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Concentration of Airborne *Staphylococcus aureus* (MRSA and MSSA), Total Bacteria, and Endotoxins in Pig Farms

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Pigs are very often colonized by *Staphylococcus aureus* and transmission of such pig-associated *S. aureus* to humans can cause serious medical, hygiene, and economic problems. The transmission route of zoonotic pathogens colonizing farm animals to humans is not well established and bioaerosols could play an important role. The aim of this study was to assess the potential occupational risk of working with *S. aureus*-colonized pigs in Switzerland. We estimated the airborne contamination by *S. aureus* in 37 pig farms (20 nursery and 17 fattening units; 25 in summer, 12 in winter). Quantification of total airborne bacterial DNA, airborne *Staphylococcus sp.* DNA, fungi, and airborne endotoxins was also performed. In this experiment, the presence of cultivable airborne methicillin-resistant *S. aureus* (MRSA) CC398 in a pig farm in Switzerland was reported for the first time. Airborne methicillin-sensitive *S. aureus* (MSSA) was found in ~30% of farms. The average airborne concentration of DNA copy number of total bacteria and *Staphylococcus sp.* measured by quantitative polymerase chain reaction was very high, respectively reaching values of 75 (± 28) × 10^7 and 35 (± 9.8) × 10^5 copy numbers m^{-3} in summer and 96 (± 19) × 10^8 and 40 (± 12) × 10^6 copy numbers m^{-3} in winter. Total mean airborne concentrations of endotoxins (1298 units of endotoxin m^{-3}) and fungi (5707 colony-forming units m^{-3}) exceeded the Swiss recommended values and were higher in winter than in summer. In conclusion, Swiss pig farmers will have to tackle a new emerging occupational risk, which could also have a strong impact on public health. The need to inform pig farmers about biological occupational risks is therefore crucial.

Keywords: airborne bacteria; bioaerosols; MRSA CC398; occupational health; quantitative PCR; swine confinement buildings

INTRODUCTION

Farm animals are colonized by a co-evolved specific bacterial community. In particular, the nostril (anterior nare) is an important niche for bacterial colonization by both opportunistic pathogens and commensal. A study performed in France (Armand-Lefevre *et al.*, 2005) was the first to show that a particular clonal complex of methicillin-resistant *Staphylococus aureus* (MRSA), namely CC398, was able to colonize healthy pig farmers and pigs. A subsequent worrying report indicated that 40% of pigs from Holland carried MRSA CC398 in their nostrils (de Neeling *et al.*, 2007; van Duijkeren

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et al., 2008). This observation has been confirmed by a number of studies in other European countries, including Belgium (Denis *et al.,* 2009), Denmark (Guardabassi *et al.,* 2007), and Germany (Witte *et al.,* 2007), in addition to Canada (Khanna *et al.,* 2008), the USA (Smith *et al.,* 2009), and Singapore (Sergio *et al.,* 2007). Several studies report that MRSA CC398 was involved in serious clinical infections affecting patients in close contact with pigs (van Loo *et al.,* 2007; Witte *et al.,* 2007; Declercq *et al.,* 2008; Lewis *et al.,* 2008; Van Hoecke *et al.,* 2009; Lozano *et al.,* 2011). Even if MRSA CC398 represents a new occupational risk that needs to be prevented, no studies have investigated the problem from an occupational health point of view. Most of the studies associated with pig MRSA have focused their attention only on nasal carriage of pigs, pig farmers, and veterinarians, without either investigating routes of transmission or factors that can influence the prevalence in animal farms or the airborne prevalence of other clonal complexes of *S. aureus*. However, apart from MRSA, pigs are also colonized by a specific *S. aureus* CC398 genotype not resistant to methicillin, called methicillin-sensitive *S. aureus* (MSSA). A recent study by our group has demonstrated that although prevalence of nasal carriage of MRSA CC398 in pigs and pig farmers was very low in Switzerland compared to other European countries (Huber *et al.,* 2011; Overesch *et al.,* 2011; Schwendener and Perreten 2011; Oppliger *et al.,* 2012), prevalence of nasally carried MSSA CC398 and other pig-associated clonal complexes of *S. aureus* (both in pigs and pig farmers) is significant (Oppliger *et al.,* 2012). This study shows also that the genotype composition of *S. aureus* from pigs and farmers was quite similar, implying that MSSA was readily transmitted from pigs to humans. The transmission route of MRSA and MSSA from animals to humans is not well established and bioaerosols could play an important role. An adult inhales 10 000 l of air per day and it is plausible that airborne bacteria perturbe the nasal bacterial communities of humans (Camarinha-Silva *et al.* 2012). Microorganisms colonizing pigs (skin, feces, and nostril) can be easily aerosolized in densely populated and enclosed farm buildings. Several studies have demonstrated that organic dust is present in high concentrations and has potential health effects on pig farmers, who can develop a variety of symptoms, including chronic cough, rhinitis, irritation, lung inflammation, and decline of lung function (Crook *et al.,* 1991; Schwartz *et al.,* 1995; Vogelzang *et al.,* 1998; Nihal Angunna Gamage *et al.,* 2007; Basinas *et al.* 2012). To better address health concerns in animal confinement buildings, we need to assess more specifically the bacterial composition of airborne dust. It is particularly important to have knowledge of the presence of pathogenic zoonotic strains such as pig-associated *S. aureus* and it is important to know whether environmental factors such as season, type of pig farm, and size of pig farms can influence the concentration of these bacterial strains.

