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A longitudinal study of *Staphylococcus aureus* colonization in pigs in Ireland

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Abstract

The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in livestock has refocused attention on *S. aureus* colonization and transmission in pigs. This study investigated the effect of the *S. aureus* colonization status of a sow on the colonization status of her piglets, and whether pigs carry the same strain of *S. aureus* throughout production. Nasal swabs were collected from the piglets of six healthy sows two days after birth and two days before and two days after they were moved into each production stage. The average prevalence of *S. aureus* colonization varied between 26% and 73%. The odds of being *S. aureus* positive were almost 12 times higher for piglets born to nasal-positive sows than for those born to nasal-negative sows, and three times higher again for piglets born to sows that were both nasal- and vaginal-positive. Isolates recovered from piglets immediately after birth were indistinguishable from those of the dam as determined by phenotypic and molecular typing, including microarray analysis and optical mapping. All isolates belonged to clonal complex 9 and the majority exhibited a novel *spa* type, t10449. The findings show that the *S. aureus* colonization status of the sow influences the colonization status of her piglets in the early production stages but strains carried by pigs change over time. Multiresistant *S. aureus* was detected, in particular post-weaning. Results suggest that sow status and management practices, including mixing of pigs and antimicrobial usage at weaning, should be considered when implementing control measures for *S. aureus* on a farm.

Word count: 249

Keywords

Staphylococcus aureus, pigs, colonization, longitudinal study, typing

Introduction

Staphylococcus aureus is a common commensal organism of the skin and mucosal membranes of both humans and animals (Werckenthin et al., 2001). Antimicrobial resistance in *S. aureus,* in particular methicillin-resistance, is a concern in both human and veterinary medicine. Livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) ST398 was first identified in Europe in the early 2000s and was found chiefly in pigs and pig handlers (Voss et al., 2005; Meemken et al., 2009). This event refocused attention on staphylococci of animal origin as a potential public health problem, in particular staphylococci of intensively farmed animals such as pigs, which are frequently exposed to high levels of antimicrobials.

Staphylococcus aureus, including MRSA, is a commensal organism in pigs and seldom results in clinical signs in this species although there are some reports of disease in pigs caused by methicillin-susceptible *S. aureus* (MSSA) or MRSA (Armand-Lefevre et al. 2005; Park et al., 2013; Schwarz et al., 2008; van Duijkeren et al., 2007). The transmission dynamics of *S. aureus* in individual pigs are of interest when examining possible control measures for MRSA and other multidrug-resistant *S. aureus* that may emerge in the future. This is especially true in light of recent findings in which colonization with MSSA was found to be protective against MRSA colonization in pig farmers (van Cleef et al., 2014). A small number of studies have investigated the host-organism relationship with *S. aureus* in pigs, focusing on LA-MRSA; however there are no previous studies on other strains of *S. aureus*. A longitudinal study by Weese et al. (2010) indicated that there was a significant association between sow and piglet colonization. In addition, their findings suggested that colonization was age related (Weese et al., 2010). Studies by Broens et al. (2011) and Verhegghe et al. (2011) also found an association between colonization status of sows and their piglets.

However, these studies examined only MRSA and did not evaluate the isolates further, either phenotypically or genotypically (Broens et al., 2011; Verhegghe et al., 2011). No study to date has investigated and characterized strains of MSSA or MRSA colonizing pigs throughout each production stage. Such studies are essential to provide a better understanding of transmission patterns of this pathogen as a basis for the design of future control programmes. A primary objective of this study was to evaluate *S. aureus* colonization in individual pigs over time, including whether pigs carry the same strain of the bacterium throughout production. A second objective was to determine the effect of a sow's colonization status on her piglets. The final objective was to fully characterize *S. aureus* isolated during the different production stages, including antimicrobial resistance patterns. At the time of the study (2011), Irish pigs were considered free of MRSA (EFSA, 2009; Horgan et al., 2011)

Materials and Methods

Animals and sampling

Pigs in a large, 2000-sow farrow-to-finish commercial unit were sampled and found to be positive for *S. aureus*. The unit was selected because it was typical of large units in Ireland and the farmer was willing to cooperate. It was a closed farm whereby all the piglets were born on the farm and no pigs were purchased. There were 30 members of staff who were each assigned to work with pigs in a particular production stage only. First stage weaned pigs received in-feed antimicrobials for the duration of their time in the first stage weaner accommodation (weeks three to seven), receiving tilmicosin (at an inclusion rate of 1 kg/tonne feed) from weeks three to five, followed by trimethoprim/sulphadiazine (inclusion rate 2 kg/tonne feed) from weeks five to seven. Second stage weaned pigs (weeks seven to 14) received in-feed antimicrobials for the first four days (trimethoprim/sulphadiazine at the same inclusion rate).

A total of 50 sows were screened for *S. aureus* colonization and six sows were selected for inclusion in this study based on their nasal and vaginal colonization status. In line with the study objectives, two sows testing nasal and vaginal negative, two sows testing nasal positive but vaginal negative and two sows testing both nasal and vaginal positive, were selected. All six sows and their litters were studied in parallel and farrowed within one to two days of each other. Each farrowing room housed approximately 14 sows; some, but not all six study sows, were housed within the same room. Both nasal and vaginal swabs (Cotton swab, VWR, Ireland) were collected seven days prior to farrowing. Sows and piglets were sampled two days after farrowing. Thereafter, each pig was individually tagged and samples (nasal swabs) were collected from piglets only. A total of 73 pigs were followed from farrow to finish. Piglets were weaned at approximately three weeks of age. Sampling was conducted on days

17 and 21, two days before and two days after moving to first stage weaner accommodation; on days 45 and 49, two days before and after moving to second stage weaner accommodation; and on days 96 and 100, before and after moving to the finishing houses. Following the farrowing stage piglets were merged, at random, into two groups of 30-40 pigs and they stayed in these groups in the first stage and second stage weaner houses. Each group was housed in a separate pen within one room in the first stage weaner house; the room contained four pens and formed one air space. There were a few pigs in each of the pens housing the two groups that did not originate from the six litters included in the study. In the finisher stage groups were sub divided into smaller groups but were not mixed with unfamiliar pen-mates. Pens housing finisher pigs were of Trobridge design, i.e. rows of pens side-by-side and opening onto an open-air passage.

