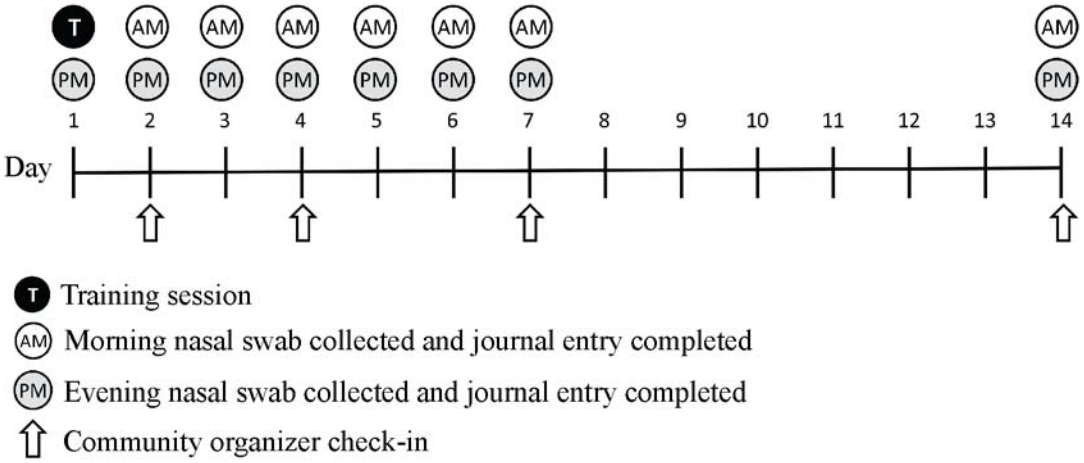
Persistence of livestock-associated antibiotic-resistant *Staphylococcus aureus* among industrial hog operation workers in North Carolina over 14 days
Supplementary File

Table of Contents

Figure S1. Graphical representation of the 14-day study design and data collection activities.



Detailed Methods and Results for *S. aureus* survival experiment

Introduction

Due to logistical constraints imposed by the design of this 14-day persistence study, participants' self-collected nasal swabs were stored for up to eight days prior to laboratory analysis. In order to determine whether false negative swabs could result from an eight-day holding time, we conducted a *Staphylococccus aureus* survival experiment prior to beginning the study. In this experiment, we examined the effect of (a) holding times between one to ten days, (b) storage temperature, and (c) initial inoculation concentration on *S. aureus* survival.

Methods

Nasal Swab Seeding

An outline of the study design is provided in **Figure S2**. On Day 0 of the study, we prepared two inoculation solutions with concentrations of 10^5 colony forming units (CFUs)/ml and 10^3 CFU/ml, respectively, using freshly grown *S. aureus* (ATCC 25923) diluted in sterile phosphate buffered saline (PBS). Both solutions were quantified by overnight culture at 37°C on tryptic soy agar (TSA) prior to use. Using sterile conditions, 63 BD BBL™ CultureSwabs™ were inoculated with 100 µl of the 10^5 CFU/ml solution and 63 nasal swabs were inoculated with 100 µl of the 10^3 CFU/ml solution. This resulted in a final concentration of 10^4 CFU/swab among 63 nasal swabs, to mimic concentrations that may be detected among persistent nasal colonizers (1), and a final concentration of 10^2 CFU/swab among the other 63 nasal swabs, to mimic concentrations that may be detected among individuals whose nasal passages are contaminated with *S. aureus*.

Three swabs seeded with 10^4 CFU and three seeded with 10^2 CFU were immediately quantified using procedures described below, in order to obtain baseline counts. For the remaining 120 swabs, half of the swabs seeded with 10^4 CFU (n=30) and half of the swabs seeded with 10^2 CFU (n=30) were stored at room temperature (25°C), in ambient light. The remaining 60 swabs were stored at 4-8°C.

