Livestock-associated MRSA in veal farming

risk factors for MRSA carriage in veal calves and humans

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Diergerelateerde MRSA in de vleeskalverhouderij

risicofactoren voor MRSA-dragerschap voor vleeskalveren en mensen

(met samenvatting in het Nederlands)

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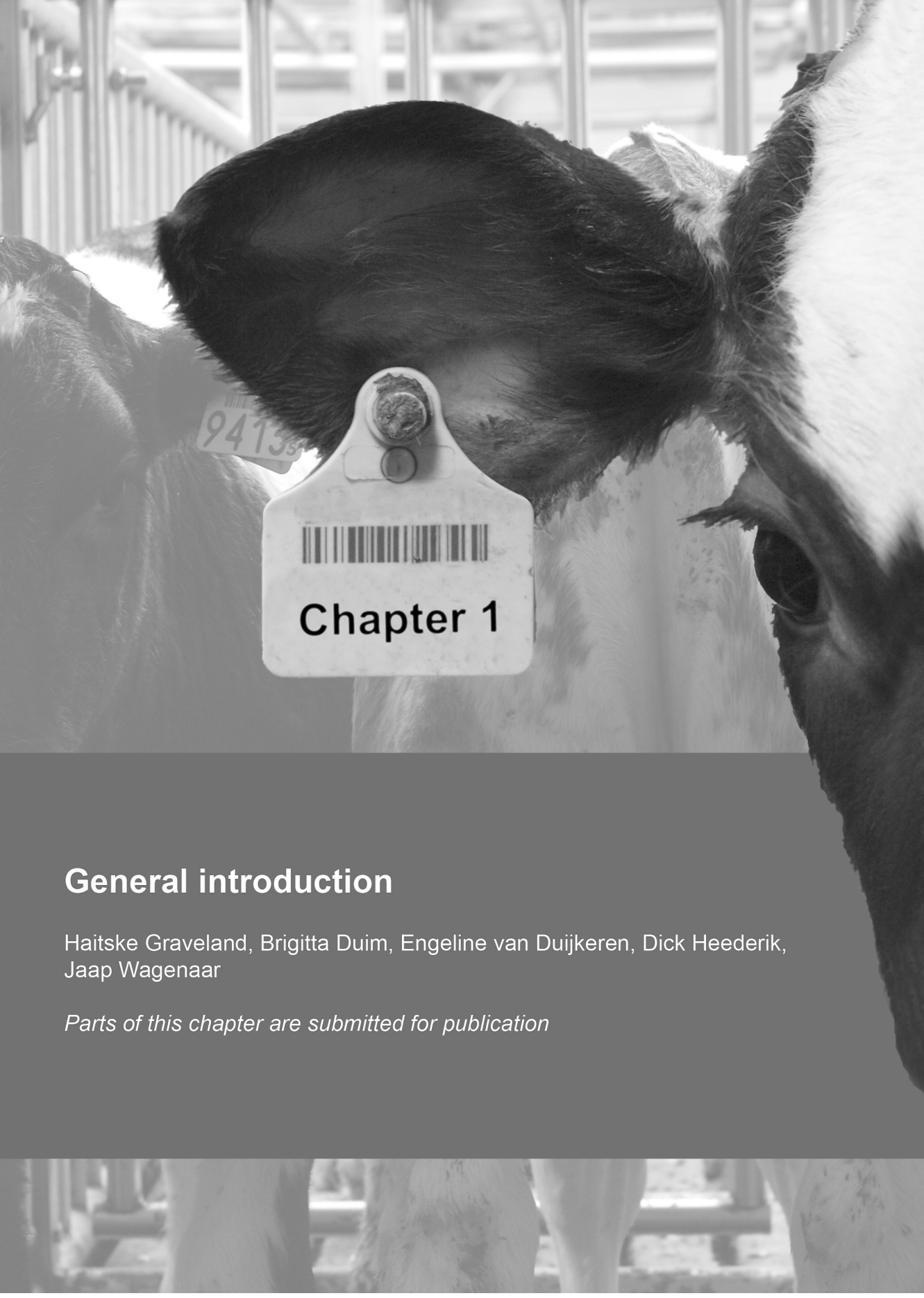
Voor mijn lieve Ouders en Oma

Contents



General introduction

Haitske Graveland, Brigitta Duim, Engeline van Duijkeren, Dick Heederik, Jaap Wagenaar

Parts of this chapter are submitted for publication

Staphylococcus aureus

Staphylococcus aureus (S. aureus) is a Gram-positive bacterium which forms part of the normal flora of humans and various animal species (30). This bacterium is a colonizer of the skin and mucosae. Under specific conditions, it can invade multiple organs. In humans, *S. aureus* is regarded the most important cause of nosocomial infections with clinical conditions ranging from common minor skin infections to severe, often life threatening infections (12, 15). Multiple body sites can be colonized in humans, but the anterior nares are the most frequent colonized sites (33). Approximately 20% of healthy individuals are persistent *S. aureus* carrier, about 30% are intermittent carriers and around 50% are never colonized with *S. aureus* (12).

In animals, *S. aureus* is one of the three major pathogenic *Staphylococcus* species, together with *S. hyicus* and *S. (pseudo)intermedius* with the latter two more restricted in host species compared to *S. aureus*. Until now, *S. aureus* plays its most significant animal pathogenic role as cause of intramammary infections in cattle and small ruminants (30). It is also the cause of joint problems in chickens (2) and it is increasingly reported in surgical site infections in small companion animals and horses (3).

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Soon after the introduction of penicillin, around 1945, the majority of the *S. aureus* population had become resistant to penicillin through the production of beta-lactamase, an enzyme that hydrolyzes penicillin. In the late 1950s, methicillin was introduced in human medicine. Methicillin was stable to hydrolyzing activity of beta-lactamases. However, soon after introduction, the first methicillin resistant isolates of *S. aureus* were reported (21).

Methicillin resistance is caused by the acquisition of the *mecA gene.* This gene encodes an alternative penicillin binding protein, called PBP2A, which has a low affinity for beta-lactam antibiotics (30). The *mecA* gene is part of a large mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* can be integrated at a specific site in the chromosome of methicillin susceptible *S. aureus* (MSSA) which is located at the 3 prime end of an open

reading frame of unknown function (*orfX)* (9). SCC*mec* carries a set of cassette chromosome recombinase genes (*ccrA, ccrB* or *ccrC*) for excision and integration into the host chromosome. According to combinations of the *mec* and *ccr* gene complexes contained by the bacterial genome, molecular typing of MRSA strains has revealed that five major SCC*mec* types and several variants have emerged worldwide. In addition to the difference in these gene complexes, the various SCC*mec* elements differ from each other in the antibiotic resistance genes against the non-beta-lactam antibiotics they carry (9).

Epidemiology of MRSA

The prevalence of MRSA infections varies widely between European countries. Especially in hospital settings MRSA prevalence is generally high in southern Europe where MRSA prevalence among clinical isolates (blood) up to 50% is documented. In contrast, in northern Europe MRSA prevalence is extremely low, <1% (23). This can partly be explained by differences in level of screening, isolation and monitoring of patients and staff in hospitals. In The Netherlands a pro-active system has been applied, called the “search and destroy” policy. This strategy consists of active screening of high risk patients and exposed healthcare workers for carriage. Risk patients involve hospitalized patients that are repatriated from countries outside the Netherlands and contacts of MRSA patients (12). In addition, in July 2006 and November 2007 people who have been in contact with live pigs or veal calves respectively, were included to the risk group (28). Furthermore strict implementation of transmission prevention measures, and treatment of carriage using topical application of mupirocin nasal cream and washing with disinfecting agents, such as chlorhexidine, is part of the strategy (12). The full strategy is described in the Dutch national guidelines (www.wip.nl).

Definitions of MRSA groups

Traditionally MRSA has been considered as a hospital-associated pathogen (HA-MRSA). If infections caused by MRSA are likely to be acquired in healthcare settings it is mentioned as HA-MRSA. They emerged at least 48h

after admission. Prolonged hospital stay, care in intensive-care units, prolonged antibiotic treatment, surgical interventions and/or close contact with infected or colonized MRSA positive individuals are assumed as risk factors for HA-MRSA (16).

Until the 1990s, infections with MRSA were rarely observed in extramural communities. However, since the mid 1990s, MRSA strains were increasingly documented in healthy people without healthcare-associated risk factors. These cases were referred as community-associated MRSA (CA-MRSA). Close contact between humans in sport settings, schools, day-care centers, the military and prisons are considered to be risk factors (3). Analysis of the genetic background of these CA-MRSA strains has shown a clear distinction from typical HA-MRSA. CA-MRSA and HA-MRSA belong to different sequence types and in addition carry different SCC*mec* types. Furthermore the carriage of virulence factors such as Panton-Valentine leukocidin (PVL) is merely associated with CA-MRSA strains (30).

MRSA has been found to be emerging in livestock (14). Animals have the capacity to act as reservoirs of MRSA, and potentially transmit this bacterium to humans in close contact with MRSA colonized animals. MRSA in this group have been referred as livestock-associated MRSA (LA-MRSA), to distinguish it from HA-MRSA and CA-MRSA types (22).

Emergence of MRSA in livestock and other animals

Between 1970-2000, MRSA was rarely isolated from animals, and if so, these strains were generally of human origin, as shown by biotyping. Therefore, it was thought that until the end of the 20th century, animal husbandry was of little relevance to MRSA causing diseases in humans. It was assumed that MRSA was a problem solely caused by antimicrobial use in human medicine (3).

In 1975 LA-MRSA was first reported in milk of cows with mastitis (7). This sporadic case was followed by only a few other cases in the next 25 years. From 2000s onwards reports became more frequent and in 2007 transmission of MRSA between cows and humans was reported (11). Since 2004, swine emerged as a novel reservoir of MRSA. In the Netherlands, the initial case of

LA-MRSA in human was described in a 6-month-old girl admitted to a hospital. Despite several decolonization attempts the girl remained MRSA positive. The girl's parents, who lived on a swine farm, were also found to be MRSA colonized (31). Since neither the girl nor her family had a history of traveling or admission to a foreign hospital, further investigations began into the source of the MRSA in regional pigs and pig farmers (10). An additional study on the occurrence of MRSA in pigs at slaughterhouse confirmed the wide spread of LA-MRSA in the Dutch pig population (6). Genotyping showed that the LA-MRSA strains as found in pigs and pig farmers were non-typable by pulsed-field gel electrophoresis (PFGE) as these were resistant to digestion with the routinely used SmaI, and therefore designated as non-typable MRSA (NT-MRSA). The strains belonged to clonal complex CC398, with the majority of strains belonging to ST398. Risk factors for humans acquiring MRSA were pig and cattle farming (26). Since 2005 onwards, LA-MRSA is more frequently reported in different food production animals including cattle (29), pigs (10, 24) and poultry (18, 19).

Besides, MRSA has also been found in companion animals, however these strains generally differ from those in livestock. The reason for this is probably because the strains carried by human owners being passed on to their animals; i.e, the acquisition of MRSA in companion animals is primarily a humanosis (17).

Molecular aspects of MRSA ST398

MRSA strains of ST398 possessing some typical features. As aforementioned the strains are non-typable with standard PFGE with SmaI digestion. This is due to the presence of a restriction/methylation system leading to protection from SmaI digestion (1).

Predominantly, the strains carry SCC*mec* element IV or V. However SCC*mec* cassette types II and III have also been reported (22). A large genetic diversity among *spa*-types has been documented in ST398. Until now, there are 25 different *spa*-types related to ST398 (30). It is suggested that ST398 has been evolved due to multiple introductions of divergent SCC*mec* elements into MSSA ST398. Studies have shown that strains with identical *spa*-types can carry different SCC*mec* elements (24). It has been suggested that coagulase-negative

Staphylococci in the farming environment could serve as a source of SCC*mec* (22).

The transfer of staphylococcal toxin genes seems to be uncommon (22). Generally, virulence factors (for instance Panton-Valentine leukocidin (PVL), *tst* and *LukM)* are absent (30). Despite the lack of virulence factors, MRSA ST398 strains have been found to cause disease in both animals (5, 25, 29) and humans (8, 20).

ST398 strains are generally resistant to tetracycline, and frequent resistance against macrolides, lincosamides, aminoglycosides and trimethoprim is documented. Fluoroquinolone resistance has also been reported, though to a lesser extent (30).

Public health consequences of MRSA ST398

Persons in direct contact with LA-MRSA positive animals have an increased risk of becoming MRSA positive. This has been documented in companion animal and equine hospitals, and livestock environments (17). It has been shown that MRSA ST398 has limited host specificity; it is able to colonize and to cause infections in various hosts. Until now, the mechanisms of host adaptation are poorly understood (4). However, incidentally reported so far, MRSA ST398 can cause serious (invasive) infections and outbreaks (13).

Potentially, there is a risk of MRSA introduction from the animal reservoir into hospitals with humans as vector. Therefore, pig and cattle farmers were included as risk groups as defined by the search and destroy policy. Consequently, in the Netherlands, the annual numbers of people at submission to the hospital, suspected of MRSA colonization requiring MRSA screening, has increased due to the emergence of LA-MRSA. This is a huge burden for the health care system (27, 32). For successful continuation of the Search and Destroy policy identification of risk factors and knowledge about persistence of LA-MRSA in humans is essential. Improved understanding of the mechanisms underlying transmission and persistence, and the role of exposure in LA-MRSA carriage in both animals and humans could have a significant impact on antibiotic and infection control policies in the hospitals. It also provides information for

evidence based guidance on the development of new strategies and preventive measures for the control of MRSA. Few studies have examined transmissibility, and from these it appears that ST398 transmit less frequently than common HA-MRSA strains (27, 32). It is unclear how long carriage persists in colonized individuals, or if persistent exposure to MRSA positive animals or environments is necessary to maintain colonization. Determinants involved in LA-MRSA carriage in both animals and humans are unknown. There is a knowledge gap regarding the spread of MRSA between farms and on the driving forces for persistence of colonization on farms. It has been suggested that the use of antimicrobials in animals on the farms is the major driver of the emergence and spread of MRSA ST398. However, to quantify this aspect, this has to be confirmed by additional empirical data (22).

MRSA ST398 research program

The increasing emergence of MRSA in animals raised questions about the possible public health threats. Therefore there was need for further research. As such, The Dutch Ministry of Agriculture, Nature and Fisheries initiated a research program on LA-MRSA in 2006, aimed at gaining insight in MRSA presence, spread in food producing animals (pigs, veal calves, poultry and dairy cattle) and further along the food chain, and the characteristics of the strains. The aims of this research program were to (i) explore the MRSA occurrence in pigs, veal calves, poultry and dairy cattle, (ii) to investigate risk factors for contamination in pigs and veal calves, (iii) to explore the occurrence of MRSA in raw meat for retail and (iv) to investigate transmission of MRSA from animals to humans. Furthermore, this program aimed to explore the genetic characterization, typing and resistance patterns of MRSA from animal origin. This study was conducted by a consortium of human and veterinary health research institutes and medical centers in the Netherlands.

Aims and outline of this thesis

This thesis focuses on MRSA in veal calf farming and was partly implemented in the Dutch MRSA research program. The main aim of this thesis is to investigate associations between determinants and ST398 MRSA carriage in both humans and veal calves and their interrelationship. Furthermore the persistence and dynamics of MRSA carriage in both human and veal calves were quantified.

Since the emergence of MRSA in livestock, screening of animals for the detection of MRSA is widely practised. Different procedures are published for animal samples but a systematic comparison of methods has not been performed. **Chapter 2** described an evaluation of three available commonly used procedures and three chromogenic agars for detecting MRSA in nasal swabs from pigs and veal calves.

Chapter 3 presents the results of a cross-sectional study in which risk factors were investigated for both veal calf and human ST398 carriage. This study focussed on the presence of MRSA among veal farmers, their family members and their animals. Specific attention has been given to associations between the presence of MRSA among animals and humans as well as identification of potential determinants of MRSA occurrence such as antibiotic use, hygiene practices at the farm and farm characteristics. In **Chapter 4** the results are presented of a refined and subsector stratified analysis of determinants for developing intervention strategies to control LA-MRSA on veal calf farms and in veal calves. The study focuses specifically on the animal data, including an extended set of potential determinants.

In **Chapter 5** the results of a longitudinal field study investigating occurrence and dynamics of MRSA in veal calves are documented. Determinants associated with MRSA carriage, such as environmental exposure and antimicrobial use, were explored. In addition, the reliability and reproducibility of nasal samples in veal calves to establish MRSA status were investigated, as well as the additional value of rectal samples.

Understanding the dynamics of MRSA carriage is essential in designing specific control strategies. We investigate the persistence and dynamics of MRSA

ST398 carriage in humans after short and long term exposure to MRSA positive animals. The results are described in **Chapter 6 and Chapter 7** respectively.

Finally, the major findings of this thesis are summarized and discussed in **Chapter 8**. Public health aspects of MRSA ST398 and the implications for the search and destroy policy will be discussed in greater detail. Furthermore, recommendations for further research and intervention strategies to control MRSA occurrence in veal calf farming are being made.

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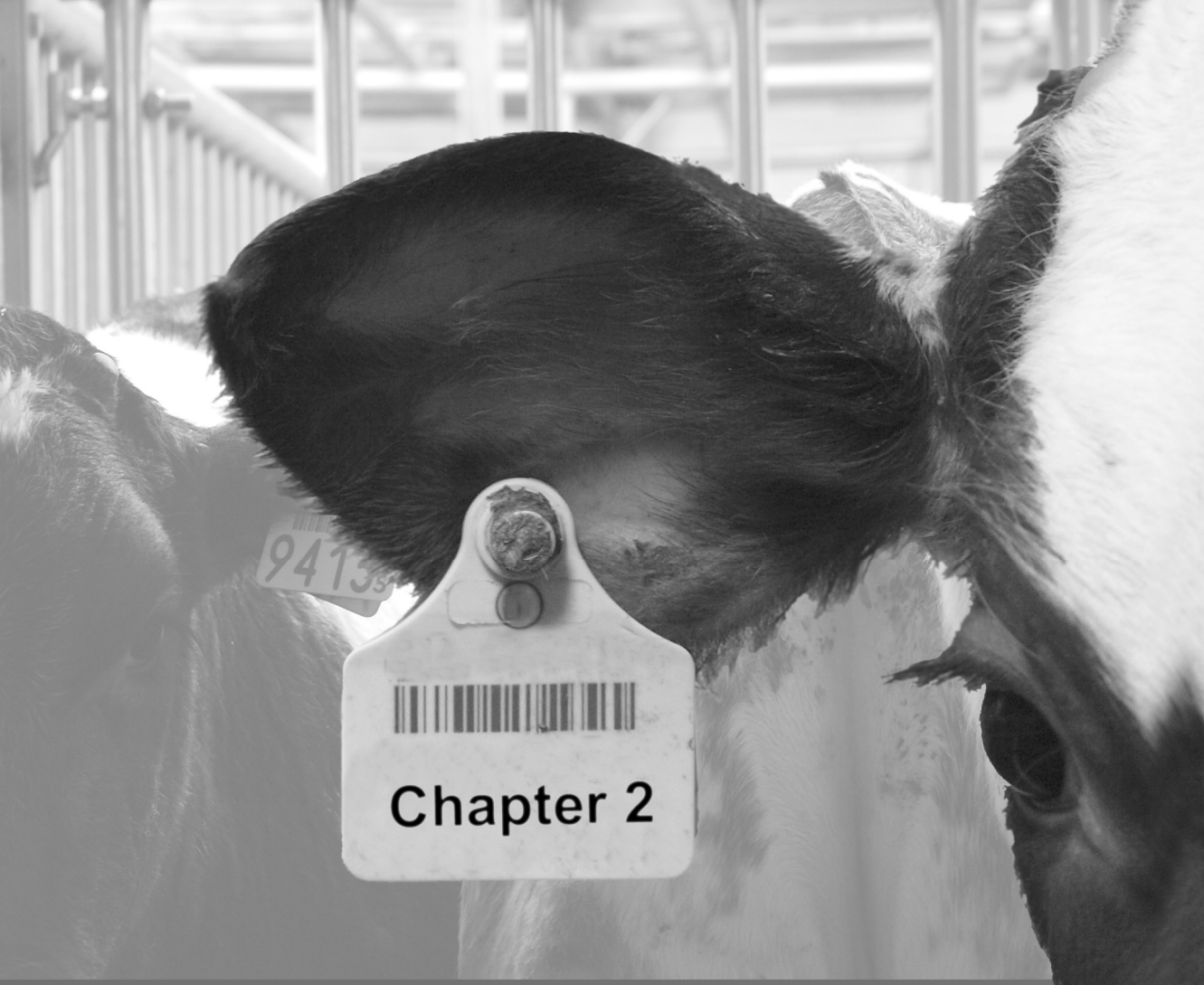

Evaluation of isolation procedures and chromogenic agar media for the detection of MRSA in nasal swabs from pigs and veal calves

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Abstract

Since the emergence of MRSA in livestock, screening of animals for the detection of MRSA is widely practised. Different procedures are published for animal samples but a systematic comparison of methods has not been performed. The objective of this study was to compare three available commonly used procedures and three chromogenic agars for detecting MRSA in nasal swabs from pigs (n=70) and veal calves (n=100). Procedures 1 and 2 used a pre-enrichment comprising Mueller Hinton broth with 6.5% NaCl followed by selective enrichment with 4 µg/ml oxacillin + 75 µg/ml aztreonam (Procedure 1) and 5 µg/ml ceftizoxime + 75 µg/ml aztreonam (Procedure 2) respectively. Procedure 3 used a selective enrichment broth only, containing 4% NaCl, 5 µg/ml ceftizoxime + 50 µg/ml aztreonam. After selective enrichment, media were streaked on to three different chromogenic agars. Significantly more MRSA were found for pig as well as for veal calf samples with procedures 1 and 2. No significant differences were found between procedures 1 and 2. For nasal swabs from pigs significantly more MRSA positive samples were found when MRSA Screen (Oxoid) or MRSA*Select* ™ (Bio-Rad) agars were used compared to MSRA ID (bioMérieux). For calf samples no significant differences between the different agars were found.

In conclusion, the results of this study show that procedures 1 and 2, both using additional high salt pre-enrichment are superior and should be recommended for MRSA detection in nasal swabs from pigs and veal calves. The preferred choice of chromogenic agar depends on the sample matrix.

Introduction

The prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) is increasing world-wide, especially since the emergence of community-acquired and animal related MRSA (8, 10, 12). Recently, a specific MRSA clone has been reported at unexpected high prevalence among pig farmers and veterinarians in different geographical areas (13,17). Strains belonging to this clone are resistant to SmaI macrorestriction and therefore referred to as non-typable (NT-MRSA). They all belong to Multi Locus Sequence Type 398 (ST398) and show closely related *spa*-types (mainly t011, t108 and t1254) (4). A case control study showed that pig and cattle farmers have an increased risk for being positive for ST398 (16). The source of these human infections can be found in the pig population and veal calves.