Microbiological analysis

Swabs were placed in 10 ml Mueller-Hinton broth (MHB) (Oxoid, UK) supplemented with 6.5 % NaCl (Sigma-Aldrich, Ireland), vortexed and incubated for 18-24 h at 37°C. After incubation samples were plated onto Baird-Parker agar plates (Oxoid, UK) and incubated statically for 24 h at 37°C. Five suspect *S. aureus* colonies from each plate were then sub-cultured onto Columbia Blood Agar Base (Oxoid, UK) supplemented with 5% defibrinated sheep blood (Oxoid, UK) and incubated for 24 h at 37°C. A single colony from the five suspect colonies displaying the typical characteristics of *S. aureus* was then selected and tested using a commercial latex agglutination test for clumping factor and protein A (Pastorex Staph-Plus, Bio-Rad, France). Identification of suspect isolates was confirmed using a polymerase chain reaction (PCR) assay, as described previously (Maes et al., 2002; Poulsen et al. 2003).

137 Antimicrobial susceptibility testing

138 One hundred and seventy of a total of 281 *S. aureus* isolates collected underwent
139 antimicrobial susceptibility testing. Isolates were selected in order to ensure pigs from all
140 litters were represented at each sampling point. Isolates from a number of pigs that were
141 positive on multiple occasions were also selected for testing in order to determine changes in
142 isolates from individual pigs over time. Antimicrobial susceptibility testing was performed
143 using The European Committee on Antimicrobial Susceptibility Testing (EUCAST)
144 methodology (EUCAST, 2014). Testing was performed for the following agents: amikacin,
145 ampicillin, chloramphenicol, clindamycin, ciprofloxacin, erythromycin, fusidic acid,
146 gentamicin, kanamycin, mupirocin, rifampicin, streptomycin, sulphonamide, tetracycline,
147 tobramycin, trimethoprim and vancomycin. The zone size interpretative criteria and disk
148 concentrations used are listed in supplemental Table S1. For those antimicrobial agents for
149 which EUCAST interpretative criteria are available, the EUCAST 2014 interpretative criteria
150 were used (EUCAST, 2014). For those antimicrobial agents for which no EUCAST
151 interpretive criteria are available, those recommended by The Clinical Laboratory Standards
152 Institute (CLSI, 2013) were used. As neither EUCAST nor CLSI interpretative criteria are
153 available for testing of streptomycin, the interpretative criteria of Rossney et al. (2007), were
154 used. The EUCAST and CLSI recommended *S. aureus* strains ATCC29213 and
155 ATCC25923 were used as a quality controls for antimicrobial susceptibility testing.

156 Susceptibility to methicillin was determined using 30 μg cefoxitin disks and EUCAST
157 methodology and interpretative criteria.

158 Molecular Typing

159 A total of 60 *S. aureus* isolates were typed using pulsed-field gel electrophoresis (PFGE)
160 following digestion of high molecular weight chromosomal DNA in agarose plugs with the

161 restriction endonuclease *Sma*I as described by O'Mahony et al, (2005). All isolates from
162 sows that were nasal-positive two days after farrowing were typed. A selection of isolates
163 recovered from the piglets from each sow were chosen for typing based on their colony
164 morphology and antimicrobial susceptibility patterns. At least one isolate representing each
165 antimicrobial resistance pattern was typed. All isolates from a single pig, which was positive
166 on all sampling days (animal no. 230), were typed. The *S. aureus* reference strain NCTC
167 8325 was used as a control strain.

168 All isolates that were analysed using PFGE were also *spa* typed. *Spa* typing was performed

169 as described by Shopsin et al. (1999). The *spa* repeat region was amplified using primers *spa*-
170 1113F (5'-AAAGACGATCCTTCGGTGAGC-3') and *spa* 1514R (5'
171 CAGCAGTAGTGCCGTTTGCTT-3'), described previously by Hasman et al. (2009). The
172 *spa* types were determined using Ridom StaphType 1.5.0 software and clustered in *spa*-
173 derived clonal complexes (*spa*-CC) by Based Upon Repeat Pattern (BURP) as described by
174 Mellmann et al. (2007).

175 A subgroup of 23 isolates from the 60 isolates was subjected to DNA microarray analysis
176 using the StaphyType Kit (Alere Technologies GmbH, Germany) as described by Monecke et
177 al. (2008). These isolates were chosen in order to ensure at least one of each antimicrobial
178 susceptibility pattern, PFGE pattern and *spa* type was analyzed. In addition, isolates obtained
179 from pigs that were repeatedly positive for *S. aureus* during production were analyzed.

180 Isolates that exhibited phenotypic resistance to particular antimicrobial agents for which
181 associated resistance genes were not detected by the DNA microarray, or for which resistance
182 genes were detected by the DNA microarray but the associated resistance phenotype was not
183 detected, were further investigated by PCR. These investigations included PCRs using

primers to detect *tet*(K), *aadE, dfrS1*, *lnu*(A), *lnu*(B) *erm*(C), *blaZ* and *vga*(A) (Supplemental Table S2).