The aim of this project was to assess the airborne concentrations of zoonotic MRSA and MSSA in pig farms by using a traditional culture-dependent method and by using molecular quantification of DNA to estimate the proportion of airborne *Staphylococcus sp*. Influence of environmental factors such as season, size, and type of pig farms has been studied. Other important classical airborne contamination indicators (fungi and endotoxins) were measured to allow comparison with other studies.

MATERIALS AND METHODS

Study population and sites of sampling

The study included 37 pig farms, which were spread over the western part of Switzerland. Each of them was investigated once in either 2008 or 2009. We visited two or three pig farms per day. Pig farms consisted either of nursery and farrowing units, where sows give birth and piglets are kept until they weigh about 20 kg (20 farms), or of fattening units, where the pigs are kept until they weigh 90–100 kg (17 farms). The number of pigs per farm varied from 4 to 280, with a mean of 90 pigs in fattening units and 20 sows in nursery/farrowing units. Pigs were kept in boxes (12–20 pigs per box; 2–12 boxes per room) on wooden grating. Sows were kept with their piglets in individual boxes (4–10 boxes per room) directly on concrete. The boxes were equipped with automatic feeding and watering. Most of the buildings had a main passageway with animal boxes on either one or both sides. Most pig buildings were totally enclosed, and three buildings were partially enclosed (pigs having the possibility to go outside). In each pig farm, air was continuously sampled for 3–4 h, during normal occupational activities in the morning hours, at one stationary point in the middle of the passageway at a height of 1.5 m from the floor. At each stationary sampling spot, samples were collected in parallel onto two separate filter devices (one for endotoxins and one for DNA) by means of pocket pumps (MSA Escort Elf, Mine

Safety Appliance Company, Pittsburgh, PA, USA; or SKC pocket pump 210–1002, SKC Inc., USA), set at a flow rate of 2.0 l min^{-1}. Airborne bacteria and fungi were collected in duplicate on nutrient media, at the same stationary point with an impactor (MAS-100 Eco, MBV; Vevey, Switzerland) at a flow rate of 100 l min^{-1}.

Microorganism analysis

Air volumes of 10 l were sampled for non-specific *Staphylococcus* onto ChromoID S.aureus plates (Biomérieux, Marcy-l'Etoile, France), 250 l for MRSA onto MRSA chromogenic plates (Biorad Laboratories, Cressier, Switzerland), and 50 l for fungi onto Sabouraud plates (Oxoid, Basel, Switzerland). These different air volumes were chosen to optimize the number of colonies on plates. Plates were incubated at 25°C for fungi and 37°C for bacteria. All plates were daily checked for 3 days to check for specific colonies. Results were expressed in colony-forming units (CFU) per cubic meter of air. Suspected *S. aureus* colonies were isolated and subsequently identified by first using an agglutination kit diagnostic test (STAPHAUREX, Oxoid, Basel, Switzerland) and then by molecular identification (see details in Sakwinska *et al.*, 2009).

