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Airborne MRSA and Total *Staphylococcus aureus* as Associated With Particles of Different Sizes on Pig Farms

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Abstract

Airborne methicillin-resistant *Staphylococcus aureus* (MRSA) have previously been found on pig farms, which may lead to nasal deposition of MRSA in humans via inhalation. The anterior nares are the main niche for *S. aureus*, and *S. aureus* can cause, e.g. wound infection and pneumonia. The aim of this study was to acquire knowledge about the potential deposition of airborne MRSA, specifically, and of total *S. aureus* (including both methicillin-sensitive *S. aureus* and MRSA, in the following called *S. aureus*) in the different parts of the airways during occupancy on pig farms. Measurements of airborne MRSA and *S. aureus* were performed on four pig farms using a six and a three-stage sampler during different work tasks, such as high-pressure cleaning and everyday inspection. MRSA were quantified using MRSA-selective agar, and *S. aureus* were quantified using *Staphylococcus* selective agar. The identity of the bacteria were confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The geometric mean (GM) concentrations of MRSA and *S. aureus* were 447 cfu/m³ air and 1.8×10^3 cfu/m³ air, respectively. The highest concentrations of MRSA and *S. aureus* were found among pigs in a weaner stable and during high-pressure cleaning of an empty stable, respectively. The lowest concentrations of MRSA and *S. aureus* were found in a stable with sick pigs and in feed-storages, respectively. Most MRSA and *S. aureus* were associated with particles between 7 and 12 μm. On average, the particle size fractions potentially depositing in the upper airways constituted 70%, in the primary and secondary bronchi 22%, and in the terminal bronchi and alveoli 8% of the inhalable MRSA and *S. aureus* concentration. Across the sampled areas, the geometric mean diameter (D_g) of particles with MRSA and *S. aureus* were 7.2 and 6.4 μm, respectively, and no significant difference was found between these D_gs. The D_g of the airborne particles with the studied bacterium was significantly associated with the different locations on the farms. The largest D_gs were found in the air samples from the aisles and on the fence to the pens, while the smallest D_gs were found in samples from the pens among the pigs and in samples taken at greater distances from the pigs: in the hallway, feed-storage, and entry room. In conclusion, airborne MRSA and *S. aureus* were

found in sample fractions potentially depositing in all six parts of the airways. However, the majority was found to potentially deposit in the upper airways. The concentration of airborne MRSA and *S. aureus* and MRSA, as well as the fraction potentially depositing in the different parts of the airways, depended on the specific work task being performed and the location on the farm.

Keywords: airway deposition; bioaerosol; dust; MRSA; occupational exposure; particle size; *Staphylococcus aureus*

Introduction

Airborne methicillin-resistant *Staphylococcus aureus* (MRSA) have previously been found in pig barns constituting 802 colony forming units (cfu)/m³ air [geometric mean value (GM)] (Friese *et al.*, 2012), and recently, it has been shown that nasal deposition of MRSA on pig farms can occur via inhalation (Angen *et al.*, 2017). The anterior nares are described as the main niche for *S. aureus* (both resistant and non-resistant to methicillin), and nasal carriage is one of the main risk factors for *S. aureus* infection (van Belkum *et al.*, 2009). *S. aureus* is an opportunistic human pathogen (Aires de Sousa and Lencastre, 2004), which can cause, e.g. wound infections (Ruhlmann et al., 2008), and, mainly for weakened individuals, also pneumonia (Witte *et al.*, 2007), thoracic empyema (Lozano *et al.*, 2011), and bacteremia (Berning *et al.*, 2015). The MRSA are of special concern because of their resistance to β-lactam antibiotics, which makes infections difficult to treat.

Exposure to MRSA on farms is an occupational health problem affecting people working on the farms (van Cleef *et al.*, 2010; Geenen *et al.*, 2013). Except for nasal deposition (Létourneau *et al.*, 2010; Angen *et al.*, 2017; Goerge *et al.*, 2017), the knowledge of deposition or potential deposition of MRSA in the different parts of the airways of exposed workers on pig farms is very limited. Yet, this knowledge is important since the place of deposition affects the potential health effects caused by the bacteria. Laboratory generated aerosols of *S. aureus* have particle sizes between 0.542 and 1.197 µm and aerodynamic particle sizes between 0.723 and 0.777 µm (Lutz, 2010). However, airborne bacteria are often present in clusters and/or associated with other particles. Thus, the deposition of bacteria in the airways depends on the aerodynamic diameter of the particle with which they are associated, and consequently determines the potential site the bacterium may initially infect (Thomas, 2013). In pig houses in the USA, mesophilic bacteria and *Staphylococcus* spp. have mainly been found on particles larger than 4 µm; in contrast, a slightly larger fraction of the total amount of *Lactobacillus* spp. was found on particles smaller than 4 µm (Predicala *et al.*, 2002). In the farm environment, we do not expect MRSA to be present only as individual bacterial cells, but also as clusters of bacteria or associated with other particles such as fragments of skin cells from the pigs. MRSA may be aerosolized into the air in the stable directly from the pigs, either from mucus or exfoliated epithelial skin particles (Zhao *et al.*, 2014), but they may also be re-aerosolized from all surfaces in the stable, resulting in a broader range of MRSA particle sizes. Furthermore, differences regarding the size distributions of particles carrying microorganisms within a specific type of environment and even within the same facility may also exist (Clauss, 2015). Therefore, it is important to acquire knowledge of the size distribution of MRSA aerosolized during different work tasks in pig farms.