Two isolates, one from pig no. 230 (230 D2) and one from its dam (no. 4069N), both obtained on day 2 after farrowing, were analysed using whole genome mapping. The isolates were gently lysed using Argus® HMW DNA isolation kit (Opgen, Inc) to generate high molecular weight DNA (HMW-DNA). HMW-DNA was loaded on a MapCard and digested *in situ* with the restriction endonuclease *Nco*I, stained with a fluorescent DNA dye, JoJo-1 (Invitrogen), and processed using the Argus Optical Mapping System (OpGen, Inc). The *Nco*I-generated restriction fragments detected by the Argus system were re-assembled into overlapping contigs to eventually create a circular restriction map. Generated restriction maps were compared with *in silico* maps of all sequenced *S. aureus* strains available in the NCBI database and edited using an *in silico* generated optical map of *S. aureus* ECT-R2 (NCBI accession no: NC_017343) as a reference. Edited maps of 230 D2 and 4069N were then compared with each other using MapSolver (OpGen, Inc).

Statistical analysis

Odds ratios were calculated to compare the odds of piglets being *S. aureus* positive for dams with different *S. aureus* status (Supplemental Table S4). A logistic mixed effects model was fitted to the data using the lmer package (Bates et al., 2012) in R (R Development Core Team, 2012) to investigate the probability of a piglet having *S. aureus*, depending on the status of its dam. Random effects were included for pig and sow. This method of analysis allows for the fact that observations from the same experimental unit (pig) are correlated. Covariates/cofactors included age, sow status and production stage. The *S. aureus* status of the sow seven days pre-farrowing was included as two indicator variables; N_pos for nasal

208 positive pre-farrowing, and NV_pos for vaginal and nasal positive. When both indicators are
209 set to zero, the model predicts the *S. aureus* status of piglets from sows that were *S. aureus*-
210 negative in both the nose and vagina.

211 Linear, quadratic and cubic terms for age and interaction effects between age and indicators
212 of sow status were included. The quadratic and cubic terms included were age^2 and age^3,
213 divided by 100 and 1000 respectively to ensure numerical stability of the model-fitting
214 algorithm.

215 To investigate the effect of moving between production stages on the probability of a piglet
216 testing positive for *S. aureus*, three indicator variables were used. Production stage 1 (birth
217 to weaning, farrowing house) was set to be the baseline level and the effect of each
218 production stage was estimated relative to stage 1. The p-values were calculated from
219 likelihood ratio tests (LRT) obtained by comparing reduced models to the full model, fitted
220 with all terms of interest. Terms of interest included age (linear, quadratic and cubic),
221 production stage (3 indicator variables), sow status and all two-way interactions between sow
222 status with age. The model was then re-fitted with only statistically significant terms
223 included.

224 An additional model employing *S. aureus* status of the pig at previous testing as a predictor
225 of current *S. aureus* status was investigated alongside the above predictors. This predictor
226 was not found to be statistically significant under likelihood ratio testing and hence results of
227 this additional model are not included here.

228 Results

229 *Sow status*

Nasal swabs from a total of 16 of the 50 sows sampled seven days prior to farrowing were positive and of these, four sows were positive in both the vagina and nares. Six of the sows were chosen for inclusion in this study as follows; two nasal positive sows, two nasal and vaginal positive sows and two negative sows. Of the six sows that were chosen and sampled two days after farrowing both of the nasal positive sows remained colonised, one of the nasal and vaginal positive sows was positive at both sites and only one of the negative sows was negative at the time of this sampling. Individual piglets from each sow were followed from farrowing to the finishing stage.

Piglet carriage of S. aureus

Figure 1 shows the average prevalence of *S. aureus* carriage in piglets from litters grouped according to the status of the sows seven days prior to farrowing. Additional information on how many individual piglets changed or maintained their carriage status between sampling points is given in Tables S3a to S3d in supplemental materials. One pig tested positive on all seven sampling occasions (pig 230) and all samples from one pig tested negative. Most piglets (60 of 73) changed status at least twice during the course of the study. The highest prevalence of *S. aureus* carriage was observed on day two following farrowing (in piglets of positive sows), but decreased prior to leaving this production stage. The lowest prevalence of *S. aureus* was observed in piglets from the negative sows (Table S3b). The prevalence of *S. aureus* increased in pigs from all sows on day 21, two days after weaning, with a further increase on day 45, just prior to leaving first stage weaner accommodation. The prevalence of *S. aureus* decreased in pigs on day 49 but another increase was observed at day 96 prior to moving to finishing houses.

Statistical analysis

The significance of the effect of the nasal status of the sow on the *S. aureus* status of the piglets was assessed from the fitted model by examining together the main effect and the two interaction effects (with the linear and quadratic terms for age) (Table 1).

The main effects alone are not statistically significant at the 0.05 level (although the main effect for nasal status of the sow is statistically significant at the 0.1 level). However, the interaction effects between age (linear and quadratic term) and nasal status of the sow, as well as the interaction between age (linear term) and vaginal status of the sow are highly significant ($p<0.01$) (Table 1).

The odds of a piglet being *S. aureus* positive at two days of age were predicted to be almost 12 times higher (Odds Ratio (OR) = 11.822) for pigs born to sows that were nasal positive at the farrowing stage, than for those born to sows that were nasal negative (Table 1). The odds of a piglet being *S. aureus* positive were estimated to be a further 3 times higher for a piglet born to a sow who was both nasal and vaginal positive, compared to piglets from sows that were nasal positive alone (OR = 3.149). The effect of the sow on the bacterial status of piglets (for sows that are nasal positive) reduced as piglets got older (Figure 2). By the time the pigs were approximately 60 days old, the predicted odds ratio was greatly reduced (to approximately 0.5), indicating that piglets from sows that were nasal positive pre-farrowing are predicted to be only half as likely to be *S. aureus* positive than piglets from sows that were *S. aureus* negative. Finally, the predicted difference in the probability of testing *S. aureus* positive between piglets from nasal positive and negative sows reduced towards zero as the pigs reached the final stage of production (i.e. the odds ratio moved towards one) (Figure 2).

