Foodborne pathogens and disease* 9 (1), S. 86–90. DOI: 10.1089/fpd.2011.0973.

Michel, O.; Nagy, A. M.; Schroeven, M.; Duchateau, J.; Nève, J.; Fondu, P.; Sergysels, R. (1997): Dose-response relationship to inhaled endotoxin in normal subjects. In: *American journal of respiratory and critical care medicine* 156 (4 Pt 1), S. 1157–1164. DOI: 10.1164/ajrccm.156.4.97-02002.

Moodley, A.; Latronico, F.; Guardabassi, L. (2011): Experimental colonization of pigs with methicillin-resistant Staphylococcus aureus (MRSA): insights into the colonization and

transmission of livestock-associated MRSA. In: *Epidemiology and infection* 139 (10), S. 1594–1600. DOI: 10.1017/S0950268810002888.

Mulders, M. N.; Haenen, A. P. J.; Geenen, P. L.; Vesseur, P. C.; Poldervaart, E. S.; Bosch, T. et al. (2010): Prevalence of livestock-associated MRSA in broiler flocks and risk factors for slaughterhouse personnel in The Netherlands. In: *Epidemiology and infection* 138 (5), S. 743–755. DOI: 10.1017/S0950268810000075.

Narita, Minoru; Kawashima, Kenji; Morozumi, Tetsuo; Takashima, Hasataka (1995): Effect of Physical Defenses of the Respiratory Tract on the Development of Pneumonia in Pigs Inoculated Endobronchially with Actionobacillus pleuropneumoniae, zuletzt geprüft am 21.12.2018.

Neeling, A. J. de; van den Broek, M. J. M.; Spalburg, E. C.; van Santen-Verheuvel, M. G.; Dam-Deisz, W. D. C.; Boshuizen, H. C. et al. (2007): High prevalence of methicillin resistant Staphylococcus aureus in pigs. In: *Veterinary microbiology* 122 (3-4), S. 366–372. DOI: 10.1016/j.vetmic.2007.01.027.

Nemeghaire, Stéphanie; Roelandt, Sophie; Argudín, M. Angeles; Haesebrouck, Freddy; Butaye, Patrick (2013): Characterization of methicillin-resistant Staphylococcus aureus from healthy carrier chickens. In: *Avian pathology : journal of the W.V.P.A* 42 (4), S. 342–346. DOI: 10.1080/03079457.2013.805183.

Nicholas, Jarrett (2017): Tiamulin Summary Report, zuletzt geprüft am 23.07.2019.

Nico T. Mutters; Christian P. Bieber; Catherine Hauck; Gerald Reiner; Veronika Malek; Uwe Frank (2016): Comparison of livestock-associated and health care–associated MRSA—genes, virulence, and resistance. In: *Diagnostic Microbiology and Infectious Disease* (86), S. 417–421, zuletzt geprüft am 14.05.2019.

Noble, W. C.; Williams, R. E. O.; Jevons, M. P.; Shooter, R. A. (1964): Some aspects of nasal carriage of staphylococci. In: *Journal of Clinical Pathology* 17 (1), S. 79–83. DOI: 10.1136/jcp.17.1.79.

Overesch, Gudrun; Büttner, Sabina; Rossano, Alexandra; Perreten, Vincent (2011): The increase of methicillin-resistant Staphylococcus aureus (MRSA) and the presence of an unusual sequence type ST49 in slaughter pigs in Switzerland. In: *BMC veterinary research* 7, S. 30. DOI: 10.1186/1746-6148-7-30.

Pete Kinross; Andreas Petersen; Robert Skov; Evelyn Van Hauwermeiren; Annalisa Pantosti; Frédéric Laurent et al. (2017): Livestock-associated meticillin-resistant Staphylococcus aureus (MRSA) among human MRSA isolates, European Union/European Economic Area countries, 2013, zuletzt geprüft am 06.05.2019.