The aerodynamic diameter of a particle also influences how long time it stays airborne and thus also for how long the potential human exposure of the airways to the airborne bacterium occurs. Furthermore, the particle size influences the ability of the bacterium to penetrate filters of dust masks and to disperse between rooms or to the outside, e.g. through cracks. The Andersen sampler has been used for decades and in several studies to measure airborne bacteria to evaluate how they potentially may deposit in the airways (Beaumont *et al.*, 1985; Buttner and Stetzenbach, 1991; Chang *et al.*, 2001; Uhrbrand *et al.*, 2017). The six-stage Andersen sampler (ASCI) samples airborne particles within six health-relevant size ranges.

In this study, we investigate the size distribution of airborne MRSA, quantified on MRSA-selective agar, and total *S. aureus* [methicillin-sensitive *S. aureus* (MSSA) and MRSA, in the following called *S. aureus*], quantified on *S. aureus* selective agar, on four pig farms in relation to deposition in the airways. The measurements were performed during miscellaneous work tasks, such as high-pressure cleaning of an empty stable, tail docking, and everyday inspection, and in different locations on the farms, including stables with pigs of different ages.

Materials and Methods

Farms and design

Airborne bacteria were sampled on four pig farms in autumn and winter in 2015–2017 in Jutland and Zealand in Denmark. The farms were specialized in

breeding pigs of different stages, and due to concern about disease transmission between sections of the farms we were not allowed to visit all areas of the farms. Also, at least 21 days occurred between visits on the different farms. Farm A, was specialized in fattening pigs and measurements were done in the autumn, 2015, in this section during an everyday inspection, and in a feed-storage room connected by a closed door to a stable with fattening pigs. Measurements on Farm B were done in the winter, 2016, during two consecutive days in three locations: An entry room, a feed-storage room from where the feed was automatically transported into the stables but otherwise connected by a closed door, and a farrowing section where a farmer docked the tails of the piglets. On Farm C, measurements were done in the winter, 2016, during three days in three locations: A weaner and a farrowing section during an everyday inspection, and a hallway outside the weaner section. On Farm D, measurements were done in the winter, 2017, in two locations: A stable with sick pigs of different ages, which had been transferred from other sections, and an empty stable during high-pressure cleaning. In general, the most active pigs were the pigs in the weaner section, followed by pigs in the farrowing section, and then the fattening pigs while the most passive were the sick pigs. All measurements were done between 7 a.m. and 7 p.m. on days with outdoor temperatures between –2 and 11°C.

Sampling of airborne bacteria using the ASCI

In total, 77 air samples were taken using the ASCI (Thermo Fisher Scientific Inc., Waltham, MA, USA). The ASCI is an active size-selective sampler, which samples directly onto six agar plates with a flow rate of 28.3 lpm. Particles of the following sizes were sampled: Stage 1: 7.0–12 μm, Stage 2: 4.7–7.0 μm, Stage 3: 3.3–4.7 μm; Stage 4: 2.1–3.3 μm; Stage 5: 1.1–2.2 μm, and Stage 6: 0.65–1.1 μm. The sum of all stages represents the inhalable bacteria, and the sum of stages 3, 4, 5, and 6 represents the respirable bacteria, while the sum of stages 1 and 2 represents the fraction potentially depositing in the upper airways. The ASCI was mounted with Brilliance MRSA 2 agar plates (in the following called MRSA-agar; Oxoid) for sampling and quantification of MRSA or with SaSelect agar plate (in the following called SA-agar; Bio-RAD, Marnes-la-Coquette, France) for sampling and quantification of airborne *S. aureus*. The two agar media were chosen as we wanted to obtain knowledge about the concentrations of *S. aureus* present both as MRSA as well as total culturable *S. aureus* (which includes MRSA) within the different size fractions. For clinical isolates, MRSA-selective agar has shown a specificity of 94% (Verkade *et al.*, 2011). In clinical samples,

MRSA-selective agar media can underestimate the number of MRSA-positive samples (Veenemans *et al.*, 2013). This may also be the case for the farm environment, and, thus, another reason to also include the SA-agar.

Sampling on MRSA-agar was done for 4–20 min and on SA-agar for 2–20 min. The detection limits depend on the duration of each sampling and were between 1.8 and 18 cfu/m^3. Other bacteria also grew on both agar types, and all colonies of MRSA and *S. aureus* were identified by color and subsequently by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). On Farm B, measurements were done using only SA-agar as we knew from a previous study that MRSA only constituted a minor fraction of the *S. aureus* (Feld *et al.*, 2018).

On farm A and D, samples were taken repeatedly, but with durations of 4, 10, and 20 min to study the effect of sampling time on the measured D_g of MRSA and *S. aureus*.

Farmers worked either in an upright position, slightly stooping, or stooping all the way down. Consequently, we measured in three heights: (i) Sampling at chest level (1.50 m) was done in the aisles, the feed-storage rooms, a windowsill, and a hallway, (ii) at approximately 1.0 m during high-pressure cleaning, and on the fence between the pen and the aisle, and (iii) 20 cm above floor level among the piglets (in the following called floor level).

Sampling using the DGI and the Respicon

A DGI-1570 Gravimetric Impactor (Dekati, Tampere, Finland) was used to sample particles according to size. Sampling was performed in the aisle once at Farms A, C, and D for 15 min at a height of 1.5 m. The sampler separates particles into four size fractions based on aerodynamic diameters with lower cut points of 0.264, 0.608, 1.200, and 2.968 μm when used at a flow rate of 50 lpm. In addition, particles < 0.264 μm is collected to the backup filter; that filter was not analyzed in this study. Airborne particles were sampled on polycarbonate filters (47 mm diameter, pore size 1 μm, Nuclepore, Whatman). The particles in the two smallest size fractions were pooled for analysis. The filters were extracted as described earlier (Madsen *et al.*, 2014), and aliquots of 500 μl and 100 μl were plated on both SA-agar and MRSA-agar. The detection limit was 27 cfu/m^3.