Screening for MRSA among various human populations with increased risk has become important for control of nosocomial infections. In human health care settings, studies have shown that different procedures employed for the detection of MRSA from clinical specimens have varying results depending on the isolation methods used (1). For animal samples less is known about differences between MRSA detection procedures, in particular on the detection of ST398 in pig and veal calf samples.

Three existing commonly used procedures are applied for MRSA screening in pig samples (4) (procedure 1) and human samples (18) with additional pre-enrichment (procedure 2), (15) (procedure 3). To ascertain the performance of these MRSA detection methods, we conducted a study to compare three different procedures for the isolation of ST398 and the usefulness of three different chromogenic agar media. Nasal swabs of pigs and veal calves were used as matrix.

Materials and Methods

Survey on the farms

Between April and May 2007, nasal swabs (Cultiplast®) were collected in duplicate from 70 pigs at seven different swine farms (10 pigs each farm) and 100 nasal swabs from veal calves were collected at three different veal farms

(approximately 30 calves each barn) in The Netherlands. On each farm the animals were selected and sampled of convenience. From each animal, two nasal swabs were taken each from both nares. Collecting animal samples was in accordance with the animal welfare law.

Bacterial procedures

A total of 70 pig samples and 100 veal calf samples were analysed using 3 different procedures and 3 different agars. In total 630 plates (70 samples x 3 procedures x 3 plating agars) were read for the pig samples and 900 plates (100 samples x 3 procedures x 3 plating agars) were read for the veal calf samples.

Swabs were transported to the laboratory and processed within 4 hours after collection. Because procedures 1 and 2 used the same pre-enrichment step, one of the duplicate nasal swabs of each animal was used for analysis in procedures 1 and 2, and the other nasal swab for analysis by procedure 3 (Figure 1). Assignment of the first and second swab of each animal over the procedures was of convenience.

Procedures 1 and 2: Swabs tested for procedures 1 and 2 were individually inoculated into tubes containing a pre-enrichment with 5 ml Mueller Hinton Broth (MH^+ broth) (Becton Dickenson), containing 6.5% NaCl. This broth was incubated at 37˚C, overnight. Thereafter, the pre-enrichment was split into 2 procedures (procedures 1 and 2).

Procedure 1: 1 ml of the pre-enrichment was transferred into 9 ml phenyl mannitol broth (PHMB/oa^+) (Brunschwig Chemie, Amsterdam) with 4 µg/ml oxacillin (Sigma) and 75 µg/ml aztreonam (ICN). This broth was freshly prepared daily. This broth was incubated overnight at 37˚C and then 10 µl of the PHMB/oa^+ broth was plated onto the agars mentioned below.

Procedure 2: 1 ml of the pre-enrichment was transferred into tubes containing 9 ml phenyl mannitol broth (PHMB/ca^+) (bioMérieux) with 5 µg/ml L ceftizoxime and 75 µg/ml aztreonam. After overnight incubation 10 µl of this PHMB/ca^+ broth was plated onto the agars mentioned below.

Procedure 3: The duplicate swab was inoculated into a tube with 5 ml MRSA broth containing, tryptic soy broth, 4% NaCl, 1% mannitol, phenol red (16 µg/ml),

aztreonam (50 µg/ml) and ceftizoxime (5 µg/ml). After incubation 48 hours at 37˚C, 10 µl of the MRSA broth was plated onto the agars mentioned below.

Chromogenic agars: Three different chromogenic agars were applied: (i) MRSA Screen (Oxoid), (ii) MRSA*Select* ™ (Bio-Rad) and (iii) MRSA ID (bioMérieux). Since Oxoid has optimised the MRSA Screen plate recently, also a selection of the calve samples was streaked out onto the Brilliance™ MRSA agar.

After 24 hours and 48 hours incubation 37˚C plates were read according to the recommendations of the respective manufactures (technical files). Characteristic MRSA colonies are blue on MRSA Screen, large and green on MRSA ID, and small and pink on MRSA*Select* ™.

Suspected colonies were subcultured on blood agar and subsequently identified using standard techniques, colony morphology and slide coagulase test. A selection of the coagulase-positive colonies were tested by PCR for the presence of the *S. aureus* specific DNA fragment (9). All coagulase-positive colonies were tested by PCR for the presence of the *mec*A gene (3).

Additionally, to investigate the effect of selective enrichment after pre-enrichment in MH+ broth, all non-selective pre-enrichment calf samples were also streaked out directly onto plates.

Furthermore, the detection limit of procedures 1 and 2 was determined by spiking MRSA-negative pig and calf samples with MRSA (clinical isolate *spa*-type t011). This was done using serial dilutions from a suspension with a optical density of 0,1 Å with parallel plating onto non-selective agar to determine the CFUs.

Typing

In a study to indentify the optimal procedure it is important to know what MRSA types are analysed. Therefore the isolates were *spa*-typed by sequencing the repetitive region of the protein A gene *spa* (6). Data were analyzed by using the Ridom Staphtype software version 1.4 (www.ridom.de/staphtype).

Statistical analysis

We tested differences for statistical significance by a logistic regression on the outcome of the analyses on procedure and agar using the GENMOD Procedure, of SAS software 9.1. A P-value of <0.05 was considered statistically significant. In all analyses correlations between repeated measurements within one animal were taken into account.

Results

Pigs

Out of 70 samples we detected 46 (66%) MRSA-positive swabs with procedure 1, 46 (66%) with procedure 2, and 32 (46%) with procedure 3. We detected statistically significant less MRSA-positive samples with procedure 3 compared to the procedures 1 and 2 (P=0.0002). Furthermore there was a statistically significant effect of the type of agar used. Statistically significant less MRSA-positive samples (P=0.0016) were found using MRSA ID. No statistically significant differences between procedures 1 and 2, and between MRSA Screen and MRSA*Select* ™ were found. We detected most MRSA positive samples from pigs with procedure 1 combined with the MRSA Screen agar and with procedure 2 and the MRSA*Select* ™ agar (both 46 (66 %)) (Table 1).

Table 1: MRSA-positive samples detected by the different detection procedures in combination with different agar plates in pig nasal swabs.

Pigs (N = 70)			
	MRSA Screen (Oxoid)	MRSA*Select* ™ (Bio-Rad)	MRSA ID* (bioMérieux)
Procedure 1	46 (66%)	40 (57%)	36 (51%)
Procedure 2	44 (63%)	46 (66%)	32 (46%)
Procedure 3*	32 (46%)	27 (39%)	21 (30%)

* P < 0.05

Calves

Out of 100 samples we found 24 (24%) positive samples with procedure 1, 31 (31%) with procedure 2 and 15 (15%) with procedure 3. Statistically significant less positive samples were detected using procedure 3 (P=0.0014). No significant differences between agars were found. Although not statistically significant, we detected most MRSA-positive samples with procedure 2 combined with the MRSA ID agar (Table 2).

Table 2: MRSA-positive samples detected by the different detection procedures in combination with different agar plates in veal calf nasal swabs.

	Calves (N = 100)		
	MRSA Screen (Oxoid)	MRSA*Select*™ (Bio-Rad)	MRSA ID (bioMérieux)
Procedure 1	21 (21%)	22 (22%)	23 (23%)
Procedure 2	29 (29%)	27 (27%)	31 (31%)
Procedure 3*	15 (15%)	14 (14%)	12 (12%)

* $P < 0.05$

Streaking out the pre-enrichment (MH^+ broth) of the calves samples directly onto plates resulted in lower yield compared to both procedures 1 and 2. On average 9% more positive samples were found after an additional selective enrichment. However, a few positive (2%) samples were detected after MH^+ enrichment, which were not detected after selective enrichment (data not shown).

No differences were observed with respect to the MRSA Screen plate and Brilliance™ (both Oxoid) when analysing veal calve samples (data not shown).

Detection limit

The detection limit of procedures 1 and 2 was determined by spiking MRSA-negative pig and calf nasal swabs. Both in pig as well as in calf samples, MRSA was recovered with a detection limit of 1-10 CFU per sample.

Discussion

This study shows that out of the three commonly used MRSA screening procedures, the procedures 1 and 2, both using an additional pre-enrichment containing Mueller Hinton with 6.5% NaCl in combination with a selective enrichment, resulted in statistically significant additional yield of MRSA in pig as well as veal calf nasal swab samples compared to the screening procedure in which the sample is directly inoculated in a selective enrichment broth. In pig samples, a higher rate of positive samples was found using MRSA Screen or MRSA*Select* ™ agar plates compared to MRSA ID agar. No statistically significant differences between plates were obtained for veal calf nasal swabs. A comparison was made between MRSA Screen plate and Brilliance™ (both Oxoid) for veal calve samples only. The results showed that the optimized Brilliance™ plate is comparable to the Screen plate for this matrix.

Spa-typing showed that all isolates were of the previously reported animal-related *spa*-types (*spa*-types mainly t011, t034, t108) belonging to clone ST398 (data not shown). NaCl-containing pre-enrichment media were used because of the inhibitory activity to many non-staphylococcal organisms and the fact that staphylococci can multiply in the presence of salt. For human samples an enhanced sensitivity and an additional yield of MRSA in human clinical specimens was also reported, using salt-containing pre-enrichment before plating (5, 11). The concentrations of salt in the broth varied widely between different studies but recommendations of using a broth with 6.5% or 7.5% NaCl are common (1). However, salt tolerance of MRSA seems to vary between strains. Jones et al., (7) found that salt enrichment broth inhibited the growth of epidemic MRSA-16, when NaCl concentrations higher than 2.5% were used. In our study, a higher yield of MRSA was found when a high salt pre-enrichment was used, compared to the yield after enrichment without NaCl. We did not systematically analyse what step(s) made procedures 1 and 2 superior to procedure 3. As animal samples may contain far more competing flora with another composition compared to human clinical samples, the pre-enrichment with salt containing broth might have played a role in the additional yield of MRSA positive samples in these animal specimens. Procedure 3 contains 4%

NaCl in the selective enrichment. This is far less than the 6.5% NaCl used in the procedure 1 and 2. Van Enk and Thompson (14) have shown that media containing 4.5% NaCl were not considered to be sufficiently selective, since the growth of non-MRSA flora is not adequately reduced. This in contrast with media containing 6.5% NaCl. The addition of a 6,5% NaCl in the selective enrichment step could potentially avoid the use of a non selective pre-enrichment and thereby save time and cost of the isolation protocol. However, combining high salt concentrations and antimicrobials in the same broth could potentially inhibit growth of certain MRSA strains. This should be evaluated in more detail.

The detection limit of the procedures with spiked nasal samples in high-salt pre-enrichment showed a high sensitivity of the procedures confirming the salt-tolerance of clone ST398.

Because of the heterogeneity of MRSA strains in general and its behaviour under particular test conditions, there is no single media that recovers all MRSA strains (1). In pig husbandry one specific clone (ST398) comprising closely related *spa*-types (t011, t108 and t1254) is present (4). This high-salt tolerant clone is also widely spread in veal calf samples (unpublished data). For use in MRSA-screening programs for pigs and veal calves, procedures 1 and 2 are recommended realising that salt-sensitive strains may be missed. It should be noted that selective enrichment increases the sensitivity of the procedure. This was also recently found by Van Loo et al., (16) who found that the use of an enrichment broth prior to plating increased the number of MRSA strains detected by 12% in human clinical samples compared to the absence of selective enrichment. The difference in antimicrobials used in the selective broths potentially influenced the MRSA yield. However, since no differences were found between procedure 1 and 2 this is not likely. A more plausible explanation could be the difference in antimicrobial concentrations used. Procedure 3 used just 50 µg/ml aztreonam compared to 75 µg/ml aztreonam in the other procedures. It is possible that the lower aztreonam concentration is not able to reduce the other competing flora and therefore results in lower MRSA yield. This has to be evaluated in more detail.

With regard to plating, a significant higher yield was found in pig samples when MRSA Screen or MRSA*Select* ™ plates were used after selective enrichment. This is in accordance with the results with human clinical samples as reported by Cherkaoui et al., (2). In our study, the MRSA*Select* ™ plates resulted in more false positive colonies (suspected based upon colony morphology, but *mecA* negative). The light sensitivity of the MRSA ID plates makes them less practical for use.

In conclusion, out of the three commonly used procedures, for MRSA screening of nasal swabs from pigs or veal calves, the procedures 1 and 2, both using pre-enrichment containing Mueller Hinton and 6.5% NaCl prior selective enrichment, should be recommended. No significant differences were found between the procedures using either oxacillin or ceftizoxime in the selective broth. MRSA Screen is the plate of choice in this study taking into account practical reasons and performance.

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Methicillin resistant *Staphylococcus aureus* ST398 in veal calf farming: Human MRSA carriage related with animal antimicrobial usage and farm hygiene

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Abstract

Recently a specific MRSA sequence type, ST398, emerged in food production animals and farmers. Risk factors for carrying MRSA ST398 in both animals and humans have not been fully evaluated. In this cross-sectional study, we investigated factors associated with MRSA colonization in veal calves and humans working and living on these farms. A sample of 102 veal calf farms were randomly selected and visited from March 2007 – February 2008. Participating farmers were asked to fill in a questionnaire (n = 390) to identify potential risk factors. A nasal swab was taken from each participant. Furthermore, nasal swabs were taken from calves (n = 2151). Swabs were analysed for MRSA by selective enrichment and suspected colonies were confirmed as MRSA by using slide coagulase test and PCR for presence of the *mecA* gene. *Spa*-types were identified and a random selection of each *spa*-type was tested with ST398 specific PCR. The Sequence Type of non ST398 strains was determined. Data were analyzed using logistic regression analysis. Human MRSA carriage was strongly associated with intensity of animal contact and with the number of MRSA positive animals on the farm. Calves were more often carrier when treated with antibiotics, while farm hygiene was associated with a lower prevalence of MRSA.

This is the first study showing direct associations between animal and human carriage of ST398. The direct associations between animal and human MRSA carriage and the association between MRSA and antimicrobial use in calves implicate prudent use of antibiotics in farm animals.

Introduction

The continuing emergence of pathogenic organisms resistant to antimicrobials is a global concern. Infections with antibiotic resistant bacteria have been associated with frequent treatment failure and increased severity of disease (2, 8, 24). Both human and non-human antimicrobial usage may result in increased occurrence of bacterial resistance (1). Transfer of antimicrobial resistance (genes) from animals to humans may occur through transmission of zoonotic pathogens or commensals through food or direct contact (2). Methicillin Resistant *Staphylococcus aureus* (MRSA) has traditionally been considered a nosocomial pathogen. More recently MRSA emerged in the community (28) and since 2004 a specific sequence type of MRSA emerged, which was observed in food producing animals and farmers and is referred to as livestock-associated MRSA (LA-MRSA) (4, 11, 13, 22, 25, 26). This MRSA sequence type is characterized by being non-typable by use of Pulsed Field Gel Electrophoresis with SmaI, and was therefore previously called non-typable MRSA (NT-MRSA). All NT-MRSA belong to one clonal complex, in particular multilocus sequence type 398 (ST398) (28). A high prevalence of ST398 was found among farm animals, predominantly pigs and veal calves, and people occupationally in contact with these animals (22, 28). Carriers of MRSA may develop an MRSA (wound) infection after disruption of the skin (scarification) or after surgery and are therefore at risk. Additionally, the impact of this MRSA sequence type on farming communities has been considerable because carriers have been treated in isolation in the health care system as part of the infection control measures: the search and destroy policy, common in low prevalence countries (31). Many case studies show parallel occurrence of MRSA ST398 in both animals and humans (11, 13, 15, 21, 27). Risk factors for carrying MRSA ST398 in both animals and humans have not been fully evaluated. Evidence for animal to human transmission of MRSA is often indirect and based on parallel observations in genetic (17, 23) or resistance patterns (7) among isolates or else indications obtained by *in vitro* experiments (3).

This study focused on the presence of MRSA among veal farmers, their family members and their animals. Specific attention has been given to associations

between the presence of MRSA among animals and humans as well as identification of potential determinants of MRSA occurrence such as antibiotic use, hygiene practices at the farm and farm characteristics.

Materials and Methods

Study design

We conducted a cross-sectional study on 102 randomly selected veal calf farms. All farms have been visited from October 2007 to March 2008. Nasal swabs (from both anterior nares) were taken from veal calf growers, family members and employees. All participants were asked to fill in a questionnaire containing items about activities on the farm, intensity and duration of animal contact and MRSA history. Questions on potential confounders such as age, sex and smoking were included. In addition questions on farm structure, antibiotic intake (group- and individual treatments, timing and duration of treatment and kind of antibiotics administered) of the calves and farm hygiene were incorporated. The study protocol was approved by the Medical Ethical Committee of Utrecht University. All participants completed an informed consent.

On each farm the square root of the number of veal calves (a minimum of 10 and a maximum of 25 calves for each farm) was randomly selected and sampled using a sterile cotton-wool swab (Cultiplast®). From each calf, one nasal swab was taken from both nares by rubbing the swab in each nostril. Collecting animal samples was in accordance with the Dutch Law on Animal Health and Welfare. The swabs were immediately transported to the laboratory and processed within four hours after collection.

Laboratory analysis

The nasal swabs were analysed individually using a pre-enrichment containing Mueller Hinton broth with 6.5% NaCl (10). After overnight incubation at 37°C, 1 mL of the pre-enrichment was transferred into 9 mL selective enrichment of phenyl red mannitol broth (bioMérieux, France) with 75 mg/L aztreonam and 5 mg/L ceftizoxime followed. Ten µL of the selective enrichment broth was inoculated onto sheep blood agar (Biotrading, The Netherlands) and a MRSA

Brilliance™ agar (Oxoid, The Netherlands). All suspected colonies were identified as *S. aureus* using standard techniques: colony morphology and slide coagulase assay with rabbit serum (10). In addition, the presence of the *mecA* gene was confirmed by PCR as described previously (9). A random selection of the *mecA*-positive colonies (n = 208) was confirmed to be MRSA by PCR of the *S. aureus* specific DNA-fragment Martineau (19).

For all MRSA human isolates (n = 62), and a random selection of MRSA strains isolated from veal calves with a maximum of three per farm (n = 207), *spa*-types were determined. The strains were *spa*-typed by sequencing of the repetitive region of the protein A gene *spa* (12). Data were analyzed by using the Ridom Staphtype software version 1.4 (www.ridom.de/staphtype). A random selection of three strains per *spa*-type was tested with ST398 specific PCR (30). Non-ST398 strains were analysed by multilocus sequence typing (MLST) (6) (www.saureus.mlst.net).

Data analysis

Statistical analysis was performed using SAS software 9.1. Descriptive analyses were undertaken followed by logistic regression analysis (GLIMMIX procedure) to identify determinants of MRSA carriage on individual level (human/calf). Risk factors for MRSA carriage were first identified by univariate logistic regression analysis. Thereafter, multivariate analysis was done by stepwise, forward entry including factors associated with MRSA positivity in humans and calves ($P<0.2$). Hierarchical structure of the data was taken into account to adjust for the fact that observations of human and calves on the same farm may not be independent. Relationships between MRSA carriage in humans and veal calves were further studied by assessing the shape of these relationships by means of nonparametric regression modelling (smoothing) using generalized additive models (PROC GAM). A P-value <0.05 was considered statistically significant.

Results

Humans

Nasal swabs of 390 individuals, working or living on 102 veal calf farms were tested for the presence of MRSA. The response rate was 81%: 97 farmers, 259 family members and 34 employees were included. Reasons for non-participation were no interest, lack of time, or retirement from farming. MRSA prevalence in farmers was 33% and 8% in family members (Table 1). This large difference in MRSA prevalence could be explained by the difference in time spent in the stables, even after adjustment for smoking, age and gender (Table 1).

The risk for being MRSA carrier increased with increasing number of working hours spent per week in stables (Odds Ratio (OR) = 1.4, expressed per 10 hours/week). The shape of this relationship was investigated, showing a strong increase in prevalence with increasing time spent with animals (Figure 1A). In addition, those who spent more time on feeding calves (OR = 1.5), veterinary care (OR = 1.6) and stable management (OR = 2.0) were more often MRSA carrier (Table 2).

MRSA carriage in humans was associated with the prevalence of MRSA in calves. Farmers were more often MRSA carrier when they had more MRSA positive calves. The estimated prevalence in humans was approximately 1% when less than 20% of the calves were carrier. On farms with a higher carrier prevalence in calves MRSA prevalence in humans was above 10%. (Figure 1B). We adjusted for potential confounding variables, like age, gender and smoking habits; age was positively associated (OR = 1.2 per 10 years) and males were significantly more often MRSA colonized compared to females (OR = 3.0). Smoking was negatively associated (OR = 0.5), however not statistically significant. The associations between human MRSA carriage and any of the described determinants (task, duration animal contact, prevalence among animals) did not change when potential confounders were included in the model.

Calves

Nasal swabs were taken from 2151 veal calves of 102 farms (Table 3) and MRSA prevalence was 28%. On 88% of the farms MRSA could be detected in

Table 1: Characteristics of 390 farmers, family members, employees and determinants for MRSA carriage in human (multiple logistic regression analysis).

	No. (Prevalence %)	
Total	390 (16)	
General charcteristics		
Farmers	97 (33)	
Family members	259 (8)	
Employees	34 (26)	
Multiple regression model		**OR (95% Confidence interval)**
Gender		
Female	177 (7)	1
Male	213 (24)	3.0 (1.4 -6.8)*
Age (yr)		
0-18	130 (6)	
19-65	248 (21)	
66-85	12 (25)	
Per 10 years		1.3 (1.1 -1.5)*
Smoking habits		
No smoking	334 (17)	1
Smoking	55 (11)	0.5 (0.2 -1.4)
Level of animal contact / # working hours in veal calf stable/week		
< 20 hours a week	250 (7)	
20-40 hours a week	56 (23)	
> 40 hours a week	72 (42)	
Per 10 hours/week		1.4 (1.2 –1.7)*
Percentage of positive calves on farm		
< 28% (below mean)	232 (12)	
> 28% (above mean)	158 (22)	
Difference between 0-28%		2.1 (1.4 -3.0)*

* P <0.05

one or more calves. MRSA carriers were more often seen in calves treated with antibiotics (group treatment) (OR = 1.8), compared to calves not treated. Since veal calves were frequently treated with different kinds of antibiotics, even during one treatment, it was not possible to unravel the effect of individual antibiotics or antibiotic classes. Besides this, older calves were more often MRSA positive than calves of younger age (OR = 1.3 (per 10 weeks). The shape of the relationship with age is shown in Figure 2. MRSA carriage with age is higher

(P<0.05) in calves treated with antibiotics compared to untreated calves. Calves from large farms (farms with many animals) were significantly more often colonized compared to calves from smaller farms (OR = 2.7 per 500 calves/farm. Moreover, a negative association was found between MRSA carriage and farm hygiene (OR = 0.3). i.e. cleaning of stables before entrance of new calf populations to the farm. Disinfection was applied in less than 20% of the farms and was not associated with MRSA carriage in calves. No associations were found with other possible determinates like country of origin of calves, drinking- or feeding systems and numbers of animals used.