Quantification of *S. aureus* on seeded nasal swabs

We assayed three swabs from each of the four experimental groups (**Figure S2**) on days 1 through 10. Swabs were clipped into 500 µl of PBS and vortexed for 30-60 seconds at high speed. 100 µl of the neat sample and serial 10-fold dilutions there-of were spread on TSA plates using a sterilized spreader and incubated for 24 hours at 37°C. Colonies with *S. aureus* morphology were counted manually or by a destructive counter. The detection limit using this method was 5 *S. aureus* CFU/swab.

Statistical Analyses

We examined the effect of storage time on *S. aureus* survival by constructing time series curves for each initial inoculation concentration. To assess the effect of storage temperature on *S. aureus* survival, we used unpaired, two-sided student t-tests and the Satterthwaite approximation to evaluate the hypothesis that the average *S. aureus* CFU/swab recovered from refrigerated swabs was equivalent to the average *S. aureus* CFU/swab recovered from swabs stored at room temperature (H_o: $\mu_1=\mu_2$) for each day elapsed. We evaluated this hypothesis separately for each initial inoculation concentration. All analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC).

Results

Ten-day survival curves for *S. aureus* seeded onto nasal swabs at concentrations of 10^4 CFU and 10^2 CFU are presented in **Figure S3**. *S. aureus* was recovered throughout the ten-day period from swabs seeded with 10^4 CFU, regardless of storage temperature. However, *S. aureus* was only consistently recovered (*S. aureus* CFU/swab $\geq$ detection limit for all three replicates) from swabs seeded with 10^2 CFU through days 1-4.

The effect of storage temperature on survival of *S. aureus* was unclear among swabs inoculated with 10^2 CFU. However, among swabs inoculated with 10^4 CFU, we observed that storage at 4-8°C resulted in greater survival of *S. aureus* compared to storage at room temperature. This effect was statistically significant at p=0.05 on day 5 and after day 7 (**Table S1**).

Conclusions

We conclude that swabs inoculated with 10^4 CFU or higher will reliably be detected by culture following a holding time of up to eight days whether stored at 4-8°C or 25°C. However, swabs inoculated with 10^2 CFU or lower may not be reliably detected by culture after five or more days of storage whether stored at 4-8°C or 25°C. To minimize *S. aureus* die-off before laboratory analysis, we determined that nasal swabs should be stored at 4-8°C following participant self-collection.

Figure S2. Outline of *S. aureus* survival study design.