Pletinckx, L. J.; Verhegghe, M.; Dewulf, J.; Crombé, F.; Bleecker, Y. de; Rasschaert, G. et al. (2011): Screening of poultry-pig farms for methicillin-resistant Staphylococcus aureus: sampling methodology and within herd prevalence in broiler flocks and pigs. In: *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* 11 (8), S. 2133–2137. DOI: 10.1016/j.meegid.2011.07.008.

Price, Lance B.; Stegger, Marc; Hasman, Henrik; Aziz, Maliha; Larsen, Jesper; Andersen, Paal Skytt et al. (2012): Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock. In: *mBio* 3 (1). DOI: 10.1128/mBio.00305-11.

Quinn, Gerry A.; Cole, Alexander M. (2007): Suppression of innate immunity by a nasal carriage strain of Staphylococcus aureus increases its colonization on nasal epithelium. In: *Immunology* 122 (1), S. 80–89. DOI: 10.1111/j.1365-2567.2007.02615.x.

Romberger, D. J.; Bodlak, V.; Essen, S. G. von; Mathisen, T.; Wyatt, T. A. (2002): Hog barn dust extract stimulates IL-8 and IL-6 release in human bronchial epithelial cells via PKC activation. In: *Journal of applied physiology (Bethesda, Md. : 1985)* 93 (1), S. 289–296. DOI: 10.1152/japplphysiol.00815.2001.

Roque, Katharine; Shin, Kyung Min; Jo, Ji Hoon; Lim, Gyeong Dong; Song, Eun Seob; Shin, So Jung et al. (2018): Association between endotoxin levels in dust from indoor swine housing environments and the immune responses of pigs. In: *Journal of veterinary science* 19 (3), S. 331–338. DOI: 10.4142/jvs.2018.19.3.331.

Rosen, Kerstin; Roesler, Uwe; Merle, Roswitha; Friese, Anika (2018): Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model. In: *Frontiers in microbiology* 9, S. 1542. DOI: 10.3389/fmicb.2018.01542.

Ryu, Sunhyo; Song, Peter I.; Seo, Chang Ho; Cheong, Hyeonsook; Park, Yoonkyung (2014): Colonization and infection of the skin by S. aureus: immune system evasion and the response to cationic antimicrobial peptides. In: *International journal of molecular sciences* 15 (5), S. 8753–8772. DOI: 10.3390/ijms15058753.

Sakwinska, Olga; Blanc, Dominique S.; Lazor-Blanchet, Catherine; Moreillon, Martine; Giddey, Marlyse; Moreillon, Philippe (2010): Ecological temporal stability of Staphylococcus aureus nasal carriage. In: *Journal of clinical microbiology* 48 (8), S. 2724–2728. DOI: 10.1128/JCM.02091-09.

SanMiguel, Adam J.; Meisel, Jacquelyn S.; Horwinski, Joseph; Zheng, Qi; Grice, Elizabeth A. (2017): Topical Antimicrobial Treatments Can Elicit Shifts to Resident Skin Bacterial Communities and Reduce Colonization by Staphylococcus aureus Competitors. In: *Antimicrobial agents and chemotherapy* 61 (9). DOI: 10.1128/AAC.00774-17.

9. References

Schierl, Rudolf; Heise, Antje; Ehher, Ulrich; Schneider, Friedhelm; Eichelser, Robert; Neser, Stefan; Nowak, Dennis (2007): ENDOTOXIN CONCENTRATION IN MODERN ANIMAL HOUSES IN SOUTHERN BAVARIA, zuletzt geprüft am 04.12.2018.

Schulz, J.; Formosa, L.; Seedorf, J.; Hartung, J. (2011): Measurement of culturable airborne staphylococci downwind from a naturally ventilated broiler house. In: *Aerobiologia* 27 (4), S. 311–318. DOI: 10.1007/s10453-011-9202-6.