Figure S1 shows that piglets from sows that were vaginal positive pre-farrowing have a higher predicted odds of testing *S. aureus* positive than those piglets from sows that were not, but after they are around 60 days old, their odds of being *S. aureus* positive are predicted to become lower than for piglets from sows that were either *S. aureus* negative, or only nasal positive.

In comparison to piglets in the farrowing house, the probability of pigs testing positive for *S. aureus* was estimated to be lowest in second stage weaned pigs. The indicator variable for second stage weaner house was the only significant indicator variable of the three included for production stage, and hence is the only one of the three included in the final model (Table 1). The estimated odds ratio for pigs being nasal positive on entering the second stage weaner production stage is approximately 0.25 (p=0.03), i.e. the odds of piglets being *S. aureus* positive during production stage weaner 2 was estimated to be about four times less compared to all other stages of production.

Antimicrobial Resistance

In total, 170 isolates identified as *S. aureus* by PCR assay were investigated for methicillin resistance and underwent antimicrobial susceptibility testing. The results are summarized in Table 2, with more details provided in Supplemental Table S4. No MRSA were detected based on susceptibility to cefoxitin. All isolates from sows were resistant to tetracycline alone and 88 % of isolates from piglets on day 2 had the same resistance pattern as that of the sows. The resistance patterns post weaning were different from those pre-weaning. Resistance to ampicillin, erythromycin and clindamycin was commonly observed in isolates from first and

second stage weaners. Tetracycline resistance was not observed in the *S. aureus* isolates from first stage weaners but isolates from six second stage weaned pigs on day 96 and from four finisher pigs showed resistance to tetracycline only. Nine different resistance patterns were detected in isolates from pigs in the finishing stage (Table 2 and Supplemental Table S4).

Molecular Typing

Table 3 summarizes the genotypic characteristics of 23 isolates. A total of 60 isolates were typed using pulsed field electrophoresis (PFGE) and two *Sma*I- PFGE patterns, A and B, were identified. There were several band differences between the two patterns (Supplemental Figure S2). All isolates except one were assigned to pattern A ($n = 59$). The same 60 isolates which were typed using PFGE analysis were subjected to *spa* typing. Two different *spa* types were identified with t10449 ($n = 55$) being the dominant type over t1334 ($n = 5$).

A total of 23 isolates were characterised using DNA microarray profiling for assigning isolates to MLST clonal complexes (CCs) and sequence types (STs) and for the detection of antibiotic resistance and virulence genes (Table 3). All isolates belonged to clonal complex 9 (CC9) and were negative for (i) the Panton-Valentine leukocidin (PVL) toxin genes, (ii) the exfoliative and enterotoxin genes, (iii) the immune evasion complex genes, and (iv) *mec* and SCC*mec* associated genes.

The majority of isolates (22/23, 95.7%) harboured at least one antimicrobial resistance gene including those encoding resistance to macrolide-lincosamide-streptogramin B (MLS_B) compounds (*erm*(C) 10/23, 43%), β-lactams (*blaZ*, 10/23, 43%), tetracycline (*tet*(K), 11/23, 48%), trimethoprim (*dfrS1*, 3/23, 13%) and streptomycin (*aadE,* 3/23, 13%) antimicrobials

(Table 3). One isolate from a piglet (205 D2) whose dam was negative for *S. aureus,* did not harbour any detectable resistance genes and was susceptible to all antimicrobial agents tested. In the majority of instances, a good correlation between the resistance phenotype and the presence of a particular resistance gene was detected (Table 3). However, no corresponding resistance gene could be detected in five isolates that exhibited phenotypic susceptibility to erythromycin and clindamycin resistance (Table 3).

Six different combinations of resistance genes were identified among the isolates with the presence of *tet*(K) only predominating (8/23, 35%) (Table 3). Isolates obtained post weaning had the most resistance genes.

Pig 230 was the only pig that was positive for *S. aureus* on all seven sampling occasions. Isolates from this pig harboured different resistance genes and exhibited different resistance phenotypes at different production stages and isolates obtained post weaning had a greater number of resistance genes (Table 3). However, one piglet and one weaner isolate exhibited the same resistance pattern and carried the same resistance genes (*tet*(K), Table 3).

Pig 155, whose dam was both nasal and vaginal positive, was positive for *S. aureus* on four of the seven sampling occasions. Similar to pig 230, isolates from pig 155 had a greater number of resistance genes post weaning.

Whole genome mapping (WGM) was employed to further corroborate the clonal relatedness of *S. aureus* isolates recovered from piglet no. 230 and its dam, sow 4069, immediately after birth (230 D2 and 4069N, respectively). Matrix similarity cluster using unweighted-pair group method using arithmetic averages (UPGMA) showed 100% map similarity between the two strains (Figure 3a). Moreover, comparison of the two maps with those of *in silico*

346 generated optical maps of *S. aureus* reference strains identified their close homology to *S.*
347 *aureus* ECT-R2 (97.5% of map similarity) (Figure 3b), which recently caused a clonal
348 outbreak in Sweden (Lindqvist et al., 2012). *S. aureus* ECT-R2 was originally isolated from
349 a human wound and is a multiresistant methicillin-susceptible *S. aureus* (Lindqvist et al.,
350 2012).

