



Dynamics of MRSA carriage in veal calves: A longitudinal field study

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ABSTRACT

Colonization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in food producing animals has public health implications, but intervention targets have not yet been identified. In this field study occurrence and dynamics of MRSA in veal calves were investigated longitudinally on three farms. Determinants generally associated with MRSA carriage, such as environmental exposure and antimicrobial use, were explored. In addition, the reliability and reproducibility of MRSA detection in nasal samples from veal calves were investigated as well as the additional value of rectal samples to establish MRSA status of an individual animal.

On these three farms, MRSA prevalence and MRSA air loads in stables rapidly increased during the production cycle, especially after releasing calves from their individual houses, but not simultaneously with or directly after treatment with antimicrobials. These observations constitute the hypothesis that antimicrobial use may not necessarily be the only condition for MRSA transmission in veal calves, but indicate that other factors may contribute to transmission as well. MRSA in calves was present both nasally and rectally. The reproducibility and repeatability of the nasal samples were moderate.

The results of this study give a better understanding of the dynamics of MRSA in a field situation.

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1. Introduction

The emerging problem of livestock-associated (LA)-MRSA colonization in food producing animals, such as pigs and veal calves, has put pressure on intensive livestock farming to control this spread. In addition, it has an impact on occupational health among people in regular contact with food producing animals and potential risks of carriage or disease in the general population (Catry

et al., 2010; Graveland et al., 2011b; Vanderhaeghen et al., 2010). Therefore, it is of importance to reduce LA-MRSA occurrence in food producing animals and consequently in occupationally exposed humans.

Risk factors for LA-MRSA carriage in humans in close contact with food producing animals have been investigated in cross-sectional studies (Smith et al., 2009; Van den Broek et al., 2009; Graveland et al., 2010). A longitudinal study in veal farmers and their family members showed that LA-MRSA carriage was associated with the intensity and duration of animal contact. LA-MRSA prevalence in people working or living on veal farms decreased during absence of animal exposure, and the detection of LA-MRSA in nasal swabs varied greatly over time (Graveland et al., 2011a). This suggests that both colonization and transient contamination of LA-MRSA occur in humans. This variable

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status may also occur in animals. Little is known about dynamics, spread and possible changes in carrier status of LA-MRSA in animal populations. Data on dynamics of LA-MRSA in individual animals and at herd-level, and association with risk factors, are relevant to develop control strategies. Knowledge of the reliability and reproducibility of the diagnostic procedure when collecting these data are a prerequisite for correct interpretation of results, but currently lacking.

The aim of this longitudinal study was (i) to describe the dynamics of LA-MRSA in veal calves by analyzing nasal and rectal samples, (ii) to describe the dynamics of environmental contamination, and (iii) to determine the reliability and reproducibility of MRSA detection in nasal samples.

2. Materials and methods

2.1. Study design and data collection

The study population consisted of 402 veal calves housed on three veal farms in The Netherlands. No other food producing animals than calves were present on these farms. A positive MRSA history was confirmed by MRSA positive dust samples from calf stables at the end of the previous production cycle. Included calves were housed in closed compartments (i.e. a separate, closed area within the stable). Each compartment has a separate ventilation system. Within each compartment the calves were housed in multiple pens. In total, 5 compartments were studied: two compartments containing 112 calves in total on farm 1, one compartment containing 150 calves on farm 2 and two compartments containing 140 calves in total on farm 3. All calves were approximately 2 weeks of age at the start of the study period. All three farms had additional compartments in the same stables with calves of the same age, but these were physically separated.

Nasal and rectal samples were taken from these 402 veal calves during a period of 18 weeks between October 2009 and March 2010. Eight samplings were performed from the moment of arrival of the calves (T0) and in weeks 3, 6, 8, 10, 12, 15, and 18 after arrival. Nasal and rectal swabs were collected during week 0 till week 10, and from week 12 onward only nasal swabs were taken. After T8, solely rectal carriers were rarely found.