The Respicon (TSI Incorporated, MN, USA) sampled in the aisles for 3 h, at a height of 1.5 m, once at each farm; after 1 h of sampling, a sample was also collected using the ASCI for 10 min. The Respicon has been shown to meet the ACGIH/ISO/CEN particle size-selective sampling criteria (Koch *et al.*, 2002; Tatum *et al.*, 2002). Stage 1 of the device collects respirable particles and

stage 2 collects tracheobronchial particles. Extrathoracic particles are collected on stage 3 of the Respicon. Therefore, particles collected with the Respicon sampler are categorized by the appropriate combination of the results from the three stages; thus, thoracic = respirable (stage 1) + tracheobronchial (stage 2), and inhalable = thoracic + extrathoracic (stage 3). The Respicon was fitted with Teflon filters and used at a flow rate of 3.1 lpm. The dust on the filters was extracted in 5.0 ml solution (0.85% NaCl and 0.05% Tween 80) by orbital shaking (500 rpm) for 15 min at room temperature, and aliquots of 500 µl and 100 µl were plated on four SA-agar and four MRSA-agar plates. The detection limit for each agar type was 3.6 cfu/m^3.

Species identification by MALDI-TOF MS

To verify the species identity of bacterial colonies with specific morphology on the selective agar plates, we used MALDI-TOF MS. Isolates resembling MRSA and *S. aureus* were identified from MRSA-agar and SA-agar, respectively (Madsen *et al.*, 2016). On SA plates with samples from Dekati Gravimetric Impactor (DGI) filters, all colonies were identified. A toothpick was used to transfer a small amount of the bacterial colony onto the target plate (MSP 96 target polished steel BC, Bruker Daltonics, Bremen, Germany). The sample was then overlaid with 70% formic acid and allowed to dry before addition of an HCCA matrix solution (α-cyano-4-hydroxycinnamic acid, Bruker Daltonics). The MALDI-TOF MS analysis was performed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Bruker Biotyper 3.1 software with the BDAL standard library. A bacterial test standard (Bruker Daltonics) was used to calibrate the instrument.

In the following, bacteria identified as *S. aureus* on MRSA-agar are presented as MRSA, and bacteria identified as *S. aureus* on SA-agar are presented as *S. aureus* or total *S. aureus*, even though unknown quantities of the latter are MRSA. The MRSA on all farms were previously determined to be CC398 (Feld *et al.*, 2018).

Treatment of data

SAS version 9.4 (SAS Institute, Cary, NC, USA) was used for statistical analysis. The bacteria measured by the ASCI are presented in several different ways: As the D_g calculated from sizes and quantities; as concentrations (cfu/m^3) of the six size fractions; as the percentage each fraction constitutes out of the total concentration; as the fraction depositing in the upper airways (Stages 1 and 2), in the primary and secondary bronchi (Stages 3 and 4), in the terminal bronchi and alveoli (Stages 5 and 6); and as respirable bacteria (Stages 3, 4, 5, and 6). Three samples

on SA-agar from the empty stable were overgrown, and data from these samples are presented as minimum values in Fig. 1, but are excluded from the calculations of D_g and percentage each fraction constitute. The data on the percentages, each fraction constituted, were normally distributed. The average, as well as the standard deviation (SD), was calculated from the fraction percentages.

The effect of sampling duration (4, 10, and 20 min) on the measured D_g of particles with *S. aureus* and MRSA was compared for 3 × 4 samples using General Linear Models (GLM). The effect of farm and sampling day on D_g of particles with *S. aureus* and MRSA as measured among pigs was studied using GLM.

The D_gs of airborne *S. aureus* and MRSA in different locations were compared across farms. To do this, the sampled locations were divided into the following categories (where *n* = number of D_gs of total *S. aureus* + number of D_gs of MRSA): (i) the pen, among piglets, floor level, *n* = 8 + 4; (ii) the pen fence, *n* = 8 + 9; (iii), in the aisle between the pens, *n* = 16 + 14; (iv) in an empty stable during high-pressure cleaning, *n* = 3 (MRSA only); (v) hallway, between weaner sections, *n* = 3 + 2; (vi) in the feed-storages and entry room, *n* = 1 + 4. In total, 72 D_gs were used for the comparison. The D_gs were compared using GLM. Subsequently, some locations were pooled further into three groups based on the D_gs, and using GLM, each size fraction of *S. aureus* and MRSA as sampled by the ASCI was compared across the groups.

The bacteria measured by the Respicon are presented as the concentrations (cfu/m^3) of the three size fractions and as the percentage, each fraction constitutes out of the total concentration. The concentrations and the size fractions (%) of respirable MRSA and *S. aureus* as measured using the Respicon and the ASCI were compared using a paired *t*-test. The Pearson's correlations between the concentrations and the size fractions as measured using the Respicon and the ASCI were calculated. Only values above the detection limits are included in the calculations.

Results

Initial sampling using three samplers

The concentrations as measured using the DGI on farms A, C, and D showed no presence of *S. aureus* and MRSA, but other *Staphylococcus* species, *S. epidermidis*, *S. haemolyticus*, *S. chromogenes*, *S. simulans*, and *S. warneri*, were present. Most bacteria (80–86%) were found in the fraction with the largest particles (cut point 2.968 µm).