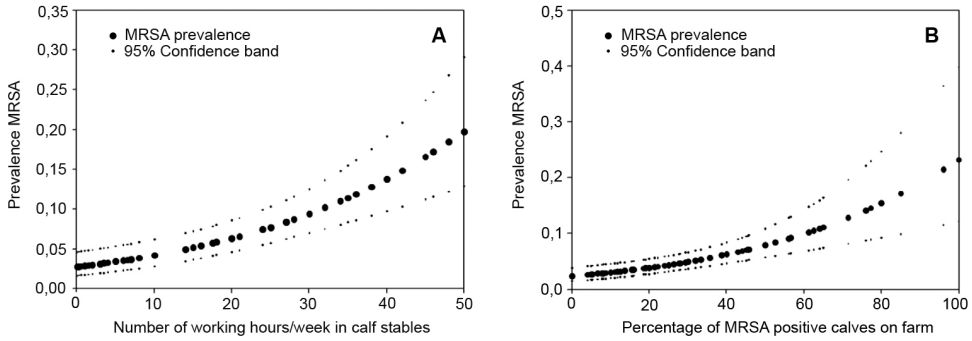

Figure 1: Human MRSA prevalence, working hours in calf stables per week and MRSA positive calves on the farm. Figure 1A shows the relationship between MRSA carrier prevalence in humans and the number of working hours / week in the calf stable, adjusted for gender, age, smoking habits and percentage of positive calves on the farm. Figure 1B shows the relationship between MRSA carrier prevalence in humans and the percentage of positive calves on the farm, adjusted for gender, age, smoking habits and number of hours working/week in calf stables. In both figures smoothed plots are given with 95% confidence bands.

Table 2: Associations between human MRSA carriage and specific tasks on the farm

Task	MRSA carriage OR (95% Confidence interval) [1▲]
Feeding calves	1.5 (1.2 -1.8)*
Veterinary care	1.6 (1.0 -2.5)**
Stable management; sorting calves	2.0 (1.3 – 3.4)*

*P <0.05, ** P 0.10-0.05. [1]OR expressed per hour/week difference, [▲]adjusted for gender, age, smoking and percentage of positive calves on farm

Table 3: Characteristics of 2151 veal calves from 102 veal farms and associations between MRSA carriage and some determinants (multivariate analysis)

	No. (Prevalence %)	
Total		
General charcteristics		
# Farms	102 (88)	
# Veal calves	2151 (28) (range 0-100%)	
Multiple regression model▲		**OR (95% Confidence interval)**
Antibiotic (group) treatment		
No	570 (21)	1
Yes	1581 (30)	1.8 (1.1 -3.0)*
Age calf (wk)		
0-6	301 (12)	
7-12	458 (36)	
> 12	1392 (28)	
Per 10 weeks		1.3 (1.1 -1.5)*
Number of calves on the farm		
<500	985 (22)	
>500	1166 (32)	
Per 100 calves		1.1 (1.0 -1.2)*
Farm hygiene		
No	375 (35)	1
yes	1776 (26)	0.3 (0.1 -0.7)*

* P <0.05, ▲adjusted for calf category (white versus rose veal), number of stables on farm, number of calves per pen, presence of other animals on the farm and rodent control

Genotyping isolates

In total 16 different *spa*-types were identified; 9 different *spa*-types in human isolates and 12 different types in veal calves (Table 4). In humans, all identified *spa*-types belonged to ST398, except four. These four non ST398 strains were identified as t002, t015, t084 and t166), respectively belonging to ST5, ST45, ST15 and CC34.

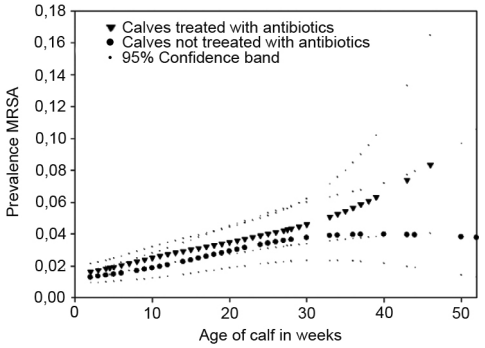

Figure 2: MRSA carriage in veal calves, age and antibiotic treatment. The relationship between MRSA carriage in veal calves and age for calves treated and not treated with antibiotics as group treatment (adjusted for age, number of calves per farm, farm hygiene, calf category (white versus rose veal), number of stables on farm, number of calves per pen, presence of other animals on the farm and rodent control). Smoothed plots are presented with 95% confidence bands.

In calves, the predominant *spa*-type found was t011 (80%); also *spa*-type t034 was frequently found (8%). Among calves, also four non ST398 strains were found, t421, t1236, t1685, t3856, respectively, ST239, ST97, ST1159 and CC425. All *spa*-types found in humans matched with the types found in calves on the same farm except for the non ST398.

Discussion

This study shows that people in close contact with veal calves have a highly elevated risk of MRSA carriage. Human carriers came more often from farms where veal calves appeared carriers as well. Overall the prevalence of MRSA was 15.9% in persons living and working on a veal calf farm which is a strong elevation compared to the general population. The prevalence of MRSA in the Dutch community is estimated to be below 1%. Moreover, strong associations with duration of animal contact and specific tasks on the farm were found. Furthermore, veal calves carrying MRSA were more often treated with antibiotics. This is the strongest evidence of a direct relationship between antibiotic use in animals and transfer of antimicrobial resistant organisms to humans at present. In addition, this evidence is strengthened by the similarity of

Table 4: *Spa*-typing and MLST typing results (non ST398 sequence types in bold)

Spa-type	No.	MLST
Humans isolates (n=62)		
t002	1	ST5
t011	51	ST398
t015	1	ST45
t084	1	ST15
t108	2	ST398
t166	1	CC34
t899	3	ST398
t1457	1	ST398
t2383	1	ST398
Calves isolates (n=207)		
t011	166	ST398
t034	17	ST398
t108	7	ST398
t421	2	**ST239**
t899	5	ST398
t1197	1	ST398
t1236	2	**ST97**
t1451	1	ST398
t1457	1	ST398
t1580	1	ST398
t1685	1	**ST1159**
t2383	2	ST398
t3856	1	**CC425**

spa-types and antimicrobial susceptibility patterns (data not shown) of the isolates found in both humans and animals.

The presence of LA-MRSA in farmers forms a potential threat for public health, for individuals carrying MRSA and for the health care system. The carriers are at risk for (wound) infections with LA-MRSA. For the health care system, the frequent occurrence of ST398 especially in countries with low and moderate MRSA prevalence put the infection control policy under pressure. In addition, a possible change in virulence (*e.g.* introduction of toxin genes) will change the

public health perspective considerably and further challenges the search and destroy strategy. The unexpected and sudden increase of LA-MRSA incidence in hospitals which occurred since 2007, resulted in a shortage of isolation facilities. An additional problem is the treatment of carriers, which is also part of this strategy (29). Decolonization of patients who are constantly exposed to MRSA in their work and home settings is not effective: at present the 'destroy' element is impossible to execute.

The observation that MRSA carriage in humans is associated with MRSA prevalence among their calves is remarkable. This might indicate that the prevalence in humans in close contact with animals follows the prevalence of MRSA among animals over time. Evidence for an association between the use of antimicrobials in animals and increase in occurrence of resistant bacteria in humans is often not as direct as observed in this study. Parallel occurrence of resistant bacteria in humans and animals is observed in ecological studies. The risk of transfer of resistance from animals to humans is seldom observed directly. For instance, vancomycin-resistant *enterococci* (VRE) acquired their resistance gene (*vanA*) after glycopeptide use as growth promoter in animals and later emerged in hospitals. However, detailed genetic analysis demonstrated that the isolates in animals and humans were not genetically related, and a direct association has never been demonstrated (33). Similarly, use of fluoroquinolones in poultry co-incided with an increased occurrence of food borne infections in humans with fluoroquinolone resistant *Campylobacter jejuni* (20).

Our results suggest that occurrence of ST398 in humans is transient and varies over time within individuals, because of the correlation with the prevalence in animals. The number of positive calves on the farm is a characteristic that changes over time as shown in this study. Veal calves live between 7–10 months before being slaughtered and new animals are brought in from many different dairy farms, from across the European Union. The prevalence in a new herd of animals is likely to differ from herd to herd. Also the results from fieldworkers who took nasal swabs directly after the farm visits and two days thereafter suggest transient carriage. They were rarely tested MRSA positive

during several hundred days of fieldwork but few appeared colonized and when this occurred the duration of colonization was never more than one day (data not shown).

Few studies investigated risk factors for the occurrence of ST398 in humans. High animal to human transmission of ST398 has been reported in pig farming (5, 14, 16, 18, 22). In these studies also a large difference in MRSA prevalence is observed between the farmer and family members, which is indicative of a comparable role for intensive animal contact as risk factor for ST398 occurrence (14, 18).

A major finding is that the prevalence of ST398 in veal calves appeared associated with antimicrobial (group) treatments. Also hygiene and some other farm characteristics, such as herd size, were associated with ST398 prevalence. These associations may give further direction to preventive strategies and indicate that is a potential to influence the occurrence of ST398 MRSA in humans and animals.

The large difference in MRSA prevalence between farmers (33%) and family members (8%) may indicate infrequent human to human transmission of ST398. This was also suggested in studies where comparisons were made of ST398 and non-ST398 MRSA regarding the occurrence of secondary cases in hospitals (29, 32). However, in our study, we did not investigate contact patterns between humans as an explanatory variable. Our observation of a likely low human to human transmission needs further exploration.

MRSA was more frequently observed on veal calf farms as on pig farms (22). However a larger variety in *spa*-types appears present in veal farming, not only in veal calves but also in human isolates. The large number of dairy farms supplying young calves for veal production and their distribution across Europe, and thereby the large variety in origin of veal calves compared to pigs, may play a role in this finding. A limitation of our study is the cross-sectional design in which both cause and effect are measured at the same time. For that reason, results from this study need to be confirmed in a longitudinal study in which occurrence of MRSA carriage over time is associated with the use of antibiotics and other determinants. A longitudinal design may also provide valuable

information about dynamics and persistence of MRSA carriage in both animals and humans. Nevertheless, results of this study indicate that prudent use of antibiotics is warranted. This is recognized by all parties responsible for use of antimicrobials in veterinary practice; the Dutch calf producers and the Dutch Government therefore recently agreed on a covenant to reduce antibiotic resistance.

In conclusion, this is the first study showing direct associations between animal and human carriage of ST398. MRSA carriage in calves was positively associated with use of antibiotics while farm hygiene was associated with a lower prevalence of MRSA. For optimal design and implementation of infection control strategies in both animals and humans, detailed combined human-animal studies exploring direct associations are essential. These not only refers to MRSA, but to all (resistant) pathogens in general, where direct associations between animals and humans may be expected.

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Livestock-associated MRSA prevalence in veal calf production is associated with farm hygiene, use of antimicrobials, and age of the calves

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Abstract

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is highly prevalent in pork and veal production chains. In this study, we used data from a cross-sectional study on 2151 calves from 102 veal calf farms, to identify determinants to enable the development of intervention strategies, and reduce MRSA prevalence. Overall, calves from rose veal farms had a lower risk of LA-MRSA carriage than calves from white veal farms. Data were analysed separately for white and rose veal calves, because management systems differed between the two production chains. Rodent control and age of the calves were risk factors for LA-MRSA carriage, while cleaning of the stables was negatively associated. Group treatments with antimicrobials appeared to be a risk factor for MRSA carriage mainly in white veal calves, although the relation was no longer statistically significant after adjustment for age in a multiple regression model. However, the strong correlation between antimicrobial use and age makes interpretation of these results difficult. In rose veal calves the number of start treatment days was positively associated with LA-MRSA carriage. In conclusion, antimicrobial use was associated with LA-MRSA carriage in calves, but other age related factors may also be important. The findings further emphasize the need for prudent use of antimicrobials, and point to improvement of farm hygiene as an important control measure.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a potential threat for public health, but until recently MRSA was mainly confined to hospital settings, the so-called hospital-associated MRSA (HA-MRSA). Since 2004, another type of MRSA is increasingly found in humans: livestock-associated MRSA (LA-MRSA), which belongs predominantly to multilocus sequence type ST398 (24). This type has been shown to be highly prevalent in meat production animals worldwide, such as pigs, veal calves, and recently in broilers (4, 6, 16, 18). LA-MRSA has emerged in humans along with the increase in livestock (2, 15, 19).

The majority of LA-MRSA studies published so far, focused on prevalence and characteristics of the specific lineage involved (4, 9, 22, 24). Risk factors for colonization of humans with LA-MRSA have been determined to be cattle farming, the presence of both sows and finishing pigs on the farm, and regular contact with pigs or veal calves (5, 8, 12, 21, 27). Furthermore, recent studies have shown that MRSA is also prevalent in meat from various sources; in a Dutch study the highest prevalence was found in veal, chicken, and turkey meat (3, 17, 13). However, this is supposed to play a minor role in the transmission of LA-MRSA to humans if meat is cooked properly and hygienic measures are taken (10). Until now, LA-MRSA has not been shown to spread efficiently between humans (22 - 25).

Because of the potential public health impact, it is necessary to reduce LA-MRSA prevalence in the primary meat production chain. Wenzel et al., (26) noted in their review on MRSA that decision-making so far is based on uncertainty and limited data. Control programmes should be based on knowledge of risk factors and determinants, leading to evidence based intervention strategies. It is known that LA-MRSA colonization in pigs is associated with the use of antimicrobial drugs as group medication on a farm and the number of pigs present on the farm (1, 6, 22), but determinants should be identified and specified for the development of intervention strategies. Until now, most studies involved pig farming, as ST398 is widely spread in pigs

worldwide. However, recently it was shown that carrier prevalence in veal calves is also high, and associated to human LA-MRSA carriage. Antimicrobial use in calves and farm hygiene were identified as determinants of carriage in calves (6).

This paper describes a refined and subsector stratified analysis of determinants for developing intervention strategies to control LA-MRSA on veal calf farms and in veal calves. The study focuses specifically on the animal data, including more potential determinants. A previously conducted large cross-sectional study among 2,151 calves on 102 veal calf farms provided a unique dataset, containing data on farm and animal characteristics, hygiene protocols, antimicrobial use, and LA-MRSA status of the animals.

Materials and methods

Background information on veal calf production chain

The veal production chain can be roughly divided into two subcategories with distinctly different management practices: white and rose veal. This distinction is based on the diet of the calves: white veal calves are fed calf milk replacer and limited roughage; whereas rose veal calves are fed calf milk replacer during the first weeks, and after that they are fed roughage and concentrates. This leads to a difference in meat colour: white veal generally has a pale pink colour, and rose veal generally is red pink coloured. White veal calves are also generally slaughtered at younger age, around 6-8 months, and rose veal calves around 8-12 months. In the Netherlands, calves from various European countries arrive on the fattening farm when they are around two weeks of age. The calves are individually housed until they are 6-8 weeks old. Hereafter, they are sorted based on their size and/or feed uptake, and generally housed in groups of 4-10 animals per pen, although much larger groups are not uncommon. Some farms have calves in different age groups, but this is more common in rose veal farming; in white veal farming an all in – all out system is more commonly used. Among rose veal farms starters (calves stay on the farm until they are 12 weeks

of age), fatteners (calves arrive on the farm at 12 weeks of age), or a combination of the two can be distinguished.
When necessary, calves will be relocated to other pens during their fattening period, based on their size and/or feed uptake. This is done to make the groups of calves as homogenous as possible, which ensures the calves to be fed as balanced as possible. Farms can have more than one stable, and a stable can consist of multiple separate ("closed") areas, i.e. compartments. Within one compartment housing conditions and management are the same for all calves. Each compartment is divided into pens, which are lined up in rows. Relocation of the calves can be within a row, within a compartment, within stables, but also between stables.

In general, the first weeks after arrival on the farm, calves are fed from buckets. When the calves are placed into groups, they are generally fed from a gully (on the floor), or from a trough. Sometimes a different feeding system is used, such as a teat. Calves can get drinking water from a water bowl, the feed trough, a nipple, or none.

In this study, a group treatment is defined as a treatment with antimicrobials of all calves in a stable, not being a start treatment. A start treatment is defined as a (preventive) group treatment with antimicrobials, which the calves usually receive one day after arrival on the farm for 5-10 days. In general, during the following weeks group treatments can be given against respiratory problems or scour. After 12 weeks of age, little antimicrobial group treatments are usually given; if the calves need to be treated they usually will be treated individually. It should be noted that calves on rose fattener farms may have received start and / or group treatments on the starter farms they originated from, but this information is not available. Because no information is available on the use of antimicrobials prior to arrival on the fattener farm, misclassification might affect associations, and therefore these fattener farms are excluded in a more detailed analysis.

Data collection

Data were collected in a cross-sectional study among 2,151 veal calves (6). From May 2007 until March 2008, 102 randomly selected Dutch veal calf farms were visited. A sample of the animals per farm (in general: square root of the number of calves on the farm, with a minimum of 10 and a maximum of 43 animals) were tested for MRSA carriage by means of a nasal swab and a previously described method (7). Farmers filled out a questionnaire together with a researcher on farm characteristics, hygiene protocols, housing and calf characteristics, and animal antimicrobial use. A Dutch study showed an increase over the course of the study in MRSA prevalence among pig farms (1), and in US beef calf farming, the month of arrival on the farm influenced the health of the calves (14). Therefore, sampling month and data on weather conditions (sun hours per day, rain in mm per day, and the mean temperature) on the day of sampling were included as explanatory mechanisms behind possible influence of the sampling time on LA-MRSA carriage in the calves. Weather data were collected from the website of the Royal Netherlands Meteorological Institute (11).

Statistical analysis

A generalized linear mixed model was used to analyse the MRSA status of each calf, including compartment and farm as random effects to adjust for clustering. Analyses were performed using the GLIMMIX procedure in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA), assuming a binary distribution and logit link. The denominator degrees of freedom for the tests of fixed effects were estimated using the Satterthwaite method. Analyses were adjusted for clustering on compartment and on farm level, by treating these as random effects. Categorical variables with two categories were only included if each category occurred at least among 10% of the animals in the dataset. Age was categorized, based on general housing system (individual housing until they are around 6 weeks of age) and antimicrobial use (generally little after 12 weeks of age). Because of the management differences between rose and white veal farming, we analysed the data for these two farming systems separately.

To avoid colinearity problems, we calculated Pearson's correlation coefficient for all variables that were selected for inclusion in the multiple regression model (i.e. P-value <0.25 in the univariate analyses). For (pairs of) variables with a correlation coefficient >0.40, only one variable was included in the multiple regression model, and the choice was based on the results of the univariate analyses and the biological plausibility. Multiple regression analysis was performed using stepwise-backward regression. First, we included all variables in the full model that had a P-value <0.25 in the univariate analyses, and were not excluded because of correlation. After running the full model, the variable with the highest P-value was removed. This was repeated until all variables included in the final model had a P-value <0.05. Outcomes with a P-value <0.05 were considered to be statistically significant. Results are given in odds ratios with 95% confidence intervals, as determined by SAS.

Results

Descriptive characteristics of the farms and calves

Overall MRSA prevalence in the calves was 28%. MRSA was detected in 31% of the calves from white veal farms, 21% of the calves from rose veal farms (18% if fatteners were excluded) and in 32% of the calves from mixed farms. On 78% of the farms, one or more of the sampled calves was MRSA positive (in contrast to the 88% prevalence previously reported, which was based on both environmental dust samples and individual calf samples (6); prevalence for white veal farms was 82%, for rose veal farms 75% (73% if fatteners were excluded), and for mixed farms 75%.

Table 1 shows the descriptive characteristics for the farms and calves included in this study. The median number of calves per farm was 441, ranging from 25 to 2,200, housed in on average 2 stables, ranging from 1 to 6. Of the 102 farms, 50 farms had white veal calves, with a median of 625 calves. Further, 44 farms had rose veal calves, with a median of 310 calves. The eight mixed farms had both types of veal calves. The age of the calves on the day of visit ranged from 2 – 29 weeks (median: 15) for white veal farms, and from 2 – 104 weeks (median: 19) for rose veal farms. In the latter case, four rose veal calves from one farm were

an exception with an age of 104 weeks. Without these four calves the maximum age of rose veal calves on the day of visit was 52 weeks.

On white veal farms, 11% of the calves only had received a start treatment, 3% only had received a group treatment at some later stage, and 86% of the calves had received both. This implies that all calves from white veal farms had received antimicrobials at least once during their life. The number of additional group treatments for these calves ranged from 0 – 7, with a median of 2. From the calves from rose veal starter and combination farms 26% had only received a start treatment, 3% only a later group treatment, 57% both, and 14% had received no group nor start treatment. The number of additional group treatments ranged from 0 – 6, with median of 1.

Univariate analyses

Antimicrobial use

We specifically considered whether calves had received a start treatment, the number of antimicrobial drugs used in the start treatment, the duration of the start treatment in days, whether calves had received a group treatment, the number of group treatments received by the calf, and the total duration of these group treatments in days. In an analysis comprising both rose and white veal calves, group treatments were found to be significantly associated with MRSA carriage in the 2,151 calves included in this study (univariate analyses OR=2.0; P=0.02), similar to an earlier report (6). In an age stratified analysis we observed that group treated calves between 7 and 12 weeks of age showed a 4 times higher odds of MRSA carriage (P=0.08) than calves of the same age group that had not been group treated. However, this relation was much weaker in calves older than 12 weeks (OR=1.6; P=0.19), and was even inversed for calves under 6 weeks of age (OR=0.5; P=0.17). When data from rose veal fattener farms were excluded from the analysis, the group treated calves had even higher odds of being MRSA carrier, with OR: 2.6 (95% CI: 1.3 – 5.0; P=0.005).

Table 1: Descriptive characteristics of veal calves from randomly selected Dutch veal farms in a cross-sectional study

Variable	White veal	Rose veal (without fatteners)	Rose veal	All
# Farms	50	26	44	102
# Calves	1182	475	764	2151
MRSA prevalence – farm level	82%	73%	75%	78%
MRSA prevalence – animal level	31%	18%	21%	28%
Number of calves per farm (range (median))	90 – 2200 (625)	25 – 1700 (280)	25 – 1700 (310)	25 – 2200 (441)
Age of sampled calves (range weeks (median))	2 – 29 (15)	2- 104 (13)*	2 – 104 (19)*	2 – 104 (16)
Only start treatment given (calves)	125 (11%)	122 (26%)		
Only group treatment given (calves)	35 (3%)	15 (3%)		
Both start and group treatment given (calves)	1022 (86%)	273 (57%)		
No start or group treatment given (calves)	0	65 (14%)		

* Four calves from one farm were 104 weeks old, which is an exception. Without these calves, the maximum age of rose veal calves was 52 weeks.