Schulz, Jochen; Friese, Anika; Klees, Sylvia; Tenhagen, Bernd A.; Fetsch, Alexandra; Rösler, Uwe; Hartung, Jörg (2012a): Longitudinal study of the contamination of air and of soil surfaces in the vicinity of pig barns by livestock-associated methicillin-resistant Staphylococcus aureus. In: *Applied and environmental microbiology* 78 (16), S. 5666–5671. DOI: 10.1128/AEM.00550-12.

Schulz, Jochen; Friese, Anika; Klees, Sylvia; Tenhagen, Bernd A.; Fetsch, Alexandra; Rösler, Uwe; Hartung, Jörg (2012b): Longitudinal study of the contamination of air and of soil surfaces in the vicinity of pig barns by livestock-associated methicillin-resistant Staphylococcus aureus. In: *Applied and environmental microbiology* 78 (16), S. 5666–5671. DOI: 10.1128/AEM.00550-12.

Schwarz, Stefan; Kadlec, Kristina; Strommenger, Birgit (2008): Methicillin-resistant Staphylococcus aureus and Staphylococcus pseudintermedius detected in the BfT-GermVet monitoring programme 2004-2006 in Germany. In: *The Journal of antimicrobial chemotherapy* 61 (2), S. 282–285. DOI: 10.1093/jac/dkm487.

Seedorf, J.; Hartung, J.; Schröder, M.; Linkert, K. H.; Phillips, V. R.; Holden, M. R. et al. (1998): Concentrations and Emissions of Airborne Endotoxins and Microorganisms in Livestock Buildings in Northern Europe. In: *Journal of Agricultural Engineering Research* 70 (1), S. 97–109. DOI: 10.1006/jaer.1997.0281.

Sellera, Fábio P.; Lincopan, Nilton (2019): Zooanthroponotic transmission of high-risk multidrug-resistant pathogens: A neglected public health issue. In: *Journal of infection and public health* 12 (2), S. 294–295. DOI: 10.1016/j.jiph.2018.12.013.

Skerrett, Shawn J.; Braff, Marissa H.; Liggitt, H. Denny; Rubens, Craig E. (2017): Toll-like receptor 2 has a prominent but nonessential role in innate immunity to Staphylococcus aureus pneumonia. In: *Physiological reports* 5 (21). DOI: 10.14814/phy2.13491.

Slifierz, Mackenzie Jonathan; Friendship, Robert M.; Weese, J. Scott (2015): Methicillin-resistant Staphylococcus aureus in commercial swine herds is associated with disinfectant and zinc usage. In: *Applied and environmental microbiology* 81 (8), S. 2690–2695. DOI: 10.1128/AEM.00036-15.

Sørensen, Anna Irene Vedel; Toft, Nils; Boklund, Anette; Espinosa-Gongora, Carmen; Græsbøll, Kaare; Larsen, Jesper; Halasa, Tariq (2017): A mechanistic model for spread of livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) within a pig herd. In: *PloS one* 12 (11), e0188429. DOI: 10.1371/journal.pone.0188429.

Souza Xavier Costa, Natália de; Ribeiro Júnior, Gabriel; Dos Santos Alemany, Adair Aparecida; Belotti, Luciano; Zati, Douglas Hidalgo; Frota Cavalcante, Marcela et al. (2017): Early and late pulmonary effects of nebulized LPS in mice: An acute lung injury model. In: *PloS one* 12 (9), e0185474. DOI: 10.1371/journal.pone.0185474.

Spohr, M.; Rau, J.; Friedrich, A.; Klittich, G.; Fetsch, A.; Guerra, B. et al. (2011): Methicillin-resistant Staphylococcus aureus (MRSA) in three dairy herds in southwest Germany. In: *Zoonoses and public health* 58 (4), S. 252–261. DOI: 10.1111/j.1863-2378.2010.01344.x.

Springer, Burkhard; Orendi, Ulrike; Much, Peter; Höger, Gerda; Ruppitsch, Werner; Krziwanek, Karina et al. (2009): Methicillin- resistant Staphylococcus aureus: a new zoonotic agent? . In: *The Middle European Jurnal of Medicine* 2009 (121), S. 86–90, zuletzt geprüft am 24.08.2018.