Until week 6, calves were housed individually in boxes placed in pens. Thereafter, they were released from their individual boxes and housed in pens with an average 6 calves. At this occasion, relocating of calves between pens in the study compartments was done to harmonize per pen for size and/or feed intake of the calves. On all three farms, calves were sampled in week 6 the day before they were released from their individual housing. Calves that were relocated to areas outside the study compartment(s) were excluded from the study. As a rule, no animals from a non-study compartment were allowed to move into the study compartments. However, on farm 1 three calves entered the study compartments at a later stage (two calves at T6, one calf at T10); they all tested negative before entering.

Swabs were taken using a sterile cotton-wool swab (Cultiplast®). From each calf, the nasal swab was taken from both nares by rubbing the swab in each nostril. Rectal swabs

were taken by rubbing the swab in the rectum of the calf. The swabs were immediately transported to the laboratory and processed at the same day. To investigate the reproducibility of MRSA status by nasal swabs in veal calves, 32 randomly selected veal calves originating from farm 3 were repeatedly sampled in their nose (5 times) at T12 and these samples were analyzed individually.

Questionnaires were used to register farm characteristics and hygiene practices, as well as antimicrobial use of the calves, gender and when and where calves were relocated within the study compartments. Collection of animal samples was in accordance with the Dutch Law on Animal Health and Welfare. The study protocol was approved by the Animal Ethical Committee of Utrecht University.

Calves were considered to be MRSA positive when MRSA was isolated from the nasal and/or rectal swabs. If no MRSA was present in both swabs, the calf was recorded as MRSA negative.

2.2. Environmental sampling

To determine the MRSA load in the air of the calf stables, air samples were collected one day after each animal sampling took place. Air samples were collected using mobile samplers, consisting of a GilAir-5 air sampling pump (Gilian) and a GSP conical sampler. Cassettes containing 37 mm GFA glass fiber membrane filters (Whatman) were placed in the GSP samplers. Air samples were collected at a flow of 2 L/min for 5–8 h. In each compartment, on 3 different locations, duplicate air samples were collected resulting in 30 samples in total per sampling moment.

2.3. Laboratory analysis

All swabs were analyzed as previously described (Graveland et al., 2009). In short: swabs were inoculated in a non-selective pre-enrichment containing Mueller Hinton broth with 6.5% NaCl (MH+). After overnight aerobic incubation at 37 °C, 1 mL of pre-enrichment was transferred into 9 mL selective enrichment broth containing phenyl mannitol broth with 5 µg/mL ceftizoxime and 75 µg/mL aztreonam (BioMérieux, France). After overnight aerobic incubation at 37 °C, 10 µL of this selective enrichment broth was plated onto sheep blood agar (Biotrading, The Netherlands) and MRSA Brilliance™ agar (Oxoid, The Netherlands). All suspected colonies were identified as *Staphylococcus aureus* using standard techniques: colony morphology and coagulase assay (slide) using rabbit plasma (Becton Dickinson, The Netherlands). The presence of the *mecA*-gene was confirmed by PCR as previously described (Fluit et al., 2001). Randomly, a selection of strains ($n = 168/2183$) was confirmed by *S. aureus* ST398 specific PCR (van Wamel et al., 2010).

Air filter membranes were similarly analyzed by culturing. The glass fiber filter membranes were divided in two equal parts. One half was stored at –20 °C, the other half was extracted by vigorous vortexing in 5 mL MH+. Subsequently, 0.5 mL of this suspension was diluted 10, 100 and 1000-fold in MH+. Further analysis was performed as described above for nasal and rectal swabs. By diluting

samples until MRSA was non-detectable (after culturing), MRSA concentration ranges could be obtained, giving an indication of the MRSA load.

2.4. Statistical analysis

Data were analyzed by simple descriptive statistics using the statistical software program SAS®, version 9.2. Reproducibility (agreement) was measured by kappa values.

