Risk factors for livestock-associated MRSA persistent carriage and environmental exposure in veal calf farmers and their family members: an observational longitudinal study.

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Title:
Risk factors for livestock-associated MRSA persistent carriage and environmental exposure in veal calf farmers and their family members: an observational longitudinal study.

Short Title: LA-MRSA persistent carriage and environmental exposure in pig farming.

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ABSTRACT

Objectives: LA-MRSA emergence is a major public health concern. This study is aimed at assessing risk factors for persistently carrying MRSA in veal calf farmers and their family members. We also evaluate the dynamics of MRSA environmental load during the veal-calf production cycle.

Design: Observational, longitudinal, repeated cross-sectional study.

Setting: 52 veal calf farms in the Netherlands.

Participants: Between the end of 2010 to the end of 2011, a total of 211 farmers, family members and employees were included in the study.

Primary outcome and secondary outcome measures: Nasal swabs were taken from participants on days 0, 4, 7 and week 12. A persistent MRSA carrier was defined as a person positive for MRSA on days 0, 4 and 7. Participants filled in an extensive questionnaire to identify potential risk factors and confounders. For estimation of MRSA prevalence in calves and environmental contamination, animal nasal swabs and Electrostatic Dust Collectors (EDCs) were taken on day 0 and week 12.

Results: The presence of potential animal reservoirs (free-ranging farm cats and sheep) and the level of contact with veal calves was positively associated with persistent MRSA carriage. Interestingly, at the end of the study (week 12), there was a two-fold rise in animal prevalence and a significantly higher MRSA environmental load in the stables was found on farms with MRSA carriers.

Conclusions: This study supports the hypothesis that environmental contamination with MRSA plays a role in the acquisition of MRSA in farmers and their household members and suggests that other animal species should also be targeted to implement effective control strategies.

ARTICLE SUMMARY

Article focus:

- Determinants for persistent LA-MRSA carriage in humans and for a possible true colonization have not been thoroughly assessed.
- It is unclear whether bacterial contamination in the farm environment plays a role in LA-MRSA transmission in humans

Key messages:

- The presence of other animals in the farm might be of importance in acquisition and persistence LA-MRSA in humans. There is a need for detailed molecular-epidemiological analysis of MRSA specimens in various animal species and humans in the veal-calf farming community.

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- During the veal-calf production cycle, there is a parallel increase in animal prevalence and environmental MRSA load which is linked to higher risk for human carriage.

Strengths and limitations of this study:

The longitudinal nature of the data allows to establish dynamic epidemiological inferences.

No other animals apart from veal-calves were sampled in this study. The self-sampling of noses by individuals might influence the sensitivity for MRSA detection.

INTRODUCTION

In recent years, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), specifically sequence type (ST) 398, has emerged in food-producing animals and people in contact with these animals.(1-4) Illness associated to ST398 in humans is rare and only a small proportion of MRSA infections can be attributed to LA-MRSA.(5,6) Nonetheless, invasive infections and hospital outbreaks of MRSA ST398 have been reported in Europe, the United States and Asia.(5,7,8)

LA-MRSA strains have been found mainly in pigs and veal calves, but they have the capacity to colonize a wide spectrum of hosts, including sheep and poultry.(9) Farmers are easily contaminated and in general the carriage prevalence in farmers is high. Frequency of transmission between farmers and their family members and among hospitalized humans appears to be low.(2,10,11) However, this belief might be contradicted by recently described LA-MRSA transmission events in Dutch patients with neither risk factors nor livestock contact.(12) The potential public health threat posed by these strains is emphasized in a recent metapopulation model in which the likelihood of persistent carriage in the livestock-exposed population was the key parameter for LA-MRSA spreading to the community.(13)

Previous studies have been mainly based on cross-sectional designs and have shown that intensity of animal contact and MRSA prevalence among animals are positively associated to LA-MRSA human carriage.(14) Associations between animal carriage and farm hygiene and antimicrobial use have also been shown.(15,16) A longitudinal study including periods of high and low exposure to animals showed that LA-MRSA carriage was mainly transient. It was suggested that LA-MRSA is a poor persistent colonizer in humans, which was confirmed by a study on short term occupational exposure.(10,14) However, risk factors for persistent LA-MRSA carriage and for a possible true colonization have not been thoroughly assessed. Furthermore, little is still known about the dynamics of environmental contamination with MRSA in the farm and its role in transmission to humans. A recent study showed a steep increase in prevalence among calves and in MRSA air load during the production cycle.(17)

The aim of the current study is twofold. Firstly, to assess risk factors and dose-response relationships for persistently carrying MRSA over a period of one week at the beginning of the production cycle in veal calf farmers and their family members. Secondly, to evaluate the deposition of MRSA-containing dust inside the farm and its relationship with animal and human MRSA carriage.

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MATERIALS AND METHODS

Study design and population

A longitudinal cohort study was performed over a period of 12 weeks in 52 veal calf farms starting at the beginning of the production cycle. All farms were visited from the end of 2010 to the end of 2011. On each farm there were 2 sampling moments for animal and environmental samples (day 0 and week 12) and 4 sampling moments for human samples (days 0, 4, 7 and week 12). Nasal swabs from both anterior nares of calves were taken and analysed in 10 pools of 6 swabs each (60 animals per farm). Swabs were also collected from farmers, family members and employees (n=211). On day 0, quantitative nasal and throat swabs were taken by field workers in the majority of participants or by self-sampling. On days 4, 7 and on week 12, dry cotton swabs (Copan, Brescia, Italy) were used to self-sample the nose. Swabs were given to participants with instructions including photographs in case of self-sampling. Nasal swabs in animals and humans were introduced in the nostril and rotated once. Throat swabs in humans sampled the area of the inner cheek including the tonsils. The swabs were immediately taken to the laboratory or sent by post and processed within 24 hours after arrival. Furthermore, environmental samples were taken by placing 4 Electrostatic Dust Collectors (EDCs) (Zeeman, Utrecht, The Netherlands) on different surfaces inside the stables and one on the highest cupboard in the living room or kitchen of the house. The EDCs were left in place during a period of 2 weeks and sent by post to the laboratory. Upon arrival, EDC samples were stored at -20°C until quantitative analysis.(18) A schematic overview of the study design is displayed in the Supplemental Figure 1.

All participants completed an informed consent and filled in an extensive questionnaire including items related to individual health status, household and farm characteristics, activities performed on the farm and hygiene practices. The protocol of the study was approved by Medical Ethical Committee of Utrecht University. The collection of animal samples was in compliance with the Dutch Law on Animal Health and Welfare.

For the assessment of MRSA persistent carriage, we selected the beginning of the veal calf production cycle, just after the stables were empty and when animal prevalence is lower. In this period, deposition of MRSA-containing dust particles in human nasal cavities and mechanical carriage was assumed to be less likely. Therefore and for the purpose of this study, a person was defined to be a persistent MRSA carrier when each of the nasal swabs collected on days 0, 4 and 7 were positive for MRSA presence.

Laboratory analysis

Swabs in liquid transport medium (ESwab, Copan, Brescia, Italy) were used for quantitative cultivation. Serial dilutions (1:10) of the transport medium (concentration 10^0) were made by adding 100 µl sample to 900 µl phosphate buffered saline (PBS) to a final concentration of 10^{-4} of the original sample. Each dilution was cultured on chromID *S. aureus* and chromID MRSA agar plates (BioMérieux, La Balme Les Grottes, France) at 37°C for 18-24 hours. Plates with 10-100 colony-forming units (CFU) were used to calculate the original amount of CFU per swab. In order to detect positive samples without bacterial growth in the first day, the remaining transport medium and swab were enriched overnight in Mueller Hinton broth with 6.5% NaCl (MH+), and consequently cultured on chromID *S. aureus* and chromID MRSA agar plates. The theoretical lower limit of quantification (LLOQ) of MRSA CFU was 10. Dry cotton swabs (Copan) were inoculated directly onto chromID *S. aureus*,

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chromID MRSA and MH+. Confirmation of MRSA presence in the 3 sampling moments was done by Real-Time PCR targeting *mecA*, *femA* and *nuc* genes.(19,20) Methicillin-susceptible *Staphylococcus aureus* (MSSA) presence was tested when the bacterial growth on chromID *S. aureus* was higher than on chromID MRSA. For this purpose, 10 colonies were screened for methicillin susceptibility by using the cefoxitin disk diffusion method. Confirmation of MSSA was done by Real-Time PCR. Nasal swabs from calves were analysed in pools following standard procedures previously described.(21)

To obtain an estimate of exposure in CFU per EDC, EDCs were analysed by Real-Time quantitative PCR (qPCR). EDC samples were suspended in 10 mL EDTA saline buffer (150 mM NaCl, 1 mM EDTA) and mixed in a Stomacher (Seward Ltd., London, United Kindom) for 10 minutes. Two mL of the resulting suspension was stored at -20°C for the analysis. For DNA isolation, 200 µL of the suspension was incubated at 95°C for 15 minutes. Phosphate buffered saline (PBS) was added and a Versant kPCR molecular system (Siemens Healthcare Diagnostics, The Hague, The Netherlands) was used for DNA purification with an elution volume of 50 µL. Five µL of the purified sample were used for detection of *mecA*, *femA* and *nuc* genes by the means of a LightCycler 480-II system (Roche Diagnostics, Almere, The Netherlands). For MRSA quantification, a standard curve was established for all targets. A standard control sample was included in each run to correct the curve for run-to-run variation. For interpretation of the results, CFU counts per PCR were transformed to CFU counts per EDC (1 CFU/PCR = 200 CFU/EDC). The theoretical limit of detection (LOD) was 20 CFU/EDC.

Data analysis

Statistical analysis was performed using SAS software version 9.2 (SAS institute Inc., Cary, North Carolina, USA). Descriptive analysis determined the cross-sectional human prevalences on each of the 4 sampling moments and the longitudinal carriage patterns (persistent, intermittent or non-carriers).

Risk factors for nasal MRSA persistent carriage were investigated with univariate and multivariate analysis. PROC GENMOD was used for Generalized Estimating Equations (GEE) modelling to take clustering of data at farm level into account. The mean response was modelled with a Poisson regression with robust standard errors. Crude and age-sex adjusted prevalence ratios were obtained. Eligibility criteria for variables to be considered in multivariable analysis included univariate p-values below 0.2, less than 10% of missing data in relation with the outcome, and at least 2 persistent carriers falling in each of the categories of the explanatory categorical variables. Bivariate correlation structure of all eligible variables was studied with PROC CORR and Spearman correlation coefficients were obtained. Thereafter, eligible variables were added in a stepwise backward selection approach and retained in the final model when $P<0.15$. A p-value<0.05 was considered statistically significant.

The shape of the relationships between MRSA persistent carriage and numerical variables was studied by means of nonparametric or semiparametric regression modelling (smoothing) using PROC GAM to relax the assumption of linearity. For this purpose, the number of CFU from quantitative nasal swabs positive for MRSA but below LLOQ was set to 5.

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To assess the environmental exposure during the first week, farms were classified in 3 categories: i) farm with persistent carrier, when there was at least one MRSA persistent carrier working and/or living on the farm; ii) farm with intermittent carrier, when there was at least one MRSA intermittent carrier and there was no persistent carrier on the farm; iii) non-carrier farm, when all people at the farm were MRSA-negative on the first 3 sampling moments. On week 12 farms were classified as carrier and non-carrier farms when there was at least one MRSA carrier on the farm, and when all people on the farm were MRSA-negative on week 12 respectively. Proportions of MRSA-positive EDCs were calculated per farm category and sampling moment. For calculation of average exposure levels, CFU counts per EDC were log-transformed since they followed a highly right-tailed distribution. PROC LIFEREG was used for left-censored regression (tobit) modelling to obtain an accurate estimate of the mean exposure level accounting for the large proportion of undetectable values. Thereafter geometric means (GM) were calculated.

RESULTS

Descriptive results

Nasal swabs were collected from 211 participants on 52 farms. The total population average nasal MRSA prevalence for the 4 sampling moments was 17.6% and in farmers it was twice as high (29.7%) as in family members (13.0%). Cross-sectional nasal MRSA prevalences per sampling moment are displayed in Supplemental Figure 2.

Nasal carriage patterns for MRSA, MSSA and *S. aureus* in general (including both MSSA and MRSA) were assessed over the one week period. The MRSA and MSSA persistent carrier prevalence followed opposite directions in farmers as compared to family members. For MRSA persistent carriage the prevalence in farmers (15.5%) was twice as high as in family members (7.6%). MSSA persistent carriage prevalence was three times higher in family members than in farmers (15.3% and 5.2%, respectively). Regarding *S. aureus,* there were not significant differences between the subpopulations of farmers and family members and 22.8% of all individuals were persistently carrying the bacteria, 29.6% were intermittent carriers and the remaining 47.6% never carried *S. aureus.* Supplemental Table 1 shows these longitudinal carriage patterns in more detail.

Microbiological status and persistent MRSA nasal carriage

CFU counts were determined in 42 participants from quantitative nasal swabs on day 0. Figure 1 shows the shape of the relationship between the probability of being a persistent MRSA nasal carrier and the log-transformed MRSA concentration (CFU/swab suspension). The median CFU count was 43.65 with an interquartile range (IQR) 5.01-1,096.48. In addition, the univariate logistic regression analysis in this population resulted in 1.68 times higher risk (95% Confidence Interval (CI)=1.34-2.10, $P<0.001$) for persistent MRSA carriage per 10 CFU increase.

No MSSA was found in MRSA-positive samples at day 0. In order to obtain an estimation of the prevalence ratio (PR) for the outcome when MSSA is present at day 0, data was manipulated by placing a MSSA positive result for one of the persistent carriers. This way an adjusted PR of 0.14 (95% CI=0.02-1.06, P=0.06) was obtained.

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People found positive for MRSA in throat swabs at day 0 were at higher risk for being persistent nasal carriers (adjusted PR=12.2, 95%CI=5.2-28.8, P<0.0001). The spearman correlation coefficient between this variable and the outcome was 0.6 (P<0.0001).

Univariate and multivariate analysis for persistent MRSA nasal carriage

Crude and age-sex-adjusted PRs in determinants meeting the specified criteria are presented in Table 1. Sex and smoking habits were not clearly associated with the outcome (P>0.2). Because these variables together with age are considered potential confounders, sensitivity analysis was performed with smoking habits added to sex and age for adjustment. This did not result in significant changes in estimates (results not shown) when compared to adjustment without smoking habits.

Statistically significant risk factors for persistent MRSA carriage were identified (table 2). Pet ownership showed a PR of 2.7 (P=0.05). The number of working hours per week in the farm was positively associated with the outcome (adjusted PR=2.5 expressed per 20 hours/week increase, P=0.001). An increasing probability for MRSA persistent carriage with number of hours working in the farm was also demonstrated through semiparametric regression modelling (Supplemental Figure 3). Administration of antimicrobials to calves through milk and injection in the past month preceding sampling was also a significant risk factor (adjusted PR= 3.4, P=0.01). Other associations with the outcome did not show statistical significance. These include protective factors such as people living on farms with a changing room available (adjusted PR=0.5, P=0.07) or on farms where clean towels are used after work (adjusted PR=0.6, P=0.11) and risk factors, such as people living in farms where baby boxes are cleaned at the beginning of the production cycle (adjusted PR=1.3, P=0.54). Other determinants such as the prevalence of MRSA in animals at the farm level did not show an association with persistent human MRSA carriage (PR=1.0, 95%CI=1.0-1.0, P=0.96). There was also no association found with variables regarding individual health status.