The concentrations as measured using the Respicon and ASCI showed presence of *S. aureus* on all farms (Table 1). The ASCI and the Respicon sampled in parallel
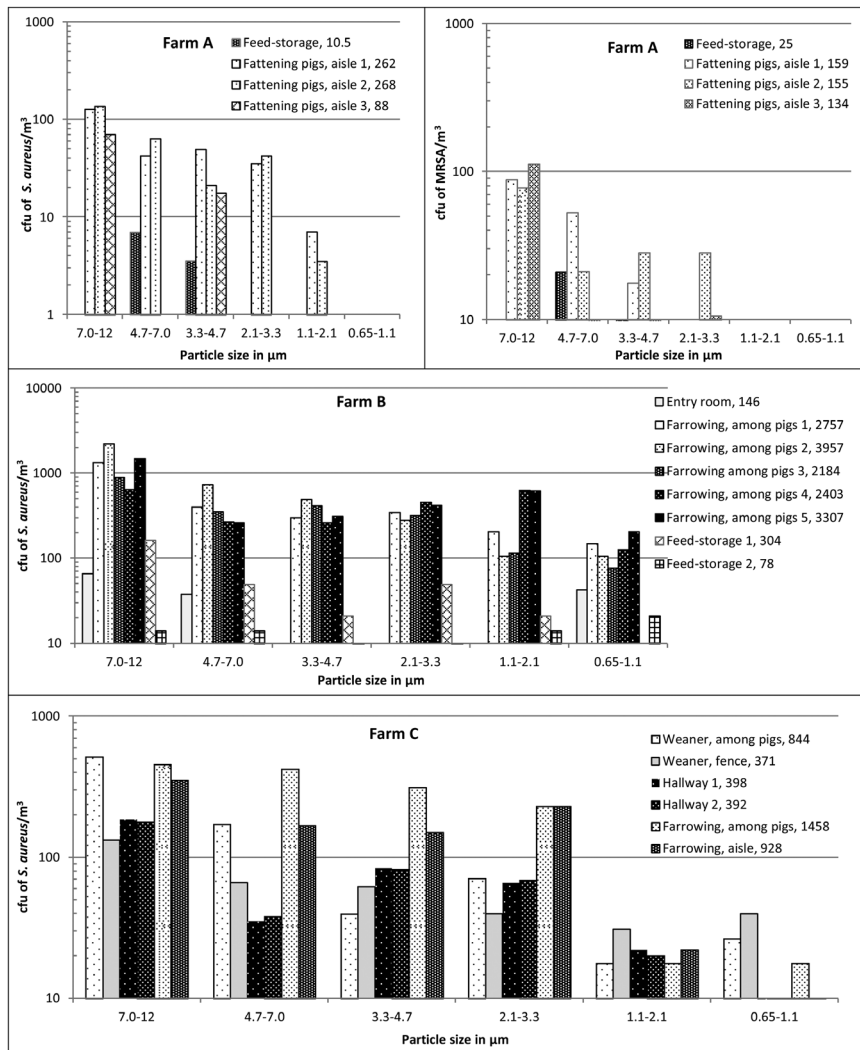
Figure 1. The concentration of *S. aureus* (both methicillin-sensitive *S. aureus* and MRSA) and MRSA as a function of particle size in different areas and during different work tasks on pig farms A to D. On Farm A, measurements were done in a stable with fattening pigs and in a feed-storage next to that stable. On Farm B, measurements were done in an entry room next to the door to the outside, in the farrowing section among the piglets, and in a feed-storage next to the stable. On Farm C, in the windowsill in a weaner section, on the fence between the pig-pen and the aisle in the weaner and farrowing section, in the hallway outside the weaner section, in the farrowing section among the pigs, and in the aisle. On Farm D, in an empty stable during high-pressure cleaning and in a stable with sick pigs of different ages. The last numbers in the legends are the sums (cfu/m³) of the cfu of *S. aureus* and MRSA in the six size fractions. Where no bars are present, measurements were below the detection level. The solid black line in the *S. aureus* figure for Farm D illustrates the highest measurable level, and thus 12 measurements were above this level.

in the same area and at the same time; the Respicon, however, sampled for 3 h, while the ASCI sampled for 10 min. The respirable ($P = 0.22$, $n = 6$) and inhalable ($P = 0.62$, $n = 6$) concentrations and the respirable fractions ($P = 0.82$, $n = 6$) of *S. aureus* and MRSA were not significantly affected by sampling method. The concentrations ($r = 0.99$, $P < 0.0001$, $n = 6$) and fractions

($r = 0.87$, $P = 0.024$, $n = 6$) of respirable *S. aureus* and MRSA as sampled by the Respicon correlated significantly with concentrations and fractions obtained by the ASCI. The same was found for the inhalable concentrations ($P < 0.0001$).