The transmission rate parameter β for within-pen spread of MRSA was estimated as indicator of the number of calves infected by one infectious calf per time unit. It was assumed that a calf was infectious (i.e. a carrier shedding the bacteria) from the day it was tested positive until the next sampling, and susceptible (i.e. non-carrier) from the day it was tested negative until the next sampling. Calves were assumed to be either susceptible or infectious and could not become immune, and were further assumed to mix randomly in pens, to be equally infectious throughout the infectious period, and for all susceptible calves to be equally susceptible. For calculation of the transmission rate parameter β only pens without animal movements were eligible. A common, previously described method was adapted and used to estimate the transmission rate parameter β (Bos et al., 2009; Schouten et al., 2009), which makes use of GENMOD procedure in SAS®, version 9.2, to program the model as a GLM with a binomial distribution of the stochastic outcome variable ($C(t)/S(t)$) and a complementary-log-log link function. $C(t)$ and $S(t)$ denote the number of cases (in this study the number of infectious calves that were negative at the previous sampling time) and susceptible calves present per pen at the sampling time, respectively. $\text{Log}(I(t)/N(t))$ was used as offset variable, with $I(t)$ and $N(t)$ denoting the number of infectious and total number of calves present per pen at the sampling time, respectively. Pen was inserted as a repeated subject to account for clustered data. The outcome of the model is $\log(\beta)$; therefore, β is derived by taking the antilog.

3. Results

3.1. Farm and animal characteristics

All farms belonged to the category white veal and applied an all-in-all-out system. They differed slightly in farm structure with regard to the buildings and the total number of animals on the farm. All stables were divided into different compartments. Stable compartments of farm 1 were naturally ventilated; whereas in the stable compartments of both farms 2 and 3 mechanical ventilation was applied. Cleaning, but not disinfection, of the stables before the calves enter the stables was applied on all three farms.

The included calves of farm 2 were all heifers ($n = 150$), whereas the included calves of farm 1 ($n = 109$) and 3 ($n = 140$) were all bulls. All animals originated from Dutch farms. The mean weight of the calves at the start of the study (T0) was slightly different between the farms (40.8, 37.4 and, 48.9 kg respectively). The number of calves lost

for follow-up due to death was higher on farm 1 (5 calves) compared to both farm 2 and farm 3 (1 calf). The number of calves relocated between pens once or more, differed from farm to farm, and varied between 75% and 89%. This indicates that just a few animals were not relocated during the production cycle. The majority of the relocations took place when the calves were released from their individual houses and housed in groups. Thereafter, fewer calves were relocated, varying between 14.3 and 44.0%. Table 1 summarizes general farm and animal characteristics.

The number of antimicrobial group treatments of the calves (i.e. all calves in the study compartment were given the same antimicrobial treatment orally) was comparable between farms regarding the number of treatments, but differed in timing and type of antimicrobials used. Antimicrobials were administered mainly during the first 30 days after arrival on the farm. Tetracycline and macrolide antimicrobials were the most frequently used on all three farms. Type, duration and timing of antimicrobial group treatments are shown in Fig. 1. Observed antimicrobial use on these three Dutch veal calf farms cannot be generalized because this study involved only one production cycle in winter season and is subject to considerable systematic and random variation.

3.2. MRSA carriage

MRSA prevalence in calves ranged from 9 to 14% directly after arrival on the farm (T0), and increased over time to 63–96% at the end of the study period (T18) (Fig. 1). All typed MRSA strains found in these veal calves belonged to ST398. On farm 1, there was an increase of MRSA prevalence from 14% to 91% in the first 3 weeks. In contrast, for farms 2 and 3 there was no clear increase in MRSA prevalence over the first 6 weeks of the study period. On these farms, a considerable increase in MRSA prevalence occurred between 6 and 8 weeks after arrival on the farm. After this strong increase in MRSA, prevalence leveled off (Fig. 1). The change in MRSA prevalence is mirrored by the calculated transmission rate for different periods during follow-up. For the period just after the release of the calves into group pens (T8–T12) β was 1.6/calf/week. Hereafter (T15–T18), β was 0.9/calf/week (farms 2 and 3 together). The transmission rate could not be estimated for farm 1 because of the continuous high prevalence (i.e. there were no new cases).