Springorum, A. C.; Clauß, M.; Hartung, J. (2011): A Temperature-Controlled AGI-30 Impinger for Sampling of Bioaerosols. In: *Aerosol Science and Technology* 45 (10), S. 1231–1239. DOI: 10.1080/02786826.2011.588275.

Szabó, István; Beck, Britta; Friese, Anika; Fetsch, Alexandra; Tenhagen, Bernd-Alois; Roesler, Uwe (2012): Colonization kinetics of different methicillin-resistant Staphylococcus aureus sequence types in pigs and host susceptibilities. In: *Applied and environmental microbiology* 78 (2), S. 541–548. DOI: 10.1128/AEM.05327-11.

Tacconelli, Evelina; Angelis, Giulia de; Cataldo, Maria A.; Pozzi, Emanuela; Cauda, Roberto (2008): Does antibiotic exposure increase the risk of methicillin-resistant Staphylococcus aureus (MRSA) isolation? A systematic review and meta-analysis. In: *The Journal of antimicrobial chemotherapy* 61 (1), S. 26–38. DOI: 10.1093/jac/dkm416.

Takai, H.; Pedersen, S.; Johnsen, J. O.; Metz, J.H.M.; Groot Koerkamp, P.W.G.; Uenk, G. H. et al. (1998): Concentrations and Emissions of Airborne Dust in Livestock Buildings in Northern Europe. In: *Journal of Agricultural Engineering Research* 70 (1), S. 59–77. DOI: 10.1006/jaer.1997.0280.

Takeuchi, O.; Hoshino, K.; Akira, S. (2000): Cutting Edge: TLR2-Deficient and MyD88-Deficient Mice Are Highly Susceptible to Staphylococcus aureus Infection. In: *The Journal of Immunology* 165 (10), S. 5392–5396. DOI: 10.4049/jimmunol.165.10.5392.

Terzieva, Silva; Donnelly, Jean; Ulevicius, Vidmantas; Grinshpun, Sergey A.; Willeke, Klaus; Stelma, Gerald N.; Brenner, Kristen P. (1996): Comparison of Methods for Detection and

Enumeration of Airborne Microorganisms Collected by Liquid Impingement, S. 2264–2272. Online verfügbar unter https://www.ncbi.nlm.nih.gov/pmc/articles/PMC168007/pdf/622264.pdf, zuletzt geprüft am 23.08.2018.

Thorn, J. (2001): The inflammatory response in humans after inhalation of bacterial endotoxin: a review. In: *Inflammation research : official journal of the European Histamine Research Society … [et al.]* 50 (5), S. 254–261. DOI: 10.1007/s000110050751.

Tipper, Donald J. (1985): Mode of action of β-lactam antibiotics. In: *Pharmacology & Therapeutics* 27 (1), S. 1–35. DOI: 10.1016/0163-7258(85)90062-2.

Uehara, Y.; Nakama, H.; Agematsu, K.; Uchida, M.; Kawakami, Y.; Abdul Fattah, A. S.; Maruchi, N. (2000): Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp. In: *The Journal of hospital infection* 44 (2), S. 127–133. DOI: 10.1053/jhin.1999.0680.

Urbain, B.; Gustin, P.; Charlier, G.; Coignoul, F.; Lambotte, J. L.; Grignon, G. et al. (1996a): A morphometric and functional study of the toxicity of atmospheric ammonia in the extrathoracic airways in pigs. In: *Veterinary Research Communications* 20 (4), S. 381–399. DOI: 10.1007/BF00366545.

Urbain, Bruno; Beerens, Dominique; N´Guyen, T.Quyen; Goddeeris, Bruno; Ansay, M.; Gustin, P. (1999): Effect of inhalation of dust and endotoxin on respiratory tracts of pigs., zuletzt geprüft am 12.12.2018.