Because of the management differences between rose and white veal farming, we analysed the data for these two farming systems separately. Tables 2 and 3 show results from univariate analyses for white and rose veal farms, respectively, with P-value <0.25. Other variables were univariately analysed, but did not result in an estimate with P-value <0.25. These were sampling month, sunshine intensity and mean temperature at the day of sampling. We also analysed possible associations between MRSA status of the calf and the number of calves on the farm, whether the farm had multiple locations, the presence of an all-in-all-out system, or a disinfection container at the entrance, and the number of times calves were relocated between pens. The presence of other farm animals was analysed, also distinguishing between pigs, cattle, and horses. Calf characteristics included the number of calves per pen and the drinking system.

White veal farms

Calves under 6 weeks of age were 2.5 times less likely to be MRSA carrier then calves over 12 weeks of age, with OR=0.4 (95% CI: 0.2 – 0.9; P<0.05). Calves between 7 – 12 weeks of age showed a trend of having twice the odds for MRSA carriage, OR=2.2 (95% CI: 0.9 – 5.1; P<0.1).

Performing rodent control (either by the farmer or a professional) was determined to be a significant risk factor for MRSA carriage in calves, resulting in a ten-fold increase in the risk of MRSA carriage (OR=9.7 (95% CI: 1.6 – 59), P<0.05). Feeding from a bucket resulted as a protective factor when compared to feeding from a trough (OR=0.3 (95% CI: 0.1 – 0.9), P<0.05). A positive trend was found for the amount of rain on the sampling day (OR=1.1 (95% CI: 1.0 – 1.2), P<0.1). Another risk factor was group treatment with antimicrobials (OR=2.8 (95% CI: 1.0 – 7.6), P<0.05). The association between group treatment and MRSA carriage appeared to be stronger in calves aged 7 – 12 weeks when compared to calves aged 0 – 6 weeks, with OR's of 9.3 (P=0.2) and 0.6 (P=0.4), respectively. In calves over 12 weeks of age only 7 calves had not received a group treatment and no statistical analysis was possible.

Table 2: Results of univariate analyses for white veal calves; variables on animal and farm level MRSA status of the sampled calf is used as dependent variable; 1,182 calves from 50 Dutch white veal calf farms. Only the variables with P<0.25 are presented, with the results given as the OR with 95% confidence intervals.

Variable	Category	# rec[a]	# farms	OR[b]	95% CI[c]
# Mm rain on sampling day ***	Per Mm	1182	50	1.11	(1.00 – 1.24)
Rodent control*	Yes*	1020	44	9.70	(1.59 – 59.1)
	No or natural enemies (ref[d])	162	6	1.00	
Presence of companion animals	Yes	987	42	2.73	(0.56 – 13.2)
	No (ref)	195	8	1.00	
Gender	Female	151		0.46	(0.15 – 1.35)
	Male (ref)	1031		1.00	
Age calf category**	1-6 weeks*	206		0.40	(0.18 – 0.91)
	7-12 weeks[t]	236		2.15	(0.90 – 5.13)
	> 12 weeks (ref)	740		1.00	
Feeding system	Bucket*	163		0.31	(0.10 – 0.92)
	Gully	54		1.46	(0.14 – 15.4)
	Other	46		3.73	(0.23 – 61.5)
	Trough (ref)	919		1.00	
Group treatment*	Yes *	1057		2.79	(1.03 – 7.56)
	No (ref)	125[e]		1.00	

[a] rec: calves in dataset, [b] OR: odds ratio, [c] CI: confidence interval, [d] ref: reference category, * P<0.05; ** P<0.01; *** P<0.1, [e] The calves that had not received a group treatment, had received a start treatment with antimicrobials. This means that all calves had received at least one treatment with antimicrobials in life.

Rose veal farms (starter and combination)

Cleaning showed to have a strong negative association with MRSA carriage in calves from rose veal farms, resulting in a 5-fold reduction (OR=0.2 (95% CI: 0.1 – 0.7), P<0.05). Again, calves younger than 6 weeks showed an indication for having less risk of MRSA carriage when compared to calves older than 12 weeks, resulting in an OR of 0.4 (95% CI: 0.1 – 1.3, P=0.12). The number of start treatment days had a positive association with MRSA carriage, with an OR of 1.3 (95% CI: 1.1 – 1.4, P<0.01).

Table 3: Results of univariate analyses for rose veal calves; variables on animal and farm level MRSA status of the sampled calf is used as dependent variable; 475 calves from 26 Dutch rose veal calf (starter and combination) farms. Only the variables with P<0.25 are presented, with the results given as the OR with 95% confidence intervals.

Variable	Category	# rec[a]	# farms	OR[b]	95% CI[c]
# Hours sun	Per hour	475	26	1.14	(0.91 – 1.41)
All in – all out	Yes	63	3	3.9	(0.69 – 22.3)
	No (ref[d])	412	23	1.00	
Cleaning*	Yes*	357	20	0.20	(0.06 – 0.69)
	No (ref)	118	6	1.00	
Age calf category	1-6 weeks	68		0.37	(0.11 – 1.28)
	7-12 weeks	166		0.58	(0.21 – 1.58)
	> 12 weeks (ref)	241		1.00	
Start treatment	Yes	395		2.82	(0.55 – 14.4)
	No (ref)	80		1.00	
# Start treatment days**	Per day	475	26	1.25	(1.10 – 1.43)
# Group treatment days	Per day	475	26	1.05	(0.97 – 1.13)

[a] rec: calves in dataset, [b] OR: odds ratio, [c] CI: confidence interval, [d] ref: reference category, * P<0.05; ** P<0.01

Group treatments with antimicrobials was not picked up by the univariate model. When forced into the model, group treatment resulted in an OR of 1.4 (95% CI: 0.5 – 3.8, P=0.5). The association was strongest and positive only in older calves, with OR's of 0.4 (P=0.5), 0.6 (P=0.6), and 1.8 (P=0.4) for calves aged 0 – 6 weeks, 7 – 12 weeks, and older than 12 weeks, respectively.

Multiple regression analysis

Table 4 shows the results of the full multiple regression model for white veal farms. Variables included in the final model for white veal farms were performing rodent control (OR=8.1 (95% CI: 1.3 – 50), P<0.05), and age of the calves (calves under 6 weeks: OR=0.4 (95% CI: 0.2 – 1.0), P<0.05). No association was found between antimicrobial use and MRSA carriage in these models. Correlation within the white veal data was found between age of calves and feeding system (white veal calves under 6 weeks were generally fed from buckets, whereas calves over 12 weeks were generally fed from troughs),

showing that the apparent univariate effect for feeding system is likely an artefact related to age. Age of white calves was also strongly correlated with group treatment with antimicrobials (white calves over 12 weeks were more likely to have received a group treatment). For the rose veal data, only the number of start treatment days was included in the final regression model obtained after stepwise backward regression. The number of start treatment days was strongly correlated with cleaning, start treatment, and using an all-in–all-out system.

Table 4: Result of full multiple regression model on white veal data. Variables on animal and farm level with MRSA status of the sampled calf as dependent variable; 1,182 calves from 50 Dutch white veal calf farms were included in the study and the results are given as the OR with 95% confidence interval. Variables set in italic make up the final model after stepwise backward regression.

Variable	Category	OR[a]	95% CI[b]
# Mm rain on sampling day	Per Mm	1.09	(0.98 – 1.21)
*Rodent control**	*Yes**	*8.08*	*(1.29 – 50.5)*
	No or natural enemies (ref[c])	*1.00*	
Presence of companion animals	Yes	4.02	(0.88 – 18.3)
	No (ref)	1.00	
Gender[t]	Female[t]	0.44	(0.15 – 1.29)
	Male (ref)	1.00	
*Age calf category**	*1-6 weeks**	*0.42*	*(0.18 – 0.96)*
	7-12 weeks	*1.78*	*(0.74 – 4.24)*
	> 12 weeks (ref)	*1.00*	

[a] OR: odds ratio, [b] CI: confidence interval, [c] ref: reference category, * P<0.05, [t] P<0.1

Discussion

In this study we identified determinants for MRSA carriage in veal calves, and quantified the influence of farm and animal level factors on the presence of LA-MRSA in veal calves. Overall, calves from rose veal farms had a lower risk of LA-MRSA carriage than calves from white veal farms. In the whole dataset, group treatments with antimicrobials appeared to be a risk factor for MRSA carriage, although the strong association between antimicrobial use and age of the calves makes interpretation of this result difficult. Risk in group treated calves appeared to be highest between 7-12 weeks of age. In white veal calves,

group treatment appeared to be a risk factor in the univariate analysis, but this relation was no longer statistically significant after adjusting for the age of the calves. Other factors that were shown to have a positive association with LA-MRSA carriage in white veal calves in multiple regression analysis were rodent control and age of the calves (older calves had a higher risk). In calves from rose veal farms the number of start treatment days was positively associated with LA-MRSA carriage. A protective (negative) association with LA-MRSA carriage was found for cleaning of stables in rose veal calves in univariate analysis only.

The results of the current and previous studies indicate that MRSA carriage might be related to age of the animals. In a pilot study conducted among 209 pigs in one production system in the United States, older pigs had lower odds of MRSA carriage than younger pigs (18). This result, however, could not be repeated in another production system included in the same study. A small longitudinal Canadian study on an antibiotic-free herd sampled 100 pigs from 1 day to 10 weeks of life to determine the presence of MRSA (28). The risk of becoming MRSA positive reached an optimum around 50 days of age, and after this age the risk of being positive for MRSA decreased. Comparative results for the latter study were also previously found in calves based on the current data, where age was analysed as a continuous variable by smoothing. MRSA prevalence in calves not treated with antimicrobials seemed to reach a plateau just after 30 weeks of age (6). In the current study, age of the calves is considered a proxy of changes that occur during life, and therefore modeled as a categorical variable. First, the way calves are being housed changes over time. Second, age is related to antimicrobial use, and third, we can not exclude that age is a proxy of other biological changes over time, such as a change in feed and gut flora. This, however, needs to be studied in a longitudinal study.
Calves are housed individually during approximately the first 4 – 6 weeks after arrival on the farm. Hereafter, calves are housed in small groups (circa 6 calves per pen). In this study, we found a significant contrast between calves younger than six weeks and calves older than 12 weeks, with the young calves having a

2 - 3 times lower risk of being MRSA carrier. This was found for both the rose and the white veal management systems. Group-housed calves have more high risk contacts with more calves and thus a higher risk of becoming infected with MRSA, if MRSA is transmitted between calves. Furthermore, calves can be relocated between pens, thus increasing the possibility of placing a MRSA carrier into a susceptible group of calves, hence increasing the risk of transmission of MRSA. This relocating of calves is generally done regularly until the calves are approximately 14-15 weeks of age. Further, after arrival on the farm, calves are usually given a start treatment, and in the following weeks group treatments will be given. After 12 weeks of age, the use of antimicrobial group treatments declines because of decreased disease incidence, or because more often individual treatments are given. This might explain why the point estimate for the group of 7 – 12 week-old calves was the highest in the white veal model, albeit not significantly. This result, however, was not found in the rose veal data.

Group treatments with antimicrobials (ignoring whether or not the calf had received a start treatment) was a significant risk factor for calves from white veal farms in the univariate analysis, with a similar effect for all calves taken together. Previously we reported a difference in MRSA prevalence between group treated and untreated calves, adjusted for number of calves per farm, farm hygiene, sector (white versus rose veal), number of stables on farm, number of calves per pen, presence of other animals on the farm and rodent control (6). This difference could mainly be attributed to the larger difference in MRSA carriage prevalence at higher age. In the current more detailed stratified analysis a strong difference in MRSA carriage was observed in white veal calves aged between 7 - 12 weeks, depending on whether or not they were group treated. These results together indicate that the use of antimicrobials is very likely modified by other factors that determine the effect on a population level, such as well practiced relocation of the calves and feeding management. It seems relevant to further explore interactions between age, relocation of animals, and use of antimicrobials in experimental designs and intervention studies.

The data showed that all calves from white veal farms had received antimicrobials at least once in their lives, either as start treatment or a group treatment. This complicates exploring direct associations between use of antimicrobials in a population and MRSA status in a cross sectional study, since few animals do not receive treatment at all and the contrast in use is extremely limited. The associations between age, use of antimicrobials and MRSA prevalence suggest that group treatments with antimicrobials are a condition for MRSA to remain present on a farm after introduction, but that other factors determine transmission of MRSA, such as placing the calves in small groups (and thus increasing the number of high risk contacts). This can also explain why calves older than 12 weeks generally have a high risk of being a MRSA carrier while they do not regularly receive group treatments.

We expected month of sampling to influence MRSA carriage risk, as similar indications were found in studies aimed at Dutch pig farms and US beef calves (1, 14). Therefore we included month of sampling and explanatory variables reflecting the weather conditions on the day of sampling in the analyses. However, none of these factors were associated with MRSA occurrence.
We did find a significant effect for performing rodent control in white veal calves, and a similar (non-significant) effect was found in rose veal data. Our results indicate that performing no rodent control at all (or with natural enemies) would be preferred over performing rodent control, either by the farmer or by a professional. However, reversed causality might explain these findings. Rodent control might be the result of poor hygiene and presence of high numbers of rodents, with poor hygiene also likely to cause health effects in calves which in turn might lead to a higher risk of MRSA carriage, due to a higher antimicrobial use. On farms where no rodent control is performed, hygiene might be of a high level with a lower MRSA risk as a result. This result seems to be in line with the strongly protective result of cleaning of the stables in the rose veal data, another farm hygiene factor (a similar point estimate was found in white veal data (OR=0.4 (95% CI: 0.1 – 2.9), P=0.35)). However, it is also very likely that farms where rodent control is performed have a higher hygiene standard, which would

contradict the results. Future studies should consider cleaning or disinfection practices on the farms in greater detail.

Because of the sampling design (square root of the number of calves present on a farm), larger farms will have a larger influence in the analyses, as these are represented by more calves. However, we have run various models with various dependent variables (e.g. proportion of positive calves per farm, farm status with different cut-off values for considering the farm positive, farm level variables only), and the results are consistent with the findings shown here, indicating results are robust. For some analysis in rose veal farms, data is more sparse. Although data used in this study comes from a uniquely large dataset, compared to other studies, results from the stratified analyses show that when variables are strongly correlated, problems occur resulting from further stratifications.

Conclusions

Use of antimicrobials was associated with LA-MRSA carriage in calves, but other age related factors may also be important. This needs further exploration and study in experimental designs or intervention studies, but the findings further emphasize the need for prudent use of antimicrobials. Increasing farm hygiene should be incorporated into control programmes, and will not only be helpful in controlling MRSA but also many other pathogens. Further research should be done on how to improve and increase farm hygiene levels, but based on the current results regularly cleaning and disinfecting the stables, at least between production rounds, should be applied on all farms. Determinants such as the type of veal and age of the calves provide important information on potential additional risk factors but are less easily incorporated into control programmes.

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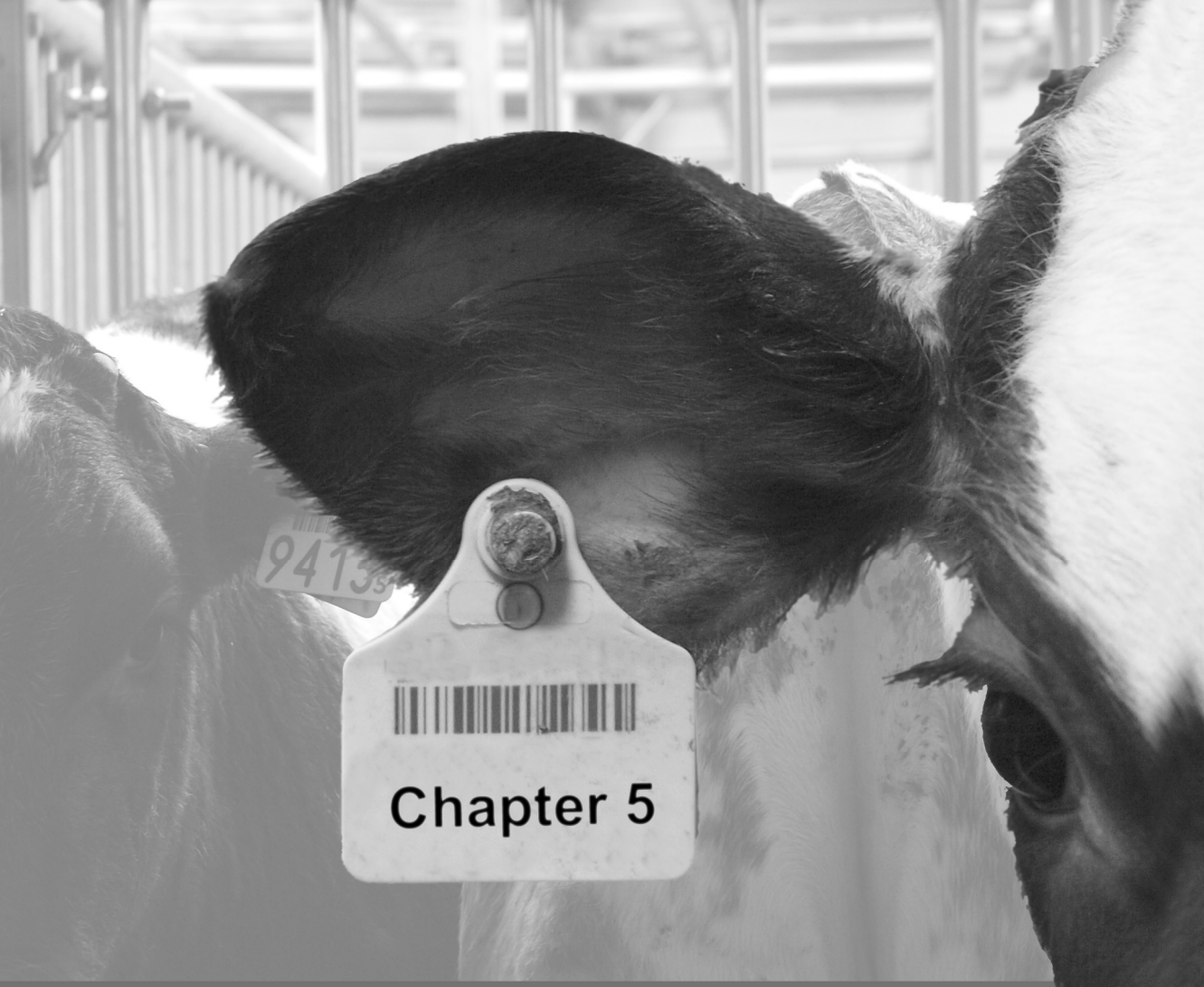

Dynamics of MRSA carriage in veal calves; a longitudinal field study

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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. Determinants associated with MRSA carriage, such as environmental exposure and antimicrobial use, were evaluated. 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.

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. This suggests that antimicrobial use is not necessarily the main condition for MRSA transmission in veal calves but other factors seemed to determine transmission as well. MRSA in calves was present both nasally and rectally. Relatively more positive rectal samples were found in the first 6 weeks. Therefore, we hypothesise that rectal MRSA carriage contributes to a higher environmental MRSA load and thereby influences MRSA spread into the population.

The results of this study are relevant for developing potential intervention and control strategies since they give a better understanding of the dynamics of MRSA in a field situation.

Introduction

The emerging problem of livestock-associated (LA)-MRSA colonization in food producing animals, such as pigs and veal calves, and its link with human carriage, has put pressure on the intensive 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 (4, 20). 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 (7, 15, 17). A longitudinal study in veal farmers and their family members, showed that LA-MRSA carriage was dependent on the intensity and duration of animal contact. LA-MRSA prevalence in people working or living on veal farms declined during absence of animal exposure, and the detection of LA-MRSA in nasal swabs varied greatly over time (6). This suggests that both colonization and transient contamination of LA-MRSA occurs in humans. This variable 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 the 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, but currently lacking.

The aim of this longitudinal study was (i) to describe the dynamics of LA-MRSA in veal calves by analysing nasal and rectal samples, (ii) to describe the dynamics of environmental contamination, (iii) to explore the presence of risk factors associated with MRSA carriage, and (iv) to determine the reliability and reproducibility of MRSA detection in nasal samples.

Materials and Methods

Study design and data collection

The study population consisted of 402 veal calves living 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. 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. Nasal and rectal swabs were collected during week 0 till week 10, and from week 12 onward only nasal swabs were taken.

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 on 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. Basically, no animals from outside could move into the study compartments. However, if still necessary, MRSA status of the introduced calf was established 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 determinants including farm characteristics and hygiene practices, as well as antibiotic use of the calves, gender and when and where calves were relocated within the study compartments. Collecting 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 nasal and/or rectal swabs. If no MRSA was present in both swabs the calf was considered to be MRSA negative.

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 hours. In each compartment, on 3 different locations, duplicate air samples were collected resulting in 30 samples in total per sampling moment.

Laboratory analysis

All swabs were analysed as previously described (8). 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). Ten 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 *S. 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 (5). Randomly, a selection of strains (n=168/2183) was confirmed by *S. aureus* ST398 specific PCR (19).

Air filter membranes were similarly analyzed by culturing. 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 is non-detectable (after culturing), MRSA concentration ranges could be obtained, giving an indication of the MRSA load.

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 assumed to mix randomly in pens, to be equally infectious throughout the infectious period, and all susceptible calves were 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 (2, 14), 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. 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(β); therefore, β is derived by $e^{outcome}$.

Results

Farm and animal characteristics

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

The included calves of farm 2 where all heifers (n=150), whereas the included calves of farm 1 (n=109) and 3 (n=140) were all bulls. 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 once or more differed between farms, and varied between 75% and 89%, indicating 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 far less calves were relocated, varying between 14.3 - 44.0%. Table 1 summarizes general farm and animal characteristics.

The number of antibiotic group treatments of the calves was comparable between farms regarding the number of treatments, but differed in timing and type of antibiotics used. Antimicrobials were administered mainly during the first 30 days after arrival on the farm. In general, tetracycline and macrolide antibiotics were the most frequently used on all three farms. Type, duration and timing of antibiotic group treatments are shown in Figure 1. Observed

antimicrobial use on these three Dutch veal calf farms can not be generalized because this study involved only one production cycle in winter season and is subject to considerable systematic and random variation.