351

352 Discussion

353 This study investigated the transmission and persistence of *S. aureus* in individual pigs
354 throughout the production cycle. Significant findings include the identification and detailed
355 characterization for the first time of *S. aureus* isolates from Irish pigs and demonstration of
356 the influence of the *S. aureus* colonization status of the sow on the status of her piglets. In
357 addition, this study provided further information on the possible influence of management
358 practices at weaning on *S. aureus* colonization patterns. It documented changes in the
359 antimicrobial resistance profile of *S. aureus* over time and provided data that suggest that
360 antimicrobial use may be an important factor promoting these changes. Antimicrobial use is
361 also thought to be a factor in the emergence and transmission of LA-MRSA in pigs (Crombe
362 et al., 2013) and deserves further investigation.

363

364 With regard to the impact of the colonization status of the sow on the colonization status of
365 her piglets, piglets born to sows that carried *S. aureus* prior to farrowing were more likely to
366 carry *S. aureus* at two days after birth than piglets born to negative sows. This finding
367 confirms the results of a small number of other studies that examined MRSA nasal
368 colonization of sows and piglets (Verhegghe et al., 2011; Weese et al., 2010). However this

study also examined the effect of a sow being both nasal and vaginal positive on her piglet's status, which has not been reported previously. For piglets from nasal- and vaginal-positive sows, the odds of being *S. aureus* positive were predicted to be three times higher than for piglets born to nasal positive sows, which is consistent with the suggestion by Moodley et al. (2011) that the likely source of MRSA transmission from sows to piglets was through direct contact with the snout, skin and vagina of the sows. In addition, the antimicrobial resistance patterns (Table 2), microarray and whole genome mapping (Table 3, Figure 3) data from this study strongly support the view that both the sows and the piglets on day 2 carry the same strain of *S. aureus*. On day 2, all isolates from sows were resistant only to tetracycline and 88% of isolates from piglets showed the same resistance pattern (Supplemental Table S4). In contrast, none of the isolates from pigs immediately after weaning was resistant to tetracycline (Supplemental Table S4). These results, in addition to the statistical analysis indicate that the effect of dam status decreased over time. The predicted odds ratio of a piglet from a nasal-positive sow being positive compared to a piglet from a nasal-negative sow fell below zero after approximately day 40 (Figure 2). This suggests that being born to a positive sow may be in some way protect against *S. aureus* carriage at later production stages. This finding is worthy of further study as a recent publication reported that prior colonization with MSSA appeared to be protective against acquisition of MRSA in pig farmers (van Cleef et al., 2014). However, it is acknowledged that many factors, in addition to sow colonization status, are likely to be important influences on carrier status, particularly in older pigs. Such factors might include selective pressure exerted by antimicrobial medication post weaning and differences in the environmental flora of weaner houses.

The prevalence of *S. aureus* varied in each production stage. This study found the average carriage rate of *S. aureus* was at its highest on day 2 after farrowing, followed by a decrease

394 prior to weaning. Similar findings were reported by Weese et al. (2010) and Verhegghe et al.
395 (2011) for MRSA. These results, together with the finding that the great majority of pigs
396 changed carriage status at least twice during the 100-day study suggests that piglets are
397 normally transiently rather than permanently colonized from birth. The prevalence of carriage
398 in all pigs increased post weaning on day 21 and showed a further increase when pigs were
399 sampled on day 45. Weese et al, (2010) and Dewaele et al, (2011) suggested that the increase
400 in MRSA-positive pigs recorded at weaning was due to the commingling of positive and
401 negative pigs, stress during weaning, age related susceptibility and contamination of other
402 sites on the farms. Weaning may represent a point at which controls could be implemented in
403 order to reduce transmission of *S. aureus* of public health significance such as LA-MRSA.
404 Possible control measures could include minimizing the mixing of litters at weaning and
405 reducing antimicrobial use.

406

407 The findings of this study are similar to those of previous studies with the major difference
408 that the highest prevalence of *S. aureus* in pigs was observed on days 2 and 96 rather than at
409 weaning. One possible explanation is that this study sampled sows which were positive and
410 negative for *S. aureus* prior to and two days after farrowing whereas the study by Weese et al.
411 (2010) sampled sows which were negative at the time of farrowing.

412 A previous longitudinal study by Broens et al. (2011b) suggested that pigs which received
413 antimicrobial agents such as β-lactam antibiotics and tetracyclines were at a higher risk of
414 MRSA ST398 colonization (Broens et al., 2011). Pigs in this study received macrolide in-
415 feed antibiotics (tilmicosin) upon entering first stage weaner pens and this coincided with the
416 detection of a multidrug resistant (MDR) strain of *S. aureus* resistant to the macrolides
417 (erythromycin) and carrying *erm*(C). The feed used was changed shortly before pigs entered

2nd stage weaner accommodation to a different feed medicated with potentiated sulphonamides. The reduction in prevalence of *S. aureus* observed at this time suggests that the use of antibiotics containing sulphonamides and trimethoprim to which the *S. aureus* was susceptible, may have contributed to the reduction in prevalence. However, once the use of in-feed antimicrobials ceased after day 51, increased carriage of *S. aureus* was detected on day 96. Coinciding with cessation of in-feed antimicrobials, the percentage of isolates showing multiresistance to antimicrobials decreased on days 96 and 100 and a much greater variety of antimicrobial resistance patterns was detected at this time (Table 2 and supplemental Table S4).

There was excellent correlation between the resistance phenotype and the resistance genes detected for all antimicrobial agents investigated apart from clindamycin. Five isolates were found to be clindamycin resistant and were erythromycin susceptible, but no associated resistance gene was detected (Table 3). Possible explanations include the presence of an allelic variant of a gene that was not detected by the microarray or by additional PCR analysis, or, possibly, the presence of a novel gene(s) that remains to be described.