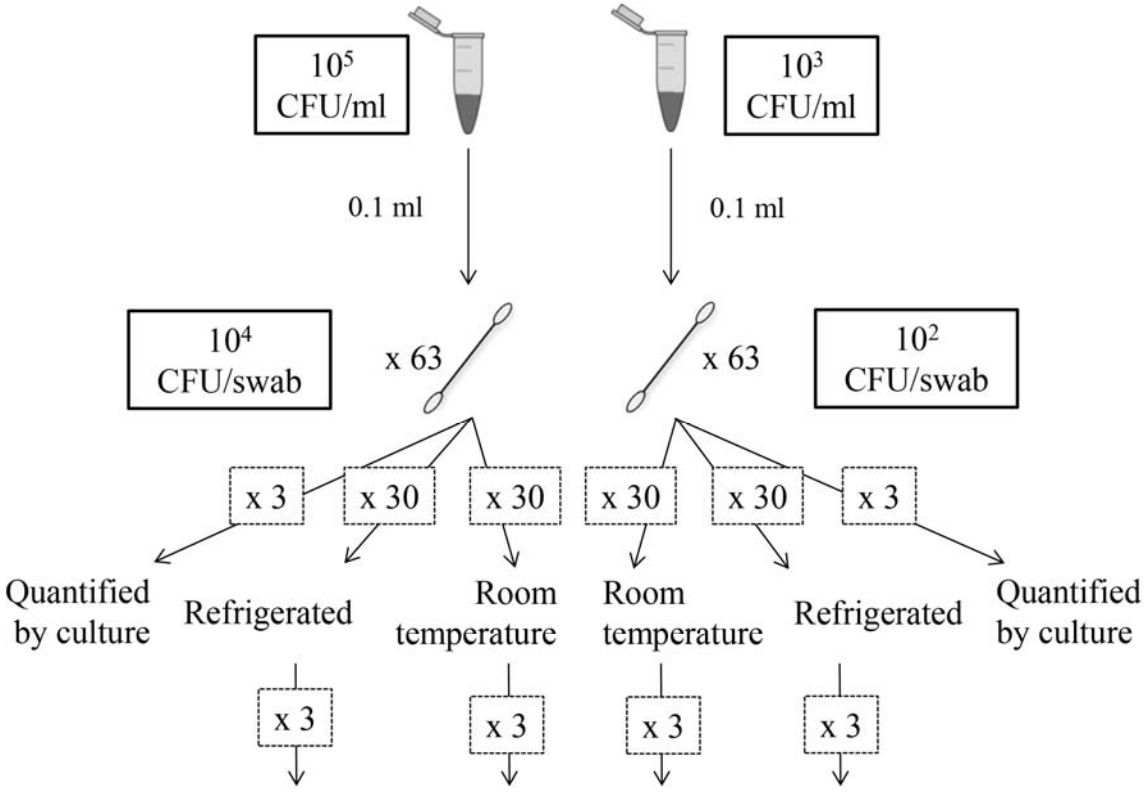


Figure S3. Survival of *S. aureus* seeded onto nasal swabs over a ten-day period.