From T0 till T10, MRSA prevalence was based on the results of nasal and rectal samples. A large variation in rectal carriage was seen between farms. At sampling T3, on average a higher MRSA prevalence was found in the rectal samples than in the nasal samples (24% versus 15%, respectively). This difference was mainly dominated by the high prevalence of rectal carriage on farm 1. The difference between nasal and rectal MRSA carriage and the number of calves which were solely MRSA positive in their rectal sample, decreased after T8 (Fig. 1).

Approximately 10% of the veal calves were LA-MRSA positive upon arrival on the farm. Twelve calves (2.5%) were persistently MRSA positive (10 calves on farm 1 and 2 on farm 2), meaning MRSA was present in nasal and/or rectal samples on all sampling moments. Nineteen calves

Table 1

Overview of general farm and animal characteristics.

	Farm 1	Farm 2	Farm 3
General farm information			
# calves on farm	942	560	486
# calves included in study	112 ^a	150 ^b	140 ^c
# stables on farm	3	1	1
# stables (compartments) in study	1 (2)	1 (1)	1 (2)
General characteristics of calves (study population)			
Gender	Bull	Heifer	Bull
Mean weight of calves at T0 (kg)	40.8	37.4	48.9
# calves lost to follow-up due to death	5	1	1
Antibiotic use			
# antimicrobial group treatments	5	4	6
# treatment days	29	27	31
# calves treated individually with antimicrobials (%)	18 (16.1%)	35 (23.3%)	10 (6.4%)
Relocating of calves			
% relocated calves (once or more)	75.0%	89.3%	74.7%
% relocated calves <i>after</i> releasing from individual houses	26.8%	44.0%	14.3%

^a Five calves died during the study period, 1 calf was removed from the study compartment, 3 calves (all MRSA negative at entrance) entered the study compartment during the study period.

^b One calf died during the study period, 2 calves were removed from the study compartment.

^c One calf died during the study period, 1 calf was removed from the study compartment.

(4.7%) were MRSA negative during the whole study period (4 calves on farm 2 and 15 calves on farm 3). The majority of calves ($n = 371$, 92.3%) were MRSA positive at one or more sampling moments but not all. However, we observed that 51% (189 calves) of these calves became positive on a certain moment and were persistently positive for the rest of the study period. The other 182 calves (49%) were more irregularly found to be MRSA positive and therefore defined as intermittent carriers.

At T12, 32 calves were tested repeatedly for MRSA presence. MRSA prevalence in these repeated samples (taken from the same animals on the same sampling moment) ranged from 47 to 63%. Cohen's kappa was 0.42 (95% CI: 0.27–0.57, $p < 0.05$). The observed agreement was 0.71 and expected agreement was 0.51. Thus, the level of agreement appears to be moderate and better than expected due to chance. Calculation of kappa using the results of the nasal samples of sampling moment T10 and T12 or T12 and T15