Results from the multiple logistic regression analysis are presented in Table 2. In model A, all variables meeting the described criteria were eligible to entry. In this model, number of working hours per week showed the most significant association with persistent MRSA carriage (PR=1.8 expressed per 20 hours/week increase, P<0.0001). Because this variable was a very strong determinant, as a result of which potential tasks were not retained, a model was explored (model B) without the number of working hours. In consequence, stable management (sorting calves) was retained in the final model B with a statistically significant PR of 3.1 (P=0.03). In both multivariate models, the presence of cats on the farm was significantly associated with the outcome (PR=2.8, P=0.01 in model A and PR=2.6, P=0.04 in model B).

Specific tasks on the farm were adjusted for number of working hours in a bivariate analysis and the estimates obtained were not statistically significant. Only stable management remained positively associated with the outcome with a PR of 2.5 (95%CI=0.7-9.6; P=0.17); however, administration of antibiotics in the month before sampling showed no association with a PR of 1.1 (95%CI=0.2-5.9; P=0.91).

Table 1. Crude and adjusted for sex and age prevalence ratios (PR) for nasal MRSA persistent carriage in 195 veal calf farmers and household members from 51 farms.

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Determinant	Category	N	No. Persistent carriers[a] (prevalence %)	Mean (range)	PR	95% CI	PR[b] Adj	95% CI
General characteristics:								
Sex	Male	103	9 (8.7)	-	1	-	-	-
	Female	92	11 (12.0)	-	1.4	0.6-3.2	-	-
Age	-	195	-	30 (0.1-81)	1.0	1.0-1.0**	-	-
per 10 years increase	-	195	-	-	1.3	1.1-1.6**	-	-
Farm and household characteristics:								
Presence of sheep in farm	No	149	12 (8.1)	-	1	-	1	-
	Yes	46	8 (17.4)	-	2.2	1.1-4.5*	2.4	1.2-4.8*
Presence of cats on farm	No	96	5 (5.2)	-	1	-	1	-
	Yes	99	15 (15.2)	-	3.0	1.2-7.1*	2.7	1.1-6.6*
Presence of pets	No	74	4 (5.4)	-	1	-	1	-
	Yes	121	16 (13.2)	-	2.7	1.0-7.4*	2.6	1.0-6.7[t]
Tasks performed last 7 days[c] :								
Sorting calves (stable management)	No	113	5 (4.4)	-	1	-	1	-
	Yes	82	15 (18.3)	-	4.2	1.5-12.3**	4.7	1.3-16.8*
Healthcare / control [d]	No	132	9 (6.8)	-	1	-	1	-
	Yes	63	11 (17.5)	-	2.6	1.1-6.1*	2.3	0.8-7.3
Feeding calves	No	72	2 (2.8)	-	1	-	1	-
	Yes	123	18 (14.6)	-	7.2	0.9-58.6[t]	5.4	0.6-52.3
Work at farm, hygiene cleaning and disinfection								
Administration of antibiotics during last month	No	131	8 (6.1)	-	1	-	1	-
	Yes	64	12 (18.8)	-	3.2	1.4-7.1**	3.4	1.3-9.1*
# working hours per week	-	195	-	16.5 (0-80)	1.0	1.0-1.0***	1.0	1.0-1.1**
per 20 hours increase	-	-	-	-	1.8	1.4-2.4***	2.5	1.4-4.2**
Clean towel	No	45	7 (16.7)	-	1	-	1	-
	Yes	150	13 (8.67)	-	0.6	0.3-1.3	0.6	0.3-1.1
Changing room available	No	18	3 (16.7)	-	1	-	1	
	Yes	177	17 (9.7)	-	0.6	0.3-1.2	0.5	0.2-1.0[t]
Cleaning of baby boxes	No	184	18 (9.8)	-	1	-	1	-
	Yes	11	2 (18.2)	-	1.9	1.0-3.5*	1.3	0.6-2.8

[a] A person is considered a persistent carrier when all nasal swabs at days 0, 4 and 7 are positive for MRSA.
[b] Prevalence ratios adjusted for sex and age.

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[c] Tasks performed in the week before time 0.
[d] The task healthcare and control includes the administration of antibiotics.
[t] Nonsignificant trend (P-value 0.05-0.10). [*] P-value 0.01-0.05. [**] P-value 0.0001-0.01. [***] P-value <0.0001.

Table 2. Results from multiple logistic regression analysis for nasal MRSA persistent carriage in veal calf farmers and their household members (N=195). Model A: final model in which all variables meeting eligibility criteria were added to the automatic selection. Model B: final model in which all the variables in model A were added to the automatic selection except # working hours.

Determinant	Category	PR	95% CI	P-value
MODEL A				
# working hours per week	-	1.03	1.02-1.04	0.000*
per 20 hours increase		1.81	1.49-2.19	-
Presence of cats on farm	No	1	-	-
	Yes	2.80	1.23-6.36	0.014*
Presence of sheep in farm	No	1	-	-
	Yes	1.83	0.89-3.77	0.100
Changing room available	No	1	-	-
	Yes	0.48	0.20-1.13	0.094
Cleaning of baby boxes	No	1	-	-
	Yes	3.96	1.59-9.90	0.003*
MODEL B				
Age	-	1.02	1.00-1.05	0.037*
per 10 years increase	-	1.26	1.01-1.56	-
Presence of cats on farm	No	1	-	-
	Yes	2.57	1.05-6.33	0.040*
Presence of sheep in farm	No	1	-	-
	Yes	1.78	0.88-3.59	0.107
Sorting calves	No	1	-	-
	Yes	3.10	1.14-8.47	0.027*

* P-value statistically significant (i.e. < 0.05).

Contamination of the environment with MRSA

At the beginning of the production cycle, MRSA was detected in only 4.6% of all EDCs placed in stables and on 6 farms. Differences in environmental exposure across persistent, intermittent and non-carrier farms were not significant (Table 3). None of the EDCs placed inside the houses were found to be positive for MRSA.

In week 12, MRSA was detected in 50.6% of all EDCs placed in the stables and on 39 farms. There was a significantly higher proportion of EDCs positive for MRSA and a trend for higher CFU counts per EDC in farms where MRSA carriers were found in week 12 (Table 4). Stratified analysis was performed in farmers and family members. The same trends

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for higher MRSA environmental load were found only in farmers, however not statistically significant (results not shown). MRSA was found in EDCs from 10 houses (Table 4).

The mean pooled MRSA prevalence in calves rose from 18.7% at day 0 to 46% in week 12. A simple linear regression between the EDC MRSA levels (maximum log-transformed MRSA CFU/EDC per farm) and animal prevalence showed a positive and significant association (β=0.006, P=0.0014). Furthermore, there was a 60% increased probability for detecting a MRSA-positive EDC in farms where animal prevalence in week 12 was above the mean (PR=1.6, 95%CI=1.09-2.38, P=0.02). With regards to human carriage in relation to animal prevalence, no association between being a MRSA carrier and the prevalence in calves was found on day 0. However, a significant association was found at the last sampling moment. Individuals from farms with MRSA prevalence in calves above the mean were at 2 times higher risk for carrying MRSA in week 12 (PR=2.12, 95%CI=1.12-4.01, P=0.02).

Table 3. Environmental MRSA samples (EDCs) taken in stables at the beginning of the production cycle in 51 farms with persistent, intermittent or non-MRSA carrying veal calf farmers and household members.

	Persistent[a]	Intermittent[a]	Non-carrier[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	2/18 (11.11)	2/12 (16.67)	2/21 (9.52)	0.86
No. MRSA positive EDCs / total no. EDCs (%)	2/69 (2.90)	4/47 (8.51)	3/78 (3.85)	0.38
GM MRSA CFU/EDC (p-value)[c]	<1 (0.75)	<1 (0.29)	<1 (ref.)	-

[a] A farm was categorised as persistent when there was at least one persistent carrier living and/or working on the farm, non-carrier farms had no individual positive for MRSA in nasal swabs on days 0,4,7 and intermittent farms were the remaining.
[b] P-values among proportions were calculated with Fisher's exact test. Mean values had not an overall assigned p-value since they could not be tested with non-parametric tests.
[c] Geometric mean (antilogged results from tobit regression). P-values indicate the difference with the reference category (non-carrier farm).

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Table 4. Environmental MRSA samples (EDCs) taken in stables on week 12 in 49 farms with MRSA carriers and non-carriers.

	Location EDC	Carrier farms[a]	Non-carrier farms[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	Stable	22/25 (88.00)	17/24 (70.83)	0.14
	House	3/25 (12.00)	7/24 (29.17)	0.17
No. MRSA positive EDCs / total no. EDCs (%)	Stable	54/90 (60.00)	35/86 (40.70)	0.01
	House[d]	-	-	-
GM[c] MRSA CFU/EDC	Stable	27.54	16.98	0.06
	House	2.29	5.50	0.29

[a] A farm was categorised as carrier when there was at least one carrier on week 12 living and/or working on the farm, non-carrier farms were the remaining.
[b] P-values among proportions were calculated with Chi-square test and Fisher's exact test when 20% of the expected cell values were <5. P-values for the GM indicate the difference with the reference category (non-carrier farms).
[c] Geometric mean (antilogged results from tobit regression).[d] There was one EDC per house, thus the values in this line are the same as the ones in "No. farms with MRSA positive EDCs / total no. farms (%)".

DISCUSSION

The associations found during the first week after arrival of the animals on the farm show that the level of exposure to veal calves and the presence of potential animal reservoirs (pets, free-ranging farm cats and sheep) are risk factors for persistent MRSA carriage in farmers and household members. Additionally, persistent MRSA carriers seem to have a different microbiological profile when compared to intermittent and non-carriers, which is characterised by higher MRSA CFU counts, presence of MSRA in throat and absence of MSSA. This study shows that as the production cycle advances, there is a rise in MRSA prevalence in calves that leads to higher contamination of the air and higher probability for human MRSA carriage.

Descriptive results confirm that high MRSA carriage prevalence (17.6%) is observed among individuals living on farms, as seen in other studies.(2,16) This percentage represents a carriage burden in countries where estimated MRSA prevalence in community is below 1% such as the Netherlands and Scandinavian countries. The large difference in prevalence between farmers and family members can be attributed to the different intensity of animal contact and is again an indication of a low LA-MRSA human-to-human transmission.(16,22) The carriage patterns of *S. aureus* presented are similar to those described by Wertheim and co-authors,(23) in which they found percentages of 20%, 30% and 50% for persistent, intermittent and non-carriers respectively among healthy individuals. The lower MRSA persistent carrier prevalence in the total study population (9.7%) as compared to the average cross-sectional MRSA prevalence (17.6%) indicates that carriage of LA-MRSA is fleeting and varies within individuals.

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Due to culturing techniques, MSSA was detected with difficulty when there was a predominant MRSA growth. The possible underestimation of MSSA asks for a cautious interpretation of the results. Nevertheless it is remarkable that no persistent MRSA carrier was positive for MSSA at day 0. This suggests that the presence of MSSA in the nose might be a protective factor for MRSA persistent carriage. Moreover, a negative association between MSSA and MRSA has been recently found in a study.(14)

In the first week of the production cycle the MRSA environmental load was lower and it can be assumed that nasal contamination with MRSA-containing dust particles and transient mechanical carriage was less likely to occur as compared to further time points in the production cycle. As shown in figure 2, there is an increased probability for persistent MRSA carriage associated with higher MRSA CFU counts in nasal swabs. Moreover, isolation of MRSA in throat swabs at day 0 was significantly associated to the outcome (PR=12.2). These findings suggest that there might be a true colonization in persistent MRSA carriers as defined here. Furthermore a recent study has shown that ST398 is capable of adequately competing for a niche with a human strain and survives in the human nose for longer periods.(24)

Direct association between administration of antibiotics and MRSA persistent carriage in farmers and their family members, as defined in our study, was shown in univariate results (PR=3.2). It is known that when antimicrobials are administered to animals, substantial quantities of these drugs can be present in manure, on surfaces of animal houses and in dust as a potential risk source.(25) We could hypothesize that aspiration of dust containing antibiotics, either from a contaminated environment or directly from a powder formulation, would exert a selective pressure in the anterior nares leading to higher risk for MRSA persistent carriage in people occupationally exposed. However, this association was not confirmed in multivariate models and it needs further exploration. Number of working hours and other tasks were correlated and may have more influence on persistent carriage. This was also shown when adjustment for number of working hours was done in a bivariate fashion.

This study supports that close contact with animals is a major risk factor for persistent LA-MRSA carriage in humans. This is made clear by the final set of variables retained in the multivariate models. The number of working hours was most strongly associated with persistent carriage as indicated by the model A and by the smoothed exposure-response relation shown in the Supplemental Figure 3. Moreover, when the number of working hours was removed for model B, another variable representing close contact with animals (stable management) was retained by the backward procedure.

In recent years, several reports have suggested a potential role for pet animals, specifically cats and dogs, in household MRSA transmission and relapse of human MRSA infections. This transmission seems to be of anthropozoonotic origin. Thus, pets can acquire human strains from humans and they can cause colonization or infection in human cohabitants.(26-31) In most cases, the distribution of the clones in pet animals has mirrored the epidemiology of human clones and mainly shared hospital-associated (HA) and community-associated (CA) MRSA strains have been reported. It is remarkable that in this study, having a pet in the household was strongly associated with MRSA carriage in veal farmers and household members. Moreover, there is a demonstrated spread of LA-MRSA between animal species, humans and the farm environment.(32) In this study the presence of free-ranging farm cats and sheep were significantly associated and retained in multivariate models. These animals might represent an intermittent source of LA-MRSA that might

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contribute to LA-MRSA persistent carriage in humans. However, no other animals apart from veal calves were sampled in this study.

Other farm characteristics and hygiene practices were also associated with persistent MRSA carriage, although not significantly. Having a changing room in the farm and using a clean towel after working in the stables were found as protective factors. This might give a direction to specific preventive strategies. On the other hand, cleaning of baby boxes at the beginning of the production cycle was a risk factor for the outcome (PR=4 in multivariate model A and PR=1.9 in univariate analysis). This hygiene practise could give rise to transitory spread in the air of accumulated MRSA.

Environmental contamination with dust particles containing MRSA is much lower in veal calf farming as compared to pig farming and associations are less evident.(33) As shown in table 3, no difference in the environmental MRSA load was found across persistent, intermittent and non-carrier farms at the beginning of the production cycle. However, the two-fold rise in animal prevalence at the end of the study was associated with a considerably higher environmental MRSA load and a significantly higher proportion of MRSA-positive EDCs was found on farms with MRSA carriers on week 12. This finding supports that contamination of the environment plays a role in the acquisition of MRSA in people living or working in the farm.

A possible limitation of the study is the self-sampling of nose and throat by individuals which might be lacking of accuracy for MRSA detection. This is however believed to be a minor bias. A recent pilot study has shown high degree of agreement between self-samples and investigator samples (93% agreement, kappa 0.85 for nasal swabs and 83% agreement, kappa 0.60 for throat swabs).(34) Another limitation is the previously described underestimation of MSSA presence but this is of negligible impact in the results because detection of MRSA and *S. aureus* remains unaffected. Finally, there were many missing values in some variables and they were excluded from the analysis. There were 5 individuals (/211=2%) with missing nasal samples but sensitivity analysis did not reveal significant changes in estimates.

In conclusion, people living and/or working in veal calf farms who persistently carry MRSA seem to be defined by a differential microbiological profile. The associations found here with the presence of free-ranging farm cats and multispecies farming ask for improved internal and external biosecurity measures. Detailed molecular-epidemiological analysis of MRSA specimens on the farm in various animal species and humans is also essential to identify reservoirs and transmission routes for LA-MRSA. Finally, environmental contamination with MRSA has to be thoroughly studied to assess the extent of its importance in the transmission of MRSA within the veal-calf farming community.