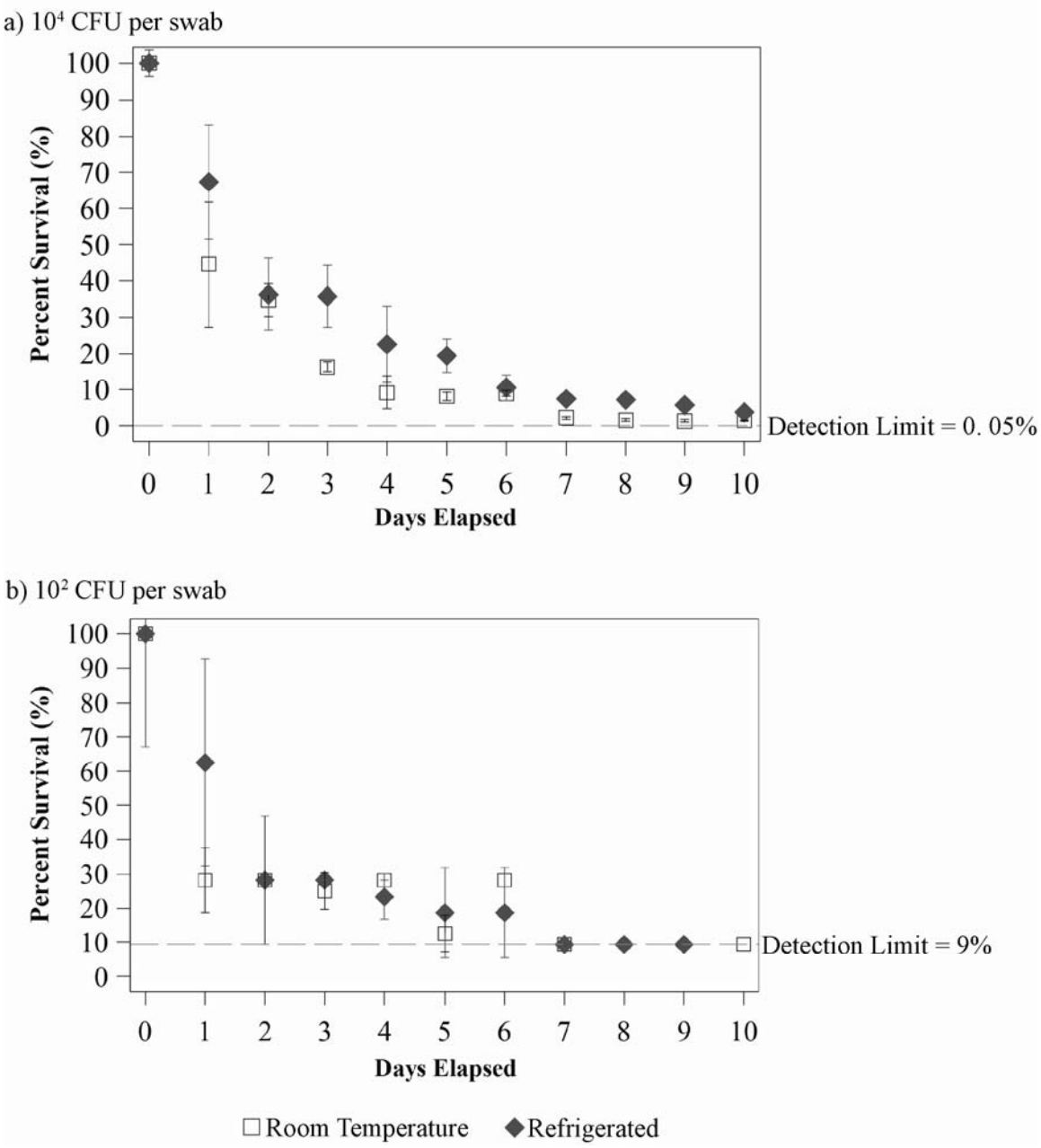


Table S1. Differences in survival of *S. aureus* seeded onto nasal swabs when stored at room temperature (25°C) versus refrigeration (4-8°C) over a 10-day period.

	S. aureus CFU/swab	
	10^4	10^2
Day	p-value[a]	p-value[a]
1	0.1697	0.1790
2	0.8112	1.000
3	0.0549	0.4226
4	0.1442	1.000
5	0.0458*	0.6244
6	0.5254	-[b]
7	0.0119*	-
8	0.0162*	-
9	0.0401*	-
10	0.0054*	-

[a]p-value comparing mean *S. aureus* CFU/swab recovered from swabs stored at room temperature versus refrigeration using unpaired, two-sided student t-test and the Satterthwaite approximation.
[b]p-value cannot be computed because observations are too few or because there is not enough variation among observations within a group.
*Statistically significant difference in survival at the 0.05 level.

Detailed methods for *S. aureus* detection and susceptibility testing

Detection of *S. aureus* and MRSA

Upon arrival in the laboratory, swabs were inoculated into 10 ml of Mueller-Hinton broth containing 6.5% NaCl, then incubated overnight at 37°C. To isolate presumptive *S. aureus*, a loopful of Mueller-Hinton broth was streaked onto Baird Parker and CHROMagar™ Staph aureus media (BD, Franklin Lakes, NJ) and incubated at 37°C for 24 hours. Both media were used in parallel to increase detection of *S. aureus* from our study population.(2) Colonies with morphological characteristics of *S. aureus* were confirmed through catalase testing, tube coagulase testing with rabbit plasma (BD BBL™, Franklin Lakes, NJ), and multiplex PCR detection of a *Staphylococcus*-specific region of the 16S rRNA gene and the *S. aureus*-specific *nuc* gene.(3) *S. aureus* isolates that were positive for *mec*A were classified as MRSA.

Approximately one-third of swabs collected were not immediately processed upon arrival at UNC due to time and labor constraints. Immediately upon receipt, these swabs were shipped overnight at 4°C to Johns Hopkins University (JHU) and archived in tryptic soy broth with 20% glycerol (w/v) at -80°C. After approximately four months of storage at -80°C, swabs were thawed and assessed for *S. aureus* and MRSA using the same procedures as above. Laboratory protocols and training of personnel were identical in both labs. We observed no systematic differences in the data produced.