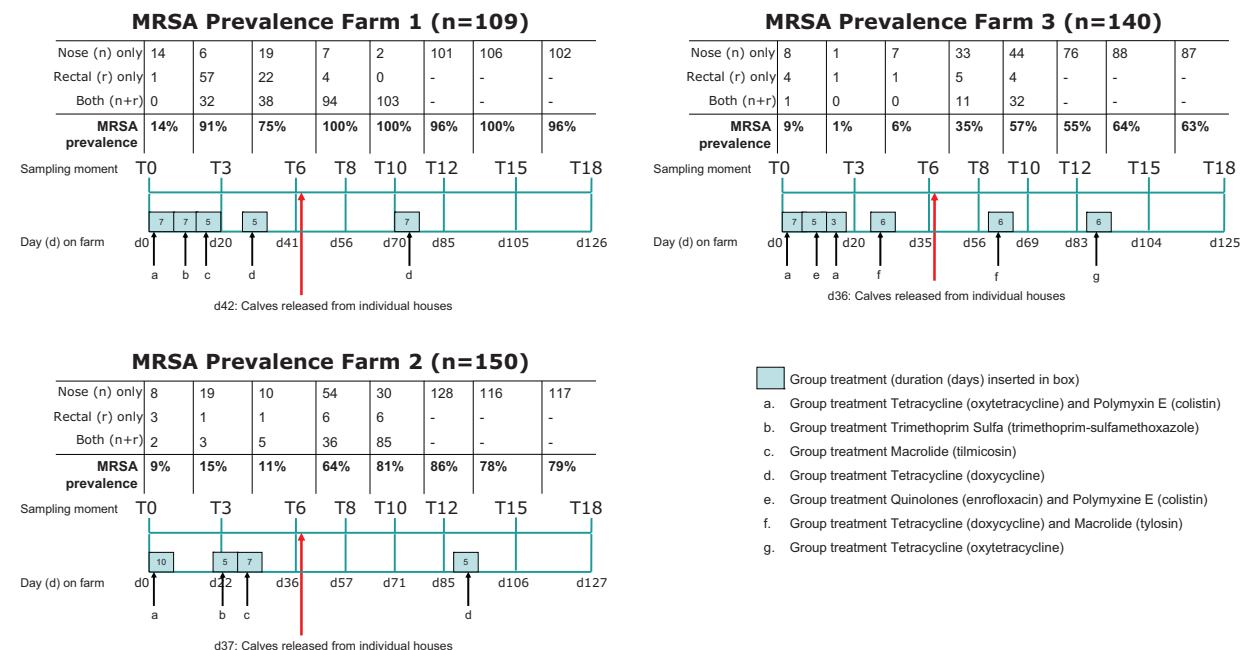


Fig. 1. Schematic overview of MRSA prevalence in veal calves over time per farm. Per farm, the number of calves MRSA positive in solely nasal or rectal samples as well as the number of calves MRSA positive in both nasal and rectal samples are mentioned, together with the total MRSA prevalence per sampling moment. In addition, timing and duration of antimicrobial group treatments are illustrated in boxes.

for the same 32 calves resulted in comparable kappa values (0.41 and 0.40, respectively).

In total, the results of 14/32 calves (44%) were consistent over 5 samples. Ten of them were consistently positive and 4 calves were MRSA negative in all 5 samples that were taken. In 13/32 calves (41%) the result of the first nasal sample was negative, followed by one or more positive samples. In 5/32 (16%) calves the result of the first nasal sample was MRSA positive, followed by one or more MRSA negative samples.

3.3. Environmental sampling

Air samples were mainly negative when MRSA prevalence in veal calves was low (T0–T6), but the percentage of MRSA positive environmental samples increased parallel to the increasing prevalence of MRSA in veal calves (Table 2). Not only the proportion of positive samples increased over time, but also the amount of MRSA in the samples as tested with diluted samples (data not shown). Until T6, MRSA in air samples was mainly detected in undiluted samples only, whereas after T6 MRSA was more frequently detected in samples which were diluted 10- or 100-fold, indicating an increase in MRSA levels in air (data not shown). In farms 2 and 3 no air samplings were performed on sampling moment T8 due to bad weather conditions on the sampling days.

4. Discussion

This study presents the results of repeated MRSA sampling in veal calves, which give insight in the spread and dynamics of MRSA in a veal calf herd. Approximately 10% of the veal calves were LA-MRSA positive upon arrival on the farm. The origin of the LA-MRSA in these calves is currently unknown, but may be the dairy farm where the calves originate from, or it may be a result of contact with positive calves during transport. In this study only LA-MRSA belonging to ST398 was detected, which is in accordance with a previous cross-sectional study that showed ST398 as the most prevalent MRSA type in veal calves (Graveland et al., 2010).