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COMPETING INTERESTS

None declared

CONTRIBUTORSHIP

Alejandro Dorado-García performed the statistical analyses and interpretation of the data, and drafted the manuscript. Marian EH Bos collected the data, contributed to the interpretation of the data, and contributed to the critical revision of the manuscript. Haitske Graveland participated in the conception and design of the study, collected the data and contributed to the critical revision of the manuscript. Brigitte AGL van Cleef and Jan AJW Kluytmans contributed to the critical revision of the manuscript. Koen M Verstappen carried out the laboratory analysis. Jaap A Wagenaar conceived the study and contributed to the critical revision of the manuscript. Dick JJ Heederik conceived the study and contributed to the interpretation of the data and the critical revision of the manuscript. All authors read and approved the final manuscript.

DATA SHARING

Data will not be publicly accessible.
Interested individuals may contact the authors

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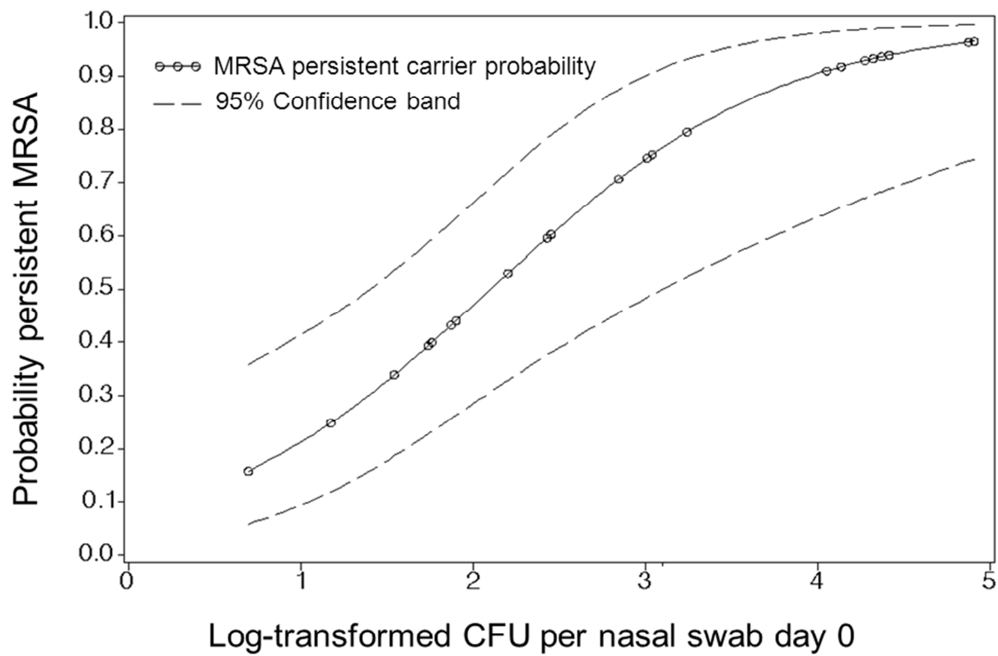

Figure 1. Probability of nasal MRSA persistent carriage and its relationship with the log-transformed CFU from MRSA positive nasal swabs at day 0. Nonparametric regression modelling.
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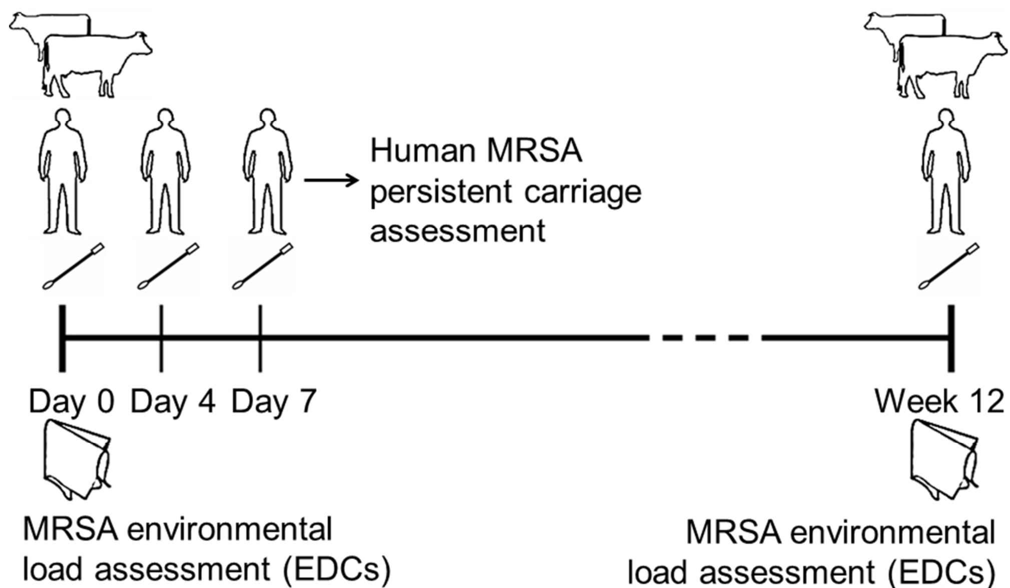

Supplemental Figure 1. Schematic overview of the study design.
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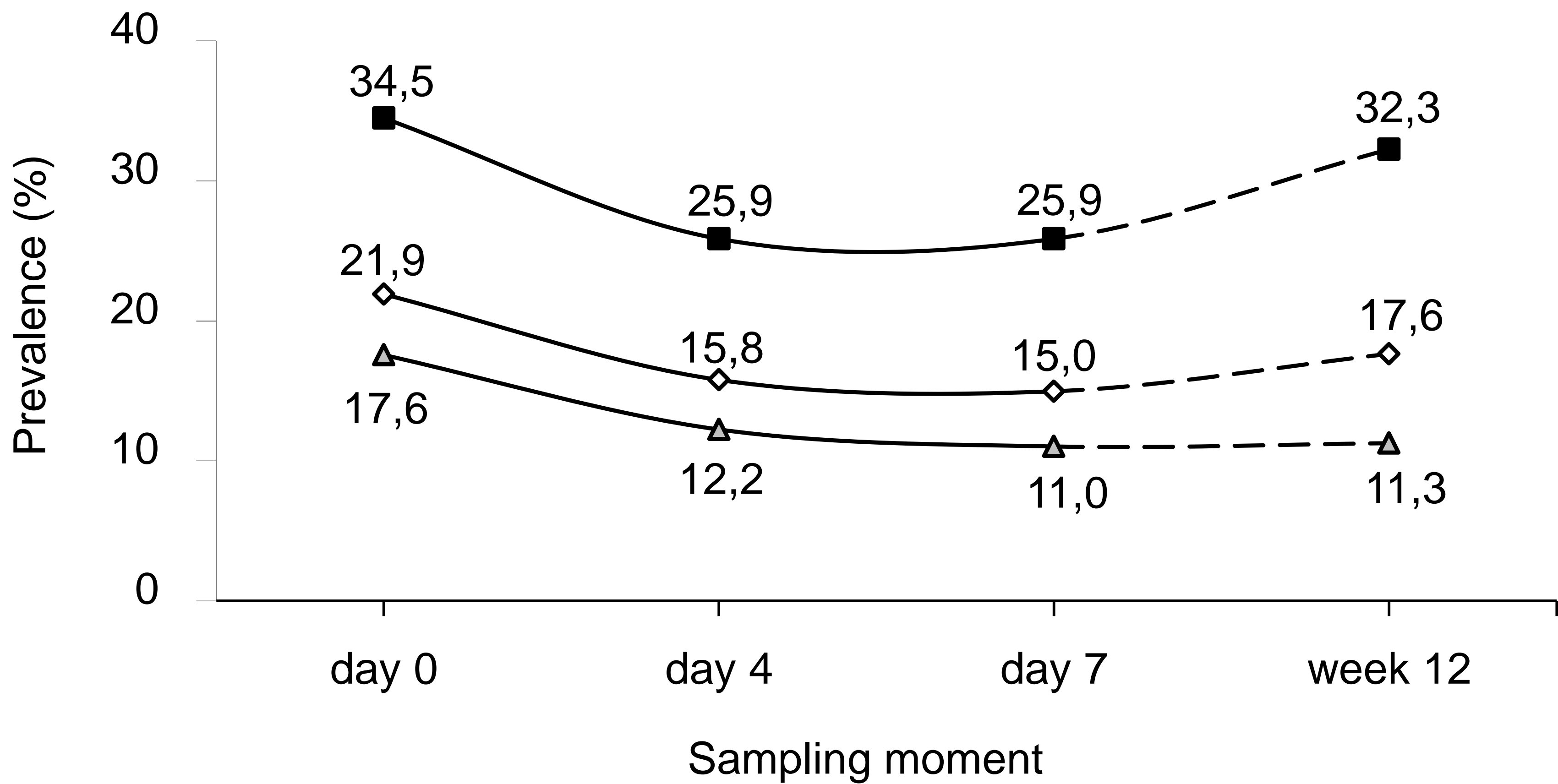
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MRSA in family members

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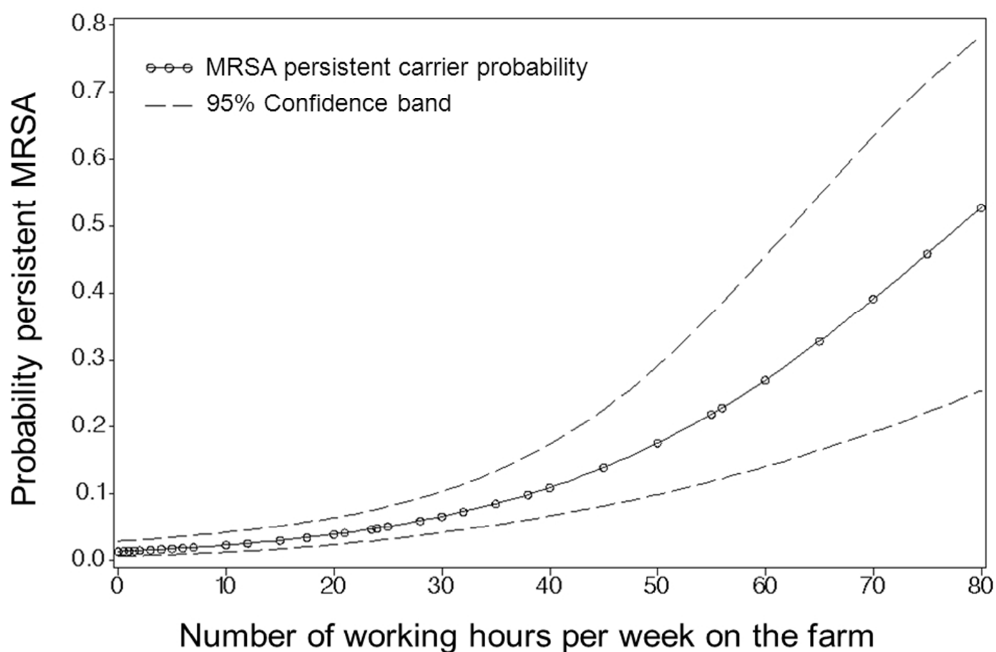

Supplemental Figure 3. Probability of nasal MRSA persistent carriage and its relationship with number of working hours in the farm. Semiparametric regression modelling setting sex and age as parametric components for adjustment.
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Supplemental Table 1. Patterns for one week nasal carriage of *S. aureus*, MRSA and MSSA in the total study population and subpopulations of farmers, household members and employees.

	No. persistent (%)[a]	No. intermittent (%)[a]	No. non-carrier (%)[a]	Total no.
MRSA in nose:				
Total population [b]	20 (9.7)	35 (17.0)	151 (73.3)	206
Farmers	9 (15.5)	15 (25.9)	34 (58.6)	58
Family members	11 (7.6)	20 (13.9)	113 (78.5)	144
Employees	0 (0.0)	0 (0.0)	4 (100.0)	4
MSSA in nose:				
Total population [b]	25 (12.1)	36 (17.5)	145 (70.4)	206
Farmers	3 (5.2)	13 (22.4)	42 (72.4)	58
Family members	22 (15.3)	22 (15.3)	100 (69.4)	144
Employees	0 (0.0)	1 (25.0)	3 (75.0)	4
S. aureus in nose:				
Total population [b]	47 (22.8)	61 (29.6)	98 (47.6)	206
Farmers	14 (24.1)	21 (36.2)	23 (39.7)	58
Family members	33 (22.9)	39 (27.1)	72 (50.0)	144
Employees	0 (0.0)	1 (25.0)	3 (75.0)	4

[a] A person was persistent carrier when each of the nasal swabs collected on days 0, 4 and 7 was positive for MRSA; non-carriers had no positive swabs; intermittent carriers were the remaining persons.
[b] there were 5 missing values (total study population = 211).

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up Farms and participants willing to participate *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at Not reported in manuscript.
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
	NA.	(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Section/Topic	Item #	Mark	Recommendation
Results			
Participants	13*	✓	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		NA	(b) Give reasons for non-participation at each stage
		NA	(c) Consider use of a flow diagram
Descriptive data	14*	✓	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
			(b) Indicate number of participants with missing data for each variable of interest
			(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*	✓	*Cohort study*—Report numbers of outcome events or summary measures over time
			Case-control study—Report numbers in each exposure category, or summary measures of exposure
			Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16	✓	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		✓	(*b*) Report category boundaries when continuous variables were categorized
		NA	(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	✓	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion			
Key results	18	✓	Summarise key results with reference to study objectives
Limitations	19	✓	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	✓	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	✓	Discuss the generalisability (external validity) of the study results
Other information			
Funding	22	✓	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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Risk factors for livestock-associated MRSA persistent carriage and environmental exposure in veal calf farmers and their family members: an observational longitudinal study.

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Title:

Risk factors for livestock-associated MRSA persistent carriage and environmental exposure in veal calf farmers and their family members: an observational longitudinal study.

Short Title: LA-MRSA persistent carriage and environmental exposure in pig farming.

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Key words (max 5): Livestock-Associated MRSA, persistent carriage, longitudinal study, veal calves, environmental contamination

Word count excluding title page, abstract, references, figures and tables: 4.168

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ABSTRACT

Objectives: LA-MRSA emergence is a major public health concern. This study is aimed at assessing risk factors for persistently carrying MRSA in veal calf farmers and their family members. We also evaluate the dynamics of MRSA environmental load during the veal-calf production cycle.

Design: Observational, longitudinal, repeated cross-sectional study.

Setting: 52 veal calf farms in the Netherlands.

Participants: Between the end of 2010 to the end of 2011, a total of 211 farmers, family members and employees were included in the study.

Primary outcome and secondary outcome measures: Nasal swabs were taken from participants on days 0, 4, 7 and week 12. A persistent MRSA carrier was defined as a person positive for MRSA on days 0, 4 and 7. Participants filled in an extensive questionnaire to identify potential risk factors and confounders. For estimation of MRSA prevalence in calves and environmental contamination, animal nasal swabs and Electrostatic Dust Collectors (EDCs) were taken on day 0 and week 12.

Results: The presence of potential animal reservoirs (free-ranging farm cats and sheep) and the level of contact with veal calves was positively associated with persistent MRSA carriage. Interestingly, at the end of the study (week 12), there was a two-fold rise in animal prevalence and a significantly higher MRSA environmental load in the stables was found on farms with MRSA carriers.

Conclusions: This study supports the hypothesis that environmental contamination with MRSA plays a role in the acquisition of MRSA in farmers and their household members and suggests that other animal species should also be targeted to implement effective control strategies.