Antibiotic susceptibility testing

One isolate from each *S. aureus*-positive nasal swab was assessed for susceptibility to 12 classes of antibiotics: aminoglycosides, β-lactams, cephalosporins, floroquinolones, glycopeptides, lincosamides, macrolides, oxazolidones, rifamycin, streptogramins, sulfonamide/methoprim, and tetracyclines (see **Table S2** for a listing of antibiotics used). Among *S. aureus* isolates identified at UNC-Chapel Hill (n=151), the Kirby-Bauer disk diffusion method was used to assess susceptibility to all given antibiotic classes except glycopeptides (assessed using brain heart infusion agar supplemented with 5 mg/L teicoplanin. (4)) Diameter interpretations were based on Clinical and Laboratory Standards Institute (CLSI) guidelines. (5) Inducible clindamycin resistance was evaluated in erythromycin-resistant isolates using the D-zone test (6). Among *S.*

aureus isolates identified at Johns Hopkins University (n=67), the Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD) was used to assess susceptibility to all 12 antibiotic classes.(7) Testing was completed by the Clinical Microbiology Laboratory at the Johns Hopkins Hospital.

Molecular analyses

The staphylococcal protein A (*spa*) gene was amplified and sequenced for one isolate from each *S. aureus*-positive nasal swab using methods described previously.(8) All isolates were characterized by *spa* typing using the Ridom Staph Type standard protocol (http://www.ridom.com) and the Ridom SpaServer (http://spa.ridom.de/index.shtml). The putative clonal complex (CC) to which each *spa* type belonged was inferred based on the existing literature. Isolates that could not be assigned to a putative CC with a high degree of certainty based on *spa* type and the existing literature alone were additionally analyzed by multilocus-sequence typing (MLST) (9). For these isolates, CCs were determined using eBURST (version 3; http://eburst.mlst.net) and the stringent group definition (6/7 shared alleles).(10)

We used PCR to determine whether the *scn* gene was absent from *S. aureus* isolates.(11) We also used PCR to determine whether the *tet*(M) gene was present among *S. aureus* isolates, as *tet*(M) is a proposed marker of livestock association among CC398 isolates, specifically.(12) Both targets were assessed simultaneously in one isolate from each *S. aureus*-positive nasal swab using a novel duplex PCR assay (the same isolate for which we tested antibiotic susceptibility).(13)

Table S2. Antibiotics used for susceptibility testing of *S. aureus* isolates

Antibiotic class	**Antibiotic tested**
aminoglycosides	gentamicin
β-lactams	ampicillin
	oxacillin
	penicillin
cephalosporins	ceftriaxone
floroquinolones	ciprofloxacin[a]
	gatifloxacin[a]
	levofloxacin[a]
	moxifloxacin[b]
glycopeptides	teicoplanin[a]
	vancomycin[b]
lincosamides	clindamycin
macrolides	erythromycin
oxazolidones	linezolid
rifamycin	rifampin
streptogramins	quinupristin/dalfopristin
sulfonamide/methoprim	sulfamethoxazole/ trimethoprim
tetracycline	tetracycline
	minocycline[b]

[a]Tested at UNC-Chapel Hill only

[b]Tested at Johns Hopkins only

Additional Results

Table S3. Distribution of *spa* types, MLST results, and putative CCs among *S. aureus* isolated from 22 industrial hog operation workers in North Carolina over a 14-day study period, stratified by absence of the *scn* gene.

a) *scn*-negative *S. aureus*

spa type	MLST	CC[a]	N[b]	References
t034	---	398	61	(14)
t337	---	9	47	(15)
t12116[c]	398	398	28	---
t5883	398	398	14	---
t571	---	398	8	(14)
t2582	---	398	3	(16)
t1430	---	9	5	(17)
t4652	398	398	1	---
t3446	9	9	1	---
t2963	20	20	1	---

b) *scn*-positive *S. aureus*

spa type	MLST	CC[a]	N[b]	References
t008	---	8	15	(18)
t021	---	30	15	(18)
t363	30	30	7	---
t2963	20	20	6	---
t346	---	15	3	(19)
t5026	72	8	1	---
t878	779[c]	779	1	---
t8890	9	9	1	---