Endotoxin analysis

Endotoxins were sampled in 30 pig farms and were measured as described in a previous study (Oppliger *et al.*, 2005). Briefly, airborne dust collected onto polycarbonate filters (37-mm diameter, 0.4-µm pore size) placed in a ready-to-use polystyrene cassette (endofree cassette, Aerotech Laboratories, Inc., Phoenix, AZ, USA) was transported within 2 h of sampling to the laboratory in a cold box and was then stored at –20°C for 1–3 months to await endotoxin measurement. Endotoxins were extracted by shaking the filters at room temperature for 1 h in 10 ml of pyrogen-free water in a 50 ml conical polypropylene tube. The filter extraction solutions were then analyzed with a quantitative kinetic chromogenic LAL (Limulus Amebocyte Lysat) assay; Biowhittaker, Cambrex Bio Science, Verviers, Belgium) at 37°C. *Escherichia coli* O55:B5 endotoxin (Biowhittaker) was used as a calibration standard. Results were expressed in units of endotoxin (EU) per cubic meter of air.

Sampling of airborne bacterial DNA and nucleic acid extraction

Airborne bacterial DNA samples were collected onto a 3-µm pore size 25-mm gelatin filter (SKC, Inc., Eighty Four, PA, USA), placed in a button aerosol sampler (SKC, Inc.) at a flow rate of 4 l min^{-1} for 4 h, and were stored at –20°C until further use. Gelatin filters were dissolved and treated with lysozyme and proteinase K in 20 µl as described in Verreault *et al.* (2010). Then, DNA was directly extracted from the lysate with the FastDNA® SPIN Kit for Soil (Bio101, Carlsbad, CA, USA). The protocol was slightly modified from the manufacturer's instructions: 650 µl of sodium phosphate buffer and 80 µl of MT Buffer (furnished with the kit, unknown composition) were added to the lysate, because previous results have demonstrated better recovery of DNA when applying this modification. The sample was processed in a Fastprep® system and centrifuged. The supernatant was recovered in a new tube. The pellet was dissolved in 400 µl of sodium phosphate buffer and 50 µl of MT Buffer and then processed a second time in the Fastprep® system to ensure maximal recovery of DNA. Following potassium acetate treatment, the supernatant was treated with 500 µl of Binding Matrix solution and 500 µl of 6 M guanidium thiocyanate. Finally, the DNA was eluted from the matrix with 200 µl of double-distilled water (ddH_2O).

Real-time polymerase chain reaction amplification and quantification

All polymerase chain reaction (PCR) assays were performed on a Rotor-Gene 3000 thermal cycler (Qiagen, Switzerland) and results were analyzed by Rotor-Gene software, version 6.1(build 93) (Qiagen, Switzerland). All DNA samples were run in triplicate. Highly concentrated DNA samples were 10-fold diluted for easier analysis and to assess the presence of inhibitors. Standards were included in each run. Negative controls with ddH_2O instead of template DNA were included in triplicate in each run.

Total bacterial load assessment. A 466-bp fragment of the bacterial 16S rDNA 331–797 (according to *E coli* position) was amplified with a universal primer and probe set (Nadkarni *et al.*, 2002). The PCR was performed essentially as described in Rinsoz *et al.* (2008). Quantification was achieved by using calibration curves generated with a serially diluted plasmid.

Staphylococcus sp. load assessment. A 370-bp fragment of the *Staphylococcus sp. tuf* gene, which encodes the elongation factor Tu, was amplified with a primer set specific for the *Staphylococcus* genus (Martineau *et al.*, 2001). The PCR was

performed in a total volume of 20 µl as described in Rinsoz *et al.* (2008). Quantification was achieved by using calibration curves generated with a serially diluted plasmid.

Standard curves. The 466-bp PCR fragment of the 16S rDNA and the 370-bp PCR fragment of the *tuf* gene were ligated into a pGEM-T Easy Vector system (Promega). The amount of each extracted plasmid was measured with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies) and the corresponding copy number was calculated. For each plasmid, a six-point calibration curve [quantification cycle (Cq) value versus log of initial plasmid copy number] was generated for quantitative PCR using 10-fold serial dilution of the plasmid. Based on the calibration curves, both total bacterial load and staphylococcal load were expressed as gene copy number per unit volume.