Table 1: Overview of general farm and animal characteristics

General farm information	**Farm 1**	**Farm 2**	**Farm 3**
# calves on farm	942	560	486
# calves included in study	112*	150**	140***
# stables on farm	3	1	1
# stables (compartments) in study	1 (2)	1 (1)	1 (2)
General characteristics 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			
# antibiotic group treatments	5	4	6
# treatment days	29	27	31
# individual treated calves with antibiotics (%)	18 (16.1%)	35 (23.3%)	10 (6.4%)
Relocating of calves			
% of relocated calves (once or more)	75.0%	89.3%	74.7%
% of relocated calves *after* releasing from individual houses	26.8%	44.0%	14.3%

* Five calves died during study period, 1 calf was removed out of the study compartment, 3 calves (all MRSA negative at entrance) entered the study compartment during the study period, ** One calf died during study period, 2 calves were removed out of the study compartment, *** One calf died during the study period, 1 calf was removed out of the study compartment

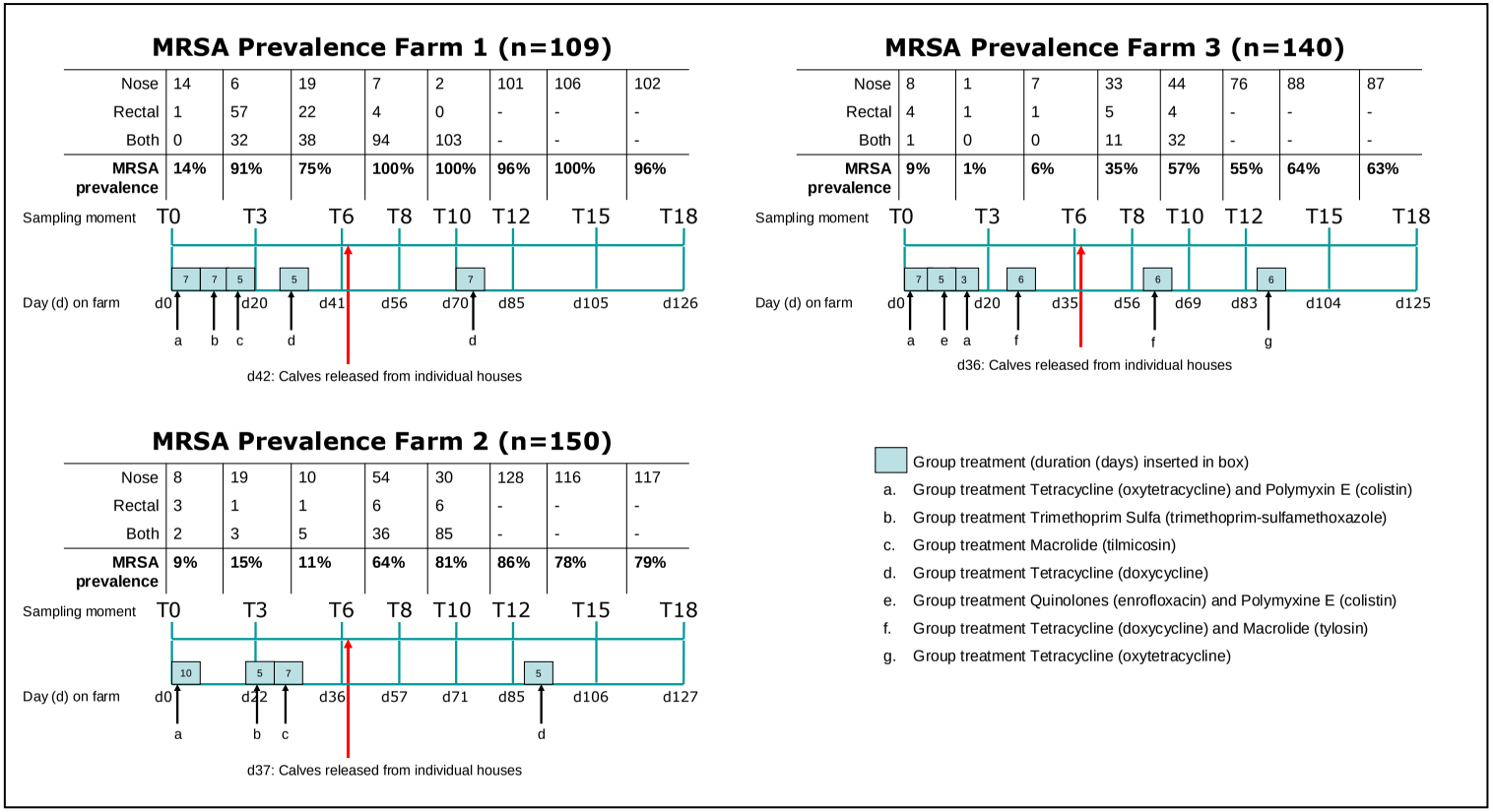

Figure 1: Schematic overview of MRSA occurrence in veal calves over time per farm. Per farm the number of calves MRSA positive in solely nasal or rectal samples, the number of calves MRSA positive in both nasal and rectal samples, as well as total MRSA prevalence per sampling moment are mentioned. In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

MRSA carriage

MRSA prevalence in calves ranged from 9-14% directly after arrival on the farm (T0) and increased over time to 63-96% at the end of the study period (T18) (Figure 1). All typed MRSA strains found in these veal calves belonged to ST398. At 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 in the first 6 weeks of the study period when antibiotic group treatments were most regularly given. A considerable increase in MRSA prevalence occurred at all three farms between 6 and 8 weeks after arrival on the farm. This increase coincided with the release of calves from their individual houses. After this strong increase in MRSA prevalence levelled off (Figure 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 (farm 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. Especially at T3, MRSA was more present in rectal samples compared to nasal samples (on average 24% vs. 15%, respectively). Differences in MRSA prevalence between rectal and nasal carriage were largest in farm 1 on sampling moment T3 (55% vs 37% respectively) (Figure 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 (Figure 1).

Approximately 10% of the veal calves were LA-MRSA positive upon arrival on the farm. 12 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. Only 19 calves (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 were (371 calves (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 from the same animals taken on the same sampling moment ranged from 47-63%. 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 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.

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 fraction of samples increased over time but also the amount of MRSA in the samples as tested with diluted samples. 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 farm 2 and 3 no air samplings were performed on sampling moment T8 due to bad weather conditions on the sampling days.

Table 2: MRSA prevalence in calves and air samples.

	Farm 1 (12 air samples per sampling moment)		**Farm 2 (6 air samples per sampling moment)**		**Farm 3 (12 air samples per sampling moment)**	
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	- *	35	- *
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)

* No air sampling performed due to bad weather conditions

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 directly or due to cross-contamination. 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 (7). MRSA prevalence was not constant over time.

At farm 1 there was a strong increase of MRSA prevalence (up to >90%) in 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 antibiotic treatments. At farms 2 and 3 MRSA prevalence increased rapidly after calves were released from the individual housing, a trend also observed in farm 1 but due to the relatively high prevalence just before release (75%) the increase was less prominent. This suggests that antibiotic use is a condition for MRSA carriage in veal calves, as previously demonstrated (7), but additionally other factors seem to have an important role in transmission as well.

It has been shown that rapid selection of antibiotic resistant bacteria can occur after antibiotic treatments in animals. For example, in pigs an increase in MRSA prevalence was observed after the pigs where treated with oxytetracycline (18). However, the occurrence and persistence of antimicrobial resistant bacteria like MRSA are the result of complex interactions between antimicrobial drugs, microorganisms, host factors and environmental factors (3). Therefore it is difficult to explain the observation that MRSA prevalence does not increase simultaneously with or directly after antibiotic treatments in calves. An explanation could be that different antibiotic treatments possibly counteract each

others' effects on the selection of resistant microorganisms. The majority of the studied calves were additionally treated with trimethoprim/sulfa (TMPS) after tetracycline therapy. Generally, MRSA ST398, the most predominant strain found in veal calves (6, 7) is resistant to tetracycline but susceptible for TMPS (9). Because it is not clear how rapid various antibiotic treatments affect MRSA selection and MRSA presence in animals, 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 explore the dynamics behind the effect of antibiotic treatments on MRSA occurrence in veal calves in-depth.

Results from this study indicate that different factors may contribute to transmission, and we hypothesize a mechanism which is dependent on rectal colonization by MRSA. This study indicated that rectal colonization of MRSA significantly contributed to higher environmental contamination of MRSA, and resulted in a strongly increased MRSA prevalence. Comparable observations are seen in humans where intestinal or perineal carriage of MRSA has been implicated as an important contributor to environmental dissemination as well (1). The high rectal MRSA shedding at younger age, especially at farm 1, could contribute to a strong boost in environmental MRSA load and be an explanation of the strong increase in MRSA prevalence at this farm. This is strengthened by the observations from farm 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 of these farms were also less frequently MRSA positive. The observation of rectal MRSA carriage in veal calves is relevant since it is a likely source of environmental contamination through the air which has not been taken into account earlier.

The large number of intermittent carriers found in this study suggests that calves could become MRSA positive due to contact to their positive pen mates (especially after T6), or through the exposure to MRSA presence 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 in times when 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 must play a role.

A previous cross-sectional study (7) showed a direct association between MRSA prevalence in calves and humans. Here, we show that this could be explained by a rising MRSA prevalence in calves and MRSA air load during the production cycle. It emphasizes the need to reduce exposure to reduce MRSA 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 (7). Examples exist of spontaneous decolonization of MRSA positive animals in cleaned and disinfected environments. For example in dogs it was shown that MRSA carriage resolved spontaneously in a regularly cleaned kennel (12). Taken together, these findings implicate that as long as few calves are MRSA positive at arrival, MRSA transmission can be limited by improvement of hygiene practices. Furthermore, measures to reduce exposure, for instance by wearing protective masks, might contribute to prevention of human colonization.

We can not 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. Comparable data are available for pigs (10) and humans (1) where rectal MRSA carriage without nasal MRSA carriage was also shown in especially younger subjects. Intestinal micro flora in animals can be strongly influenced by diet and can be disturbed by the effect of antimicrobial compounds (16). Rectal carriage might be explained by a change in feed (addition of roughage at a later age), or the higher amount of antimicrobials calves receive when they are under 12 weeks of age. Stress in animals caused by extreme fluctuations in temperature (13), transport or overcrowding and disease (11), influenced shedding of resistant *Enterobactericae* in the environment. For MRSA however, there is limited data available on the effect of these factors on shedding in animals, and therefore this needs further research.

We found 31 calves which had a constant 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 constantly positive or negative is low. A possible explanation could be that there are differences in MRSA load between calves. Calves that were constantly MRSA positive are likely to shed the bacteria in higher amounts than calves that where intermittent carriers. We did not investigate differences in MRSA load between calves in this study. The effect of MRSA load on test performance needs confirmation in other studies.

In conclusion, this first observational longitudinal study on MRSA in veal farming showed that MRSA prevalence rapidly increases 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 suggesting that other probably more important factors contribute to MRSA transmission in veal calf farming. The new findings that MRSA prevalence is not constant over time, different carriage patterns were observed, and the observation that calves were MRSA positive in both nasal and rectal samples gives hypothesis-forming insights. Further research is necessary to explore these insights in more detail and to explore efficient tools to reduce exposure for both animal and human. The results of this study are very relevant to successfully develop and implement intervention and control strategies since they give a better understanding of the dynamics of LA-MRSA in a field situation.

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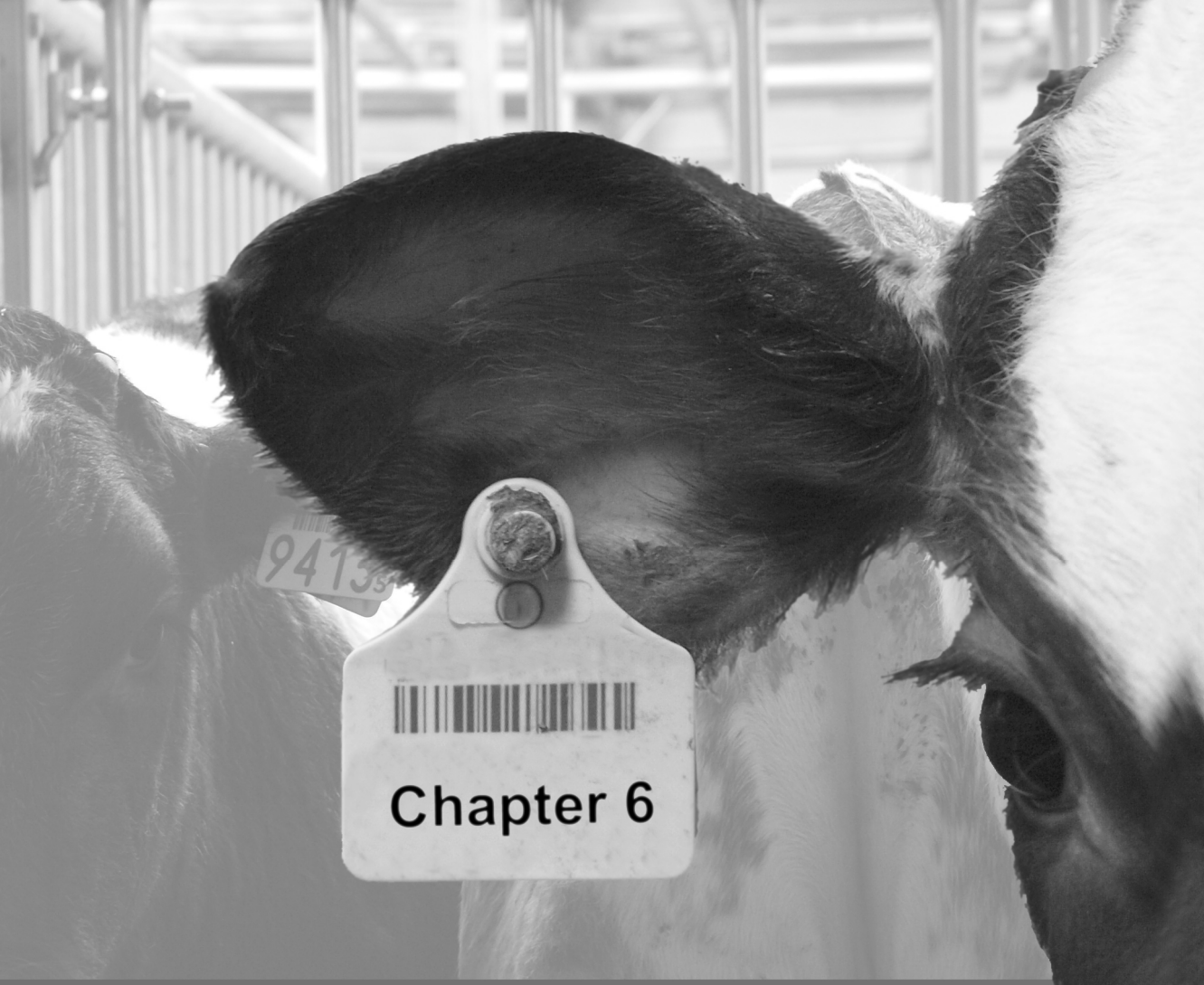

Persistence of livestock-associated Methicillin resistant *Staphylococcus aureus* in field workers after short-term occupational exposure to pigs and veal calves

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Abstract

The prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) carriage in pig and veal calf farmers in the Netherlands is estimated at 25 to 35%. However, no information is available about MRSA carriage in humans after short-term occupational exposure to pigs or veal calves. This study examines the prevalence and duration of MRSA acquisition after short-term intensive exposure to pigs or veal calves for persons not exposed to livestock on a daily basis. The study was performed with field workers who took samples from the animals or the animal houses in studies on MRSA prevalence in pig and veal farms. They were tested for MRSA by taking nasal samples before, directly after, and 24 h after they visited the farms. There were 199 sampling moments from visits to 118 MRSA-positive farms. Thirty-four of these visits (17%) resulted in the acquisition of MRSA. Thirty-one persons (94%) appeared negative again after 24h. There were 62 visits to 34 MRSA-negative farms; none of the field workers acquired MRSA during these visits. Except for that from one person, all *spa*-types found in the field workers were identical to those found in the animals or in the dust in animal houses and belonged to the livestock-associated clone. In conclusion, MRSA is frequently present after short-term occupational exposure, but in most cases the strain is lost again after 24h.

Introduction

Beginning in 2003, a specific clone of Methicillin-resistant *Staphylococcus aureus* (MRSA) associated with animal husbandry has emerged (15). This livestock-associated MRSA (LA-MRSA) clone belongs to multilocus sequence typing (MLST) clonal complex (CC) 398 (7), and humans in close contact with pigs are often colonized. Humans in contact with other animals, such as veal calves and poultry, may also have a significantly higher prevalence of MRSA carriage than the general population (3, 5, 8, 9, 12, 17).

So far, the prevalence of LA-MRSA carriage is known only for persons with long-term exposure to livestock, such as persons living or working on pig or veal calf farms or livestock veterinarians (5, 12, 19). In the Netherlands, a vigorous search and destroy policy is maintained, successfully controlling MRSA in health care settings by screening persons at risk for MRSA presence (14) (www.wip.nl). As part of this policy, all persons with livestock contact are screened for the presence of MRSA upon admission to a hospital. Since it is not clear whether persons with short-term exposure to livestock acquire MRSA, the necessity of screening these persons is questionable. This study examines the prevalence and duration of MRSA acquisition after intensive short-term exposure to pigs or veal calves in persons not exposed to livestock on a daily basis.

Materials and Methods

Study design and study population

During two cross-sectional studies investigating the prevalence of LA-MRSA on randomly selected pig and veal farms in the Netherlands (5, 12), dust samples from the animal houses and nasal swabs from pigs and veal calves were taken by field workers on the same day. These field workers (n = 40) all had short (up to a maximum of 3h per day) but intensive contact with animals and dust on the farms and were therefore at risk of acquiring MRSA on MRSA-positive farms. Intensive contact was defined as direct physical contact with the animals during the farm visit. Acquisition was defined as a MRSA-negative initial swab, followed by a MRSA-positive swab. Standard personal protective equipment included boots and overalls provided by the farm, gloves, mouth masks, and hair nets.

Hygienic procedures, including hand washing and showering, were mandatory when the field workers left the animal houses. Nasal swabs were taken from these field workers before, directly after, and 24h after their farm visits and were tested for MRSA presence. Field workers who had livestock exposure other than that on the farms concerned were excluded from the analysis. In addition, data on farm characteristics (i.e., farm type, number of animals, other animals present, and hygiene measures) were collected by questionnaire and have been described previously (5, 12). The study protocols of both cross-sectional studies were approved by the medical ethics committees of the institutes involved as required by the law of the Netherlands (5, 12).

All farms were visited by more than one field worker. For the veal calf farms, more than one farm could be visited on one field day. One sampling moment refers to a set of three individual nasal swabs (taken before, directly after, and 24h after the field day) pertaining to a field day on which one or more farms were visited by a field worker. Therefore, the number of field days could be different from the number of sampling moments and the number of farms visited. A farm was considered to be MRSA positive when MRSA was found in one or more animals or in dust samples taken on that particular farm. When only MRSA-negative farms were visited, the field day was considered to be MRSA negative. If one or more MRSA-positive farms were visited, the field day was considered to be MRSA positive. A schematic overview of the study design is given in Figure 1.

Laboratory analysis

Nasal swabs from the veal calf field workers were analyzed individually as published previously (4). Briefly, swabs were inoculated in a pre-enrichment medium containing Mueller-Hinton broth with 6.5% NaCl. After overnight aerobic incubation at 37°C, selective enrichment in phenol red mannitol broth (bioMérieux, France) with 75 mg/liter aztreonam and 5 mg/liter ceftizoxime was performed. Ten microliters of the selective enrichment broth was inoculated onto sheep blood agar (Biotrading, Netherlands) and Brilliance MRSA agar (Oxoid,

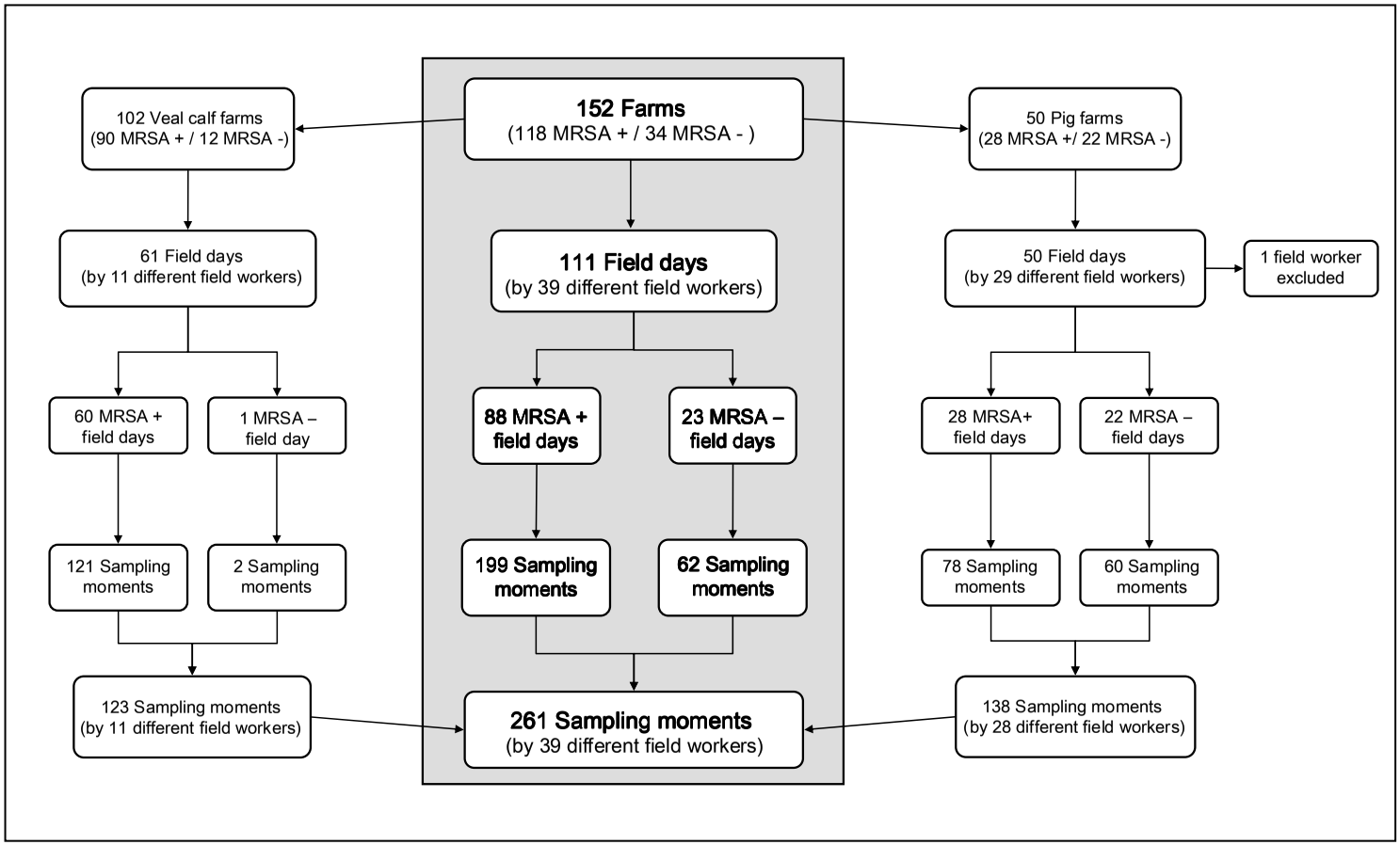

Figure 1. Schematic overview of study design.

Netherlands). The nasal swabs of the pig farm field workers were analyzed similarly; however, the selective enrichment step was excluded because of different protocols in the laboratories analyzing these samples. All suspected colonies were identified as *S. aureus* by using standard techniques: colony morphology and coagulase assays. The presence of the *mecA* gene was confirmed by PCR. The strains were *spa*-typed by sequencing of the repetitive region of the protein A gene *spa* (6). The strains of all positive dust and pooled pig samples and a random selection of samples from three MRSA-positive veal calves per farm were *spa*-typed. Data were analyzed by using Ridom Staphtype software, version 1.4.