ARTICLE SUMMARY

Article focus:

- Determinants for persistent LA-MRSA carriage in humans and for a possible true colonization have not been thoroughly assessed.
- It is unclear whether bacterial contamination in the farm environment plays a role in LA-MRSA transmission in humans

Key messages:

- The presence of other animals in the farm might be of importance in acquisition and persistence LA-MRSA in humans. There is a need for detailed molecular-epidemiological analysis of MRSA specimens in various animal species and humans in the veal-calf farming community.

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- During the veal-calf production cycle, there is a parallel increase in animal prevalence and environmental MRSA load which is linked to higher risk for human carriage.

Strengths and limitations of this study:

The longitudinal nature of the data allows to establish dynamic epidemiological inferences.

No other animals apart from veal-calves were sampled in this study. The self-sampling of noses by individuals might influence the sensitivity for MRSA detection.

INTRODUCTION

In recent years, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), specifically sequence type (ST) 398, has emerged in food-producing animals and people in contact with these animals.(1-4) Illness associated to ST398 in humans is rare and only a small proportion of MRSA infections can be attributed to LA-MRSA.(5,6) Nonetheless, invasive infections and hospital outbreaks of MRSA ST398 have been reported in Europe, the United States and Asia.(5,7,8)

LA-MRSA strains have been found mainly in pigs and veal calves, but they have the capacity to colonize a wide spectrum of hosts, including sheep and poultry.(9) Farmers are easily contaminated and in general the carriage prevalence in farmers is high. Frequency of transmission between farmers and their family members and among hospitalized humans appears to be low.(2,10,11) However, this belief might be contradicted by recently described LA-MRSA transmission events in Dutch patients with neither risk factors nor livestock contact.(12) The potential public health threat posed by these strains is emphasized in a recent metapopulation model in which the likelihood of persistent carriage in the livestock-exposed population was the key parameter for LA-MRSA spreading to the community.(13)

Previous studies have been mainly based on cross-sectional designs and have shown that intensity of animal contact and MRSA prevalence among animals are positively associated to LA-MRSA human carriage.(14) Associations between animal carriage and farm hygiene and antimicrobial use have also been shown.(15,16) A longitudinal study including periods of high and low exposure to animals showed that LA-MRSA carriage was mainly transient. It was suggested that LA-MRSA is a poor persistent colonizer in humans, which was confirmed by a study on short term occupational exposure.(10,14) However, risk factors for persistent LA-MRSA carriage and for a possible true colonization have not been thoroughly assessed. Furthermore, little is still known about the dynamics of environmental contamination with MRSA in the farm and its role in transmission to humans. A recent study showed a steep increase in prevalence among calves and in MRSA air load during the production cycle.(17)

The aim of the current study is twofold. Firstly, to assess risk factors and dose-response relationships for persistently carrying MRSA over a period of one week at the beginning of the production cycle in veal calf farmers and their family members. Secondly, to evaluate the deposition of MRSA-containing dust inside the farm and its relationship with animal and human MRSA carriage.

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128 **MATERIALS AND METHODS**

Study design and population

A longitudinal cohort study was performed over a period of 12 weeks in 52 veal calf farms starting at the beginning of the production cycle. All farms were visited from the end of 2010 to the end of 2011. All farms met the following inclusion criteria: implemented all-in-all-out system; no other livestock in large scale apart from veal calves; an unique location for all the stables or farm; veal calf farmers not working in another animal sector (e.g. transport of pigs) and not operating in other farms. Preference for selection was given to farms in the proximity of Utrecht, the Netherlands. On each farm there were 2 sampling moments for animal and environmental samples (day 0 and week 12) and 4 sampling moments for human samples (days 0, 4, 7 and week 12). Nasal swabs from both anterior nares of calves were taken and analysed in 10 pools of 6 swabs each (60 animals per farm). Swabs were also collected from farmers, family members and employees (n=211). On day 0, quantitative nasal and throat swabs were taken by field workers in the majority of participants or by self-sampling. On days 4, 7 and on week 12, dry cotton swabs (Copan, Brescia, Italy) were used to self-sample the nose. Swabs were given to participants with instructions including photographs in case of self-sampling. Nasal swabs in animals and humans were introduced in the nostril and rotated once. Throat swabs in humans sampled the area of the inner cheek including the tonsils. The swabs were immediately taken to the laboratory or sent by post and processed within 24 hours after arrival. Furthermore, environmental samples were taken by placing 4 Electrostatic Dust Collectors (EDCs) (Zeeman, Utrecht, The Netherlands) on different surfaces inside the stables and one on the highest cupboard in the living room or kitchen of the house. The EDCs were left in place during a period of 2 weeks and sent by post to the laboratory. Upon arrival, EDC samples were stored at -20°C until quantitative analysis.(18)

All participants completed an informed consent and filled in an extensive questionnaire including items related to individual health status, household and farm characteristics, activities performed on the farm and hygiene practices. The protocol of the study was approved by Medical Ethical Committee of Utrecht University. The collection of animal samples was in compliance with the Dutch Law on Animal Health and Welfare.

For the assessment of MRSA persistent carriage, we selected the beginning of the veal calf production cycle, just after the stables were empty and when animal prevalence is lower. In this period, deposition of MRSA-containing dust particles in human nasal cavities and mechanical carriage was assumed to be less likely. Therefore and for the purpose of this study, a person was defined to be a persistent MRSA carrier when each of the nasal swabs collected on days 0, 4 and 7 were positive for MRSA presence.

Laboratory analysis

Swabs in liquid transport medium (ESwab, Copan, Brescia, Italy) were used for quantitative cultivation. Serial dilutions (1:10) of the transport medium (concentration 10^0) were made by adding 100 µl sample to 900 µl phosphate buffered saline (PBS) to a final concentration of 10^{-4} of the original sample. Each dilution was cultured on chromID *S. aureus* and chromID MRSA agar plates (BioMérieux, La Balme Les Grottes, France) at 37°C for 18-24 hours. Plates with 10-100 colony-forming units (CFU) were used to calculate the original amount of CFU per swab. In order to detect positive samples without bacterial growth in the

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first day, the remaining transport medium and swab were enriched overnight in Mueller Hinton broth with 6.5% NaCl (MH+), and consequently cultured on chromID *S. aureus* and chromID MRSA agar plates. The theoretical lower limit of quantification (LLOQ) of MRSA CFU was 10. Dry cotton swabs (Copan) were inoculated directly onto chromID *S. aureus*, chromID MRSA and MH+. Confirmation of MRSA presence in the 3 sampling moments was done by Real-Time (RT) PCR targeting *mecA*, *femA* and *nuc* genes.(19,20) Methicillin-susceptible *Staphylococcus aureus* (MSSA) presence was tested when the bacterial growth on chromID *S. aureus* was higher than on chromID MRSA. For this purpose, 10 colonies were screened for methicillin susceptibility by using the cefoxitin disk diffusion method. Confirmation of MSSA was done by Real-Time PCR. Nasal swabs from calves were analysed in pools following standard procedures previously described.(21)

To obtain an estimate of exposure in CFU per EDC, EDCs were analysed by Real-Time quantitative PCR (qPCR). EDC samples were suspended in 10 mL EDTA saline buffer (150 mM NaCl, 1 mM EDTA) and mixed in a Stomacher (Seward Ltd., London, United Kindom) for 10 minutes. Two mL of the resulting suspension was stored at -20°C for the analysis. For DNA isolation, 200 µL of the suspension was incubated at 95°C for 15 minutes. Phosphate buffered saline (PBS) was added and a Versant kPCR molecular system (Siemens Healthcare Diagnostics, The Hague, The Netherlands) was used for DNA purification with an elution volume of 50 µL. Five µL of the purified sample were used for detection of *mecA*, *femA* and *nuc* genes by the means of a LightCycler 480-II system (Roche Diagnostics, Almere, The Netherlands). For MRSA quantification, a standard curve was established for all targets. A standard control sample was included in each run to correct the curve for run-to-run variation. For interpretation of the results, CFU counts per PCR were transformed to CFU counts per EDC (1 CFU/PCR = 200 CFU/EDC). The theoretical limit of detection (LOD) was 20 CFU/EDC.

RT-PCR targeted at C01 gene was done for confirmation of ST398 in all MRSA positive human, animal and environmental samples.

Data analysis

Statistical analysis was performed using SAS software version 9.2 (SAS institute Inc., Cary, North Carolina, USA). Descriptive analysis determined the cross-sectional human prevalences on each of the 4 sampling moments and the longitudinal carriage patterns (persistent, intermittent or non-carriers).

Risk factors for nasal MRSA persistent carriage were investigated with univariate and multivariate analysis. PROC GENMOD was used for Generalized Estimating Equations (GEE) modelling to take clustering of data at farm level into account. The mean response was modelled with a Poisson regression with robust standard errors. Crude and age-sex adjusted prevalence ratios were obtained. Eligibility criteria for variables to be considered in multivariable analysis included univariate p-values below 0.2, less than 10% of missing data in relation with the outcome, and at least 2 persistent carriers falling in each of the categories of the explanatory categorical variables. Bivariate correlation structure of all eligible variables was studied with PROC CORR and Spearman correlation coefficients were obtained. Thereafter, eligible variables were added in a stepwise backward selection approach and retained in the final model when $P<0.15$. A $p\text{-value}<0.05$ was considered statistically significant.

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220 The shape of the relationships between MRSA persistent carriage and numerical
221 variables was studied by means of nonparametric or semiparametric regression modelling
222 (smoothing) using PROC GAM to relax the assumption of linearity. For this purpose, the
223 number of CFU from quantitative nasal swabs positive for MRSA but below LLOQ was set
224 to 5.
225
226 To assess the environmental exposure during the first week, farms were classified in 3
227 categories: i) farm with persistent carrier, when there was at least one MRSA persistent
228 carrier working and/or living on the farm; ii) farm with intermittent carrier, when there was at
229 least one MRSA intermittent carrier and there was no persistent carrier on the farm; iii) non-
230 carrier farm, when all people at the farm were MRSA-negative on the first 3 sampling
231 moments. On week 12 farms were classified as carrier and non-carrier farms when there was
232 at least one MRSA carrier on the farm, and when all people on the farm were MRSA-
233 negative on week 12 respectively. Proportions of MRSA-positive EDCs were calculated per
234 farm category and sampling moment. For calculation of average exposure levels, CFU counts
235 per EDC were log-transformed since they followed a highly right-tailed distribution. PROC
236 LIFEREG was used for left-censored regression (tobit) modelling to obtain an accurate
237 estimate of the mean exposure level accounting for the large proportion of undetectable
238 values. Thereafter geometric means (GM) were calculated.
239

240 **RESULTS**

241 **Descriptive results**

242 Nasal swabs were collected from 211 participants on 52 farms. The average nasal MRSA
243 prevalence for the 4 sampling moments was twice as high in farmers (29.7%) as compared to
244 family members (13.0%). Cross-sectional nasal MRSA prevalences per sampling moment are
245 displayed in Supplemental Figure1.

246 Nasal carriage patterns for MRSA, MSSA and *S. aureus* in general (including both
247 MSSA and MRSA) were assessed over the one week period. The MRSA and MSSA
248 persistent carrier prevalence followed opposite directions in farmers as compared to family
249 members. For MRSA persistent carriage the prevalence in farmers (15.5%) was twice as high
250 as in family members (7.6%). MSSA persistent carriage prevalence was three times higher in
251 family members than in farmers (15.3% and 5.2%, respectively). Regarding *S. aureus,* there
252 were not significant differences between the subpopulations of farmers and family members
253 and 22.8% of all individuals were persistently carrying the bacteria, 29.6% were intermittent
254 carriers and the remaining 47.6% never carried *S. aureus.* Supplemental Table 1 shows these
255 longitudinal carriage patterns in more detail.

256 The RT-PCR targeted at C01 gene showed that ST398 was present in 90.5% of the
257 human MRSA isolates, in 97.9% of the MRSA positive animal pools and 90.9% of the
258 MRSA positive EDCs.

259 **Microbiological status and persistent MRSA nasal carriage**

260 CFU counts were determined in 42 participants from quantitative nasal swabs on day 0.
261 Figure 1 shows the shape of the relationship between the probability of being a persistent
262 MRSA nasal carrier and the log-transformed MRSA concentration (CFU/swab suspension).
263 The median CFU count was 43.65 with an interquartile range (IQR) 5.01-1,096.48. In

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264 addition, the univariate logistic regression analysis in this population resulted in 1.68 times
265 higher risk (95% Confidence Interval (CI)=1.34-2.10, P<0.001) for persistent MRSA carriage
266 per 10 CFU increase.

267 No MSSA was found in MRSA-positive samples at day 0. In order to obtain an
268 estimation of the prevalence ratio (PR) for the outcome when MSSA is present at day 0, data
269 was manipulated by placing a MSSA positive result for one of the persistent carriers. This
270 way an adjusted PR of 0.14 (95% CI=0.02-1.06, P=0.06) was obtained.

271 People found positive for MRSA in throat swabs at day 0 were at higher risk for being
272 persistent nasal carriers (adjusted PR=12.2, 95%CI=5.2-28.8, P<0.0001). The spearman
273 correlation coefficient between this variable and the outcome was 0.6 (P<0.0001).

274 Sensitivity analysis was done restricted to ST398 and it yielded similar results as
275 described above.

276 **Univariate and multivariate analysis for persistent MRSA nasal carriage**

277 Crude and age-sex-adjusted PRs in determinants meeting the specified criteria are
278 presented in Table 1. Sex and smoking habits were not clearly associated with the outcome
279 (P>0.2). Because these variables together with age are considered potential confounders,
280 sensitivity analysis was performed with smoking habits added to sex and age for adjustment.
281 This did not result in significant changes in estimates (results not shown) when compared to
282 adjustment without smoking habits.

283 Statistically significant risk factors for persistent MRSA carriage were identified (table
284 2). Pet ownership showed a PR of 2.7 (P=0.05). The number of working hours per week in
285 the farm was positively associated with the outcome (adjusted PR=2.5 expressed per 20
286 hours/week increase, P=0.001). An increasing probability for MRSA persistent carriage with
287 number of hours working in the farm was also demonstrated through semiparametric
288 regression modelling (Supplemental Figure2). Administration of antimicrobials to calves
289 through milk and injection in the past month preceding sampling was also a significant risk
290 factor (adjusted PR= 3.4, P=0.01). Other associations with the outcome did not show
291 statistical significance. These include protective factors such as people living on farms with a
292 changing room available (adjusted PR=0.5, P=0.07) or on farms where clean towels are used
293 after work (adjusted PR=0.6, P=0.11) and risk factors, such as people living in farms where
294 baby boxes are cleaned at the beginning of the production cycle (adjusted PR=1.3, P=0.54).
295 Other determinants such as the prevalence of MRSA in animals at the farm level did not
296 show an association with persistent human MRSA carriage (PR=1.0, 95%CI=1.0-1.0,
297 P=0.96). There was also no association found with variables regarding individual health
298 status.

299 Results from the multiple logistic regression analysis are presented in Table 2. In model
300 A, all variables meeting the described criteria were eligible to entry. In this model, number of
301 working hours per week showed the most significant association with persistent MRSA
302 carriage (PR=1.8 expressed per 20 hours/week increase, P<0.0001). Because this variable
303 was a very strong determinant, as a result of which potential tasks were not retained, a model
304 was explored (model B) without the number of working hours. In consequence, stable
305 management (sorting calves) was retained in the final model B with a statistically significant
306 PR of 3.1 (P=0.03). In both multivariate models, the presence of cats on the farm was

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307 significantly associated with the outcome (PR=2.8, P=0.01 in model A and PR=2.6, P=0.04
308 in model B).