All isolates investigated in this study were identified as belonging to CC9-MSSA, a lineage not previously reported in Ireland. Previous studies have reported *S. aureus* ST9 colonization in pigs in China and Malaysia (Neela et al., 2009; Wagenaar et al., 2009), including methicillin-resistant strains. This lineage appears to predominate among pigs in Asia whereas CC398 is dominant in North America and Europe (De Neeling et al., 2007; Khanna et al., 2008). However, *S. aureus* CC9 has been reported in France and Italy in both pigs and humans working with pigs (Armand-Lefevre et al., 2005; Battisti et al., 2009). Other studies

442 have also reported this lineage in pigs, cattle and poultry in Europe and occasionally in
443 healthy humans (Grundmann et al., 2002; Hasman et al., 2009, Richter et al., 2012; Monecke
444 et al., 2013).

445

446 The majority of the 60 isolates that were *spa* typed belonged to a new *spa* type, t10449. This
447 *spa* type has not been reported previously in animals or humans and belongs to CC9. A small
448 number of isolates in this study belonged to another *spa* type, t1334 (four repeat differences
449 from t10449). This *spa* type has been previously found in the USA and clusters with t337, a
450 dominant *spa* type associated with CC9 (Dressler et al., 2012). Only two PFGE patterns
451 were identified among the isolates typed using enzymatic digestion followed by pulsed-field
452 gel electrophoresis, with the vast majority of strains belonging to a single pattern. The
453 probable explanation of this finding is that the herd was closed with no animals having been
454 brought into the herd for several years. Thus, there was minimal opportunity for the
455 introduction of new strains of *S. aureus* that were adapted to pigs. Utilizing high resolution
456 WGM, we found the two strains isolated from piglet 230 and its dam to be identical and
457 closely related to the human outbreak strain ECT-R2, which harbors only remnants of an
458 SCC*mec* element and is a multiresistant MSSA (Lindqvist et al., 2012). All three *S. aureus*
459 isolates belonged to the same WGM cluster, which is defined as a set of isolates having a
460 map distance of less than 5%, a cut-off point established by Shukla et al. (2012) to cluster
461 clonally related *S. aureus* strains.

462

463 In conclusion, this study found that the colonization status of the sow has an important
464 influence on the colonization status of her piglets but that this effect decreases as the piglets
465 get older and move through the production stages. The findings suggest that consideration of

sow status and management practices such as mixing of pigs at weaning and antimicrobial usage, would be useful in the formulation of strategies to control LA-MRSA or other strains of MSSA of public health significance which may emerge in pigs in the future.

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Figure captions

Figure 1. Mean percentage prevalence of *S. aureus*-positive piglets from 2 negative sows (n = 25 piglets), 2 nasal-positive sows (n = 24 piglets) and 2 nasal- and vaginal-positive sows (n = 24 piglets) from day 2 to day 100

Figure 2. The odds ratio of a piglet from a nasal positive sow being *S. aureus* positive to that of a piglet from a negative sow plotted against age (x-axis), illustrating the estimated main effect of sows being nasal positive as well as the interactions of this variable with age.

Figure 3. A: Comparison of whole-genome maps of *S. aureus* from piglet no.230 (230 D2) and its dam (4069N); B: Comparison of WGMs of 230 D2 and 4069N to an *in silico* map of ECT-R 2; C: Map similarity cluster generated for 230 D2, 4069N and ECT-R 2 by unweighted-pair group method using arithmetic averages.

Table 1. Parameter estimates, odds ratios with 95% confidence intervals and p-values from likelihood ratio tests for the fitted model assessing the significance of nasal status of the sow on the *S. aureus* status of the piglets.

	Estimate		95% confidence interval		Likelihood ratio test
Parameter		**Odds ratio**	**lower**	**upper**	**p-value**
age	-0.202	0.847	0.75	0.891	<0.001
age^2/100*	0.675	1.965	1.543	2.501	<0.001
age^3/1000*	-0.046	0.955	0.94	0.971	<0.001
2nd stage weaner house	-1.403	0.246	0.135	0.448	0.033
N_pos	2.47	11.822	3.554	39.329	0.096
VN_positive	1.147	3.149	1.263	7.852	0.465
age:N_pos	-0.089	0.915	0.87	0.963	0.006
age:VN_pos	-0.019	0.981	0.967	0.996	0.009
(age^2/100):N_pos	0.064	1.066	1.018	1.115	0.006

*The quadratic and cubic terms age^2 and age^3 were divided by 100 and 1000 respectively to ensure numerical stability of the model-fitting algorithm
N_pos = nasal positive, VN_pos = nasal and vaginal positive

Table 2. Days sampled, the most common antimicrobial resistance pattern identified among *S. aureus* isolates at each production stage and the number of isolates showing each pattern from a total of 170 isolates tested.

Days	Group medication	**Number of antimicrobial resistance patterns detected**	The most common antimicrobial resistance pattern detected	Number of isolates exhibiting pattern
Sows	None			
Positive Sows		1	Te	6/6
Piglets	None			
Day 2		1	Te	21/24
Day 17		4	Te	8/13
1st Stage Weaners	Tilmicosin			
Day 21	(days 21 to 35)	3	ApDaEr	14/20
	Trimethoprim/			
	Sulphadiazine			
Day 45	(days 36-47)	2	ApDaEr	26/28
2nd Stage Weaners	Trimethoprim/ Sulphadiazine			
Day 49	(days 48 to 52)	2	ApDaEr	15/16
Day 96		7	ApDaEr	17/33
Finishers	None	9	ErDa	10/30
Day 100				

Isolates were recovered from four *S. aureus*-positive and two *S. aureus*- negative sows sampled on day 2 and from their piglets on all 7 sampling occasions.