Data analysis

Statistical analysis of complete data sets was performed using SAS software, version 9.1 (10). Descriptive analyses were undertaken, followed by logistic multivariate multilevel regression analysis (GLIMMIX and LOGISTIC procedures) to identify determinants of MRSA carriage. A P-value of <0.05 was considered statistically significant.

Results

In total, 152 farms (50 pig and 102 veal calf farms) were visited by 40 different field workers. One field worker was excluded because he was continuously exposed to livestock at home, and analyses were performed on data related to the remaining 39 field workers. None of them was MRSA positive on the initial swab. The 152 farms were visited in 111 field days. In total, 261 individual sampling moments were obtained. A total of 118 (78%; 28 pig and 90 veal calf farms) MRSA-positive farms were visited in 88 field days, and 34 (22%; 22 pig and 12 veal calf farms) MRSA-negative farms were visited in 23 field days. Figure 1 and Table 1 summarize the farm and field day characteristics for the pig and veal calf farms. More-extensive farm characteristics have been published in previous studies (5, 12).

Table 1: Overview of characteristics for pig and veal calf farms.

Source	No. of farms visited	Average no. of animals (range)	Average MRSA prevalence in animals (range %)	Average MRSA prevalence in dust samples (range %)[d]
Pigs	50	932 (0-3200)[a]	33% (0-100)[b]	34% (0-100)
Veal calves	102	565 (25-2200)	28% (0-100)[c]	47% (0-100)

[a] Only sows and finisher pigs were counted.

[b] Data were obtained from pooled pig samples (10 pools of 6 pigs per farm).

[c] Data were obtained from individual veal calf samples (10 to 43 samples per farm)

[d] Five dust samples were taken per farm.

In total, 199 individual sampling moments were present for 34 different field workers visiting the MRSA-positive farms (Table 2). These 34 different field workers acquired MRSA on 34 out of 199 visits (17%; 95% confidence interval [CI], 13-22%). Overall, 16 field workers (48%; 95% CI, 33-65%) acquired MRSA at least once. Five of them acquired MRSA twice (field workers 3, 7, 25, 26, and 27), and three acquired MRSA on more than two visits (field workers 4, 23, and 24). The field workers who acquired MRSA more than once visited, on average, more positive farms than field workers who acquired MRSA once (median number of positive farms visited per field worker, 10 and 1, respectively). Although the correlation between the number of sampling moments and the number of MRSA acquisitions was high, its statistical significance was only borderline (Spearman's rho, 0.87; P=0.09).

After 24h, 31 of the 33 field workers who had acquired MRSA (94% [95% CI, 83-98%]) were negative again. Only one field worker who was negative directly after exposure was found to be positive after 24h; he tested negative after subsequent farm visits. The *spa*-types found in field workers were t011 (n = 25), t108 (n = 8), and t567 (n = 1), all of which belong to CC398. All MRSA isolates except for that from one field worker (field worker 5) had *spa*-types identical to those isolated either from animals or from dust on the same farms on the same visit. Persons who acquired MRSA more than once were positive for different *spa*-types at different moments, depending on the farm visited. The 34 MRSA-negative farms were visited by 19 different field workers. None of them acquired

Table 2: Overview of sampling moments pertaining to positive field days and MRSA acquisition before, directly after, and 24 hours after sampling.

Farm type	Field worker(s) [a]	No. field days (No. farms visited)	No. positive field days	No. positive samples *before visit*	No. positive samples directly after visit [b]	No. positive samples 24h after visit [b]
Pig	1	9 (9)	2	0	1 (t011)	0
	2	1 (1)	1	0	1 (t011)	0
	3	15 (15)	11	0	2 (t011 [1], t108 [1])	0
	4	18 (18)	13	0	3 (t011 [1], t108 [2])	0
	5	1 (1)	1	0	1 (t108)[c]	0
	6	1 (1)	1	0	0	1[d] (t108)
	7	7 (7)	6	0	2 (t108 [1], t567 [1])	0
	8	3 (3)	1	0	1 (t108)	0
	9	1 (1)	1	0	1 (t011)	1[e] (t011)
	10	1 (1)	1	0	1 (t011)	0
	11	1 (1)	1	0	1 (t108)	0
	12-13	74 (74)	39	0	0	0

Veal calf	24	55 (82)	54	0	8 (t011)	0
	25	39 (77)	39	0	5 (t011)	0
	26	2 (5)	2	0	2 (t011)	1[d,f]
	27	5 (10)	5	0	2 (t011)	0
	28	7 (10)	7	0	2 (t011)	0
	29-34	15 (14)	14	0	0	0
Total	34	261	199	0	33[g]	3

[a] Field workers who visits to positive farms did not result in MRSA acquisition are grouped together.

[b] Designations in parentheses are *spa*-types. Where different *spa*-types are present fore one individual, the number of samples with particular *spa*-type is given in brackets.

[c] This *spa* type is not identical to the *spa*-type on the farm visited.

[d] The individual tested MRSA negative following subsequent visits to negative farms.

[e] Individual was not tested again.

[f] The sample was not *spa*-typed.

[g] Excluding field worker 6 who acquired MRSA after 24 hours.

Table 3: MRSA acquisition in field workers in relation to the MRSA prevalence among farm animals.

Source [a]	Odds ratio (per 10% increase in prevalence)[b]	95% CI[c]
Pigs	2.04	1.24-3.34
Veal calves	1.28	1.06-1.53

[a] Pig samples were pooled (10 pools of 6 pigs per farm). Samples were taken from individual calves. The number of veal calves sampled per farm (ranging from 10 to 43 samples per farm) was equal to the square root of the total number of veal calves on that farm.

[b] Adjusted for number of MRSA-positive dust samples on the farms.

[c] CI: confidence interval, $P<0.05$.

MRSA on the 62 field days. Further statistical analysis showed that field workers acquired MRSA more often when they had visited farms where more MRSA-positive animals were present. Similar associations were found for pig and veal calf field workers (Table 3). No significant associations were found with other farm characteristics.

Discussion

This study indicates that short-term occupational exposure to pigs or veal calves on MRSA-positive farms frequently results in the acquisition of MRSA. However, within 24h after exposure, 94% of those who had acquired MRSA tested negative again; the majority of people who acquire LA-MRSA during short-term occupational exposure lose the strain within 24h. Possibly, the high prevalence of MRSA carriage in livestock farmers and livestock veterinarians found in cross-sectional surveys is partly the result of repeated contamination instead of real colonization (5, 12, 19). Further longitudinal studies are needed to clarify these and other possible types of carriage and to determine the true dynamics and determinants of LA-MRSA carriage in humans.

It is questionable whether the nasal presence of MRSA should be considered true colonization or whether it is better described as contamination. We presume that in the animal houses on MRSA-positive farms, high concentrations of MRSA are present in the dust, and it is well known that *S. aureus* can survive in dust for long periods (2). People who work in these animal houses inhale MRSA-contaminated dust particles that may persist in the nares for hours to days

without truly colonizing the epithelial cells (16). Therefore, there is a risk of overestimating colonization rates, and other cross-sectional studies could overestimate colonization for the same reason. For *S. aureus*, it is confirmed that persistent colonization occurs only in 20% of persons; 60% are intermittent carriers, and 20% are noncarriers (18).

In this study, some persons acquired MRSA more frequently than others; 52% of the field workers never acquired MRSA despite their visits to MRSA-positive farms (17/33), while 24% of the field workers acquired MRSA once and another 24% acquired MRSA more than once. This could not be attributed to the number of sampling days or to sampling on specific farms, possibly due to a lack of statistical power. An explanation for this difference in acquisition may be differences in susceptibility to MRSA. Many different studies have been performed to reveal host susceptibility patterns for both *S. aureus* and MRSA, indicating that this could somehow play a role in MRSA acquisition (11). In this specific setting, hygienic behavior during work and the use of personal protective equipment may have influenced the potential for acquiring MRSA. This was not evaluated in this study and needs further investigation.

The *spa*-type of one field worker (field worker 5) was different from that found on the particular farm visited (t108 versus t011); he did not report any other contact with livestock. The most plausible explanation is that more than one *spa*-type was present on the farm, as found in another study (13). Due to the analytical method applied, this was not detected in the dust samples.

Part of the search and destroy policy is to screen health care workers who have been exposed to MRSA-positive patients without taking transmission-based precautions. Those who are persistently colonized are temporarily suspended from work. Samples are taken not during the work shift on which the health care worker has been exposed but during the next work shift (14) (www.wip.nl). This is done to limit the number of false-positive results due to contamination. This is consistent with our study results, which show that the presence of MRSA after short-term occupational exposure to livestock rarely persists for more than 24h.

The small sample size was the main limitation of our study; however, significant associations between MRSA acquisition and positive animal and dust samples

were found. Another limitation of this study is the difference between the analytical methods applied for the examination of the swabs from the pig and veal farms (5, 12). Studies on hospital-acquired MRSA strains in human samples suggest that selective enrichment broth with large amounts of antimicrobials can inhibit the growth of *S. aureus* in general (1). However, the detection of LA-MRSA using additional enrichment, as in this study, does not affect MRSA growth (4). Since we found only LA-MRSA strains in the nasal swabs of both pig and veal calf field workers, and all suspected strains were confirmed by *mecA* gene PCR in both protocols, it is not likely that this difference has influenced the study results.

In conclusion, LA-MRSA is frequently acquired after short-term occupational exposure. However, the majority of people who acquire LA-MRSA during occupational exposure test negative for MRSA again within 24h. This calls into question whether these individuals are colonized or contaminated. Screening of individuals upon hospital admission within 24h after exposure to livestock does not seem reasonable.

Acknowledgements

We thank the field workers of the Food and Consumer Product Safety Authority (VWA), the Pig Health Department of the Animal Health Service (GD), and the Institute for Risk Assessment Sciences (IRAS), all located in the Netherlands. We thank Matthé van den Broek for managing the field workers of the VWA; Marian Broekhuizen-Stins, Isabella Oosting-Schothorst, and Anky Schoormans for microbiological analysis; and Xander Huijsdens for *spa*-typing.

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Persistence of livestock-associated MRSA CC398 in humans is dependent on intensity of animal contact

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Abstract

The presence of livestock-associated MRSA (LA-MRSA) in humans is associated with intensity of animal contact. It is unknown whether the presence of LA-MRSA is a result of carriage or retention of MRSA-contaminated dust. We conducted a longitudinal study among 155 veal farmers in which repeated nasal and throat swabs were taken for MRSA detection. Periods with and without animal exposure were covered. Randomly, 51 veal calf farms were visited from June - December 2008. Participants were asked to fill in questionnaires (n=155) to identify potential risk factors for MRSA colonisation. Nasal and throat swabs were repeatedly taken from each participant for approximately 2 months. Swabs were analysed for MRSA and MSSA by selective bacteriological culturing. *Spa*-types of the isolates were identified and a ST398 specific PCR was performed. Data were analyzed using generalized estimation equations (GEE) to allow for correlated observations within individuals. Mean MRSA prevalence was 38% in farmers and 16% in family members. Presence of MRSA in farmers was strongly related to duration of animal contact and was strongly reduced in periods with absence of animal contact (-58%). Family members, especially children, were more often carriers when the farmer was a carrier (OR=2, $P<0.05$). Only 7% (n=11) of the participants appeared to be persistent carriers. A large heterogeneity in *spa*-types was detected; however 92.7% belonged to LA-MRSA CC398. A surprisingly high fraction of the spa-types (7.3%) did not belong to CC398. The presence of LA-MRSA in farmers is strongly animal-exposure related. The rapidly decreasing MRSA prevalence during absence of animal contact suggests that LA-MRSA is a poor persistent colonizer in most humans. These results are of relevance for MRSA control strategies.

Introduction

Infections with Methicillin-Resistant *Staphylococcus aureus* (MRSA) are associated with increased morbidity and mortality, length of hospitalization and health care costs (13, 30). Surveillance data of MRSA in The Netherlands and Scandinavian countries showed that MRSA prevalence is low (<1%), whereas the prevalence in some other European countries has reached values up to 50% (25). The low prevalence in The Netherlands and Scandinavian countries in hospitals is maintained by an active search and destroy policy and restrictive antibiotic use in human healthcare. Patients with increased risk for MRSA colonization are screened at hospital admission, and cared for in isolation. Furthermore, specific hospital hygiene measures have been implemented (30, 31). This approach is costly for the health care system, but considered cost-effective.

Since 2003, MRSA belonging to Clonal Complex (CC) 398 (CC398) has emerged in livestock and this CC is by far the most prevalent livestock-associated MRSA (LA-MRSA). CC398 is now being reported from different countries around the world (2, 10, 18, 23, 26). The emergence in livestock caused a strong increase in MRSA occurrence in humans between 2001 and 2006 in The Netherlands (27-29). Identification of risk factors and knowledge about persistence of LA-MRSA in humans is essential for successful continuation of the search and destroy strategy. We recently observed a high prevalence of MRSA in veal farmers (~30%) and their family members (<10%). In particular, intensity of animal contact and MRSA occurrence among calves were risk factors for MRSA colonization in humans (6).

Studies indicate that carriage of MRSA of hospital origin may persist for several months up to years (20), although available studies had limited power and cannot be generalized easily because they involve specific patient populations (21, 22). The proportion of long-term carriers (> than 1 year) ranges between 10 – 20% (9, 12, 13, 21). Currently, no data are available about persistence and dynamics of MRSA CC398 carriage and the possible role of intensity of contact with livestock. Understanding the dynamics of MRSA carriage in farmers occupationally exposed to MRSA is essential in designing specific control

strategies. The aim of this longitudinal study was to determine the persistence and dynamics of MRSA carriage in individuals in close contact with veal calves in periods with and without animal exposure.

Materials & Methods

Study design an study population

The study population consisted of 155 individuals living or working on randomly selected veal farms (n=51) in The Netherlands. Participants included had no occupational contact with other animals than calves. Participants were followed for approximately two months between June and December 2008 during periods of both high and low or no exposure. During high exposure, veal calves were present on the farm. During low exposure, participants were on a holiday (no exposure), or animals were absent in-between production cycles (low exposure). Hereafter, 'low exposure' is used when a participant had a holiday or an empty barn period. The study period started three weeks prior to low exposure (when calves were still at the farm) and ended three weeks after this period (when the farm was populated with new calves) (Figure 1).

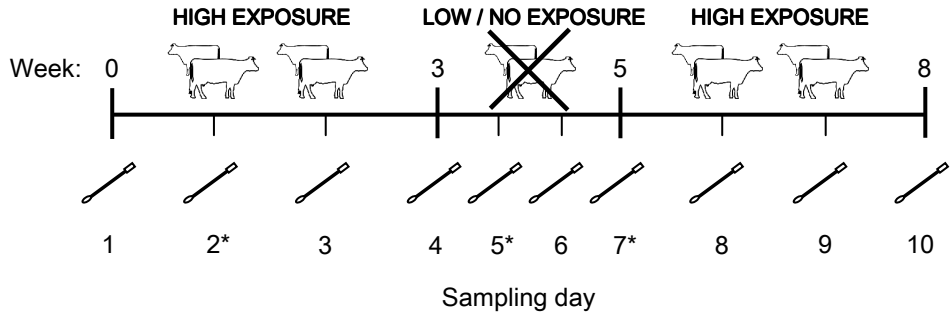

Figure 1. Schematic overview of the study design. Participants were followed for approximately two months during periods of both high and low/no exposure. The study starts 3 weeks prior to, and ends 3 weeks after, the low/no exposure period. Nasal and throat swabs were taken in the morning and evening once a week in high exposure periods, and twice a week in low exposure period.

* Nasal samples taken in the evening of sampling day 2 (high exposed), 5 and 7 (low exposed) were additionally screened for MSSA.

The study protocol was approved by the Medical Ethical Committee of University Medical Centre, Utrecht. All participants completed a written informed consent form.

Participants were considered MRSA positive and defined as "carrier" when MRSA was isolated in at least one out of four samples per day. We will use the word "carriage" or "carrier" for these individuals from here on. Persistent carriage was defined as having MRSA positive results (i.e. testing positive for MRSA presence) on all sampling days. Intermittent carriage was defined as having fewer than ten, but more than zero, positive days. Individuals with only MRSA negative swab samples were defined as non-carriers.

Laboratory analyses

All samples were analysed as previously described (5). Briefly, swabs were inoculated in a non-selective pre-enrichment containing Mueller Hinton broth with 6.5% NaCl. After overnight aerobic incubation at 37˚C, 1 mL of pre-enrichment was transferred into 9 mL selective enrichment broth (bioMérieux, France). Ten µL of this selective enrichment broth was inoculated onto sheep blood agar (Biotrading, The Netherlands) and MRSA Brilliance™ agar (Oxoid, The Netherlands). All suspected colonies were identified as *S. aureus* using standard techniques: colony morphology and coagulase assay (slide). The presence of the *mecA* gene was confirmed by PCR (4). A random selection of strains (n=478) was confirmed by *S. aureus* specific PCR (14).

Nasal samples taken in the evening of sampling day 2 (high exposed), day 5 and day 7 (low exposed) were also analysed for the presence of MSSA. Ten µL of the pre-enrichment broth were inoculated onto SA-Select agar (BioRad, The Netherlands), a *S. aureus* specific medium. Suspected colonies were identified as *S. aureus* using standard techniques: colony morphology, coagulase assay (slide) and by Pasteurex Staph-plus (Bio-Rad). The absence of the *mecA* gene was confirmed by PCR (4). A random selection of the MSSA strains (n=104) was confirmed by PCR for the *S. aureus* specific DNA-fragment Martineau (14).

Spa-types were determined for one MRSA (n=375) and one MSSA (n=104) nasal isolate per sampling day and in addition for all throat samples (n=103)

(MRSA). The strains were *spa*-typed by sequencing of the repetitive region of the protein A gene *spa* (8). A random selection of five strains per *spa*-type was tested with CC398 specific PCR (32).

Data analysis

Statistical analyses were conducted using SAS software 9.1 (SAS Institute, Inc., Cary, NC). Generalized estimation equations (GEE) were used to study associations of MRSA carriage in nose and throat with determinants, adjusting for correlations between repeated measures in the same individual and clustering of individuals by farm. Analyses were performed for nose and throat samples both, separately, and combined. Age, gender and smoking were included in the model as fixed effects, exposure was time varying. A P-value of <0.05 was considered statistically significant.

Results

MRSA carriage

Nasal (n=2864) and throat (n=2865) swabs were taken from 155 individuals. The response was 91%. Reasons given for non-participation were no interest or lack of time. The low exposure period involved a holiday for 38 participants and a period between production cycles for 117 participants (Table 1). Mean MRSA prevalence over the study period was 38% in farmers and 16% in family members. Family members were more often MRSA carriers when the farmer was an MRSA carrier, even after adjustment for the number of hours working in the calf stable in the 3 days before sampling (OR=2.0, $P<0.05$). Stratified analysis for both partners (spouses) and children showed comparable point estimates for the effect of MRSA carriage by the farmer, but the association was not statistically significant for partners. The effect of MRSA carriage of the farmer on carriage risk in family members was not statistically significant in subsets of the sampling data and could only be picked up when the complete dataset was used.

Table 1: Characteristics of study population of 155 participants.

	Number of participants (%) (n=155)
Gender	
Male	88 (57)
Female	67 (43)
Category	
Farmer	51 (33)
Partner of farmer	40 (26)
Child of farmer	57 (36.5)
Employees	7 (4.5)
Age	
Age (mean, range)	155 (33, 1-79)
age <18	40 (26)
age 18-65	110 (71)
age >65	5 (3)
Smoking	32 (21)
No Smoking	123 (79)
Exposure	
Number of hours working in calf stable in 3 days before sampling (mean, (range))	155 (6, (0-49))
< 6 hours	109 (70)
> 6 hours	46 (30)
Duration of low exposure period in days (mean (range))	155 (13, (3-32))
Duration of empty barn period in days (low exposure) (mean,(range))	117 (14, (3-32))
< 14 days	63 (41)
> 14 days	54 (35)
Duration of holiday period in days (no exposure) (mean,(range))	38 (11, (4-21))
< 10 days	16 (10)
> 10 days	22 (14)

MRSA prevalence was strongly reduced during low exposure periods. The reduction was strongest for holiday periods; during holiday periods the prevalence was 11% compared to 26% in exposed periods (-58%). The average prevalence of MRSA during low exposed periods, was reduced from 25% to 19% (- 24%) (Table 2).

Table 2: Mean daily MRSA and MSSA prevalence

	Mean prevalence, % (95% CI^)
MRSA Carriage	
Nasal MRSA carriage (total n=155)*	16.7 (14.7 – 18.5)
Throat MRSA carriage (total n=155)*	3.6 (2.7 - 4.6)
High exposed (total n=155)**	24.7 (22.2 – 27.4)
High exposed (holiday n=38)**	25.6 (18.4 – 30.4)
High exposed (empty barn n=117)**	24.4 (21.7 – 27.6)
Low exposed (total n=155)**	19.0 (14.9 – 23.1)
No exposed (holiday n=38)**	11.1 (4.1 – 18.1)
Low exposed (empty barn n=117)**	21.7 (16.3 – 25.9)
MSSA carriage (total n=155)***	23.0 (19.1 – 26.9)

^CI: confidence interval, * mean prevalence over 10 sampling days based on nasal/throat swabs taken in morning, ** mean prevalence over sampling days in either low (on average 3.4 sampling days) or high exposed period (on average 5.8 sampling days), *** mean prevalence over 3 sampling days based on nasal swabs taken in evening

MRSA carriage was less frequently seen in throat samples compared to nasal samples (Table 2, $P<0.05$). Farmers who worked more hours in stables on days before sampling were more often carriers compared to individuals who worked fewer hours (Odds Ratio (OR) =1.5, (P=0.03) expressed per 3 working days of 8 hours each). The longer farmers were non-exposed, the lower MRSA carriage prevalence was (OR=0.5, (P=0.01) for those exposed above and below median duration of 12 days; Table 3), both for nasal and throat carriage. Potential confounding variables like age, gender and smoking habits had associations in the expected direction but hardly changed associations between MRSA and duration of exposure; age was positively associated (OR=1.2 (P=0.04) per 10

years) and males were significantly more often MRSA colonized compared to females (OR=1.9, (P=0.04)). Smoking was negatively associated (OR=0.6 (P=0.22)), although not statistically significantly (Table 3).

Table 3: Associations between MRSA nasal and throat carriage and determinants in a multivariate regression analysis.