309 Specific tasks on the farm were adjusted for number of working hours in a bivariate
310 analysis and the estimates obtained were not statistically significant. Only stable management
311 remained positively associated with the outcome with a PR of 2.5 (95%CI=0.7-9.6; P=0.17);
312 however, administration of antibiotics in the month before sampling showed no association
313 with a PR of 1.1 (95%CI=0.2-5.9; P=0.91).

314 Sensitivity analysis was done restricted to ST398 and it yielded similar univariate and
315 multivariate results.

316 Table 1. Crude and adjusted for sex and age prevalence ratios (PR) for nasal MRSA
317 persistent carriage in 195 veal calf farmers and household members from 51 farms.

Determinant	Category	N	No. Persistent carriers[a] (prevalence %)	Mean (range)	PR	95% CI	PR[b] Adj	95% CI
General characteristics:								
Sex	Male	103	9 (8.7)	-	1	-	-	-
	Female	92	11 (12.0)	-	1.4	0.6-3.2	-	-
Age	-	195	-	30 (0.1-81)	1.0	1.0-1.0**	-	-
per 10 years increase	-	195	-	-	1.3	1.1-1.6**	-	-
Farm and household characteristics:								
Presence of sheep in farm	No	149	12 (8.1)	-	1	-	1	-
	Yes	46	8 (17.4)	-	2.2	1.1-4.5*	2.4	1.2-4.8*
Presence of cats on farm	No	96	5 (5.2)	-	1	-	1	-
	Yes	99	15 (15.2)	-	3.0	1.2-7.1*	2.7	1.1-6.6*
Presence of pets	No	74	4 (5.4)	-	1	-	1	-
	Yes	121	16 (13.2)	-	2.7	1.0-7.4*	2.6	1.0-6.7[t]
Tasks performed last 7 days[c] :								
Sorting calves (stable management)	No	113	5 (4.4)	-	1	-	1	-
	Yes	82	15 (18.3)	-	4.2	1.5-12.3**	4.7	1.3-16.8*
Healthcare / control [d]	No	132	9 (6.8)	-	1	-	1	-
	Yes	63	11 (17.5)	-	2.6	1.1-6.1*	2.3	0.8-7.3
Feeding calves	No	72	2 (2.8)	-	1	-	1	-
	Yes	123	18 (14.6)	-	7.2	0.9-58.6[t]	5.4	0.6-52.3
Work at farm, hygiene cleaning and disinfection								
Administration of antibiotics during	No	131	8 (6.1)	-	1	-	1	-

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last month	Yes	64	12 (18.8)	-	3.2	1.4-7.1**	3.4	1.3-9.1*
# working hours per week	-	195	-	16.5 (0-80)	1.0	1.0-1.0***	1.0	1.0-1.1**
per 20 hours increase	-	-	-	-	1.8	1.4-2.4***	2.5	1.4-4.2**
Clean towel	No	45	7 (16.7)	-	1	-	1	-
	Yes	150	13 (8.67)	-	0.6	0.3-1.3	0.6	0.3-1.1
Changing room available	No	18	3 (16.7)	-	1	-	1	
	Yes	177	17 (9.7)	-	0.6	0.3-1.2	0.5	0.2-1.0[t]
Cleaning of baby boxes	No	184	18 (9.8)	-	1	-	1	-
	Yes	11	2 (18.2)	-	1.9	1.0-3.5*	1.3	0.6-2.8

[a] A person is considered a persistent carrier when all nasal swabs at days 0, 4 and 7 are positive for MRSA.
[b] Prevalence ratios adjusted for sex and age.
[c] Tasks performed in the week before time 0.
[d] The task healthcare and control includes the administration of antibiotics.
[t] Nonsignificant trend (P-value 0.05-0.10). [*] P-value 0.01-0.05. [**] P-value 0.0001-0.01. [***] P-value <0.0001.

Table 2. Results from multiple logistic regression analysis for nasal MRSA persistent carriage in veal calf farmers and their household members (N=195). Model A: final model in which all variables meeting eligibility criteria were added to the automatic selection. Model B: final model in which all the variables in model A were added to the automatic selection except # working hours.

Determinant	Category	PR	95% CI	P-value
MODEL A				
# working hours per week	-	1.03	1.02-1.04	0.000*
per 20 hours increase		1.81	1.49-2.19	-
Presence of cats on farm	No	1	-	-
	Yes	2.80	1.23-6.36	0.014*
Presence of sheep in farm	No	1	-	-
	Yes	1.83	0.89-3.77	0.100
Changing room available	No	1	-	-
	Yes	0.48	0.20-1.13	0.094
Cleaning of baby boxes	No	1	-	-
	Yes	3.96	1.59-9.90	0.003*
MODEL B				
Age	-	1.02	1.00-1.05	0.037*
per 10 years increase	-	1.26	1.01-1.56	-
Presence of cats on farm	No	1	-	-
	Yes	2.57	1.05-6.33	0.040*
Presence of sheep in farm	No	1	-	-
	Yes	1.78	0.88-3.59	0.107

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Sorting calves	No	1	-	-
	Yes	3.10	1.14-8.47	0.027*

* P-value statistically significant (i.e. < 0.05).

Contamination of the environment with MRSA

At the beginning of the production cycle, MRSA was detected in only 4.6% of all EDCs placed in stables and on 6 farms. Differences in environmental exposure across persistent, intermittent and non-carrier farms were not significant (Table 3). None of the EDCs placed inside the houses were found to be positive for MRSA.

In week 12, MRSA was detected in 50.6% of all EDCs placed in the stables and on 39 farms. There was a significantly higher proportion of EDCs positive for MRSA and a trend for higher CFU counts per EDC in farms where MRSA carriers were found in week 12 (Table 4). Stratified analysis was performed in farmers and family members. The same trends for higher MRSA environmental load were found only in farmers, however not statistically significant (results not shown). MRSA was found in EDCs from 10 houses (Table 4).

The mean pooled MRSA prevalence in calves rose from 18.7% at day 0 to 46% in week 12. A simple linear regression between the EDC MRSA levels (maximum log-transformed MRSA CFU/EDC per farm) and animal prevalence showed a positive and significant association (β=0.006, P=0.0014). Furthermore, there was a 60% increased probability for detecting a MRSA-positive EDC in farms where animal prevalence in week 12 was above the mean (PR=1.6, 95%CI=1.09-2.38, P=0.02). With regards to human carriage in relation to animal prevalence, no association between being a MRSA carrier and the prevalence in calves was found on day 0. On week 12 there was a slight increase in prevalence among farmers as compared to the previous sampling moment (see supplemental figure 1) and individuals from farms with MRSA prevalence in calves above the mean were at 2 times higher risk for carrying MRSA (PR=2.12, 95%CI=1.12-4.01, P=0.02).

Table 3. Environmental MRSA samples (EDCs) taken in stables at the beginning of the production cycle in 51 farms with persistent, intermittent or non-MRSA carrying veal calf farmers and household members.

	Persistent[a]	Intermittent[a]	Non-carrier[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	2/18 (11.11)	2/12 (16.67)	2/21 (9.52)	0.86
No. MRSA positive EDCs / total no. EDCs (%)	2/69 (2.90)	4/47 (8.51)	3/78 (3.85)	0.38
GM MRSA CFU/EDC (p-value)[c]	<1 (0.75)	<1 (0.29)	<1 (ref.)	-

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358 [a] A farm was categorised as persistent when there was at least one persistent carrier living and/or
359 working on the farm, non-carrier farms had no individual positive for MRSA in nasal swabs on days
360 0,4,7 and intermittent farms were the remaining.
361 [b] P-values among proportions were calculated with Fisher's exact test. Mean values had not an overall
362 assigned p-value since they could not be tested with non-parametric tests.
363 [c] Geometric mean (antilogged results from tobit regression). P-values indicate the difference with the
364 reference category (non-carrier farm).
365

366 Table 4. Environmental MRSA samples (EDCs) taken in stables on week 12 in 49 farms with
367 MRSA carriers and non-carriers.

	Location EDC	Carrier farms[a]	Non-carrier farms[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	Stable	22/25 (88.00)	17/24 (70.83)	0.14
	House	3/25 (12.00)	7/24 (29.17)	0.17
No. MRSA positive EDCs / total no. EDCs (%)	Stable	54/90 (60.00)	35/86 (40.70)	0.01
	House[d]	-	-	-
GM[c] MRSA CFU/EDC	Stable	27.54	16.98	0.06
	House	2.29	5.50	0.29

368 [a] A farm was categorised as carrier when there was at least one carrier on week 12 living and/or
369 working on the farm, non-carrier farms were the remaining.
370 [b] P-values among proportions were calculated with Chi-square test and Fisher's exact test when 20%
371 of the expected cell values were <5. P-values for the GM indicate the difference with the reference
372 category (non-carrier farms).
373 [c] Geometric mean (antilogged results from tobit regression).[d] There was one EDC per house, thus the
374 values in this line are the same as the ones in "No. farms with MRSA positive EDCs / total no. farms
375 (%)".
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377 DISCUSSION

378 The associations found during the first week after arrival of the animals on the farm show
379 that the level of exposure to veal calves and the presence of potential animal reservoirs (pets,
380 free-ranging farm cats and sheep) are risk factors for persistent MRSA carriage in farmers
381 and household members. Additionally, persistent MRSA carriers seem to have a different
382 microbiological profile when compared to intermittent and non-carriers, which is
383 characterised by higher MRSA CFU counts, presence of MSRA in throat and absence of
384 MSSA. This study shows that as the production cycle advances, there is a rise in MRSA
385 prevalence in calves that leads to higher contamination of the air and higher probability for
386 human MRSA carriage.

387 Descriptive results confirm that high MRSA carriage prevalence (17.6%) is observed
388 among individuals living on farms, as seen in other studies.(2,16) This percentage represents
389 a carriage burden in countries where estimated MRSA prevalence in community is below 1%
390 such as the Netherlands and Scandinavian countries. The large difference in prevalence
391 between farmers and family members can be attributed to the different intensity of animal

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392 contact and is again an indication of a low LA-MRSA human-to-human transmission.(16,22)
393 Swabs in liquid transport medium were used only on day 0 for the purpose of quantification.
394 The fact that higher prevalences are observed on day 0 as compared to days 4 and 7 might be
395 due to highest sensitivity for MRSA detection as compared to dry cotton swabs
396 (supplemental figure 1). The carriage patterns of *S. aureus* presented are similar to those
397 described by Wertheim and co-authors,(23) in which they found percentages of 20%, 30%
398 and 50% for persistent, intermittent and non-carriers respectively among healthy individuals.
399 The lower MRSA persistent carrier prevalence in the total study population (9.7%) as
400 compared to the average cross-sectional MRSA prevalence (17.6%) indicates that carriage of
401 LA-MRSA is fleeting and varies within individuals.

402 Confirmation of only ST398 was done in the laboratory and it was predominant (higher
403 than 90%) among the MRSA isolates from humans, animal pools and EDC samples. MRSA
404 positive subjects negative for ST398 did not visit a hospital during the previous 12 months of
405 the study and there was other than ST398 MRSA present in animal and environmental
406 samples. All MRSA was considered to be circulating and transmitted in the farm since it is
407 very likely that other livestock-associated sequence types were present as in previous studies
408 (14, 16).

409 Due to culturing techniques, MSSA was detected with difficulty when there was a
410 predominant MRSA growth. The possible underestimation of MSSA asks for a cautious
411 interpretation of the results. Nevertheless it is remarkable that no persistent MRSA carrier
412 was positive for MSSA at day 0. This suggests that the presence of MSSA in the nose might
413 be a protective factor for MRSA persistent carriage. Moreover, a negative association
414 between MSSA and MRSA has been recently found in a study.(14)

415 In the first week of the production cycle the MRSA environmental load was lower and it
416 can be assumed that nasal contamination with MRSA-containing dust particles and transient
417 mechanical carriage was less likely to occur as compared to further time points in the
418 production cycle. As shown in figure 2, there is an increased probability for persistent MRSA
419 carriage associated with higher MRSA CFU counts in nasal swabs. Moreover, isolation of
420 MRSA in throat swabs at day 0 was significantly associated to the outcome (PR=12.2). These
421 findings suggest that there might be a true colonization in persistent MRSA carriers as
422 defined here. Furthermore a recent study has shown that ST398 is capable of adequately
423 competing for a niche with a human strain and survives in the human nose for longer
424 periods.(24)

425 Direct association between administration of antibiotics and MRSA persistent carriage in
426 farmers and their family members, as defined in our study, was shown in univariate results
427 (PR=3.2). It is known that when antimicrobials are administered to animals, substantial
428 quantities of these drugs can be present in manure, on surfaces of animal houses and in dust
429 as a potential risk source.(25) We could hypothesize that aspiration of dust containing
430 antibiotics, either from a contaminated environment or directly from a powder formulation,
431 would exert a selective pressure in the anterior nares leading to higher risk for MRSA
432 persistent carriage in people occupationally exposed. However, this association was not
433 confirmed in multivariate models and it needs further exploration. Number of working hours
434 and other tasks were correlated and may have more influence on persistent carriage. This was
435 also shown when adjustment for number of working hours was done in a bivariate fashion.

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This study supports that close contact with animals is a major risk factor for persistent LA-MRSA carriage in humans. This is made clear by the final set of variables retained in the multivariate models. The number of working hours was most strongly associated with persistent carriage as indicated by the model A and by the smoothed exposure-response relation shown in the Supplemental Figure 3. Moreover, when the number of working hours was removed for model B, another variable representing close contact with animals (stable management) was retained by the backward procedure.

In recent years, several reports have suggested a potential role for pet animals, specifically cats and dogs, in household MRSA transmission and relapse of human MRSA infections. This transmission seems to be of anthropozoonotic origin. Thus, pets can acquire human strains from humans and they can cause colonization or infection in human cohabitants.(26-31) In most cases, the distribution of the clones in pet animals has mirrored the epidemiology of human clones and mainly shared hospital-associated (HA) and community-associated (CA) MRSA strains have been reported. It is remarkable that in this study, having a pet in the household was strongly associated with MRSA carriage in veal farmers and household members. Moreover, there is a demonstrated spread of LA-MRSA between animal species, humans and the farm environment.(32) In this study no other animals apart from veal calves were sampled, however the presence of free-ranging farm cats and sheep were significantly associated and retained in multivariate models. A previous large cross-sectional study sampled 35 cats from 25 farms, 26 of them came frequently in the veal stables. Only one of these cats was found to be MRSA positive with a spa type t011 (ST398).(33) Cats might act as reservoirs but this is more suggestive of cats acting as mechanical vectors. These animals might represent an intermittent source of LA-MRSA that might contribute to LA-MRSA persistent carriage in humans.

Other farm characteristics and hygiene practices were also associated with persistent MRSA carriage, although not significantly. Having a changing room in the farm and using a clean towel after working in the stables were found as protective factors. This might give a direction to specific preventive strategies. On the other hand, cleaning of baby boxes at the beginning of the production cycle was a risk factor for the outcome (PR=4 in multivariate model A and PR=1.9 in univariate analysis). This hygiene practise could give rise to transitory spread in the air of accumulated MRSA.

Environmental contamination with dust particles containing MRSA is much lower in veal calf farming as compared to pig farming and associations are less evident.(34) As shown in table 3, no difference in the environmental MRSA load was found across persistent, intermittent and non-carrier farms at the beginning of the production cycle. However, the two-fold rise in animal prevalence at the end of the study was associated with a considerably higher environmental MRSA load and a significantly higher proportion of MRSA-positive EDCs was found on farms with MRSA carriers on week 12. This finding supports that contamination of the environment plays a role in the acquisition of MRSA in people living or working in the farm.