Urbain, Bruno; Prouvost, J. F.; Beerens, D.; Michel, O, Nicks, B; Ansay, M.; Gustin, P.: Chronic exposure of pigs to airborne dust and endotoxins in an environmental chamber: technical note, zuletzt geprüft am 09.10.2018.

Urbain, Bruno; Prouvost, Jean-Francois; Beerens, Domonique; Ansay, Michel; Gustin, Pascal (1996b): Acute Effects of Endotoxin Inhalation on the Respiratory Tract in Pigs: Interaction with Ammonia. In: *Inhalation Toxicology* 8 (9), S. 947–968. DOI: 10.3109/08958379609034272.

van Cleef, B. A. G. L.; Broens, E. M.; Voss, A.; Huijsdens, X. W.; Züchner, L.; van Benthem, B. H. B. et al. (2010): High prevalence of nasal MRSA carriage in slaughterhouse workers in contact with live pigs in The Netherlands. In: *Epidemiology and infection* 138 (5), S. 756–763. DOI: 10.1017/S0950268810000245.

van Cleef, Brigitte A. G. L.; Graveland, Haitske; Haenen, Anja P. J.; van de Giessen, Arjen W.; Heederik, Dick; Wagenaar, Jaap A.; Kluytmans, Jan A. J. W. (2011): Persistence of livestock-associated methicillin-resistant Staphylococcus aureus in field workers after short-

term occupational exposure to pigs and veal calves. In: *Journal of clinical microbiology* 49 (3), S. 1030–1033. DOI: 10.1128/JCM.00493-10.

van den Broek, I. V. F.; van Cleef, B. A. G. L.; Haenen, A.; Broens, E. M.; van der Wolf, P. J.; van den Broek, M. J. M. et al. (2009): Methicillin-resistant Staphylococcus aureus in people living and working in pig farms. In: *Epidemiology and infection* 137 (5), S. 700–708. DOI: 10.1017/S0950268808001507.

van Duijkeren, E.; Ikawaty, R.; Broekhuizen-Stins, M. J.; Jansen, M. D.; Spalburg, E. C.; Neeling, A. J. de et al. (2008): Transmission of methicillin-resistant Staphylococcus aureus strains between different kinds of pig farms. In: *Veterinary microbiology* 126 (4), S. 383–389. DOI: 10.1016/j.vetmic.2007.07.021.

van Duijkeren, Engeline; Greko, Christina; Pringle, Märit; Baptiste, Keith Edward; Catry, Boudewijn; Jukes, Helen et al. (2014): Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health. In: *The Journal of antimicrobial chemotherapy* 69 (8), S. 2022–2031. DOI: 10.1093/jac/dku123.

Vanderhaeghen, W.; Hermans, K.; Haesebrouck, F.; Butaye, P. (2010a): Methicillin-resistant Staphylococcus aureus (MRSA) in food production animals. In: *Epidemiology and infection* 138 (5), S. 606–625. DOI: 10.1017/S0950268809991567.

Vanderhaeghen, Wannes; Cerpentier, Tineke; Adriaensen, Connie; Vicca, Jo; Hermans, Katleen; Butaye, Patrick (2010b): Methicillin-resistant Staphylococcus aureus (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. In: *Veterinary microbiology* 144 (1-2), S. 166–171. DOI: 10.1016/j.vetmic.2009.12.044.

Verhegghe, M.; Pletinckx, L. J.; Crombé, F.; Vandersmissen, T.; Haesebrouck, F.; Butaye, P. et al. (2013): Methicillin-resistant Staphylococcus aureus (MRSA) ST398 in pig farms and multispecies farms. In: *Zoonoses and public health* 60 (5), S. 366–374. DOI: 10.1111/zph.12007.

Verkade, Erwin; van Benthem, Birgit; den Bergh, Marjolein Kluytmans-van; van Cleef, Brigitte; van Rijen, Miranda; Bosch, Thijs; Kluytmans, Jan (2013): Dynamics and determinants of Staphylococcus aureus carriage in livestock veterinarians: a prospective cohort study. In: *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 57 (2), e11-7. DOI: 10.1093/cid/cit228.