Statistical methods

All bioaerosol data were log-transformed prior to analyses. The influence on bioaerosols in terms of type of pig farm (nursery or fattening), size of pig farm (small with <20 pigs; medium with 20–50 pigs, and big with >50 pigs), and season (winter or summer) were tested with an analysis of variance (ANOVA). All statistics were performed by using Systat or STATA software (SYSTAT Software Inc. products Canada; StataCorp, TX, USA). The data are presented as arithmetic mean values and ranges (min–max). Geometric means are presented only in the table.

RESULTS

Prevalence of airborne MRSA and MSSA

Airborne MSSA were isolated from 32.4% (12/37) of farms, with a mean of 1564 CFU m^{-3} air (range 100–4000 CFU m^{-3}). They were found significantly more frequently in winter (7/12) than in summer (5/25) (Yates-corrected chi-square = 3.8, $P = 0.05$). Airborne MRSA CC398 (300 CFU m^{-3} air) was discovered on one farm.

Effect of season, size, and type of production on bioaerosol levels

Results of annual and seasonal levels of bioaerosols are presented in Table 1. The mean airborne endotoxin concentrations in winter were about three times higher than the Swiss recommended limit of 1000 UE m^{-3} (Anonymous 2012), whereas the mean summer concentrations were slightly below this threshold. Ninety percent (27/30) of the measured endotoxins sampled exceeded 90 UE m^{-3}, which is the recent recommended exposure limit suggested by an expert committee associated with the Health Council of the Netherlands (DECOS 2010). In winter, mean airborne concentrations of fungi were 15 times higher than the Swiss recommended limit of 1000 CFU m^{-3}. Concerning the DNA copy number of total bacteria and *Staphylococcus sp.*, mean airborne concentrations were very high, and DNA copy number of *Staphylococcus sp.* represents between 0.005 and 12.8% (with a mean of 2.4%) of the DNA copy number of the total bacteria. Analysis of variance (mixed-effect model) showed that the season had a significant influence on the mean quantities of

Table 1. Arithmetic mean/geometric mean, range (min–max), and sample size of the different bioaerosols measured in pig farms in Switzerland.

Parameters	Mean		
	Total	Summer	Winter
Cultivable *S. aureus*[a], CFU m^{-3}	1616/1011 (100–4000) $N = 12$	580/323 (100–1700) $N = 5$	2357/2279 (2000–4000) $N = 7$
DNA copy number (cn)			
Staphylococcus sp., cn m^{-3}	$1.6 \times 10^7/8.6 \times 10^5$ $(2 \times 10^3 - 4 \times 10^8)$ $N = 34$	$3.6 \times 10^6/4.3 \times 10^5$ $(1.9 \times 10^3 - 4.7 \times 10^7)$ $N = 23$	$4.1 \times 10^7/3.5 \times 10^6$ $(5.9 \times 10^4 - 4 \times 10^8)$ $N = 11$
Total bacteria, cn m^{-3}	$3.6 \times 10^9/7.7 \times 10^7$ $(1.6 \times 10^5 - 6.1 \times 10^{10})$ $N = 34$	$7.5 \times 10^8/3.5 \times 10^7$ $(6.1 \times 10^4 - 1.4 \times 10^{10})$ $N = 23$	$9.6 \times 10^9/4.1 \times 10^8$ $(1.5 \times 10^6 - 6.1 \times 10^{10})$ $N = 11$
Fungi, CFU m^{-3}	5707/770 (20–5 2560) $N = 37$	1049/404 (20–5370) $N = 25$	1 5412/2943 (30–5 2560) $N = 12$
Endotoxin, EU m^{-3}	1289/636 (17–6149) $N = 30$	798/444 (17–2678) $N = 24$	3253/2666 (721–6149) $N = 6$

[a]Totally, 37 pig farms were sampled, but mean has been calculated only with the positive farms.

endotoxins, fungi, and DNA copy numbers of both total bacteria and *Staphylococcus sp.,* with higher levels in winter, whereas size of pig farm and type of pig production (nursery versus fattening) had no effect (Table 2).