Sampling time did not affect the measured D_g of particles with *S. aureus* and MRSA as sampled using
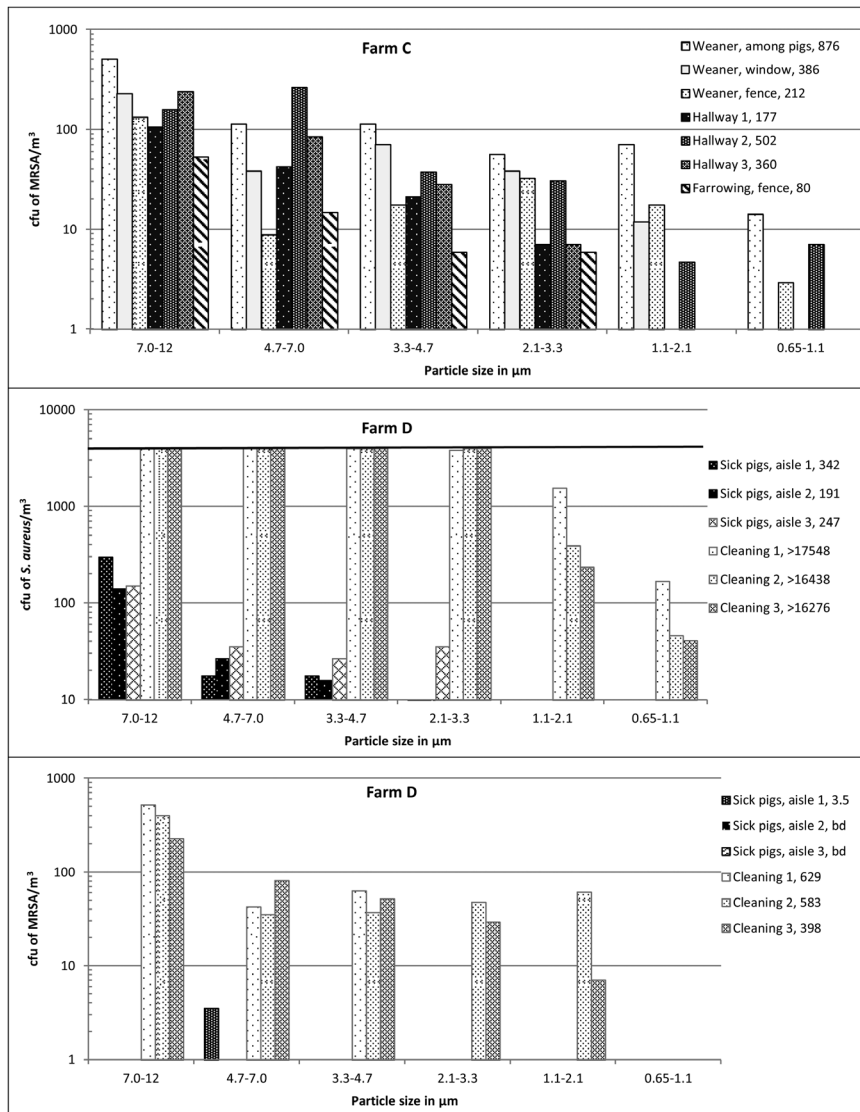
Figure 1. Cont

the ASCI (P = 0.42). Thus, the average D_g for a 4-min sampling was 7.9 µm (SD = 0.50 µm, n = 4), for 10 min 7.3 µm (SD = 0.88 µm, n = 4) and for 20 min 7.7 µm (SD = 0.63 µm, n = 4). Consequently, data obtained using the ASCI but with different sampling durations are treated together.

Concentrations of airborne *S. aureus* and MRSA as measured using the ASCI

Airborne *S. aureus* were found in all 44 measurements with the ASCI on the four farms while MRSA were detected in 31 of 33 measurements on three farms. On Farm B, we did not measure MRSA with the ASCI as

previous measurements showed a very low concentration of MRSA.

In [Fig. 1](#), concentrations of *S. aureus* and MRSA are presented for one sampling day at each farm. The GM concentration of inhalable *S. aureus* and MRSA were 1.8×10^3 cfu/m³ air (average = 5.1×10^3 cfu/m³; range: 77 to > 1.7×10^4 cfu/m³; n = 44) and 447 cfu/m³ air (average = 1.1×10^3 cfu/m³; range: below detection level (bd) to 6.6×10^3 cfu/m³; n = 33), respectively. The highest concentrations of *S. aureus* and MRSA were found during high-pressure cleaning of an empty stable and among pigs in a weaner stable, respectively. The lowest concentrations were found for *S. aureus* in feed-storages

Table 1. Concentrations (CFU/m³ and percentage[a]) of inhalable, thoracic, and respirable *S. aureus* and MRSA as measured using the Respicon, and inhalable and respirable *S. aureus* and MRSA as measured using the ASCI in the same area in the aisles.

Farm	Place	*S. aureus*[b]					MRSA				
		Respicon			ASCI		Respicon			ASCI	
		Inhalable	Thoracic	Respirable	Inhalable	Respirable	Inhalable	Thoracic	Respirable	Inhalable	Respirable
A	Fattening pigs	227	113	91	246	80	219	95	61	266	88
			46%	37%		30%		43%	28%		33%
B	Farrowing	2.6×10^3	850	732	2.9×10^3	990	100	34	29	Nm	Nm
			33%	29%		34%		31%	27%		
C	Weaner	327	194	138	441	172	202	113	93	151	85
			59%	42%		45%		56%	46%		41%
D	Sick pigs	211	62	32	188	43	16	5.6	3.6	Bd	Bd
			32%	17%		19%		36%	23%		

[a]Percentage out of total inhalable *S. aureus* or MRSA.

[b]Both methicillin-sensitive *S. aureus* and MRSA.

nm = not measured, ASCI = Andersen six-stage sampler, bd = below the detection limit, nm = not measured.

(average = 131 cfu/m³; SD = 154 cfu/m³; *n* = 3). For MRSA, the lowest concentration was found in a stable with sick pigs (average = 1.2 cfu/m³; SD = 1.7 cfu/m³; *n* = 3). In a farrowing section where pigs were being tail-docked, an average concentration of 3.0×10^3 cfu *S. aureus*/m³ (SD = 642 cfu/m³; *n* = 5) was found. The concentration of respirable *S. aureus* and MRSA were between 17.7 and > 9.5×10^3 cfu/m³ and bd and 975 cfu/m³, respectively. As an example, the average concentration of respirable *S. aureus* in the feed-storages was 48 cfu/m³ (SD = 44 cfu/m³; *n* = 3).