MRSA prevalence was not constant over time. On farm 1, there was a strong increase in MRSA prevalence (to >90%) during the first 3 weeks. During these weeks the animals received a group treatment for 19 out of 21 days, which may have been a trigger for MRSA proliferation. However, group treatments were given on the other farms as well; on farm 2 for 10 out of 21 days and on farm 3 for 15 out of 21 days. On both these farms, no increase in MRSA prevalence was observed simultaneously with or directly after antimicrobial treatments. On farms 2 and 3, MRSA prevalence increased rapidly after calves were released from the individual housing, a trend also observed on farm 1 but due to the relatively high prevalence just before release (75%), this increase was less prominent.

It is not clear how soon various antimicrobial treatments affect MRSA selection and MRSA amplification in animals. However, in pigs it has been shown that rapid selection of antimicrobial resistant bacteria can occur after antimicrobial treatments. Prevalence of MRSA in groups of pigs

increased after antimicrobial treatment (van Duijkeren et al., 2008). The observations on these 3 farms could be the result of the (co)selection and amplification by some classes of antimicrobials on one hand and the therapeutic treatment by other antimicrobials on the other. This balance is hard to predict and analyze due to different susceptibility patterns of MRSA strains and potential co-selection between different antimicrobials.

It is possible that the intervals of 2–3 weeks between samplings were too long to detect the effects of these treatments. Further research is needed to study in-depth the dynamics behind the influence of duration and type of antimicrobial treatments on MRSA occurrence in veal calves.

Results from this study indicate that various factors may contribute to transmission. It is plausible that the type of ventilation system in stables (natural versus mechanical) may have influenced environmental MRSA transmission. However, no associations have been observed between ventilation system and MRSA occurrence in veal calves so far (Graveland et al., 2010). Farm size however has been shown to be positively associated with MRSA occurrence in veal farming (Graveland et al., 2010). Farm 1 has the largest farm size as well as the largest number of MRSA positive calves at arrival on the farms (14% on farm 1 versus 9% on farms 2 and 3) which may influence the MRSA prevalence and transmission during further production cycle. These observations may probably result in the differences in increase of MRSA prevalence between farm 1 and the other 2 farms.

We additionally hypothesize a mechanism which is dependent on rectal colonization by MRSA. This study indicated that rectal colonization of MRSA contributed to higher environmental contamination of MRSA, and may have resulted in a strongly increased MRSA prevalence. Comparable observations were seen in humans where intestinal or perineal carriage of MRSA had been implicated as an important contributor to environmental dissemination as well (Acton et al., 2009). The high rectal MRSA prevalence at younger age, especially on farm 1, could contribute to a strong boost in environmental MRSA load and be an explanation of the strong increase in MRSA prevalence and spread on this farm. This is strengthened by the observations from farms 2 and 3, where nasal carriage of MRSA was predominant over rectal carriage and MRSA prevalence was more gradually increasing over time. In addition, air samples from farms 2 and 3 were also less frequently MRSA positive. Not only the proportion of positive samples, but also the number of MRSA in the samples was less (data not shown). The observation of rectal MRSA carriage in veal calves is relevant, since it is a likely source of the increase in environmental contamination through the air, which has not been taken into account previously.

We cannot explain why rectal carriage is especially seen at younger age and why the number of calves solely carrying MRSA in their rectum decreases over time. The development of their gut flora or immune system may play a role. Comparable data are available for pigs (Khanna et al., 2008) and humans (Acton et al., 2009), where rectal MRSA carriage without nasal MRSA carriage was also shown in especially younger subjects. Intestinal micro flora

Table 2
MRSA prevalence in calves and air samples.

Sampling moment	Farm 1 (12 air samples per sampling moment)		Farm 2 (6 air samples per sampling moment)		Farm 3 (12 air samples per sampling moment)	
	MRSA prevalence in calves (%)	# MRSA positive air samples (%)	MRSA prevalence in calves (%)	# MRSA positive air samples (%)	MRSA prevalence in calves (%)	# MRSA positive air samples (%)
T0	14	0/12 (0)	9	0/6 (0)	9	1/12 (8)
T3	91	2/12 (17)	15	1/6 (17)	1	0/12 (0)
T6	75	7/12 (58)	11	0/6 (0)	6	1/12 (8)
T8	100	11/12 (92)	64	— ^a	35	— ^a
T10	100	12/12 (100)	81	5/6 (83)	57	5/12 (42)
T12	96	12/12 (100)	87	5/6 (83)	55	6/12 (50)
T15	100	10/12 (83)	78	3/6 (50)	64	5/12 (42)
T18	96	12/12 (100)	80	4/6 (66)	63	6/12 (50)