DISCUSSION

The most important result of this study is the high prevalence of pig farms contaminated with airborne pig-associated MSSA. Indeed about one-third of the air samples of the investigated farms are contaminated with MSSA. The worrying concern is that a previous study (Oppliger *et al.* 2012) carried out in the same pig farms has demonstrated that MSSA isolated from the nasal swabs of pigs and pig farmers, even if they were sensitive to methicillin, harbored high levels of multi-drug resistance to other antibiotic drugs, in particular to tetracyclines and macrolides. As instance, among isolates from pig farmers, 52% were resistant to tetracycline, whereas in persons without contact with farm animals, no isolates were resistant to tetracycline, an antibiotic used both in veterinary and human medicine. Tetracycline-resistance genes have also been found in the nasal flora of pig farmers and in air samples from swine confinement houses in Canada (Létourneau *et al.,* 2010). Thus, potential airborne environmental dissemination of drug-resistant bacteria from pig houses concerns not only methicillin-resistance genes but also tetracycline-resistance genes.

Our results show also for the first time, in Switzerland, the presence of MRSA CC398 in the air of a pig farm. Only one research group in Germany (Schulz and Hartung 2009; Friese *et al.,* 2012; Schulz *et al.,* 2012) has isolated airborne MRSA associated with the clonal complex 398 in pig-fattening units housing pigs with positive nasal carriage of MRSA, and a conference report of an agricultural forum in the USA signals the presence of MRSA, but without details on the prevalence and the genotype of the isolated strains, in air samples of pig farms (Harper *et al.,* 2010). In regard to these few studies, we can suppose that airborne pig-associated *S. aureus* (MRSA and/or MSSA) are present in each pig farm housing pigs colonized by *S. aureus*. The presence of airborne MRSA and MSSA in pig farms means that the traditional routes of transmission of *S. aureus* from a colonized carrier to another animal or human could not be restricted to direct contact, as currently accepted. It seems highly likely that *S. aureus* could colonize the human nostrils via simple inhalation of contaminated air. The precise mechanisms whereby *S. aureus* colonizes the nostril are still unknown, but several bacterial surface proteins are implicated in promoting adhesion to desquamated epithelial cells. Competition with other commensal microbes is necessary, as are mechanisms of adhesion, invasion, and immune evasion (Edwards *et al.* 2012). Elimination of the other nasal strains could be favored by the presence of high levels of airborne endotoxins, which are well known for their pro-inflammatory capability. Indeed, pro-inflammatory cytokines released in the upper respiratory tract in response to endotoxin exposure (Wang *et al.,* 1997) could promote elimination of the commensal bacterial strains, thus favoring the settlement of bacteria

Table 2. Relative effect (Coef. = coefficient), *F* value, and *P* value of season, size, and type of pig farms on bioaerosol concentrations in Swiss pig farms.

		Endotoxin, $N = 30$			Cultivable fungi, $N = 37$			DNA copy no. of total bacteria, $N = 34$			DNA copy no. of *Staphylococcus sp.*, $N = 34$		
		Coef.	*F*	*P*	Coef.	*F*	*P*	Coef.	*F*	*P*	Coef.	*F*	*P*
Season	Summer (ref.)	0	5.02	0.03*	0	9.67	0.004*	0	4.96	0.03*	0	6.97	0.01*
	Winter	1.38			2.09			2.68			2.24		
Size	Small (ref.)	0	0.97	0.39	0	3.04	0.06	0	0.79	0.46	0	1.60	0.21
	Medium	–0.16			1.69			–1.81			–1.81		
	Big	0.63			0.34			–1.63			–1.70		
Type	Nursery (ref.)	0	0.18	0.67	0	0.79	0.38	0	0.39	0.54	0	0..99	0.32
	Fattening	0.24			–0.64			0.80			–0.90		
Intercept	—	5.94	—		5.42	—		18.2	—		14.57	—	

Mean values of each season for each bioaerosol are presented in Table 1.
*$P < 0.05$.

such as *S. aureus*, which are able to escape an immune response. This hypothesis needs further investigations.

Beside *S. aureus*, results showed that other *Staphylococci* species (coagulase negative) are well present in pig farms. But it was found to be impossible to apply standard culture-dependent methods using impaction of bacteria onto nutrient media to enumerate them. Indeed, collection of only 10 l of air onto culture media specific for *Staphylococci* resulted in an overcrowded growth (impossible to count) of different phenotypes of *Staphylococcus sp*.. However, quantification of these strains, by using PCR amplification, showed that they represented only 2.4% of the total bacteria. This is higher than the proportion of 0.8% of *Staphylococci* found in poultry houses using the same qPCR methods (Oppliger *et al.* 2008). The number of the DNA copy number of total bacteria is in the same range as the results of a Canadian study (Nehmé *et al.,* 2009) that found between 10^6 and 10^8 DNA copy numbers of total bacteria m^{-3} air in a swine confinement building. Molecular quantification yields higher numbers than culture-dependent methods, which confirm that most of the environmental bacteria are non-cultivable and that one needs to use molecular methods to quantify and also to identify bacterial communities in such complex environments.