Size distribution of airborne MRSA and *S. aureus* as measured using the ASCI

Most airborne *S. aureus* and MRSA were associated with particles between 7 and 12 μm (Fig. 1). Across the six areas, the GM of the D_gs for *S. aureus* and MRSA were 6.4 and 7.2 μm, respectively, and no significant difference was found between D_gs for *S. aureus* and MRSA (*P* = 0.78). The D_g was measured among pigs in the farrowing section five times at farm B and two times at farm C, and no effect of farm was found (*P* = 0.30). The D_g was measured on farm C during 2 days in the weaner section among the pigs (*P* = 0.67; 7 samples), during 3 days in the aisle (*P* = 0.83; 18 samples), and with the ASCI placed on the pen fence (*P* = 0.99; 14 samples) and no significant effect of sampling day was found.

When all data for D_gs of MRSA and *S. aureus* were studied together in one model, the D_g was not affected by date of sampling (*P* = 0.92), but by location on the farms (*P* = 0.0018). The largest D_gs were found in the samples from the aisle, the fence to the pens, and during high pressure cleaning, while the smallest D_gs were found in the samples from the pens among the pigs and samples taken at greater distances from the pigs, such as in the hallway, feed-storage, and entry room (Fig. 2).

On average, the fractions of *S. aureus* and MRSA potentially depositing in the upper airways constituted 70% (SD = 15%; max = 93%), in the primary and secondary bronchi 22% (SD = 12%; max = 41%), and in the terminal bronchi and alveoli 8% (SD = 11%; max = 45%) of the concentration of inhalable *S. aureus* and MRSA. For the particles depositing in the nose, the fraction of *S. aureus* and MRSA present in the samples from the aisle and from the fence to the pens was greater than in the samples from inside the pens among the pigs and the samples from the hallway, feed-storage, and entry room considered together. The opposite was seen for the fractions potentially depositing in the pharynx and alveoli (Table 2).

Discussion

This study shows that airborne MRSA and *S. aureus* are present on particles with aerodynamic diameters that can be deposited in the human upper airways, the primary, secondary, and terminal bronchi, and the alveoli. *S. aureus* and MRSA were measured as culturable bacteria, as only viable bacteria are able to colonize and infect the airways. The fact that the largest fractions potentially deposit in the upper airways is interesting since the anterior nares are the main niche for *S. aureus*, and persistent nasal carriage is a main risk factor for *S. aureus* infection (Wertheim *et al.*,
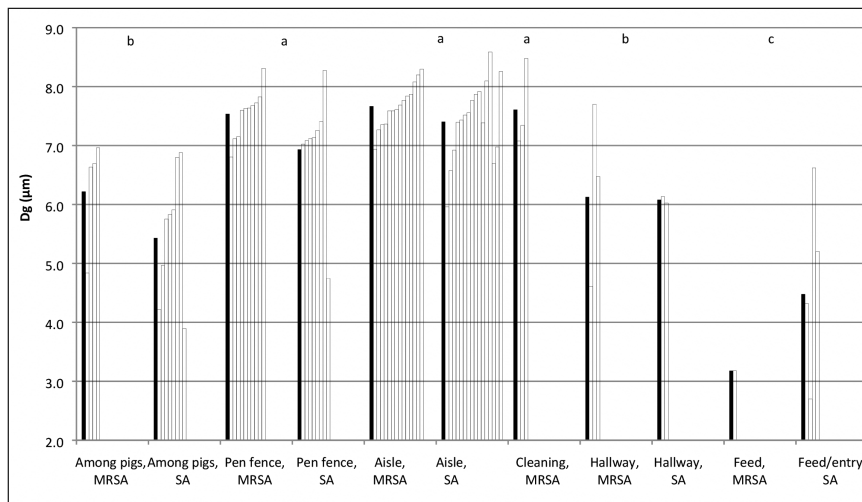
Figure 2. Geometric mean diameter (D$_g$) of airborne MRSA and *S. aureus* (SA) in six areas (among pigs, floor level, *n* = 4 + 8; pen fence, on the fence separating the pens from the aisles, 1.0 m above the floor, *n* = 9 + 8; Aisle, in the aisle between the pens, 1.5 m above floor, *n* = 14 + 16; Cleaning, in an empty stable during high-pressure cleaning, MRSA only, n = 3; hallway, between stable sections, 1.5 m above the floor, *n* = 3 + 2; feed/entry, in the feed-storages and entry room, 1.5 m above floor, *n* = 1 + 4). The white bars are individual measurements and the black bars are the geometric mean values. Bars with the same letters are not statistically different, analyzed in GLM and the data for D$_g$ of MRSA and SA are considered together.

Table 2. Average fractions (% of total) of MRSA and *S. aureus* particle size ranges delimited by their potential site of deposition in the six parts of the airways as measured in selected locations on the four farms.

	Aisle and pen fence *n* = 47	Among pigs *n* = 12	Feed-storage, entry, and hallway *n* = 10	*P*-value**
7.0–12 µm Nasal cavity	62.3 [a]*	45.4 [b]	42.7 [b]	0.0047
4.7–7.0 µm Pharynx	14.2 [b]	19.7 [ab]	30.8 [a]	0.0097
3.3–4.7 µm Trachea and primary bronchi	11.3 [a]	13.4 [a]	11.8 [a]	0.85
2.1–3.3 µm Secondary bronchi	8.1 [ab]	11.4 [a]	6.7 [b]	0.16
1.2–2.1 µm Terminal bronchi	2.1 [b]	7.0 [a]	3.4 [b]	0.12
0.65–1.2 µm Alveoli	1.2 [b]	3.2 [a]	4.9 [a]	0.0067

*Numbers in the same row followed by the same letter are not statistically significantly different.
**P*-value for the effect of location as measured using GLM.