A possible limitation of the study is the self-sampling of nose and throat by individuals which might be lacking of accuracy for MRSA detection. This is however believed to be a minor bias. A recent pilot study has shown high degree of agreement between self-samples and investigator samples (93% agreement, kappa 0.85 for nasal swabs and 83% agreement, kappa 0.60 for throat swabs).(35) Another limitation is the previously described underestimation of MSSA presence but this is of negligible impact in the results because

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detection of MRSA and *S. aureus* remains unaffected. Finally, there were many missing values in some variables and they were excluded from the analysis. There were 5 individuals (/211=2%) with missing nasal samples but sensitivity analysis did not reveal significant changes in estimates.

In conclusion, people living and/or working in veal calf farms who persistently carry MRSA seem to be defined by a differential microbiological profile. The associations found here with the presence of free-ranging farm cats and multispecies farming ask for improved internal and external biosecurity measures. Detailed molecular-epidemiological analysis of MRSA specimens on the farm in various animal species and humans is also essential to identify reservoirs and transmission routes for LA-MRSA. Finally, environmental contamination with MRSA has to be thoroughly studied to assess the extent of its importance in the transmission of MRSA within the veal-calf farming community.

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CONTRIBUTORSHIP STATEMENT

Alejandro Dorado-García performed the statistical analyses and interpretation of the data, and drafted the manuscript. Marian EH Bos collected the data, contributed to the interpretation of the data, and contributed to the critical revision of the manuscript. Haitske Graveland participated in the conception and design of the study, collected the data and contributed to the critical revision of the manuscript. Brigitte AGL van Cleef and Jan AJW Kluytmans contributed to the critical revision of the manuscript. Koen M Verstappen carried out the laboratory analysis. Jaap A Wagenaar conceived the study and contributed to the critical revision of the manuscript. Dick JJ Heederik conceived the study and contributed to the interpretation of the data and the critical revision of the manuscript. All authors read and approved the final manuscript.

DATA SHARING

Data will not be publicly accessible. Interested individuals may contact the authors.

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COMPETING INTERESTS

None declared

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Title:
Risk factors for livestock-associated MRSA persistent carriage and environmental exposure in veal calf farmers and their family members: an observational longitudinal study.

Short Title: LA-MRSA persistent carriage and environmental exposure in pig farming.

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Key words (max 5): Livestock-Associated MRSA, persistent carriage, longitudinal study, veal calves, environmental contamination

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ABSTRACT

Objectives: LA-MRSA emergence is a major public health concern. This study is aimed at assessing risk factors for persistently carrying MRSA in veal calf farmers and their family members. We also evaluate the dynamics of MRSA environmental load during the veal-calf production cycle.

Design: Observational, longitudinal, repeated cross-sectional study.

Setting: 52 veal calf farms in the Netherlands.

Participants: Between the end of 2010 to the end of 2011, a total of 211 farmers, family members and employees were included in the study.

Primary outcome and secondary outcome measures: Nasal swabs were taken from participants on days 0, 4, 7 and week 12. A persistent MRSA carrier was defined as a person positive for MRSA on days 0, 4 and 7. Participants filled in an extensive questionnaire to identify potential risk factors and confounders. For estimation of MRSA prevalence in calves and environmental contamination, animal nasal swabs and Electrostatic Dust Collectors (EDCs) were taken on day 0 and week 12.

Results: The presence of potential animal reservoirs (free-ranging farm cats and sheep) and the level of contact with veal calves was positively associated with persistent MRSA carriage. Interestingly, at the end of the study (week 12), there was a two-fold rise in animal prevalence and a significantly higher MRSA environmental load in the stables was found on farms with MRSA carriers.

Conclusions: This study supports the hypothesis that environmental contamination with MRSA plays a role in the acquisition of MRSA in farmers and their household members and suggests that other animal species should also be targeted to implement effective control strategies.

ARTICLE SUMMARY

Article focus:

- Determinants for persistent LA-MRSA carriage in humans and for a possible true colonization have not been thoroughly assessed.
- It is unclear whether bacterial contamination in the farm environment plays a role in LA-MRSA transmission in humans

Key messages:

- The presence of other animals in the farm might be of importance in acquisition and persistence LA-MRSA in humans. There is a need for detailed molecular-epidemiological analysis of MRSA specimens in various animal species and humans in the veal-calf farming community.

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- During the veal-calf production cycle, there is a parallel increase in animal prevalence and environmental MRSA load which is linked to higher risk for human carriage.

Strengths and limitations of this study:

The longitudinal nature of the data allows to establish dynamic epidemiological inferences.

No other animals apart from veal-calves were sampled in this study. The self-sampling of noses by individuals might influence the sensitivity for MRSA detection.

INTRODUCTION

In recent years, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), specifically sequence type (ST) 398, has emerged in food-producing animals and people in contact with these animals.(1-4) Illness associated to ST398 in humans is rare and only a small proportion of MRSA infections can be attributed to LA-MRSA.(5,6) Nonetheless, invasive infections and hospital outbreaks of MRSA ST398 have been reported in Europe, the United States and Asia.(5,7,8)

LA-MRSA strains have been found mainly in pigs and veal calves, but they have the capacity to colonize a wide spectrum of hosts, including sheep and poultry.(9) Farmers are easily contaminated and in general the carriage prevalence in farmers is high. Frequency of transmission between farmers and their family members and among hospitalized humans appears to be low.(2,10,11) However, this belief might be contradicted by recently described LA-MRSA transmission events in Dutch patients with neither risk factors nor livestock contact.(12) The potential public health threat posed by these strains is emphasized in a recent metapopulation model in which the likelihood of persistent carriage in the livestock-exposed population was the key parameter for LA-MRSA spreading to the community.(13)

Previous studies have been mainly based on cross-sectional designs and have shown that intensity of animal contact and MRSA prevalence among animals are positively associated to LA-MRSA human carriage.(14) Associations between animal carriage and farm hygiene and antimicrobial use have also been shown.(15,16) A longitudinal study including periods of high and low exposure to animals showed that LA-MRSA carriage was mainly transient. It was suggested that LA-MRSA is a poor persistent colonizer in humans, which was confirmed by a study on short term occupational exposure.(10,14) However, risk factors for persistent LA-MRSA carriage and for a possible true colonization have not been thoroughly assessed. Furthermore, little is still known about the dynamics of environmental contamination with MRSA in the farm and its role in transmission to humans. A recent study showed a steep increase in prevalence among calves and in MRSA air load during the production cycle.(17)

The aim of the current study is twofold. Firstly, to assess risk factors and dose-response relationships for persistently carrying MRSA over a period of one week at the beginning of the production cycle in veal calf farmers and their family members. Secondly, to evaluate the deposition of MRSA-containing dust inside the farm and its relationship with animal and human MRSA carriage.

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MATERIALS AND METHODS

Study design and population

A longitudinal cohort study was performed over a period of 12 weeks in 52 veal calf farms starting at the beginning of the production cycle. All farms were visited from the end of 2010 to the end of 2011. All farms met the following inclusion criteria: implemented all-in-all-out system; no other livestock in large scale apart from veal calves; an unique location for all the stables or farm; veal calf farmers not working in another animal sector (e.g. transport of pigs) and not operating in other farms. Preference for selection was given to farms in the proximity of Utrecht, the Netherlands. On each farm there were 2 sampling moments for animal and environmental samples (day 0 and week 12) and 4 sampling moments for human samples (days 0, 4, 7 and week 12). Nasal swabs from both anterior nares of calves were taken and analysed in 10 pools of 6 swabs each (60 animals per farm). Swabs were also collected from farmers, family members and employees (n=211). On day 0, quantitative nasal and throat swabs were taken by field workers in the majority of participants or by self-sampling. On days 4, 7 and on week 12, dry cotton swabs (Copan, Brescia, Italy) were used to self-sample the nose. Swabs were given to participants with instructions including photographs in case of self-sampling. Nasal swabs in animals and humans were introduced in the nostril and rotated once. Throat swabs in humans sampled the area of the inner cheek including the tonsils. The swabs were immediately taken to the laboratory or sent by post and processed within 24 hours after arrival. Furthermore, environmental samples were taken by placing 4 Electrostatic Dust Collectors (EDCs) (Zeeman, Utrecht, The Netherlands) on different surfaces inside the stables and one on the highest cupboard in the living room or kitchen of the house. The EDCs were left in place during a period of 2 weeks and sent by post to the laboratory. Upon arrival, EDC samples were stored at -20°C until quantitative analysis.(18) ~~A schematic overview of the study design is displayed in the Supplemental Figure 1.~~

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All participants completed an informed consent and filled in an extensive questionnaire including items related to individual health status, household and farm characteristics, activities performed on the farm and hygiene practices. The protocol of the study was approved by Medical Ethical Committee of Utrecht University. The collection of animal samples was in compliance with the Dutch Law on Animal Health and Welfare.

For the assessment of MRSA persistent carriage, we selected the beginning of the veal calf production cycle, just after the stables were empty and when animal prevalence is lower. In this period, deposition of MRSA-containing dust particles in human nasal cavities and mechanical carriage was assumed to be less likely. Therefore and for the purpose of this study, a person was defined to be a persistent MRSA carrier when each of the nasal swabs collected on days 0, 4 and 7 were positive for MRSA presence.

Laboratory analysis

Swabs in liquid transport medium (ESwab, Copan, Brescia, Italy) were used for quantitative cultivation. Serial dilutions (1:10) of the transport medium (concentration 10^0) were made by adding 100 µl sample to 900 µl phosphate buffered saline (PBS) to a final concentration of 10^{-4} of the original sample. Each dilution was cultured on chromID *S. aureus* and chromID MRSA agar plates (BioMérieux, La Balme Les Grottes, France) at 37°C for 18-24 hours. Plates with 10-100 colony-forming units (CFU) were used to calculate the original

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amount of CFU per swab. In order to detect positive samples without bacterial growth in the first day, the remaining transport medium and swab were enriched overnight in Mueller Hinton broth with 6.5% NaCl (MH+), and consequently cultured on chromID *S. aureus* and chromID MRSA agar plates. The theoretical lower limit of quantification (LLOQ) of MRSA CFU was 10. Dry cotton swabs (Copan) were inoculated directly onto chromID *S. aureus*, chromID MRSA and MH+. Confirmation of MRSA presence in the 3 sampling moments was done by Real-Time (RT) PCR targeting *mecA*, *femA* and *nuc* genes.(19,20) Methicillin-susceptible *Staphylococcus aureus* (MSSA) presence was tested when the bacterial growth on chromID *S. aureus* was higher than on chromID MRSA. For this purpose, 10 colonies were screened for methicillin susceptibility by using the cefoxitin disk diffusion method. Confirmation of MSSA was done by Real-Time PCR. Nasal swabs from calves were analysed in pools following standard procedures previously described.(21)

To obtain an estimate of exposure in CFU per EDC, EDCs were analysed by Real-Time quantitative PCR (qPCR). EDC samples were suspended in 10 mL EDTA saline buffer (150 mM NaCl, 1 mM EDTA) and mixed in a Stomacher (Seward Ltd., London, United Kindom) for 10 minutes. Two mL of the resulting suspension was stored at -20°C for the analysis. For DNA isolation, 200 µL of the suspension was incubated at 95°C for 15 minutes. Phosphate buffered saline (PBS) was added and a Versant kPCR molecular system (Siemens Healthcare Diagnostics, The Hague, The Netherlands) was used for DNA purification with an elution volume of 50 µL. Five µL of the purified sample were used for detection of *mecA*, *femA* and *nuc* genes by the means of a LightCycler 480-II system (Roche Diagnostics, Almere, The Netherlands). For MRSA quantification, a standard curve was established for all targets. A standard control sample was included in each run to correct the curve for run-to-run variation. For interpretation of the results, CFU counts per PCR were transformed to CFU counts per EDC (1 CFU/PCR = 200 CFU/EDC). The theoretical limit of detection (LOD) was 20 CFU/EDC.

RT-PCR targeted at C01 gene was done for confirmation of ST398 in all MRSA positive human, animal and environmental samples.

Data analysis

Statistical analysis was performed using SAS software version 9.2 (SAS institute Inc., Cary, North Carolina, USA). Descriptive analysis determined the cross-sectional human prevalences on each of the 4 sampling moments and the longitudinal carriage patterns (persistent, intermittent or non-carriers).

Risk factors for nasal MRSA persistent carriage were investigated with univariate and multivariate analysis. PROC GENMOD was used for Generalized Estimating Equations (GEE) modelling to take clustering of data at farm level into account. The mean response was modelled with a Poisson regression with robust standard errors. Crude and age-sex adjusted prevalence ratios were obtained. Eligibility criteria for variables to be considered in multivariable analysis included univariate p-values below 0.2, less than 10% of missing data in relation with the outcome, and at least 2 persistent carriers falling in each of the categories of the explanatory categorical variables. Bivariate correlation structure of all eligible variables was studied with PROC CORR and Spearman correlation coefficients were obtained. Thereafter, eligible variables were added in a stepwise backward selection approach and retained in the final model when $P<0.15$. A p-value<0.05 was considered statistically significant.

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The shape of the relationships between MRSA persistent carriage and numerical variables was studied by means of nonparametric or semiparametric regression modelling (smoothing) using PROC GAM to relax the assumption of linearity. For this purpose, the number of CFU from quantitative nasal swabs positive for MRSA but below LLOQ was set to 5.

To assess the environmental exposure during the first week, farms were classified in 3 categories: i) farm with persistent carrier, when there was at least one MRSA persistent carrier working and/or living on the farm; ii) farm with intermittent carrier, when there was at least one MRSA intermittent carrier and there was no persistent carrier on the farm; iii) non-carrier farm, when all people at the farm were MRSA-negative on the first 3 sampling moments. On week 12 farms were classified as carrier and non-carrier farms when there was at least one MRSA carrier on the farm, and when all people on the farm were MRSA-negative on week 12 respectively. Proportions of MRSA-positive EDCs were calculated per farm category and sampling moment. For calculation of average exposure levels, CFU counts per EDC were log-transformed since they followed a highly right-tailed distribution. PROC LIFEREG was used for left-censored regression (tobit) modelling to obtain an accurate estimate of the mean exposure level accounting for the large proportion of undetectable values. Thereafter geometric means (GM) were calculated.

RESULTS

Descriptive results

Nasal swabs were collected from 211 participants on 52 farms. The ~~total population~~ average nasal MRSA prevalence for the 4 sampling moments was ~~17.6% and in farmers it was~~ twice as high in farmers (29.7%) as compared to ~~in~~ family members (13.0%). Cross-sectional nasal MRSA prevalences per sampling moment are displayed in Supplemental Figure 1~~2~~.

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Nasal carriage patterns for MRSA, MSSA and *S. aureus* in general (including both MSSA and MRSA) were assessed over the one week period. The MRSA and MSSA persistent carrier prevalence followed opposite directions in farmers as compared to family members. For MRSA persistent carriage the prevalence in farmers (15.5%) was twice as high as in family members (7.6%). MSSA persistent carriage prevalence was three times higher in family members than in farmers (15.3% and 5.2%, respectively). Regarding *S. aureus,* there were not significant differences between the subpopulations of farmers and family members and 22.8% of all individuals were persistently carrying the bacteria, 29.6% were intermittent carriers and the remaining 47.6% never carried *S. aureus.* Supplemental Table 1 shows these longitudinal carriage patterns in more detail.

The RT-PCR targeted at C01 gene showed that ST398 was present in 90.5% of the human MRSA isolates, in 97.9% of the MRSA positive animal pools and 90.9% of the MRSA positive EDCs.

Microbiological status and persistent MRSA nasal carriage

CFU counts were determined in 42 participants from quantitative nasal swabs on day 0. Figure 1 shows the shape of the relationship between the probability of being a persistent MRSA nasal carrier and the log-transformed MRSA concentration (CFU/swab suspension).