Verstappen, Koen M.; Duim, Birgitta; van Nes, Arie; Snijders, Susan; van Wamel, Willem J. B.; Wagenaar, Jaap A. (2014): Experimental nasal colonization of piglets with methicillin-susceptible and methicillin-resistant Staphylococcus aureus. In: *Veterinary microbiology* 174 (3-4), S. 483–488. DOI: 10.1016/j.vetmic.2014.09.019.

9. References

Verstappen, Koen M.; Willems, Eveline; Fluit, Ad C.; Duim, Birgitta; Martens, Marc; Wagenaar, Jaap A. (2017): Staphylococcus aureus Nasal Colonization Differs among Pig Lineages and Is Associated with the Presence of Other Staphylococcal Species. In: *Frontiers in veterinary science* 4, S. 97. DOI: 10.3389/fvets.2017.00097.

Vincze, Szilvia; Brandenburg, Anja G.; Espelage, Werner; Stamm, Ivonne; Wieler, Lothar H.; Kopp, Peter A. et al. (2014a): Risk factors for MRSA infection in companion animals: results from a case-control study within Germany. In: *International journal of medical microbiology : IJMM* 304 (7), S. 787–793. DOI: 10.1016/j.ijmm.2014.07.007.

Vincze, Szilvia; Stamm, Ivonne; Kopp, Peter A.; Hermes, Julia; Adlhoch, Cornelia; Semmler, Torsten et al. (2014b): Alarming proportions of methicillin-resistant Staphylococcus aureus (MRSA) in wound samples from companion animals, Germany 2010-2012. In: *PloS one* 9 (1), e85656. DOI: 10.1371/journal.pone.0085656.

Voss, Andreas; Loeffen, Frans; Bakker, Juduth; Klaasen, Corne; Wulf, Mireille W. (2005): Methicillin-resistant Staphylococcus aureus in Pig Farming. In: *Emerging infectious diseases* 2005 (12), S. 1965–1966, zuletzt geprüft am 24.08.2018.

Wallgren, Per; Wilén, Inga-Lill; Fossum, Caroline (1994): Influence of experimentally induced endogenous production of cortisol on the immune capacity in swine. In: *Veterinary immunology and immunopathology* 42 (3-4), S. 301–316. DOI: 10.1016/0165-2427(94)90075-2.

Weese, J. S.; Zwambag, A.; Rosendal, T.; Reid-Smith, R.; Friendship, R. (2011): Longitudinal investigation of methicillin-resistant Staphylococcus aureus in piglets. In: *Zoonoses and public health* 58 (4), S. 238–243. DOI: 10.1111/j.1863-2378.2010.01340.x.

Weidenmaier, Christopher; Goerke, Christiane; Wolz, Christiane (2012): Staphylococcus aureus determinants for nasal colonization. In: *Trends in microbiology* 20 (5), S. 243–250. DOI: 10.1016/j.tim.2012.03.004.

Wulf, Mireille W.; van Nes, Arie; Eikekenboom-Boskamp, Andrea; Vries, Janneke de; Melchers, Willem; Klaassen, Corné, Voss, Andreas (2006): Methicillin-resistant Staphylococcus auresu in Veterinary Doctors and Sudents, the Netherlands. In: *Emerging infectious diseases* 2006 (12). Online verfügbar unter https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3291345/pdf/06-0355.pdf, zuletzt geprüft am 23.08.2018.

Wyns, Heidi; Meyer, Evelyne; Watteyn, Anneleen; Plessers, Elke; Baere, Siegrid de; Backer, Patrick de; Croubels, Siska (2013): Pharmacokinetics of dexamethasone after intravenous and intramuscular administration in pigs. In: *Veterinary journal (London, England : 1997)* 198 (1), S. 286–288. DOI: 10.1016/j.tvjl.2013.06.015.