[a]CC was inferred from spa type based on the existing literature unless a putative CC could not be assigned with a high degree of certainty based on *spa* type and the existing literature alone. If this was the case, MLST was performed and CC assignment was based on MLST results.
[b]N refers to the number of *S. aureus* isolates detected with the given *spa* type.
[c]Novel genotype.

Livestock-associated *S. aureus*, MRSA, MDRSA

We used absence of *scn* as a marker of livestock-association. All CC398 isolates (115/115), 98% of CC9 isolates (53/54), and 14% of CC20 isolates (1/7) were *scn*-negative. We observed greater within-person heterogeneity in strain type among participants carrying *scn*-positive *S. aureus* at every *S. aureus*-positive time point than among participants carrying *scn*-negative *S. aureus* at every *S. aureus*-positive time point. Tetracycline resistance was associated with presence of *tet*(M) for 85% of tetracycline-resistant isolates (117/138); specifically, for 100% of CC398 isolates (111/111) and 22% of CC9 isolates (6/27) demonstrating phenotypic resistance to tetracycline (data not shown).

Table S4. Distribution of antibiotic resistance profiles of *S. aureus* isolated from 22 industrial hog operation workers in North Carolina over a 14-day study period

		Participants		Isolates	
Category	Resistance phenotype	N=22[a,b]	%	N=218	%
Susceptible	--	1	4.5	3	1.4
Single-drug resistant	β-lactams	9	40.9	63	28.9
Double-drug resistant	tetracyclines, β-lactams	4	18.2	38	17.4
	macrolides, β-lactams	1	4.5	3	1.4
	lincosamides, β-lactams	1	4.5	1	0.5
Multidrug resistant	lincosamides, macrolides, β-lactams	3	13.6	10	4.6
	cephalosporins, tetracyclines, β-lactams	1	4.5	5	2.3
	floroquinolones, tetracyclines, β-lactams	1	4.5	1	0.5
	lincosamides, macrolides, tetracyclines, β-lactams	6	27.3	76	34.9
	floroquinolones, macrolides, tetracyclines, β-lactams	1	4.5	1	0.5
	floroquinolones, lincosamides, macrolides, tetracyclines, β-lactams	2	9.1	12	5.5
	aminoglycosides, lincosamides, macrolides, tetracyclines, β-lactams	1	4.5	5	2.3

[a] N refers to the number of participants who carried *S. aureus* demonstrating the specified resistance pattern at least once over the 14-day sampling period.

[b] Numbers do not sum to 22 because some individuals were colonized with *S. aureus* demonstrating more than one antibiotic resistance pattern over the sampling period.