2004; van Belkum *et al.*, 2009). It is simple to test for MRSA-carriage in the upper airways, and it has become almost standard to test for presence of MRSA in the upper airways in research studies in occupational settings such as farms (Létourneau *et al.*, 2010; Angen *et al.*, 2017), in population surveys (Abudu *et al.*, 2001), as well as before surgery (Rohr *et al.*, 2004; Matheson *et al.*, 2012). The observation, that the largest fractions of *S. aureus* and MRSA deposit in the upper airways, is in accordance with what has been found for MRSA in a pig farm (Ferguson *et al.*, 2016), and for culturable, mesophilic bacteria in livestock husbandry (Clauss, 2015). In a Chinese hen house, *S. aureus* was mainly carried on particles of 2.1–3.2 and 0.6–1.0 µm (reviewed by Clauss, 2015).

On average, 21.5% of the airborne *S. aureus* and MRSA were of the size potentially depositing in the primary and secondary bronchi. It has been suggested that the bronchi can act as a reservoir of *S. aureus* which may in time cause pneumonia (Kaye *et al.*, 1990; Bartlett *et al.*, 2000), and it has been shown that nasal and bronchial strains of *S. aureus* in infected patients usually are identical (Corne *et al.*, 2005). In this study, the concentration of *S. aureus* potentially depositing in the primary and secondary bronchi exceeded 8 × 10^3 cfu/m^3 during high-pressure cleaning. This means that if a farmer inhales 1.1 m^3 air/h during high-pressure cleaning for 4 h without wearing respiratory protection, > 3.6 × 10^4 cfu *S. aureus* may deposit in the bronchi. The observation that 22 % of the airborne

MRSA and total *S. aureus* potentially deposit in the bronchi, is also interesting in the relation to how this would affect the microbiota in the bronchi, especially in light of a recent study indicating that pig farming is associated with a special nose microbiota (Kraemer *et al.*, 2018).

We have found no study investigating whether exposure to airborne *S. aureus* or MRSA of alveolar size can be associated with development of pneumonia. However, it is important to be aware that the bacteria may deposit in other and deeper places in the airways than the nasopharyngeal area. Furthermore, though they may be rare, cases of pneumonia caused by LA-MRSA (Witte *et al.*, 2007; Hartmeyer *et al.*, 2010) and community associated-MRSA (Rubinstein *et al.*, 2008) have been reported. The concentration of airborne *S. aureus* on particles smaller than 1.1 µm was up to 200 cfu/m^3; this level was found in the stable during tail docking. If a farmer with no respiratory protection inhales 1.1 m^3 air/h and works for 4 h in the stable under these conditions, 870 cfu *S. aureus* may potentially deposit in the alveoli. When considering Stages 5 and 6 sampled by the ASCI together, the highest concentration was found during high-pressure cleaning of the empty stable. We found that high-pressure cleaning can cause a concentration of 1.6×10^3 cfu/m^3 air, corresponding to a potential deposition of 7.0×10^3 cfu *S. aureus* in the terminal bronchi and alveoli during 4 h work activity without airway protection.

The highest concentrations of total inhalable MRSA were also found in the empty stable during high-pressure cleaning. A review study concludes that very high exposure to bioaerosols is found during high-pressure cleaning on farms and that the material being cleaned, as well as the degree of dirtiness, highly influences the exposure level (Madsen and Matthiesen, 2013). We have found no other studies measuring concentrations of MRSA during high-pressure cleaning, or any measuring concentrations of MRSA as high as in the present study. The concentration of airborne *S. aureus* and MRSA was also high in the farrowing section where pigs were having their tails docked. However, this high concentration may partly be caused by a low ventilation rate due to the low outdoor temperature that day (–2°C, data not shown), rather than to the work activity. All the measurements in this study were performed in the winter and autumn on days with outdoor temperatures between –2 and 11°C. If the study had been performed in the summertime, lower concentrations might have been found since seasonal variations in dust concentrations are documented; the highest concentrations are found in the winter, likely due to a lower ventilation rate in the colder weather (Duchaine *et al.*, 2000; O'Shaughnessy

et al., 2009; Basinas *et al.*, 2013)— which thus might also have affected the *S. aureus* and MRSA concentration found in this study. In addition, the activity level as well as the density and sizes of pigs might also have affected the exposure levels to *S. aureus* and MRSA as these factors seem to affect the dust exposure level in pig farms (Gustafsson, 1999). In this study, the exposure to inhalable *S. aureus* was higher in the farrowing sections than in the other sections with pigs.

Even in areas where the presence of *S. aureus* was not expected, such as in the entry rooms and feed-storages, *S. aureus* was found. Thus, the concentration of airborne, respirable *S. aureus* in the feed-storage was 48 cfu/m^3, which is at the level found in public buildings in China (Li *et al.*, 2015). The presence of *S. aureus* and MRSA in these, or for MRSA some of these, areas is of importance in relation to further transmission of the bacteria out of the stable—especially in the light of an average 99.9% die-off rate of 66 days for *S. aureus* and MRSA present in sampled pig farm dust (Feld *et al.*, 2018). In entry rooms and feed-storages, *S. aureus* and MRSA particles had a smaller D_g than in, e.g. the aisle, which may allow them to stay airborne for a longer time and to pass through small cracks, e.g. between rooms.