Fungal exposure is non-negligible because about half the pig farms had concentrations greater than the Swiss recommendation (1000 CFU m^{-3}). Exposure to high levels of fungi could be problematic because a lot of species are allergenic and under certain circumstances, some species can produce mycotoxins that are toxic for human and animal health. However, medical and/or epidemiological data on the potential health risk in pigs farmers has never been investigated. Concerning endotoxins, in 36% of the pig farms, levels of airborne endotoxins exceeded the Swiss recommended occupational exposure limit of 1000 EU m^{-3}, and 90% of the pig farms exceeded the 'no adverse effects' level of chronic exposure fixed at 90 UE m^{-3} by the Dutch Expert Committee on Occupational Safety (DECOS, 2011). A review, which included 27 different studies, shows that endotoxin levels in pig farms were highly variable (Cole *et al.,* 2000) and difficult to compare due to the lack of methodology standardization. However, endotoxin levels found in this study were much lower than the values measured during hog load-out, swine building power washing (O'Shaughnessy *et al.,* 2012), or during handling of other farm animals such as poultry (Oppliger *et al.,* 2008). Chronic exposure to endotoxins can impair respiratory health (Liebers *et al.,* 2006) and it has been shown that endotoxins are a major determinant of lung function decline in pig farmers (Vogelzang *et al.,* 1998). To prevent deleterious and irreversible impairments, lung functions of farmers should be monitored regularly throughout their working life by occupational or general practitioner physicians.

The size and the type of farms (fattening or nursery/farrowing units) have no significant effects on the quantity of bioaerosols. These results were surprising because, due to the increased number of pigs in big farms and the higher density of animals in fattening units than in nursery units, we expected to observe a higher bioaerosol exposure in big farms and in fattening units than in small farms and nursery/farrowing units. It is possible that the limited sample size of the farms investigated did not allow the highlighting of these effects. On the other hand, season has a significant influence on the level of airborne MSSA, *Staphylococcus sp.*, total bacteria, endotoxins, and fungi, with higher levels found in winter. This is in agreement with other studies carried out in pig houses in Canada and the USA (Kiekhaefer *et al.,* 1995; Duchaine *et al.,* 2000). The reduced ventilation in confinement houses to avoid heat loss in winter should be responsible for the increased concentration of organic dust composed mainly of fungi, microorganisms, and their by-products such as endotoxins.

In conclusion, we can highlight that Swiss pig farmers are potentially exposed to high quantities of bioaerosols, particularly during winter months and that, like their European neighbors, they are now exposed to a new, emerging, biological occupational risk related specifically to the zoonotic *S. aureus* strain CC398. This strain is, in Switzerland, a common multi-drug resistant colonizer of pigs, which poses a problem of occupational health and also a considerable public health problem. Indeed, airborne bacteria can survive and disseminate over long distances from their emission site (Ko *et al.,* 2008; Yuan *et al.,* 2010; Schulz *et al.,* 2011) and therefore could be dispersed, colonizing new human or animal hosts living close to the pig houses.

In order to avoid transmission of *S. aureus* from animals to humans, very strict hygiene measures should be recommended (hand washing, using piggery-specific work clothing and using a changing room next to the pig buildings, taking a

shower at the end of work shifts, careful disinfection, and protection of any small cuts or grazes on the skin), as well as using gloves when handling animals themselves and wearing a respirator mask (P2 type) when carrying out activities that generate a large amount of dust.

Pig farmers should be advised that in case of hospitalization, it is important to inform their doctors that they work with animals and could be carriers of 'pig-associated *S. aureus*'. Although *S. aureus* generally poses little risk to a person in good health, the nasal carriage upon hospitalization is seen as a definitive risk factor for surgical wound infection and bloodstream infections in immunocompromised patients (e.g. Bode *et al.*, 2010).

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