	Nasal swabs (n=2804) OR (95% CI^)	Throat swabs (n=2805) OR (95% CI^)	Nasal and Throat swabs (n=5609) OR (95% CI^)
Age**	1.4 (1.1 -1.7)*	1.1 (1.1-1.7)	1.2 (1.1-1.4)*
Gender *(ref ♀)*	3.4 (1.7-6.6)*	1.3 (1.7-6.6)	1.9 (1.1-3.4)*
Smoking *(ref: no smoking)*	0.4 (0.1-1.0)*	0.6 (0.1-1.0)	0.6 (0.3-1.3)
Duration of Low exposed period*** *(ref: above median duration of 12 days)*	0.5 (0.12-1.3)	0.4 (0.2-1.3)*	0.5 (0.3-0.9)*
Duration of animal contact in 3 days before sampling****	1.7 (1.1–2.8)*	2.2 (1.1-4.5)*	1.5 (1.1-2.2)*

^CI: confidence interval, * P<0.05, ** Expressed per 10 years, *** Refers to duration in days of either empty barn (low exposure) or holiday period (no exposure), **** Expressed per 24 hours. Refers to duration of exposure to animals in 3 days before sampling

Persistence or MRSA carriage

Because MRSA carriage was strongly associated with duration of animal contact, large fluctuations in carrier status over time were seen (Table 3-5). Only 11 participants (7%), who were mainly farmers (n=9), were MRSA positive throughout the study period at each sampling point (Table 4 and 5) and were therefore defined as persistent carriers. Only one persistent carrier had a holiday, while the others still lived on the farm during a low exposure period when animals were absent. The majority of the study population (n=93) were intermittent carriers (58%). Fifty-four participants tested always MRSA negative (35%) (Table 5). The prevalence of MRSA carriage in the throat was clearly

higher in persistent carriers (35.3 %) as compared to intermittent carriers (3.6 %) (Table 5). The majority of persistent carriers were MRSA positive in their nose. One participant was MRSA negative in the nose during one out of ten sampling days. However, the throat sample of this person turned out to be MRSA positive on this day. Only one individual was a persistent MRSA carrier on the basis of the throat swabs only. All other persistent carriers were nasal carriers only, or were persistent MRSA carriers in both nose and throat.

Table 4: Persistence of MRSA carriage in farmers, family members and others.

Number of MRSA positive sampling days*	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8****	**9****	**10****	**Total**
Farmer	13	13	1	2	4	1	3	2	3+**2**	**2**	**5**	51
Partner of farmer	11	14	6	3	3	1	0	0	0	0	**2**	40
Child of farmer	25	17	7	3	0	1	3	0	1	0	0	57
Others	5	0	1	0	0	0	1	0	0	0	0	7
Total	54	44	15	8	7	3	6	2	6	2	7	155

* MRSA positive means MRSA detected in at least one out of four samples (nasal and/or throat) taken per sampling day. ** Persistent carriers in bold (Persistent carrier: MRSA positive results on all sampling days; number of sampling days dependent on duration of low exposed period)

MSSA

In total, 447 swabs of 155 participants were analyzed for presence of MSSA. A mean prevalence for MSSA of 23% was found. MSSA was found on all three sampling days in the swabs of 16 individuals (10%). MSSA was absent in the swabs of the majority of the population (64%). Forty individuals (25%) were once or twice MSSA positive out of 3 sampling days. Although not statistically significant, an inverse association was observed between MRSA carriage and MSSA carriage, in both nasal and throat swabs, adjusted for age, gender and smoking habits (Table 6). We found no MSSA carriers among persistent MRSA carriers (Table 5).

Table 5: Characteristics of persistent-, intermittent- and non-carriers of MRSA.

	MRSA* nasal carriage (Days positive/ total days sampled)	MRSA* throat carriage (Days positive/ total days sampled)	MSSA** carriage (Days positive/ total days sampled)	Mean # of working hours in calf stable in 3 days before sampling (range)	Mean # of working hours in calf stable/week (range)
Persistent carriers (n=11/155 (7%))	102/103 (99%)	36/102 (35.3%)	0/29 (0%)	12.6 (0-40)	35.2 (0-65)
Intermittent carriers (n=90/155 (58%))	209/835 (25.0%)	47/835 (5.6%)	76/261 (29.1%)	5.2 (0-49)	15.8 (0-65)
Non-carriers (n=54/155 (35%))	0/490 (0%)	0/491 (0%)	27/157 (17.2%)	4.7 (0-44)	12.7 (0-70)

* mean prevalence over 10 sampling days.

** mean prevalence over 3 sampling days.

Table 6: Associations between MRSA carriage and determinants, including MSSA nasal carriage in a multivariate analysis.

	Nasal swabs (n=431) OR (95% CI^)	Throat swabs (n=431) OR (95% CI^)	Nasal and Throat swabs (n=862) OR (95% CI^)
Age**	1.3 (1.0-1.6)	1.1 (0.7-1.7)	1.2 (0.9-1.5)
Gender *(ref ♀)*	6.5 (2.5-17.2)	1.8 (0.4-7.9)	4.4 (1.9-10.0)*
Smoking *(ref no smoking)*	0.7 (0.2-2.0)	0.2 (0.0-2.1)	0.6 (0.2-1.5)
Duration of Low exposed period*** *(ref: above median duration of 12 days)*	0.7 (0.2-2.0)	0.8 (0.2-3.5)	0.8 (0.3-1.8)
Duration of animal contact in 3 days before sampling****	1.8 (0.6-5.5)	3.9 (0.7-21.0)	1.9 (0.4-4.6)
MSSA carriage	0.6 (0.2-1.6)	0.3 (0.0-2.3)	0.7 (0.3-1.6)

Analysis based on samples taken in the evening of sampling day 2, 5 and 7. Associations shown for different dependent variables separately (nasal and/or throat carriage). ^ CI: confidence interval, * P<0.05, ** Expressed per 10 years, *** Refers to duration in days of either empty barn (low exposure) or holiday period (no exposure), **** Expressed per 24 hours. Refers to duration of exposure to animals in 3 days before sampling

Genotyping MRSA and MSSA

Spa-types were determined of 375 nasal MRSA isolates and 103 MRSA throat isolates. In total 26 different *spa*-types were identified. The majority of the *spa*-types identified belonged to CC398 (92.7%). All persistent carriers were solely CC398 carriers, however we observed that in these carriers different spa-types within CC398 could be present on different sampling days. Thirty five strains (7.3%) were identified as non-CC398 types. We found no association between carriage of non-CC398 carriage and more general risk factors for MRSA such as hospital admission or travelling in a high prevalent MRSA country. No persistent non-CC398 carriers were identified. Overall, the prevalence of non-CC398 carriage was 2% per sampling day.

In addition *spa*-types of 104 MSSA isolates were determined. Out of 24 different MSSA *spa*-types the majority of the strains (56.2%) did not belong to the CC398 lineage. In 13 individuals, MRSA and MSSA were found in the same nasal swab. In 10 of these individuals, the MRSA and MSSA strains found in those nasal samples belonged to similar *spa*-types.

Discussion

This study shows that persistence of MRSA carriage in farmers is associated with duration of animal contact. LA-MRSA prevalence drops during a low exposure period and this is strong evidence for a relation with animal exposure. The large difference in MRSA prevalence between farmers and family members and the observation that MRSA carriage is lower after a longer period of low exposure are both in line with the hypothesis that exposure to MRSA-positive animals plays a major role in MRSA carriage in farmers. The positive association between carriage status of children and MRSA carriage by the farmer, however, suggests that MRSA carriage in children might be determined more strongly by contact with highly exposed people (farmer) than by animal contact. This might be a result of the fact that on average children are less exposed to animals and therefore the effect of exposure is smaller compared to the farmers or their partners.

Furthermore, we found that the majority of the study population were intermittent carriers (58%) or non-carriers (35%), and only 11 individuals (7%) were persistent carriers. These results indicate that carriage of MRSA in a highly-exposed population is mainly transient, or that there is retention of MRSA-containing dust in the nasal cavities in absence of colonization (6). Retention of dust leading to presence of MRSA in nasal cavities seems likely, given the exposure to high levels of dust associated with animal husbandry (19). In addition, strong correlations have been found earlier in studies exploring associations between MRSA positive air samples and presence of MRSA colonized patients (35). The role and function of the anterior nares in humans (11), the region of the respiratory tract where MRSA is predominant and where dust particles are deposited, supports this hypothesis.

The few available studies for hospital-acquired MRSA (HA-MRSA) indicate long-term colonization rates over 20% (9, 12, 13, 21, 22). Thus HA-MRSA seems a more persistent colonizer than LA-MRSA, which depends on several host characteristics and also on specific staphylococcal factors (24, 33), suggesting that LA-MRSA is a poor persistent colonizer in humans.

Apart from one individual, all persistent carriers had an empty barn period (low exposure) instead of a holiday period (no exposure). Some exposure may have occurred when the barn was empty. As a result, this might have affected the number of persistent carriers observed. At the start of the study we assumed that all participants were potentially exposed to MRSA because MRSA is present on the majority of the Dutch veal farms (~90%) (6). Furthermore from our previous cross-sectional study it is shown that there is significant (but not perfect) concordance between MRSA carriage in veal calves and environmental sampling using wipes (data not shown). However, testing MRSA status of a farm by taking five wipe samples from stables at the beginning and end of the study, analysed for presence of MRSA, showed that on three farms MRSA was not detected at the beginning and end of the study period. Removal of the participants of these farms from the data analysis changed the results only marginally.

The anterior nares are considered to be the primary colonization site of *S. aureus* (9). Additional screening of the throat for presence of MRSA may lead to a significantly increased carrier prevalence (15, 16). Such an effect was not observed in this study. The throat may yield fewer organisms than an anterior nare sample, either because the sampling technique is sub-optimal for the throat, or because there are fewer organisms present at the sampling site (16). If exposure plays a major role in screening MRSA in a population of farmers, for example because of deposition of MRSA-containing dust in the nasal cavities, it may be the case that that throat samples provide a more reliable estimate of the MRSA-carriage status compared to nasal samples. However, inhaled and deposited dust is removed from nasal cavities by cilliary transport and

subsequent swallowing. Consequently, a gold standard for MRSA screening and detection is not available.

None of the persistent carriers received antibiotics over the year prior to the study. Thus an increase in throat-carriage due to antibiotic treatment associated shift in carrier site could not have occurred (34). Environmental contamination with penicillin or tetracycline might be an important risk factor for colonization of the nares, and contributes to further spread in hospital patients (3, 17). Environmental contamination with antibiotics might have occurred in stables where animals receive antibiotics. Residues of this antibiotic substance occur in manure, in air or on surfaces of animal housing (7). This may have contributed to the high MRSA nasal prevalence in farmers, but this is in need of further exploration.

Observational studies which investigate risk factors of LA-MRSA in humans are mainly based on a single measurement of one nasal swab (2, 26). The present study shows considerable variability in LA-MRSA carriage over time. Guidelines underlying the search and destroy policy have been adapted due to conclusions from observational studies based on single nasal swabs. Pig and veal calf farmers are defined as new risk populations for MRSA carriage and are actively screened when admitted to a hospital (30). The substantial increase in health-care costs due to the presence of LA-MRSA, could be reduced by changing control measures to include an exposure-free period for farmers before screening. In addition, treatment of positive farmers is not meaningful when accompanied by ongoing exposure. Treating people only in the absence of exposure will limit the number of antibiotic treatments necessary for a successful MRSA decolonization compared to continuously exposed people and therefore contributes to a restrictive antibiotic use policy.

Other studies suggested that carriage with *S. aureus* might have a protective effect on the acquisition of other strains (1, 9) possibly through colonization competition (1). The negative association between MSSA and MRSA carriage

found in this study also suggests that competition might play a role. So far, studies where risk factors for LA-MRSA colonization were investigated did not take the possible interference between *S. aureus* strains into account. In this population 64% of the participants were non-carriers, 25% were intermitted carriers and 10% were persistent MSSA carriers. These figures differ from what is generally found in hospital settings. The number of persistent and intermittent carriers there is generally higher (20% persistent – 60% intermittent carrier) and the number of non-carriers is considerably lower (20% almost never carry *S. aureus*) (9). Because exclusive throat carriage of MSSA is also reported (15, 16), it is possible that we underestimate the MSSA prevalence and number of persistent and intermittent carriers. Screening for MSSA using *S. aureus*-selective plates might also have contributed to differences, because both MRSA and MSSA may grow on these plates. It can therefore not be excluded that MSSA could have been present but was missed in samples with predominant MRSA growth.

This study showed a variety in CC398 *spa*-types and an unexpected relatively large number of non-CC398 strains were isolated. These strains could not be related to known MRSA risk factors, such as visits to health care facilities. This is remarkable, because, so far, MRSA *spa*-types other than CC398 are seldom seen in veal farmers (6). Co-colonization of CC398 and non-CC398 may potentially lead to exchange of properties, e.g. improved transmission of CC398. Although results of this study indicate limited public health risk of LA-MRSA carriage, it still remains important from a public health point of view to control spread of LA-MRSA because of this potential for adaptation.

Conclusions

The present study indicates that carriage of LA-MRSA in farmers is strongly exposure related and mainly transient. It suggests that LA-MRSA is a poor persistent colonizer in humans. Improved understanding of the role of exposure and host specificity of LA-MRSA could have a significant impact on antibiotic and infection control policies in the hospitals, and more importantly, on the development of new strategies for the control of MRSA.

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General discussion

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Parts of this chapter are submitted for publication

Introduction

Evidence for associations between the use of antimicrobials in animals and the increase in occurrence of resistant bacteria in humans is frequently indirect. Parallel increase in carriage of resistant bacteria in humans and animals is considered to be direct evidence and is mainly demonstrated in ecological studies. An ecological study is an epidemiological study in which the unit of analysis is a population rather than an individual. Ecological studies may lead to spurious inferences about the nature of specific associations because these studies make use of aggregate statistics collected for the group to which the individuals belong. Parallel increase in carriage of resistant bacteria in animals and in humans in direct contact with animals, with measurements on the individual level, is considered to be a direct form of evidence and is mainly demonstrated in cross-sectional studies. Longitudinal studies, which allow cause and effect to be distinguished, in which (development of) resistance in microorganisms which colonize animals is followed by transfer to humans, hardly exist. As a result, transfer risks of resistance from animals to humans are seldom quantified in epidemiological studies. The studies described in this thesis were designed to study direct epidemiological associations between determinants and ST398 MRSA carriage in both humans and veal calves and their inter-relationship. In addition the persistence and dynamics of MRSA carriage in both humans and veal calves were quantified.

Main findings

We observed direct associations between MRSA carriage in animals and MRSA carriage in humans in veal calf farming. Human MRSA carriage among farmers, family members and employees, was strongly associated with intensity of animal contact and with the number of MRSA positive animals on the farm. In addition, a positive farmer contributed to a higher risk of MRSA carriage among family members. On the other hand, persistent carriers of ST398 were rarely observed. The rapidly decreasing MRSA prevalence during absence of animal contact suggests that LA-MRSA is a poor persistent colonizer in most humans.

Calves were more often carrier when treated with antibiotics, while farm hygiene was associated with a lower prevalence of MRSA. However, longitudinal data showed no increase in MRSA prevalence simultaneously with, or directly after antibiotic group treatments. This suggests that antimicrobial use is not necessarily the main or only requirement for MRSA transmission in veal calves but other factors seemed to determine transmission as well. Calves under the age of 6 weeks were relatively more frequently MRSA positive in rectal samples. Rectal carriage of the calves seemed to be correlated with higher environmental MRSA loads. This observation indicates that a higher environmental MRSA load is present when rectal shedding occurs and thereby influences MRSA spread across the population. In both animals and humans, strains belonging to an other CC than CC398 were observed incidentally. This suggests that these non-398 strains were less capable to colonize and spread in this specific environment.

Human health risks

Since 2003, MRSA of ST398, which originates from livestock emerged in The Netherlands. From then onwards, MRSA was frequently reported to be present in Dutch animal husbandry, particularly in pig and veal calf farming (34). In pig farming, MRSA prevalence of up to 70% have been reported, depending on the sampling and laboratory techniques used (7), whereas on 88% of the Dutch veal calf farms MRSA was found to be present (11). Among people in close contact with living pigs or veal calves, carrier rates between 29-33% were observed. In family members of farmers, usually individuals with less frequent contact to animals, MRSA prevalence ranged between 2-8% (11, 33) (Chapter 3).

Until now, it is unknown, whether ST398 MRSA strains are equally capable of colonizing and infecting humans as other *S. aureus* strains, such as MSSA or HA-MRSA. The spectrum of infections due to ST398 which have been documented ranges from relatively minor or localized infections, including abscesses (23, 31), wound infections (40, 41) and conjunctivitis (12), as well as more serious or invasive infections such as osteomyelitis (37) and postoperative infections (19). However, despite this wide spectrum of infections, it has been

suggested that ST398 may cause less frequently disease (relative to colonization) as other human strains (28). However, up to now, the evidence is limited. A cross-sectional study among human MRSA strains originated from several European countries showed that a relatively low proportion belonged to MRSA ST398. This observation suggests that MRSA ST398 contributes to only a small fraction of all MRSA infections in humans. Furthermore it has been shown that among isolates from blood, a significantly lower proportion were MRSA ST398 than other MRSA, which also suggests that MRSA ST398 is associated with less severe disease (32). On the other hand, the population at risk is considered to be small, and by focusing mainly on skin and wound infections, other categories of ST398 related disease may be overlooked, especially in a population of farmers. In one study it was observed that ST398 was more likely to be associated with respiratory disease rather than skin infections (34). It is well documented that working in agricultural environments with high dust and microbial loads poses a serious risk for development of respiratory diseases, especially in animal production (22, 24). Farmers are exposed to a mixture of organic and inorganic dust together with fumes and gases, including allergens and microbial-associated molecular patterns with a potentially major impact on respiratory health and the immune system (10, 26). People with asthma or COPD have a higher likelihood of developing exacerbations related to respiratory infections (26). Therefore, apart from the occupational risk of MRSA exposure and carriage, these observations suggest that, unless ST398 may not cause much severe disease, farmers however, may have an increased risk for colonization as well as (severe) infections. So far, no large population surveys are performed on human ST398 infections which impede interpretation of potential disease risk. Therefore, this needs to be confirmed in large scale studies.

In addition to the increased disease risk for people occupationally exposed to MRSA positive animals, it is suggested that the general population is also at increased risk due to MRSA contaminated food. Consumption of MRSA contaminated meat may cause food poisoning or MRSA carriage/infections. However, food poisoning as well as outbreaks of MRSA infections or human

diseases which have been related to the consumption of MRSA ST398 contaminated meat have, to the authors knowledge, not been documented. To date, no enterotoxins are found in ST398 (13). Reported cases are all caused by non-ST398 MRSA strains (18). Studies have investigated the presence of MRSA ST398 contamination of meat, but it is only found in low numbers (4, 8, 35). If meat is properly prepared before consumption, it is not likely that meat is an important source of MRSA transmission of infection. Nevertheless, it was demonstrated recently, that horizontal acquisition of enterotoxin genes can occur in ST398 swine isolates carrying *seb,* or *sek* and *seq* (14, 16). This suggests that risk of MRSA transmission via meat may change in the future.

Human to human transmission of MRSA ST398

We showed a positive association between MRSA carrier status of family members and MRSA carriage of the farmer (highly exposed people) (Chapter 7). Especially the finding in children, suggests that MRSA carriage in children is more strongly determined by contact with highly exposed family members or though environmental contamination than by animal contact. This may be because they appeared to be less frequently and shorter exposed to MRSA positive animals. This might be a result of the fact that children are less exposed to animals and therefore the effect of exposure is smaller compared to the farmers or their partners. It indicates that not only exposure to MRSA positive animals is a risk factor but human to human transmission can occur as well.

A large Dutch multi-center study in hospital settings has shown that the relative risk on transmission of MRSA ST398, as compared to HA-MRSA, was 0.28 (39). Based on these data, the genotype specific single admission reproduction number (R_A value) was estimated. For LA-MRSA this was 0.16 which is clearly lower than 0.93 as found for HA-MRSA. Taken these R_A values together, this resulted in a R_A ratio between HA-MRSA and LA-MRSA of 5.9 (95% CI 2.24-23.81), indicating that LA-MRSA is 5.9 times less transmissible than HA-MRSA (6). However, these data should be interpreted with caution since several assumptions about homogeneity between HA-MRSA and LA-MRSA carriers are made and data of other potential risk factors and patient characteristics are

lacking or ignored in these calculations. Furthermore, transmission (rate) is dependent of host and environmental characteristics, indicating (as observed in Chapter 7) that human to human transmission rates outside hospital settings could be different. This is also recognized by Wassenberg and colleagues (39), who mention that farmers usually belong to a healthy population (as compared to hospitalized patients with non-ST398 MRSA) and they therefore, in hospital settings, may be less likely to transmit the pathogen to other patients. Despite the fact that the risk for transmission in clinical populations is considerably lower for ST398, public health aspects of ST398 carriage, and rapid emergence in livestock and human populations closely associated with livestock, require further attention.

ST398 evolution, genetic diversity and colonization

To date, a large genetic diversity among *spa* and SCC*mec* cassette types has been documented in ST398. ST398 appears to be evolved due to multiple acquisitions of the SCC*mec* element. It has been suggested that coagulase-negative Staphylococci in the farming environment could serve as a source for SCC*mec* (42). Furthermore, several studies have documented the presence of novel antibiotic resistance genes in ST398 such as the novel plasmid-borne trimethoprim resistance gene *dfr*K (15) and the *crf* gene (17). The presence of these resistance genes in ST398 MRSA stains are likely to represent its adaptation to selective antibiotic pressure in animal production (13).

MRSA ST398 generally lacks important virulence such as PVL or TSST and resistance genes (13). However, recently, severe cases of infections due to PVL-positive ST398 MRSA strains have been documented in several countries (29, 31). This suggests that MRSA ST398 has the potential to evolve in a more virulent strain (13).

It appears that the immune evasion complex (IEC), which plays a major role in human colonization, is not present in ST398 (38). Furthermore, host binding proteins are often missing in ST398, which suggest that colonization capacities of ST398 MRSA to human tissues may be impaired (13). The absence of the IEC and host binding proteins could at least partly explain the transient carriage

observed when farmers are non-exposed due to for example holidays or empty barn periods (Chapter 7).

Impact on search and destroy policy

The Dutch MRSA guideline was amended in July 2006 and November 2007 for pig and veal calf farmers respectively, based on the findings in livestock environments. From these moments on professional contact with pigs or veal calves was added as risk factor for MRSA carriage (1).

The presence of LA-MRSA in farmers forms a potential threat for the health care system. The frequent occurrence of ST398, especially in countries with low and moderate MRSA prevalence, put the infection control policy under pressure. In the Netherlands, in hospitals within regions with a high pig or veal calf density, MRSA ST398 is more frequently observed than healthcare associated (HA-) MRSA (36). The unexpected and sudden increase of LA-MRSA incidence in hospitals resulted in a shortage of isolation facilities. An additional problem is the treatment of carriers, which is also part of this strategy (37). Decolonisation of patients with persistent professional and intensive exposure to ST398 MRSA is not effective, because exposure as a result of farming activities continues during and after treatment: thus at present the 'destroy' element is impossible to execute. The efficiency of nasal MRSA decolonization in healthy people with very close contact with livestock is especially low due to repeated exposure (20).