Supplementary References

1. Iwase T, Uehara, Y, Shinji, H, Tajima, A, Seo, H, Takada, K, et al. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. *Nature*. 2010;465(7296):346-9.
2. Nadimpalli M, Heaney, CD, Stewart, JR. Identification of *Staphylococcus aureus* from enriched nasal swabs within 24 hours is improved with use of multiple culture media. *J Med Microbiol (in press)*. 2013.
3. Poulsen AB, Skov, R, Pallesen, LV. Detection of methicillin resistance in coagulase-negative staphylococci and in staphylococci directly from simulated blood cultures using the EVIGENE MRSA Detection Kit. *J Antimicrob Chemother*. 2003;51(2):419-21.
4. Fitzgibbon MM, Rossney, AS, O'Connell, B. Investigation of reduced susceptibility to glycopeptides among methicillin-resistant Staphylococcus aureus isolates from patients in Ireland and evaluation of agar screening methods for detection of heterogeneously glycopeptide-intermediate S. aureus. *J Clin Microbiol*. 2007;45(10):3263-9.
5. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 20th Information Supplement (M100-S200). Wayne, PA: Clinical and Laboratory Standards Institute,, 2010.
6. Steward CD, Raney, PM, Morrell, AK, Williams, PP, McDougal, LK, Jevitt, L, et al. Testing for Induction of Clindamycin Resistance in Erythromycin-Resistant Isolates of Staphylococcus aureus. *J Clin Microbiol*. 2005;43(4):1716-21.
7. Carroll KC, Borek, AP, Burger, C, Glanz, B, Bhally, H, Henciak, S, et al. Evaluation of the BD Phoenix automated microbiology system for identification and antimicrobial susceptibility testing of staphylococci and enterococci. *J Clin Microbiol*. 2006;44(6):2072-7.
8. European Union Reference Laboratory for Antimicrobial Resistance. Protocol for *spa* Typing. Kongens Lyngby, Denmark: National Food Institute, Technical University of Denmark, 2009. Available from: http://www.crl-ar.eu/data/images/tc_april-2009/7-protocols%20for%20spa%20typing.pdf.

9. Enright MC, Day, NP, Davies, CE, Peacock, SJ, Spratt, BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *J Clin Microbiol*. 2000;38(3):1008-15.
10. Feil EJ, Li, BC, Aanensen, DM, Hanage, WP, Spratt, BG. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *J Bacteriol*. 2004;186(5):1518-30.
11. van Wamel WJ, Rooijakkers, SH, Ruyken, M, van Kessel, KP, van Strijp, JA. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of Staphylococcus aureus are located on beta-hemolysin-converting bacteriophages. *J Bacteriol*. 2006;188(4):1310-5.
12. Warsa UC, Nonoyama, M, Ida, T, Okamoto, R, Okubo, T, Shimauchi, C, et al. Detection of tet(K) and tet(M) in Staphylococcus aureus of Asian countries by the polymerase chain reaction. *J Antibiot (Tokyo)*. 1996;49(11):1127-32.
13. Stegger M, Liu, CM, Larsen, J, Soldanova, K, Aziz, M, Contente-Cuomo, T, et al. Rapid Differentiation between Livestock-Associated and Livestock-Independent *Staphylococcus aureus* CC398 Clades. *PLoS ONE*. 2013;8(11):e79645.
14. Price LB, Stegger, M, Hasman, H, Aziz, M, Larsen, J, Andersen, PS, et al. Staphylococcus aureus CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. *mBio*. 2012;3(1).
15. Larsen J, Imanishi, M, Hinjoy, S, Tharavichitkul, P, Duangsong, K, Davis, MF, et al. Methicillin-Resistant *Staphylococcus aureus* ST9 in Pigs in Thailand. *PLoS ONE*. 2012;7(2):e31245.
16. Alt K, Fetsch, A, Schroeter, A, Guerra, B, Hammerl, JA, Hertwig, S, et al. Factors associated with the occurrence of MRSA CC398 in herds of fattening pigs in Germany. *BMC veterinary research*. 2011;7(1):69.
17. Feßler AT, Kadlec, K, Hassel, M, Hauschild, T, Eidam, C, Ehricht, R, et al. Characterization of Methicillin-Resistant Staphylococcus aureus Isolates from Food and Food Products of Poultry Origin in Germany. *Applied and Environmental Microbiology*. 2011;77(20):7151-7.
18. Deurenberg RH, Stobberingh, EE. The evolution of Staphylococcus aureus. *Infection, Genetics and Evolution*. 2008;8(6):747-63.

19. Ko KS, Lee, J-Y, Baek, JY, Peck, KR, Rhee, J-Y, Kwon, KT, et al. Characterization of Staphylococcus aureus nasal carriage from children attending an outpatient clinic in Seoul, Korea. *Microbial Drug Resistance*. 2008;14(1):37-44.