The median CFU count was 43.65 with an interquartile range (IQR) 5.01-1,096.48. In addition, the univariate logistic regression analysis in this population resulted in 1.68 times higher risk (95% Confidence Interval (CI)=1.34-2.10, P<0.001) for persistent MRSA carriage per 10 CFU increase.

No MSSA was found in MRSA-positive samples at day 0. In order to obtain an estimation of the prevalence ratio (PR) for the outcome when MSSA is present at day 0, data was manipulated by placing a MSSA positive result for one of the persistent carriers. This way an adjusted PR of 0.14 (95% CI=0.02-1.06, P=0.06) was obtained.

People found positive for MRSA in throat swabs at day 0 were at higher risk for being persistent nasal carriers (adjusted PR=12.2, 95%CI=5.2-28.8, P<0.0001). The spearman correlation coefficient between this variable and the outcome was 0.6 (P<0.0001).

Sensitivity analysis was done restricted to ST398 and it yielded similar results as described above.

Univariate and multivariate analysis for persistent MRSA nasal carriage

Crude and age-sex-adjusted PRs in determinants meeting the specified criteria are presented in Table 1. Sex and smoking habits were not clearly associated with the outcome (P>0.2). Because these variables together with age are considered potential confounders, sensitivity analysis was performed with smoking habits added to sex and age for adjustment. This did not result in significant changes in estimates (results not shown) when compared to adjustment without smoking habits.

Statistically significant risk factors for persistent MRSA carriage were identified (table 2). Pet ownership showed a PR of 2.7 (P=0.05). The number of working hours per week in the farm was positively associated with the outcome (adjusted PR=2.5 expressed per 20 hours/week increase, P=0.001). An increasing probability for MRSA persistent carriage with number of hours working in the farm was also demonstrated through semiparametric regression modelling (Supplemental Figure 23). Administration of antimicrobials to calves through milk and injection in the past month preceding sampling was also a significant risk factor (adjusted PR= 3.4, P=0.01). Other associations with the outcome did not show statistical significance. These include protective factors such as people living on farms with a changing room available (adjusted PR=0.5, P=0.07) or on farms where clean towels are used after work (adjusted PR=0.6, P=0.11) and risk factors, such as people living in farms where baby boxes are cleaned at the beginning of the production cycle (adjusted PR=1.3, P=0.54). Other determinants such as the prevalence of MRSA in animals at the farm level did not show an association with persistent human MRSA carriage (PR=1.0, 95%CI=1.0-1.0, P=0.96). There was also no association found with variables regarding individual health status.

Results from the multiple logistic regression analysis are presented in Table 2. In model A, all variables meeting the described criteria were eligible to entry. In this model, number of working hours per week showed the most significant association with persistent MRSA carriage (PR=1.8 expressed per 20 hours/week increase, P<0.0001). Because this variable was a very strong determinant, as a result of which potential tasks were not retained, a model was explored (model B) without the number of working hours. In consequence, stable management (sorting calves) was retained in the final model B with a statistically significant PR of 3.1 (P=0.03). In both multivariate models, the presence of cats on the farm was

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7 309 significantly associated with the outcome (PR=2.8, P=0.01 in model A and PR=2.6, P=0.04
8 310 in model B).
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10 311 Specific tasks on the farm were adjusted for number of working hours in a bivariate
11 312 analysis and the estimates obtained were not statistically significant. Only stable management
12 313 remained positively associated with the outcome with a PR of 2.5 (95%CI=0.7-9.6; P=0.17);
13 314 however, administration of antibiotics in the month before sampling showed no association
14 315 with a PR of 1.1 (95%CI=0.2-5.9; P=0.91).
15
16 316 Sensitivity analysis was done restricted to ST398 and it yielded similar univariate and
17 317 multivariate results.
18
19 318 Table 1. Crude and adjusted for sex and age prevalence ratios (PR) for nasal MRSA
20 319 persistent carriage in 195 veal calf farmers and household members from 51 farms.

Determinant	Category	N	No. Persistent carriers[a] (prevalence %)	Mean (range)	PR	95% CI	PR[b] Adj	95% CI
General characteristics:								
Sex	Male	103	9 (8.7)	-	1	-	-	-
	Female	92	11 (12.0)	-	1.4	0.6-3.2	-	-
Age	-	195	-	30 (0.1-81)	1.0	1.0-1.0**	-	-
per 10 years increase	-	195	-	-	1.3	1.1-1.6**	-	-
Farm and household characteristics:								
Presence of sheep in farm	No	149	12 (8.1)	-	1	-	1	-
	Yes	46	8 (17.4)	-	2.2	1.1-4.5*	2.4	1.2-4.8*
Presence of cats on farm	No	96	5 (5.2)	-	1	-	1	-
	Yes	99	15 (15.2)	-	3.0	1.2-7.1*	2.7	1.1-6.6*
Presence of pets	No	74	4 (5.4)	-	1	-	1	-
	Yes	121	16 (13.2)	-	2.7	1.0-7.4*	2.6	1.0-6.7[t]
Tasks performed last 7 days[c] :								
Sorting calves (stable management)	No	113	5 (4.4)	-	1	-	1	-
	Yes	82	15 (18.3)	-	4.2	1.5-12.3**	4.7	1.3-16.8*
Healthcare / control [d]	No	132	9 (6.8)	-	1	-	1	-
	Yes	63	11 (17.5)	-	2.6	1.1-6.1*	2.3	0.8-7.3
Feeding calves	No	72	2 (2.8)	-	1	-	1	-
	Yes	123	18 (14.6)	-	7.2	0.9-58.6[t]	5.4	0.6-52.3
Work at farm, hygiene cleaning and disinfection								
Administration of antibiotics during	No	131	8 (6.1)	-	1	-	1	-

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last month	Yes	64	12 (18.8)	-	3.2	1.4-7.1**	3.4	1.3-9.1*
# working hours per week	-	195	-	16.5 (0-80)	1.0	1.0-1.0***	1.0	1.0-1.1**
per 20 hours increase	-	-	-	-	1.8	1.4-2.4***	2.5	1.4-4.2**
Clean towel	No	45	7 (16.7)	-	1	-	1	-
	Yes	150	13 (8.67)	-	0.6	0.3-1.3	0.6	0.3-1.1
Changing room available	No	18	3 (16.7)	-	1	-	1	
	Yes	177	17 (9.7)	-	0.6	0.3-1.2	0.5	0.2-1.0[t]
Cleaning of baby boxes	No	184	18 (9.8)	-	1	-	1	-
	Yes	11	2 (18.2)	-	1.9	1.0-3.5*	1.3	0.6-2.8

[a] A person is considered a persistent carrier when all nasal swabs at days 0, 4 and 7 are positive for MRSA.
[b] Prevalence ratios adjusted for sex and age.
[c] Tasks performed in the week before time 0.
[d] The task healthcare and control includes the administration of antibiotics.
[t] Nonsignificant trend (P-value 0.05-0.10). [*] P-value 0.01-0.05. [**] P-value 0.0001-0.01. [***] P-value <0.0001.

Table 2. Results from multiple logistic regression analysis for nasal MRSA persistent carriage in veal calf farmers and their household members (N=195). Model A: final model in which all variables meeting eligibility criteria were added to the automatic selection. Model B: final model in which all the variables in model A were added to the automatic selection except # working hours.

Determinant	Category	PR	95% CI	P-value
MODEL A				
# working hours per week	-	1.03	1.02-1.04	0.000*
per 20 hours increase		1.81	1.49-2.19	-
Presence of cats on farm	No	1	-	-
	Yes	2.80	1.23-6.36	0.014*
Presence of sheep in farm	No	1	-	-
	Yes	1.83	0.89-3.77	0.100
Changing room available	No	1	-	-
	Yes	0.48	0.20-1.13	0.094
Cleaning of baby boxes	No	1	-	-
	Yes	3.96	1.59-9.90	0.003*
MODEL B				
Age	-	1.02	1.00-1.05	0.037*
per 10 years increase	-	1.26	1.01-1.56	-
Presence of cats on farm	No	1	-	-
	Yes	2.57	1.05-6.33	0.040*
Presence of sheep in farm	No	1	-	-
	Yes	1.78	0.88-3.59	0.107

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Sorting calves	No	1	-	-
	Yes	3.10	1.14-8.47	0.027*

333 * P-value statistically significant (i.e. < 0.05).

334 **Contamination of the environment with MRSA**

335 At the beginning of the production cycle, MRSA was detected in only 4.6% of all EDCs
336 placed in stables and on 6 farms. Differences in environmental exposure across persistent,
337 intermittent and non-carrier farms were not significant (Table 3). None of the EDCs placed
338 inside the houses were found to be positive for MRSA.

339 In week 12, MRSA was detected in 50.6% of all EDCs placed in the stables and on 39
340 farms. There was a significantly higher proportion of EDCs positive for MRSA and a trend
341 for higher CFU counts per EDC in farms where MRSA carriers were found in week 12
342 (Table 4). Stratified analysis was performed in farmers and family members. The same trends
343 for higher MRSA environmental load were found only in farmers, however not statistically
344 significant (results not shown). MRSA was found in EDCs from 10 houses (Table 4).

345 The mean pooled MRSA prevalence in calves rose from 18.7% at day 0 to 46% in week
346 12. A simple linear regression between the EDC MRSA levels (maximum log-transformed
347 MRSA CFU/EDC per farm) and animal prevalence showed a positive and significant
348 association (β=0.006, P=0.0014). Furthermore, there was a 60% increased probability for
349 detecting a MRSA-positive EDC in farms where animal prevalence in week 12 was above the
350 mean (PR=1.6, 95%CI=1.09-2.38, P=0.02). With regards to human carriage in relation to
351 animal prevalence, no association between being a MRSA carrier and the prevalence in
352 calves was found on day 0. On week 12 there was a slight increase in prevalence among
353 farmers as compared to the previous sampling moment (see supplemental figure 1) and
354 ~~However, a significant association was found at the last sampling moment.~~ iIndividuals from
355 farms with MRSA prevalence in calves above the mean were at 2 times higher risk for
356 carrying MRSA ~~in week 12~~ (PR=2.12, 95%CI=1.12-4.01, P=0.02).

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358 Table 3. Environmental MRSA samples (EDCs) taken in stables at the beginning of the
359 production cycle in 51 farms with persistent, intermittent or non-MRSA carrying veal calf
360 farmers and household members.

	Persistent[a]	Intermittent[a]	Non-carrier[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	2/18 (11.11)	2/12 (16.67)	2/21 (9.52)	0.86
No. MRSA positive EDCs / total no. EDCs (%)	2/69 (2.90)	4/47 (8.51)	3/78 (3.85)	0.38
GM MRSA CFU/EDC (p-value)[c]	<1 (0.75)	<1 (0.29)	<1 (ref.)	-

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7 361 [a] A farm was categorised as persistent when there was at least one persistent carrier living and/or
8 362 working on the farm, non-carrier farms had no individual positive for MRSA in nasal swabs on days
9 363 0,4,7 and intermittent farms were the remaining.
10 364 [b] P-values among proportions were calculated with Fisher's exact test. Mean values had not an overall
11 365 assigned p-value since they could not be tested with non-parametric tests.
12 366 [c] Geometric mean (antilogged results from tobit regression). P-values indicate the difference with the
13 367 reference category (non-carrier farm).
14 368
15 369 Table 4. Environmental MRSA samples (EDCs) taken in stables on week 12 in 49 farms with
16 370 MRSA carriers and non-carriers.

	Location EDC	Carrier farms[a]	Non-carrier farms[a]	P-value[b]
No. farms with MRSA positive EDCs / total no. farms (%)	Stable	22/25 (88.00)	17/24 (70.83)	0.14
	House	3/25 (12.00)	7/24 (29.17)	0.17
No. MRSA positive EDCs / total no. EDCs (%)	Stable	54/90 (60.00)	35/86 (40.70)	0.01
	House[d]	-	-	-
GM[c] MRSA CFU/EDC	Stable	27.54	16.98	0.06
	House	2.29	5.50	0.29

30 371 [a] A farm was categorised as carrier when there was at least one carrier on week 12 living and/or
31 372 working on the farm, non-carrier farms were the remaining.
32 373 [b] P-values among proportions were calculated with Chi-square test and Fisher's exact test when 20%
33 374 of the expected cell values were <5. P-values for the GM indicate the difference with the reference
34 375 category (non-carrier farms).
35 376 [c] Geometric mean (antilogged results from tobit regression).[d] There was one EDC per house, thus the
36 377 values in this line are the same as the ones in "No. farms with MRSA positive EDCs / total no. farms
37 378 (%)".
38 379

39 380 **DISCUSSION**

41 381 The associations found during the first week after arrival of the animals on the farm show
42 382 that the level of exposure to veal calves and the presence of potential animal reservoirs (pets,
43 383 free-ranging farm cats and sheep) are risk factors for persistent MRSA carriage in farmers
44 384 and household members. Additionally, persistent MRSA carriers seem to have a different
45 385 microbiological profile when compared to intermittent and non-carriers, which is
46 386 characterised by higher MRSA CFU counts, presence of MSRA in throat and absence of
47 387 MSSA. This study shows that as the production cycle advances, there is a rise in MRSA
48 388 prevalence in calves that leads to higher contamination of the air and higher probability for
49 389 human MRSA carriage.

50 390 Descriptive results confirm that high MRSA carriage prevalence (17.6%) is observed
51 391 among individuals living on farms, as seen in other studies.(2,16) This percentage represents
52 392 a carriage burden in countries where estimated MRSA prevalence in community is below 1%
53 393 such as the Netherlands and Scandinavian countries. The large difference in prevalence
54 394 between farmers and family members can be attributed to the different intensity of animal
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contact and is again an indication of a low LA-MRSA human-to-human transmission.(16,22) Swabs in liquid transport medium were used only on day 0 for the purpose of quantification. The fact that higher prevalences are observed on day 0 as compared to days 4 and 7 might be due to highest sensitivity for MRSA detection as compared to dry cotton swabs (supplemental figure 1). The carriage patterns of *S. aureus* presented are similar to those described by Wertheim and co-authors,(23) in which they found percentages of 20%, 30% and 50% for persistent, intermittent and non-carriers respectively among healthy individuals. The lower MRSA persistent carrier prevalence in the total study population (9.7%) as compared to the average cross-sectional MRSA prevalence (17.6%) indicates that carriage of LA-MRSA is fleeting and varies within individuals.

Confirmation of only ST398 was done in the laboratory and it was predominant (higher than 90%) among the MRSA isolates from humans, animal pools and EDC samples. MRSA positive subjects negative for ST398 did not visit a hospital during the previous 12 months of the study and there was other than ST398 MRSA present in animal and environmental samples. All MRSA was considered to be circulating and transmitted in the farm since it is very likely that other livestock-associated sequence types were present as in previous studies (14, 16).