The specific population at risk for LA-MRSA, the number of Dutch pig and veal calf farmers, is relatively small compared to the total Dutch population (approximately 0.5% of the total Dutch population, http://statline.cbs.nl). It is likely that the total risk group is slightly larger than only farmers, since additionally to farmers, people occupationally exposed to animals such as veterinarians and slaughterhouse personnel are also at risk. Nevertheless, this remains still a relatively small risk group. The findings about the low nosocomial transmission rate of ST398 MRSA implicated that less stringent control measures for ST398 MRSA carriers could be considered (6, 39). On the other hand, the ongoing evolution of ST398 may result in a possible change in virulence of MRSA ST398 (*e.g.* introduction of toxin genes) which will change

the public health perspective considerably and such an event will further challenge the search and destroy strategy. Therefore, although the impact of ST398 on public health seems limited, close (continuous) monitoring and surveillance of its evolution (including monitoring of prevalence and disease risk) over time will be required. Molecular characterization (virulence and resistance genes) of MRSA strains should be incorporated in MRSA surveillance systems.

Antimicrobial use in animals

It is shown that the extent to which antibiotics are used for veterinary purposes in food producing animals can contribute to public health risks. Antibiotic use can be an important determinant for the development of antibiotic resistance within the treated animal population (2, 27). The administration of antimicrobial agents in humans as well as in animals raises a potential risk for the selection of bacteria resistant to antibiotics (25).

We hypothesised that rectal colonization of MRSA significantly contributed to higher environmental contamination of MRSA, and thereby resulted in a strongly increased MRSA transmission which resulting in a strongly elevated prevalence (Chapter 5). It is not unlikely that rectal colonization and excretion is influenced by antibiotic treatments and that different antibiotics may have diverse effects on rectal colonization and environmental contamination. For example, for oxytetracycline and tylosin, antibiotics which are widely administered to farm animals to control intestinal and respiratory infections, it is shown that differences in dose and absorption into the gastrointestinal tract of the calf resulted in large differences in residue secretion into faeces and environment (bedding) (9).

We have shown in Chapter 3 that antimicrobial use is positively associated with LA-MRSA presence in veal calves. However, the quantitative relationship and related factors between antibiotic use in animals and the resulting increase in MRSA seems complex and has not been unravelled completely. Nevertheless, all parties responsible for use of antimicrobials in veterinary practice, the Dutch calf producers and the Dutch Government agreed in 2008 on a covenant to reduce veterinary antimicrobial use. In addition to this agreement, the Ministry

aims to reduce animal antimicrobial use with 50% in 2013. However, this decision is not evidence based. It is yet unclear to which extent this specific reduction in antimicrobial use will affect MRSA presence or presence of other resistant organisms.

In Chapter 3 en 4 we observed that antibiotic group treatments in calves were associated with higher prevalence of MRSA in calves. On the other hand, in Chapter 5, we observed that MRSA prevalence increases over time in veal calf populations, but unexpectedly not simultaneously with or directly after antibiotic treatments. This observation strengthens the suggestion that not only antibiotic treatments in general have an important role in transmission but other factors such as farm hygiene (Chapter 3 en 4), dose and timing of treatments or animal-animal contacts seemed to modify the association as well, or are at least intensively correlated with antibiotic use in calves. Studies have demonstrated that several management factors and farm and animal characteristics are associated with veal calf health and thereby indirectly associated with antibiotic treatments. For example, housing and management factors, such as ventilation of stables (3), farm size and nutrition management may influence disease occurrence in calves (30) and thereby potentially influence the need for antibiotic treatments. In addition, it has been shown that calves with an arrival weight less than 43 kg have an increased risk of disease or even dying in the first week after arrival on the farm (21). Furthermore, in many dairy operations calves are removed from their dam at the first opportunity. Consequently, these calves may not have received adequate colostrum. Numerous studies have shown that the inadequate colostrum intake results in increased morbidity and mortality in calves (5).

Future research

In Chapter 3 and 4 we described the positive association between antimicrobial use and MRSA prevalence in veal calves. However we were not able to distinguish whether specific antibiotic classes or dosages especially contribute to MRSA occurrence in veal farming. Therefore, the quantitative contribution of antibiotic use needs to be established in more detail, preferably in experimental

studies. This may provide important information that can be used to develop an adequate approach to reduce antimicrobial use in veal calves. Information about the quantitative contribution of antimicrobial use is not only relevant to interpret MRSA occurrence, but can be useful for other resistant organisms as well. Furthermore the role of management factors which could be associated with veal calf health and thereby indirectly associated with antibiotic treatments needs further investigation.

The effects of farm specific or animal related risk factors as estimated in this thesis are based on data collected in the final phase of the veal calf production cycle. The observation that approximately 10% of the veal calves were MRSA positive upon arrival on the farm (Chapter 5) suggest that MRSA enters the production chain in an earlier phase. The origin of the MRSA in these young 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; directly or due to cross-contamination. Furthermore, it is not unlikely that factors as arrival weight and colostrum intake, which can be manipulated in this early phase, may play a role for MRSA occurrence in later stages of development. However, this, and the role of other potential risk factors in this phase of the production cycle, needs further exploration.

In addition to animal antimicrobial use, we demonstrated that farm hygiene (cleaning and disinfection of stables between production cycles) was associated with a lower MRSA prevalence. From our data we can not define how cleaning and desinfection needs to be performed optimally and thus this aspect requires further investigation.

Future research should also focus on the complex structure of the veal calf production chain. Dairy farms of many countries across Europe supply calves for veal production. Consequently, a huge variety of calf origins (different dairy farms and different countries) on Dutch veal calf farms are present. Because of the complex structure of the veal calf production chain it will be difficult to eradicate MRSA from this chain. Nevertheless, restricting the number of calf origins to a farm may reduce the introduction of resistant bacteria and diseases. This may result in reduced MRSA prevalence at the start of production cycles,

which may have considerable effects of MRSA occurrence and exposure in later phases of the production cycle. Therefore, it is important to determine where, and in which part of the chain, MRSA is introduced or transmitted. Additionally, the medical history (antibiotic use and previous infections) of the calves, especially on dairy, farms needs to be more transparent. This information will allow a more systematic approach to avoid supply of numerous MRSA positive calves on Dutch veal farms.

Besides, modern farms frequently house their calves in large groups, in barns containing just one compartment. This is in contrast to less modern barns, where more (and often smaller) compartments are present. Generally, it is difficult to constrain the spread of bacteria or diseases without compartmentalisation. Therefore, in modern farms, it is difficult to treat individual calves or small groups of calves, which may consequently make it more difficult to reduce antimicrobial use.

Epilogue

Antimicrobial resistance is increasing in both zoonotic and commensal bacteria due to the extensive use of antimicrobial drugs in food producing animals. This raises great concern about the risk of transmission of resistant zoonotic bacteria (direct hazards) and resistance genes (indirect hazards) from food producing animals to humans, and the subsequent consequences for health care and public health. Farmers have already experienced the direct hazard of LA-MRSA. Nevertheless, besides LA-MRSA, other resistant organisms are emerging as well. For instance, in human and veterinary isolates, extended-beta-lactamases (ESBLs) producing Gram negative bacteria are increasingly reported and may pose another serious threat to public health.

To deal with these existing and emerging resistance problems there is need for a structural solution. Human and veterinary sectors should collaborate to adequately solve existing problem areas, to improve early detection of new resistance hazards, and preferably prevent the development and spread of resistant microorganisms. The timely exchange of information between human

and animal surveillance systems is crucial to interpret associations and to solve these issues using a systematic approach.

In summary

The findings in this thesis provide important insights that add to our understanding of LA-MRSA carriage in animals and humans. The new findings are relevant in intervention strategies which need to be developed and implemented. The direct association between animal and human MRSA carriage suggest that implementation of intervention strategies on farm and animal level will directly affect human risk for MRSA carriage. Based on the studies described in this thesis, we advise to implement intervention strategies which improve farm hygiene especially between production cycles and reduce general antibiotic use (group treatments) in calves. However, future research should be focussed on the quantitative contribution of specific antibiotic classes or dosages to MRSA occurrence livestock environments. Our data point out that environmental exposure plays a major role in MRSA carriage in humans, but human to human transmission can not be excluded and may especially play an (additional) role in low exposed people. It was observed that MRSA belonging to ST398 is not a persistent colonizer in humans.

The low nosocomial transmission rate of ST398 MRSA implicated that control measures as described in the search and destroy policy could be less stringent. However, the exact ST398 related disease risk for farmers and others occupationally exposed to ST398, is still unclear and needs further exploration. The ongoing evolution and development of ST398 MRSA suggests that adaptation to the human host might be developing and therefore close monitoring and surveillance of its evolution (including prevalence, disease risk and molecular characterization) over time is required.

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Summary

Traditionally Methicillin-resistant *Staphylococcus aureus* (MRSA) has been considered as a hospital-associated pathogen (HA-MRSA). However, since 2004, MRSA has been found to be emerging in livestock (LA-MRSA), particularly pigs and veal calves. MRSA in livestock, predominantly belongs to Clonal Complex (CC) 398. Animals have the capacity to act as reservoirs of MRSA, and potentially transmit this bacterium to humans in close contact with MRSA colonized animals. Human infections with MRSA are associated with increased morbidity and mortality, length of hospitalization and health care costs. However, the public health consequences and risk factors for MRSA carriage of CC398 are currently unknown. This thesis focuses on MRSA in veal calf farming. The main aim of this thesis is to investigate associations between determinants and ST398 MRSA carriage in both humans and veal calves and their inter-relationship. Furthermore the persistence and dynamics of MRSA carriage in both human and veal calves were quantified.

Since the emergence of MRSA in livestock, screening of animals for the detection of MRSA is widely practised. Different procedures are published for animal samples but a systematic comparison of methods has not been performed. **Chapter 2** described an evaluation of three available commonly used procedures and three chromogenic agars for detecting MRSA in nasal swabs from pigs and veal calves. The results of this study showed that using pre-enrichment containing Mueller Hinton and 6.5% NaCl prior selective enrichment, should be recommended for MRSA detection in nasal swabs from pigs and veal calves. The preferred choice of chromogenic agar depends on the sample matrix. Because of practical reasons and performance, MRSA Screen (Oxoid) is the plate of choice in the studies described in this thesis.

The sudden increasing emergence of MRSA in animals raised questions about the possible public health threats. Therefore there was need for further research. Risk factors for both veal calf and human ST398 carriage were investigated in a cross-sectional study, described in **Chapter 3**. Randomly selected, 102 veal calf farms participated in this study. Specific attention was given to the presence of

MRSA among veal farmers, their family members and their animals. We demonstrated a direct relationship between human and animal MRSA carriage. A positive association between human MRSA carriage and the number of MRSA positive calves on the farm was demonstrated. Furthermore, we observed that calves were more often MRSA carrier when treated with antibiotics. Farm hygiene however, was associated with a lower prevalence of MRSA in calves.

In **Chapter 4**, the results of a refined and subsector stratified analysis of determinants for developing intervention strategies to control LA-MRSA on veal calf farms and in veal calves are presented. Here we focuses specifically on the animal data, including an extended set of potential determinants. We demonstrated that antimicrobial use in calves was positively associated with MRSA carriage in both white and rose veal calves. This finding further emphasize the need for prudent use of antimicrobials. However, several age-related- and farm management factors seemed to be correlated with use of antimicrobials in calves and thereby complicate the interpretation of the results. These factors needs further exploration and study in experimental designs or intervention studies.

MRSA occurrence and dynamics in veal calves were investigated longitudinally. The results are documented in **Chapter 5**. Determinants associated with MRSA carriage, such as environmental exposure and antimicrobial use, were explored. In addition, the reliability and reproducibility of nasal samples in veal calves to establish MRSA status were investigated, as well as the additional value of rectal samples. 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. This suggests that antimicrobial use is not necessarily the main condition for MRSA transmission in veal calves but other factors seemed to determine transmission as well. MRSA in calves was present both nasally and rectally. Relatively more positive rectal samples were found in the first 6 weeks. Therefore, we hypothesise that rectal MRSA carriage contributes to a higher environmental MRSA load and thereby influences MRSA spread into the population.

Human persistence and dynamics of MRSA ST398 was investigated after both short and long term exposure to MRSA positive animals. Results are presented in **Chapter 6** and **Chapter 7** respectively. LA-MRSA acquisition after short term (up to 3 hours per day maximally) occupational exposure is frequent. However, the majority of people, who acquire LA-MRSA during occupational exposure, test negative for MRSA again within 24 hours. In farmers, who are long term exposed to MRSA positive animals, the presence of LA-MRSA is strongly animal-exposure related. During absence of animal contact, MRSA prevalence decreased rapidly, which suggests that LA-MRSA is a poor persistent colonizer in most humans. The study suggests that highly exposed people could be a source for MRSA for lower exposed family members.

The findings in this thesis provide important insights that add to our understanding of LA-MRSA carriage in animals and humans. Antimicrobial use in calves is associated with higher MRSA prevalence. However, future research should be focussed on the quantitative contribution of specific antibiotic classes or dosages to MRSA occurrence livestock environments. In addition, to reduce MRSA occurrence in veal farming, optimizing the complex structure of the veal calf production chain needs specific attention.

Our data point out that exposure plays a major role in MRSA carriage in humans but it seemed that MRSA of ST398 is not a persistent colonizer in humans. This is in line with other studies in which a low nosocomial transmission rate of ST398 MRSA is demonstrated. These findings implicated that control measures as described in the Search and Destroy policy could be less stringent. However, the exact ST398 related disease risk for farmers is still unclear and needs further exploration. The ongoing evolution and development of ST398 MRSA suggests that adaptation to the human host might be happening and therefore close monitoring of its evolution and surveillance (including prevalence, disease risk and molecular characterization) over time will be required.

Nederlandse samenvatting

Aanvankelijk werd Methicilline resistente *Staphylococcus aureus* (MRSA) beschouwd als een ziekenhuis geassocieerd pathogeen (hospital-associated MRSA; HA-MRSA). In de jaren 90 van de vorige eeuw werd er bij de mens een toename gesignaleerd van MRSA besmettingen die zich in de algemene bevolking voordeden (community-associated MRSA; CA-MRSA). In 2004 werd voor het eerst de veegerelateerde MRSA (livestock-associated; LA-MRSA) beschreven. Al snel bleek dat LA-MRSA op grote schaal voorkwam bij landbouwhuisdieren, met name varkens en vleeskalveren. Nagenoeg alle MRSA isolaten van landbouwhuisdieren bleken te behoren tot clonaal complex (CC) CC398.

Humane infecties met MRSA worden geassocieerd met een verhoogde morbiditeit en mortaliteit, een langere duur van ziekenhuisopname en hogere kosten voor de gezondheidszorg. Het vóórkomen van LA-MRSA bij landbouwhuisdieren en veehouders was daarom ook aanleiding tot grote ongerustheid. Het toenmalige Ministerie van LNV startte een onderzoeksprogramma om antwoord te krijgen op de vele vragen rondom LA-MRSA. Eén project van dit onderzoeksprogramma richtte zich op de vleeskalverhouderij. Resultaten hiervan worden beschreven in dit proefschrift. De scope van dit proefschrift is echter breder. Het belangrijkste doel van dit proefschrift was het identificeren van de determinanten die een rol spelen bij LA-MRSA-dragerschap bij mensen en vleeskalveren. Verder is de persistentie en de dynamiek van MRSA-dragerschap in zowel mensen als vleeskalveren in kaart gebracht.

Er waren verschillende methoden beschreven voor MRSA isolatie uit dierlijke materialen, maar een systematisch vergelijking tussen methoden was nooit uitgevoerd. **Hoofdstuk 2** beschrijft een vergelijking van drie methoden voor detectie van MRSA in neusswabs van varkens en vleeskalveren. Voorophoping van neusswabs in Mueller Hinton + 6.5% NaCl gevolgd door een selectieve ophoping gaf de beste resultaten. Als vaste (selectieve) voedingsbodem waarop de selectieve vloeibare kweek werd afgeënt, werd gekozen voor de Brilliance

agar, een chromogene agar (Oxoid). De keuze werd bepaald door kweekresultaten en gebruiksgemak.

De risicofactoren voor LA-MRSA dragerschap voor zowel mensen als kalveren werden onderzocht in een dwarsdoorsnede onderzoek. De resultaten zijn beschreven in **Hoofdstuk 3**. In totaal namen er 102 willekeurig gekozen vleeskalverhouderijen (met zowel blanke- als rosékalveren) deel aan de studie. Dit onderzoek toonde aan dat er een direct verband was tussen humaan LA-MRSA dragerschap en het aantal MRSA positieve kalveren op het bedrijf. Kalveren die een koppelkuur met antibiotica hadden gekregen bleken vaker drager te zijn van MRSA dan kalveren die geen koppelkuur hadden gekregen. Op bedrijven waar de stallen grondig gereinigd en/of gedesinfecteerd werden in de leegstandperiode werd MRSA minder frequent waargenomen bij de kalveren.

In **Hoofdstuk 4** staan de resultaten beschreven van verschillende gestratificeerde analyses van determinanten voor MRSA dragerschap in kalveren. Ook uit deze analyses bleek dat voor zowel blanke kalveren als rosé kalveren antibioticagebruik positief geassocieerd was met MRSA dragerschap. Deze bevinding benadrukt het belang van verantwoord (restrictief) antibioticumgebruik. Verschillende leeftijdsgerelateerde- en bedrijfsmanagement factoren leken gecorreleerd te zijn met het gebruik van antibiotica in kalveren. Dit maakte de interpretatie van de resultaten complex. Deze factoren moeten afzonderlijk verder onderzocht worden in experimentele onderzoeken of interventiestudies.

In **Hoofdstuk 5** staan de resultaten beschreven van een longitudinaal onderzoek in vleeskalveren. In dit onderzoek werden persistentie en dynamiek van MRSA dragerschap in kalveren onderzocht. Daarnaast werd er onderzocht hoe de determinanten zoals blootstelling aan een MRSA positieve omgeving (stof/lucht) en antibioticagebruik dragerschap in kalveren konden beïnvloeden. Ook werd onderzocht wat de herhaalbaarheid en betrouwbaarheid was van neusswab afname en MRSA analyse bij kalveren. Tevens werd de toegevoegde waarde van rectumswabs bepaald. De data lieten zien dat de MRSA prevalentie in kalveren en ook in de luchtmonsters uit de kalverstallen zeer snel stegen gedurende de mestronde. Dit gebeurde voornamelijk nadat de kalveren

losgelaten werden uit hun individuele huisvesting. Opvallend was dat de toename in MRSA prevalentie niet parallel aan of direct na een antibioticabehandeling plaatsvond. Deze bevinding geeft aan dat antibioticagebruik weliswaar een rol speelt in de spreiding en dynamiek van MRSA dragerschap in kalveren, maar dat andere factoren eveneens de transmissie lijken te beïnvloeden. Zowel neus- als rectumdragerschap werden aangetoond in dit onderzoek. Rectumdragerschap werd vaker aangetoond bij kalveren jonger dan 6 weken dan bij oudere kalveren. Deze observatie draagt bij aan de hypothese dat rectale uitscheiding mogelijk een toename kan veroorzaken in MRSA load in de stallen. Deze toename in MRSA load zou de spreiding van MRSA in de kalverpopulatie kunnen beïnvloeden.

Persistentie en dynamiek van LA-MRSA dragerschap bij de mens na zowel kortdurende als langdurige blootstelling aan MRSA positieve dieren is beschreven in de **Hoofdstukken 6 en 7.** Mensen die kortdurend (beroepsmatig) contact hebben met MRSA positieve dieren (maximaal 3 uur per dag) werden frequent drager van MRSA. De meerderheid van deze mensen die MRSA positief testten na kortdurende blootstelling, testten binnen 24 uur MRSA negatief. In kalverhouders die langdurig blootgesteld waren aan MRSA positieve dieren en omgeving, bleek de kans op MRSA dragerschap gerelateerd aan de frequentie van het directe diercontact. Gedurende perioden zonder diercontact, bijvoorbeeld tijdens leegstand- of vakantieperioden, daalde de prevalentie van MRSA bij de veehouders sterk. Deze snelle daling in prevalentie wijst erop dat de huidige circulerende kloon LA-MRSA (CC398) matig koloniseert in mensen. De resultaten suggereerden dat hoogblootgestelde mensen (kalverhouders) een risico kunnen zijn voor MRSA dragerschap voor (lager blootgestelde) familieleden.

Concluderend kan worden gesteld dat blootstelling een zeer grote rol speelt bij LA-MRSA dragerschap in mensen. Persistentie van dragerschap van LA-MRSA bij mensen is beperkt. Dit is in overeenstemming met de bevindingen van onderzoek waaruit blijkt dat de nosocomiale transmissiesnelheid van LA-MRSA laag is in vergelijking met HA-MRSA. Op basis van deze bevindingen zouden de

beheersingsmaatregelen zoals opgenomen in het search and destroy beleid heroverwogen kunnen worden. Het CC398-gerelateerde risico op ziekte voor de veehouders is echter nog onduidelijk en vereist nader onderzoek. Zoals ieder micro-organisme kan ook LA-MRSA zich ongetwijfeld aanpassen aan nieuwe gastheren en bijvoorbeeld genen opnemen die coderen voor toxinen. Ook zou een MRSA met andere eigenschappen de huidige CC398 kunnen vervangen. Continue surveillance bij mens en dier op vóórkomen van en type MRSA is een vereiste om veranderingen in epidemiologie tijdig te signaleren en hier adequaat op in te kunnen spelen.

De onderzoeksresultaten beschreven in dit proefschrift geven aanknopingspunten voor de beheersing van MRSA in de vleeskalverhouderij. Mogelijkheden liggen in het reduceren van het antibioticagebruik bij kalveren en bij het grondig reinigen en desinfecteren van de stallen in de leegstandperiode. Nader onderzoek moet worden uitgevoerd naar de kwantitatieve bijdrage van specifieke antibiotica klassen of doseringen op het voorkomen van MRSA. Daarnaast is het aan te bevelen om de complexe structuur van de vleeskalveren productieketen te optimaliseren. Dit heeft met name betrekking op de grote variëteit in de oorsprong van de kalveren, compartimentalisatie van stallen en het creëren van mogelijkheden om deelkoppels te behandelen met antibiotica.

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Haitske

Curriculum Vitae

Haitske Graveland was born in Oudewater, The Netherlands on October 15, 1981. In 2000 she graduated from secondary school at the Antonius College in Gouda and started the study Biology at Utrecht University, The Netherlands. After her internship at the Institute for Risk Assessment Sciences (IRAS), in which she conducted a study on health effects of air pollution in Dutch school children, she received her MSc degree in 2005. From December 2005 she started working as a junior researcher at IRAS on the project “Burgers informeren over lokale luchtkwaliteit”, in collaboration with Science shop Biology of Utrecht University. From December 2006 she started working on a PhD project, studying MRSA in veal farming, described in this thesis. During this PhD project she has supervised eight students. In addition, from September 2008 until May 2010 she completed a postdoctoral Master Epidemiology, with a specialization in infection and veterinary epidemiology. Currently she works as post doc at the Institute for Risk Assessment Sciences, studying MRSA.

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