9. References

Yang, Jingjing; Dai, Lei; Yu, Qinghua; Yang, Qian (2017): Histological and anatomical structure of the nasal cavity of Bama minipigs. In: *PloS one* 12 (3), e0173902. DOI: 10.1371/journal.pone.0173902.

Zejda; Jan. E.; Barber, Ernest; Dosman; James A.; Olenchock, Stephen A. et al. (1994): Respiratory Health Status in Swine Producers Relates to Endotoxin Exposure in the Presence of Low Dust Levels. In: *JOM* 1994 (1), 49-56, zuletzt geprüft am 09.10.2018.

Zhao, Yang; Aarnink, AndrÉ J. A.; Jong, Mart C. M. de; Groot Koerkamp, Peter W. G. (2014): Airborne Microorganisms From Livestock Production Systems and Their Relation to Dust. In: *Critical Reviews in Environmental Science and Technology* 44 (10), S. 1071–1128. DOI: 10.1080/10643389.2012.746064.

IV. List of publications

Publications

Rosen, K.; Roesler, U.; Merle, R.; Friese, A. Persistent and Transient Airborne MRSA Colonization of Piglets in a Newly Established Animal Model. *Frontiers in Microbiology*; 9:1542; doi: 10.3389/fmicb.2018.01542

Kerstin Rosen, Friederike Ebner, Stefanie Schmidt, Susanne Hartmann, Roswitha Merle, Anika Friese, Uwe Roesler (2018): Influence of immune status on the airborne colonization of piglets with methicillin-resistant *Staphylococcus aureus* (MRSA) clonal complex (CC) 398. *European Journal of Microbiology and Immunology;* doi: 10.1556/1886.2019.00024

Presentations

Rosen, K.; Ebner, F.; Friese, A.; Hartmann, S.; Rösler, U. Evaluation of predisposing factors for a long-time colonization of pigs with livestock-associated MRSA via the airborne route in a newly established animal model. National Symposium on Zoonoses Research 2017: 12.10.-13.10.2017; Program and Abstracts, Berlin

Rosen, K.; Ebner, F.; Friese, A.; Rösler, U. Aerogene MRSA-Kolonisierung von Schweinen - Immunsuppression als prädisponierender Faktor? 3. Tagung der DVG-Fachgruppe Umwelt- und Tierhygiene; 30.11.-01.12.2017.

Rosen, K.; Ebner, F.; Friese, A.; Rösler, U. Experimental colonization of piglets with livestock-associated MRSA via the 225/PRV airborne route. VAAM Annual Meeting 2017 69th Annual Meeting of the DGHM 05.03.2017

Rosen, K.; Ebner, F.; Friese, A.; Rösler, U. Experimentelle MRSA-Kolonisierung von Schweinen in einer Aerosolkammer - Immunsuppression als prädisponierender Faktor.
Tagung der DVG-Fachgruppe Bakteriologie und Mykologie. 30.05.-01.06.2018.

Rosen, K.; Ebner, F.; Friese, A.; Rösler, U. Airborne MRSA colonization of piglets - Investigation of predisposing factors. 70. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie; 19.02.2018

K. Rosen, F. Ebner, A. Friese, U. Roesler Influence of the immune system on the airborne LA-MRSA colonization of piglets. XIX International Congress of ISAH 2019

Poster

Rosen, K.; Rösler, U.; Friese, A. Exposure of piglets with MRSA via the airborne route. National Symposium on Zoonoses Research 2016; 13.10.-14.10.2016.

Rosen, K.; Friese, A.; Roesler, U. Airborne colonization of piglets with livestock-associated MRSA. Expertenforum „Bioaerosole - Von der Messung zur Bewertung"; 26.09.2017

Rosen, K.; Ebner, F.; Friese, A.; Roesler, U. Airborne colonization of piglets with livestock-associated MRSA. 7th Symposium on Antimicrobial Resistance in Animals and the Environment; 26.06.2017, Braunschweig

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VI. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den 10.11.2020

Kerstin Rosen