^a No air sampling performed due to bad weather conditions.

in animals can be strongly influenced by diet and can be disturbed by the effect of antimicrobial compounds (Sorum and Sunde, 2001). Rectal carriage might be explained by a change in feed (addition of roughage at a later age), or the higher number of antimicrobial treatment days calves receive when they are under 12 weeks of age. Stress in animals caused by extreme fluctuations in temperature (Moro et al., 2000), transport or overcrowding and disease (Langlois et al., 1988), influenced shedding of resistant *Enterobacteriaceae* in the environment. For MRSA however, there is limited data available on the effect of these factors on shedding in animals.

The large number of intermittent carriers found in this study suggests that calves could become MRSA positive due to contact with their positive pen mates (especially after T6), or through exposure to MRSA in the environment. The latter is supported by the finding that high MRSA prevalence in calves seemed to be associated with high MRSA air load. Furthermore, we observed no consistent clustering of MRSA positive calves when the calves were housed individually. In contrast, we observed random scattering of MRSA positive animals in the study compartments. During the period in which calves were housed individually, it is unlikely that direct contact plays a major role in transmission. Therefore, these results strengthened the hypothesis that transmission via environmental sources plays a role.

A previous cross-sectional study (Graveland et al., 2010) showed a direct association between MRSA prevalence in calves and humans. Here, we show that this could be explained by an increasing MRSA prevalence in calves and MRSA air load during the production cycle. This emphasizes the need to reduce exposure, in order to reduce MRSA carriage risk for both animals and humans. Previously, we found that MRSA prevalence in veal calves was negatively associated with farm hygiene; i.e. cleaning and disinfection of stables between two production cycles (Graveland et al., 2010). Cleaning and disinfection of stables between cycles can result in a lower environmental MRSA load at the start of a production cycle. However, the number of MRSA positive calves at arrival on the farms may also influence the MRSA prevalence during the further production cycle. This means that a high prevalence of MRSA among the calves at the start of the cycle will undo the advantage of cleaning and disinfection. Furthermore, measures like wearing protective masks, might prevent human colonization.

We found 31 calves with a consistent MRSA status over time. This is remarkable since kappa statistics showed moderate agreement between repeated nasal samples, and therefore the likelihood of finding calves which were continuously positive or negative is low. A possible explanation could be that there are differences in MRSA load between calves. It is possible that calves that were continuously MRSA positive shed the bacteria in higher amounts than calves that were intermittent carriers. We did not investigate differences in MRSA load between calves in this study. The effect of MRSA load on carriage and test performance needs confirmation in other studies.

In conclusion, this first observational longitudinal study on MRSA in veal farming showed that MRSA prevalence rapidly increased during the production cycle, especially after releasing calves from their individual houses. This study showed no clear increase in MRSA prevalence simultaneously with or directly after antimicrobial treatments, and on these farms other factors may have contributed to MRSA transmission in veal calves as well. The new findings that MRSA prevalence is not constant over time, as different carriage patterns were observed, and that calves were MRSA positive in both nasal and rectal samples, give additional hypothesis-forming insights. Further research is necessary to explore these insights in more detail, and to explore efficient tools to reduce exposure to MRSA for both animals and humans. The results of this study give a better understanding of the dynamics of LA-MRSA in a field situation.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

HG participated in the conception and design of the study, collected the data, carried out the statistical analyses and interpretation of the data, and drafted the manuscript. MB collected the data, carried out the statistical analyses and interpretation of the data and contributed to the critical revision of the manuscript. IO and KV collected data and carried out the laboratory analyses. JW and DH participated in the conception and design of the study, and contributed

to the critical revision of the manuscript. All authors read and approved the final manuscript.

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