Abbreviations: Ap, ampicillin; Er, erythromycin; Da, clindamycin; St, streptomycin; Te, tetracycline: Tp, trimethoprim.

Table 3. Characteristics of *S. aureus* isolates from selected sows and their piglets, including clonal complex, *spa* types, PFGE patterns, antimicrobial resistance patterns and resistance genes.

Strain No:		Sample origin	Typing			Antimicrobial resistance pattern‡	Resistance genes
Pig identifier including day of sampling†	*S. aureus* carriage status of sow		Clonal complex	*spa* type	PFGE pattern		
4069N†	Nasal positive	Sow	CC9	t10449	A	Te	*tet*(K)
230 D2††	Nasal positive	Piglet	CC9	t10449	A	Te	*tet*(K)
230 D17	Nasal positive	Piglet	CC9	t10449	A	Da	None detected
230 D21	Nasal positive	Weaner	CC9	t10449	A	ApDaErSt	*blaZ*, *erm*(C), *aadE*
230 D45	Nasal positive	Weaner	CC9	t10449	A	ApDaErSt	*blaZ*, *erm*(C), *aadE*
230 D49	Nasal positive	Weaner	CC9	t10449	A	ApDaEr	*blaZ*, *erm*(C)
230 D96	Nasal positive	Weaner	CC9	t10449	A	Te	*tet*(K)
230 D100	Nasal positive	Finisher	CC9	t10449	A	DaEr	*erm*(C)
237 D17	Nasal positive	Piglet	CC9	t10449	A	Te	*tet*(K)
4072N	Nasal positive	Sow	CC9	t10449	A	Te	*tet*(K)
155 D17	Nasal- and vagina-positive	Piglet	CC9	t10449	A	Te	*tet*(K)
155 D21	Nasal- and vagina-positive	Weaner	CC9	t10449	A	ApDaErSt	*blaZ*, *erm*(C), *aadE*

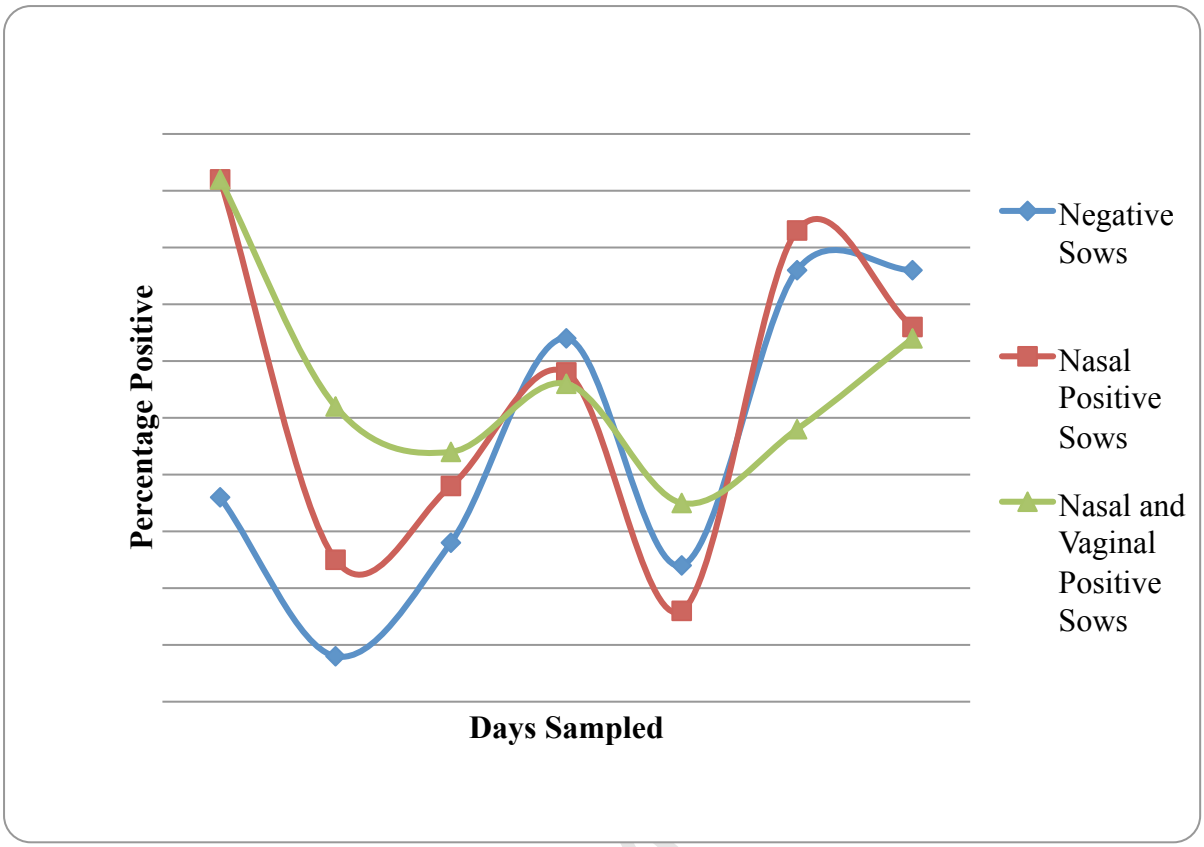
155 D49	Nasal- and vagina-positive	Weaner	CC9	t10449	A	ApDaEr	*blaZ*, *erm*(C)
155 D96	Nasal- and vagina-positive	Weaner	CC9	t10449	A	DaEr	*erm*(C)
157 D100	Nasal- and vagina-positive	Finisher	CC9	t1334	A	ApDaErTeTp	*blaZ*, *erm*(C), *tet*(K), *dfrS1*
159 D100	Nasal- and vagina-positive	Finisher	CC9	t10449	A	ApDa	*blaZ*
173 D21	Nasal- and vagina-positive	Weaner	CC9	t10449	A	ApDaEr	*blaZ*, *erm*(C)
3631N	Negative †††	Sow	CC9	t10449	A	Te	*tet*(K)
205 D2	Negative	Piglet	CC9	t10449	A	Susceptible	No resistance genes
205 D100	Negative	Finisher	CC9	t1334	A	ApDaTeTp	*blaZ*, *tet*(K), *dfrS1*
221 D96	Negative	Weaner	CC9	t1334	A	ApDaTeTp	*blaZ*, *tet*(K), *dfrS1*
212 D100	Negative	Finisher	CC9	t10449	A	DaEr	*erm*(C)
219 D17	Negative	Piglet	CC9	t10449	A	DaTe	*tet*(K)