Based on our findings in this and previous studies, we find it reasonable to suggest the use of personal dust masks to protect workers in the stable but also to reduce the risk of transmitting the bacterium to other people. This is because nasal MRSA carriage is associated with exposure level to airborne MRSA rather than to direct physical transfer between the hands and the face (Angen *et al.*, 2017), and the concentrations of viable, inhalable, airborne MRSA on pig farms are very high. Coupled with the observation that 94% of 94 volunteers with no respiratory protection were MRSA-positive after 1 h of occupancy in an MRSA-positive stable and 53% still carried MRSA 1 h after leaving the stable (Angen *et al.*, 2017), dust masks provide easily accessible and low-cost protection of workers. Personal protection by dust masks is described as an option to minimize farmers' exposure to airborne dust (Lee *et al.*, 2005). In Europe, filter dust masks are typically categorized into P1, P2, and P3, where P2 retains about 94% of all airborne particles while P3 retains about 99.95% of airborne particles. The most penetrating particle size is around 0.3 µm, i.e. smaller than *S. aureus* cells (Lee and Liu, 1980). Thus, only few MRSA-particles may penetrate the filter of a correctly used mask. However, in general, there are problems with the fit of filter masks (Lee *et al.*, 2005; Winter *et al.*, 2010), and this, together with the presence of airborne MRSA as particles smaller than 1.1 µm, may contribute to the explanation of why dust

masks sometimes fail to protect farm visitors against nasal deposition of MRSA (Wulf *et al.*, 2008).

The reasons why most of the bacteria were present in the fractions with the largest particles might be because *S. aureus* and MRSA are mainly associated with larger particles and simply that there is room for more bacteria on larger particles. However, it might also be due to a higher survival rate of *S. aureus* and MRSA on larger particles, as they may create a more protective environment for the survival of the bacteria. Scanning electron microscopy of dust from pig farms showed that dust particles larger than 5.4 µm were mainly grain meal and only a small fraction was skin (Stroik, 1987; Heber *et al.*, 1988).

S. aureus as measured on SA-agar was in this study present in the same particle size fractions as MRSA. Consequently, the data for *S. aureus* seem as relevant in relation to the ability to enter apertures, to stay airborne, as well as where in the airways deposition occurs, as the data on MRSA. The ability to stay airborne will affect the concentration of airborne *S. aureus* and MRSA. In pig farms, an important mechanism to remove dust particles from the air is to allow airborne dust to settle on surfaces (Gustafsson, 1999). The large fraction of *S. aureus* and MRSA on the 7.0–12 µm particles indicates that settling is also an important mechanism to remove them from the air. Furthermore, the presence of *S. aureus* and MRSA as particles with a smaller D_g in large distances from the pigs indicates that mainly the smaller particles are transported to these areas.

In this study, all farms were MRSA-positive, but samples from Farms B and D showed considerably lower concentrations on MRSA-agar than on SA-agar. A large fraction of the pig farms, for example in Denmark (Ministry of Environment and Food of Denmark, 2017), Germany (Dahms *et al.*, 2014), Spain (Reynaga *et al.*, 2016), and Poland (Mroczkowska *et al.*, 2017) are MRSA-positive, and the fraction has increased over time. The results of this study are therefore also relevant for other countries.

The ASCI sampler is well described and has been used for decades, and compared to the Respicon and the DGI it has the advantage that the dust extraction step is avoided. On the other hand, the Respicon and DGI samples have the advantage that they can be plated on different agar media and in different dilutions. Overall, the results obtained by the Respicon resembled the results obtained in the same areas using the ASCI. With the ASCI, it is important not to sample for > 40 min, as the agar may dry out and cause particle bouncing (Li, 1999), which causes an overestimation of the smallest particles (Park *et al.*, 2009). In this study,

we sampled with the ASCI for up to 20 min, and we saw no effect of sampling duration on the measured D_g. *Staphyloccus aureus* and MRSA were not found on the filters from the DGI after sampling, which was probably due to the high air flow the bacteria were exposed to during sampling. The sampler has previously been used successfully to sample the spore-forming, gram-positive, airborne bacterium, *Bacillus thuringiensis* (Madsen *et al.*, 2014). In the present study, we found other *Staphylococcus* species on the filters, which may indicate that these species are more robust than *S. aureus*. The observed species have previously been found in farm dust (Feld *et al.*, 2018).

This study was performed on four different farms, in stables with pigs of different ages and during either a variety of work activities or no human activity. Across farms, some general trends were found, such as the D_g of airborne *S. aureus* and MRSA being larger in the aisles than both inside the pens among the pigs as well as at larger distances from the pigs, such as in the feed-storage and in the entry room. Also, 70% (average) of the airborne *S. aureus* and MRSA were found to be present in the fraction potentially depositing in the upper airways, 22% (average) in the primary and secondary bronchi, and 8% (average) in the terminal bronchi and alveoli. The results obtained by the ASCI resembled the results obtained in the same areas using the Respicon. In conclusion, airborne *S. aureus* and MRSA were found in fractions able to deposit in the six studied parts of the airways. Thus, they might colonize or infect susceptible tissue in all parts of the airways. However, most *S. aureus* and MRSA were found to deposit in the upper airways. The concentration of airborne *S. aureus* and MRSA, as well as the fraction depositing in the different parts of the airways, depended on the specific location on the farm or the work tasks performed at the farms. Airborne MRSA were not confined to the stable facilities.

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Conflict of Interest

The authors declare no conflict of interest relating to the material presented in this Article. Its contents, including any opinions and/or conclusions expressed, are solely those of the authors.

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