Due to culturing techniques, MSSA was detected with difficulty when there was a predominant MRSA growth. The possible underestimation of MSSA asks for a cautious interpretation of the results. Nevertheless it is remarkable that no persistent MRSA carrier was positive for MSSA at day 0. This suggests that the presence of MSSA in the nose might be a protective factor for MRSA persistent carriage. Moreover, a negative association between MSSA and MRSA has been recently found in a study.(14)

In the first week of the production cycle the MRSA environmental load was lower and it can be assumed that nasal contamination with MRSA-containing dust particles and transient mechanical carriage was less likely to occur as compared to further time points in the production cycle. As shown in figure 2, there is an increased probability for persistent MRSA carriage associated with higher MRSA CFU counts in nasal swabs. Moreover, isolation of MRSA in throat swabs at day 0 was significantly associated to the outcome (PR=12.2). These findings suggest that there might be a true colonization in persistent MRSA carriers as defined here. Furthermore a recent study has shown that ST398 is capable of adequately competing for a niche with a human strain and survives in the human nose for longer periods.(24)

Direct association between administration of antibiotics and MRSA persistent carriage in farmers and their family members, as defined in our study, was shown in univariate results (PR=3.2). It is known that when antimicrobials are administered to animals, substantial quantities of these drugs can be present in manure, on surfaces of animal houses and in dust as a potential risk source.(25) We could hypothesize that aspiration of dust containing antibiotics, either from a contaminated environment or directly from a powder formulation, would exert a selective pressure in the anterior nares leading to higher risk for MRSA persistent carriage in people occupationally exposed. However, this association was not confirmed in multivariate models and it needs further exploration. Number of working hours and other tasks were correlated and may have more influence on persistent carriage. This was also shown when adjustment for number of working hours was done in a bivariate fashion.

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This study supports that close contact with animals is a major risk factor for persistent LA-MRSA carriage in humans. This is made clear by the final set of variables retained in the multivariate models. The number of working hours was most strongly associated with persistent carriage as indicated by the model A and by the smoothed exposure-response relation shown in the Supplemental Figure 3. Moreover, when the number of working hours was removed for model B, another variable representing close contact with animals (stable management) was retained by the backward procedure.

In recent years, several reports have suggested a potential role for pet animals, specifically cats and dogs, in household MRSA transmission and relapse of human MRSA infections. This transmission seems to be of anthropozoonotic origin. Thus, pets can acquire human strains from humans and they can cause colonization or infection in human cohabitants.(26-31) In most cases, the distribution of the clones in pet animals has mirrored the epidemiology of human clones and mainly shared hospital-associated (HA) and community-associated (CA) MRSA strains have been reported. It is remarkable that in this study, having a pet in the household was strongly associated with MRSA carriage in veal farmers and household members. Moreover, there is a demonstrated spread of LA-MRSA between animal species, humans and the farm environment.(32) In this study ~~However,~~ no other animals apart from veal calves were sampled, however ~~in this study.~~ the presence of free-ranging farm cats and sheep were significantly associated and retained in multivariate models. A previous large cross-sectional study sampled 35 cats from 25 farms, 26 of them came frequently in the veal stables. Only one of these cats was found to be MRSA positive with a spa type t011 (ST398).(33) Cats might act as reservoirs but this is more suggestive of cats acting as mechanical vectors. These animals might represent an intermittent source of LA-MRSA that might contribute to LA-MRSA persistent carriage in humans. ~~However, no other animals apart from veal calves were sampled in this study.~~

Other farm characteristics and hygiene practices were also associated with persistent MRSA carriage, although not significantly. Having a changing room in the farm and using a clean towel after working in the stables were found as protective factors. This might give a direction to specific preventive strategies. On the other hand, cleaning of baby boxes at the beginning of the production cycle was a risk factor for the outcome (PR=4 in multivariate model A and PR=1.9 in univariate analysis). This hygiene practise could give rise to transitory spread in the air of accumulated MRSA.

Environmental contamination with dust particles containing MRSA is much lower in veal calf farming as compared to pig farming and associations are less evident.(~~33~~34) As shown in table 3, no difference in the environmental MRSA load was found across persistent, intermittent and non-carrier farms at the beginning of the production cycle. However, the two-fold rise in animal prevalence at the end of the study was associated with a considerably higher environmental MRSA load and a significantly higher proportion of MRSA-positive EDCs was found on farms with MRSA carriers on week 12. This finding supports that contamination of the environment plays a role in the acquisition of MRSA in people living or working in the farm.

A possible limitation of the study is the self-sampling of nose and throat by individuals which might be lacking of accuracy for MRSA detection. This is however believed to be a minor bias. A recent pilot study has shown high degree of agreement between self-samples and investigator samples (93% agreement, kappa 0.85 for nasal swabs and 83% agreement, kappa 0.60 for throat swabs).(~~34~~35) Another limitation is the previously described

underestimation of MSSA presence but this is of negligible impact in the results because detection of MRSA and *S. aureus* remains unaffected. Finally, there were many missing values in some variables and they were excluded from the analysis. There were 5 individuals (/211=2%) with missing nasal samples but sensitivity analysis did not reveal significant changes in estimates.

In conclusion, people living and/or working in veal calf farms who persistently carry MRSA seem to be defined by a differential microbiological profile. The associations found here with the presence of free-ranging farm cats and multispecies farming ask for improved internal and external biosecurity measures. Detailed molecular-epidemiological analysis of MRSA specimens on the farm in various animal species and humans is also essential to identify reservoirs and transmission routes for LA-MRSA. Finally, environmental contamination with MRSA has to be thoroughly studied to assess the extent of its importance in the transmission of MRSA within the veal-calf farming community.

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COMPETING INTERESTS

None declared

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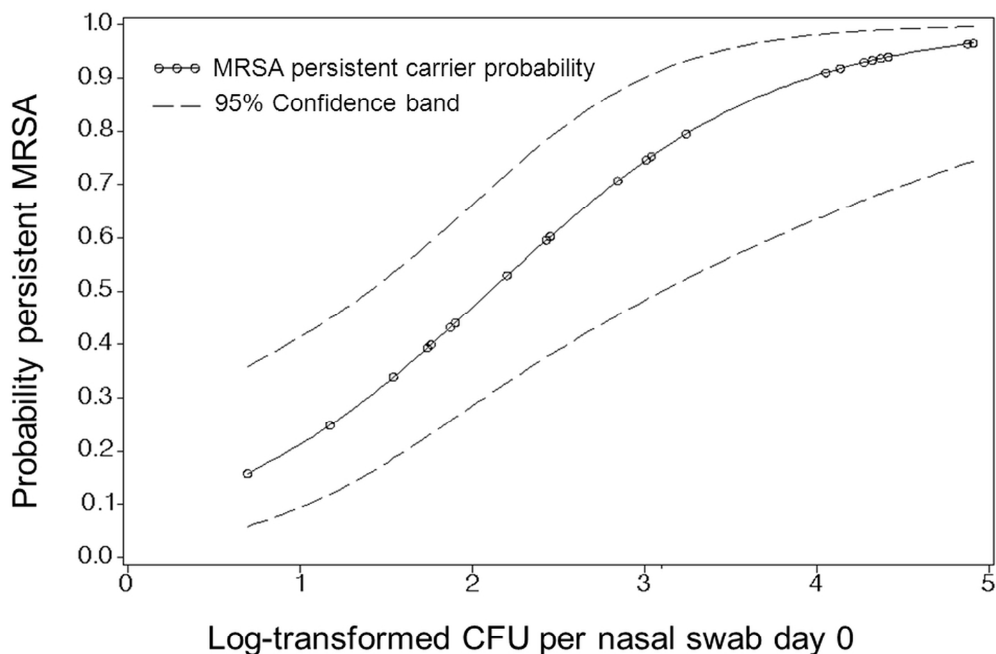

Figure 1. Probability of nasal MRSA persistent carriage and its relationship with the log-transformed CFU from MRSA positive nasal swabs at day 0. Nonparametric regression modelling.
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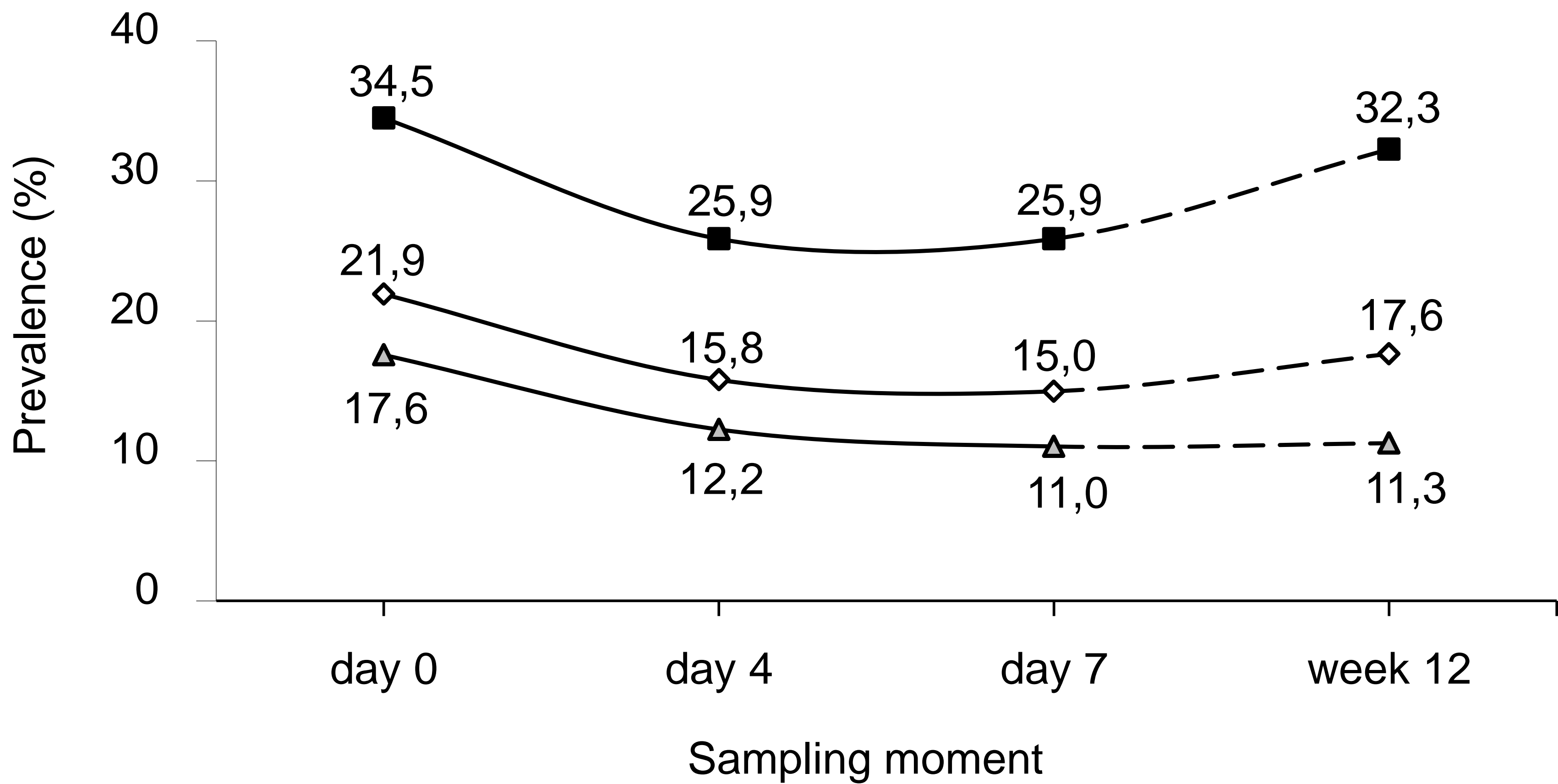

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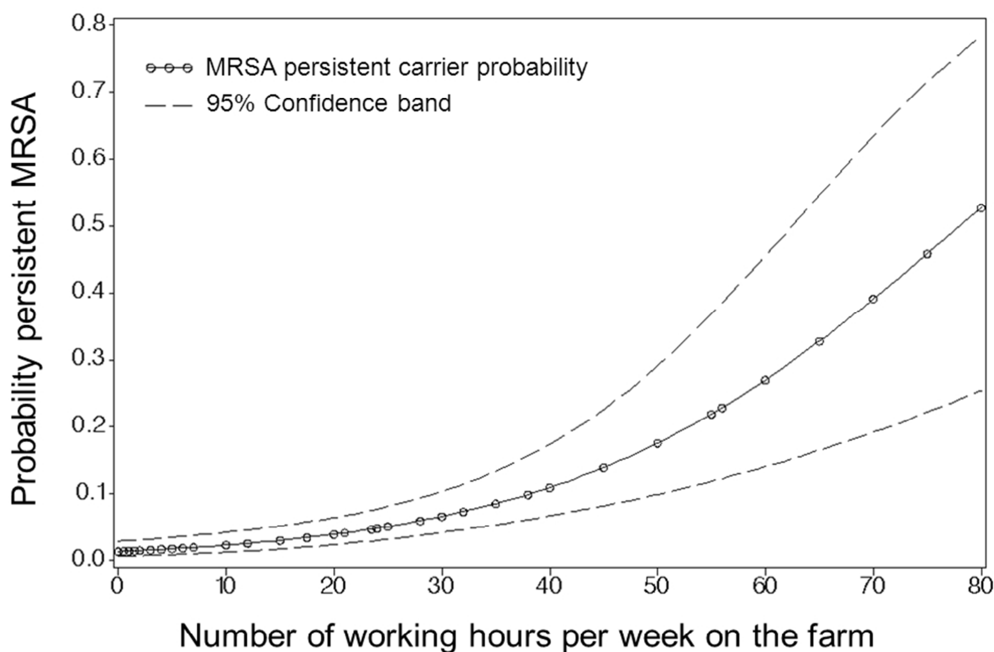

Probability of nasal MRSA persistent carriage and its relationship with number of working hours in the farm. Semiparametric regression modelling setting sex and age as parametric components for adjustment
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Supplemental Table 1. Patterns for one week nasal carriage of *S. aureus*, MRSA and MSSA in the total study population and subpopulations of farmers, household members and employees.

	No. persistent (%)[a]	No. intermittent (%)[a]	No. non-carrier (%)[a]	Total no.
MRSA in nose:				
Total population [b]	20 (9.7)	35 (17.0)	151 (73.3)	206
Farmers	9 (15.5)	15 (25.9)	38(61.3)	62144
Family members	11 (7.6)	20 (13.9)	113 (78.5)	
MSSA in nose:				206
Total population [b]	25 (12.1)	36 (17.5)	145 (70.4)	62144
Farmers	3 (5.2)	14(22.6)	45(72.5)	
Family members	22 (15.3)	22 (15.3)	100 (69.4)	
				206
S. aureus in nose:				62144
Total population [b]	47 (22.8)	61 (29.6)	98 (47.6)	
Farmers	14 (24.1)	22(35.5)	26(41.9)	
Family members	33 (22.9)	39 (27.1)	72 (50.0)	

[a] A person was persistent carrier when each of the nasal swabs collected on days 0, 4 and 7 was positive for MRSA or MSSA; For *S.aureus* carriage patterns, people intermittently positive to MRSA or MSSA were also considered as persistent as long as they were carriers of the resistant or susceptible strains on days 0,4 and 7. Non-carriers had no positive swabs; intermittent carriers were the remaining persons.

[b] there were 5 missing values (total study population = 211).

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1 ✓	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2 ✓	Explain the scientific background and rationale for the investigation being reported
Objectives	3 ✓	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4 ✓	Present key elements of study design early in the paper
Setting	5 ✓	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6 ✓	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up Farms and participants willing to particip[ate] *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	✓ 7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	✓ 8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	✓ 9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at Not reported in manuscript.
Quantitative variables	✓ 11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	✓ 12	(*a*) Describe all statistical methods, including those used to control for confounding
	✓	(*b*) Describe any methods used to examine subgroups and interactions
	✓	(*c*) Explain how missing data were addressed
	NA.	(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

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Results		
Participants	13* ✓	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
	NA	(b) Give reasons for non-participation at each stage
	NA	(c) Consider use of a flow diagram
Descriptive data	14* ✓	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15* ✓	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16 ✓	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
	✓	(*b*) Report category boundaries when continuous variables were categorized
	NA	(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17 ✓	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	✓ 18	Summarise key results with reference to study objectives
Limitations	✓ 19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	✓ 20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	✓ 21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	✓ 22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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