† 4069N signifies sow no. 4069 sampled from the nares on day 2

†† 230 D2 signifies pig no.230, sampled on day 2 etc

†††Sow 3631 was negative before farrowing but was nasal positive on day 2. The results of analysis of the day 2 isolate are shown

‡ Antimicrobial agent abbreviations: Ap, ampicillin; Er, erythromycin; Da, clindamycin; St, streptomycin; Te, tetracycline: Tp, trimethoprim.



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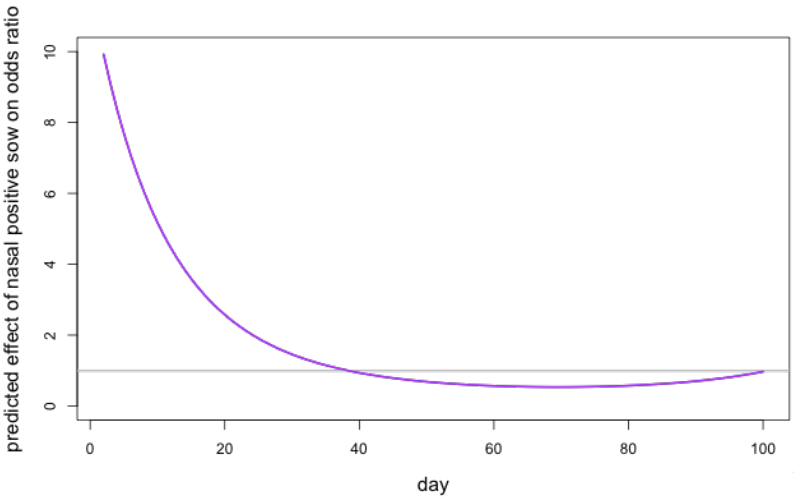
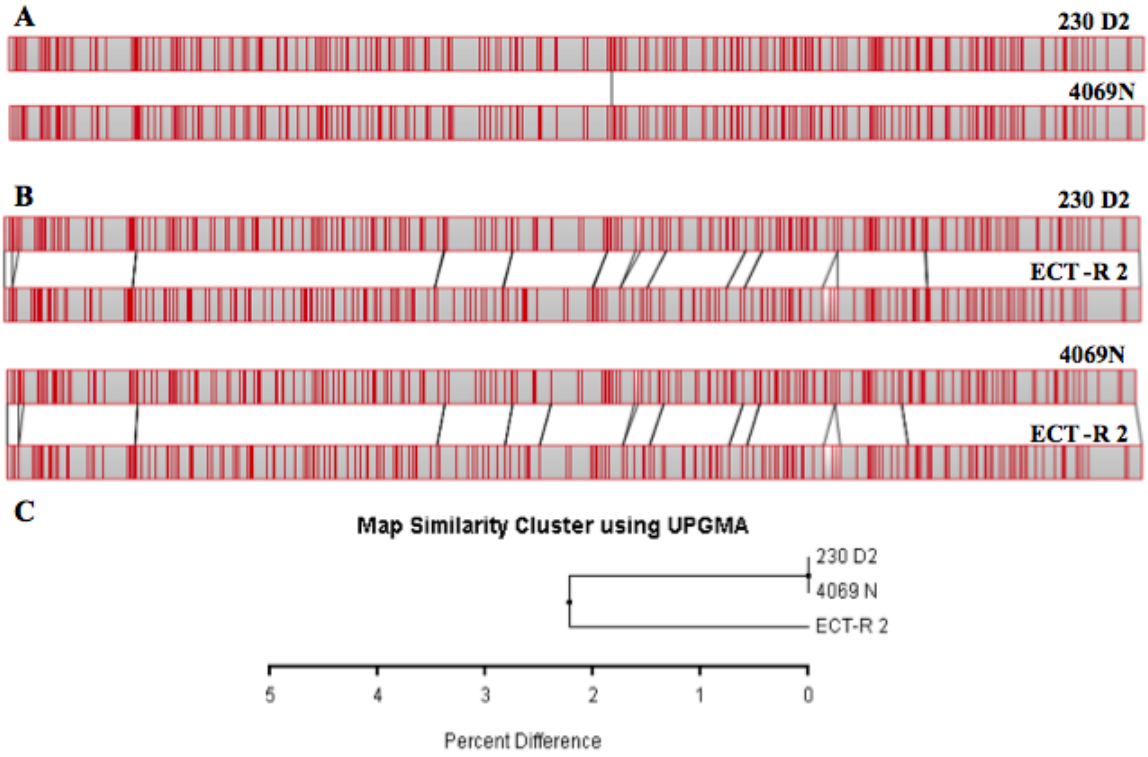


Figure 2. The odds ratio of a piglet from a nasal positive sow being *S. aureus* positive to that of a piglet from a negative sow plotted against age (x-axis), illustrating the estimated main effect of sows being nasal positive as well as the interactions of this variable with age.



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