General discussion and summary

For several decades, methicillin resistance in *S. aureus* has been considered a strictly human problem. Initially, it was confined to the healthcare sector but later it became a matter of concern in the general population too. This changed in 2005 with the isolation of MRSA CC398 from pigs and pig farmers in the Netherlands [1]. This unexpected discovery prompted research worldwide, showing the presence of MRSA CC398 in a variety of farm animals [2, 3], especially in production sectors characterized by intensive animal farming practices with high antibiotic use, being the pig, veal, dairy and broiler industry [4]. Persons in direct contact with affected animals frequently carry MRSA CC398 [5–8]. Considering the huge spread of MRSA CC398 among food production animals globally, it is unlikely that this will be eradicated easily. The emergence of this so-called 'livestock-associated MRSA' (LA-MRSA) posed a threat to the successful control strategy in the Netherlands. To determine the potential implications of MRSA CC398 it is essential to know the dynamics, transmissibility and virulence of this subtype of MRSA. The research described in this thesis aimed to gain more insight into dynamics of MRSA CC398 carriage and morbidity associated with MRSA CC398 carriage in livestock veterinarians. In addition, we aimed to determine the transmission of MRSA CC398 from livestock veterinarians to their household members and its determinants.

Detection and susceptibility testing of MRSA CC398

A large diversity of antimicrobial resistance pheno- and genotypes has been observed in MRSA CC398 isolates worldwide [9, 10]. This diversity likely results from the enhanced capability of the CC398 lineage to acquire external DNA combined with the fact that MRSA CC398 isolates worldwide may have been subjected to different antimicrobial selection pressures. Consequently, a wide variety of resistance genes has been detected in MRSA CC398, including resistance genes that are typical for human and animal staphylococci. However, the vast majority of MRSA CC398 strains are relatively susceptible for most non-beta-lactam antibiotics, with the exception of tetracycline for MRSA CC398, for which they are almost always resistant. This is most likely due to the extensive use of this antimicrobial agent in animal husbandry. Likewise, zinc and other metals are frequently used in animal feed formulations and may co-select for MRSA CC398 strains that carry the *czrC* zinc resistance gene, as suggested previously [11]. This hypothesis is supported by the fact that the vast

majority of MRSA CC398 strains carry SCC*mec* type Vc, which contains the *czrC* gene. Because tigecycline is a derivative of tetracycline, it is important to determine the activity of this new drug for MRSA CC398.

Tigecycline was shown to exhibit broad *in vitro* activity against a collection of MRSA strains collected in the Netherlands, including livestock-associated strains (chapter 2). Using the recommended methodology, we found three strains to be resistant. However, the results for tigecycline may be influenced by the concentration of manganese in the medium [12]. Additional testing showed that these strains were susceptible when Iso-Sensitest medium was used. This discrepancy warrants further investigations into the preferred test conditions because the interpretation of the *in vitro* susceptibility of tigecycline is affected significantly.

The extension of MRSA beyond its known boundaries poses an additional challenge for microbiological laboratories to improve their screening strategies. Methods to detect MRSA in clinical samples ideally should have a high sensitivity and specificity combined with a short time to reporting of the results. To identify *S. aureus* from contaminated samples more easily and reliably, selective media have been developed [13]. Chapter 3 describes the evaluation of the *in vitro* sensitivity and specificity of a new selective medium, called Oxoid *Brilliance*™ MRSA for the identification of MRSA, using a well-defined collection consisting of 266 MRSA strains, 257 methicillin-susceptible *S. aureus* (MSSA) strains, and 265 coagulase-negative staphylococci (CNS). Oxoid *Brilliance*™ MRSA was shown to be a highly reliable screening tool for the detection of MRSA.

Further clinical studies have been performed to evaluate different selective media for the detection of MRSA directly from clinical samples. Oxoid *Brilliance*™ MRSA Agar and bioMérieux chromID® MRSA medium were evaluated for their abilities to identify methicillin-resistant *Staphylococcus aureus* in clinical samples (chapter 4). Nasal and oropharyngeal samples (n = 629) were taken from veterinarians and their household members. The sensitivities of Oxoid *Brilliance*™ MRSA Agar and chromID® MRSA medium after 20 hour of incubation were 63.6 and 64.5%, and the specificities were 94.1 and 99.4%, respectively. After an enrichment step the sensitivities increased to 96.3 and 97.2%, but the specificity decreased to 88.7 and 98.5%, respectively. In another clinical evaluation described in this thesis, Bio-Rad MRSA*Select*™ medium was evaluated for its ability to recover MRSA from nasal samples of pig farmers and their household members (chapter 5). In total, 257 samples were inoculated on Bio-Rad MRSA*Select*™ medium with and without broth enrichment and on chromID® MRSA with broth enrichment. The sensitivity of Bio-Rad MRSA*Select*™ medium without broth enrichment was 63.9%. With broth enrichment, the sensitivity increased to 98.4%. The specificity was 95.4% both with and without broth enrichment. In conclusion, Oxoid

Brilliance™ MRSA Agar, Bio-Rad MRSA*Select*™ medium and chromID® MRSA medium are all sensitive method for the screening of MRSA in combination with broth enrichment, but positive results require confirmation. Despite commercial efforts to develop new media with high sensitivity, the studies described in this thesis, as well as others [14, 15], showed a substantial increase in sensitivity following enrichment compared with direct cultures. Therefore, broth enrichment remains necessary for reliable MRSA detection.

Prevalence of MRSA CC398

A case-control study performed by van Loo et al. [16] showed that contact with pigs and veal calves as the major risk factor for MRSA CC398 carriage. This study lead to an amendment in the Dutch search and destroy policy in July 2006 which stated that patients who come into contact with live pigs or veal calves have to be isolated and screened upon hospital admission. Some years later, several Dutch studies report prevalences of positive farms varying from 23% to 81%, whereas the prevalence in individual pigs varies from 11 to 39% [17, 18]. In veal production, high prevalence of MRSA CC398 were found as well: 88% of the farms and 28% of the calves tested positive [7]. The extremely high MRSA CC398 prevalence among livestock results in very high carriage in humans in contact with these animals. Broens *et al.* have shown that pig herd prevalences rose from 30% in the beginning of 2007 to 75% in the end of 2008 [19]. Similarly, the prevalence of MRSA CC398 in pig farmers found in more recently published studies are higher compared to older studies on pig farmers: from 29% in 2007 [20] to 63% in 2010 (van Cleef et al. personal communication). In addition, the carriage rate of MRSA CC398 in livestock veterinarians as reported in chapter 8 is higher than any other studies on veterinarians that has been described to date (MRSA prevalences in veterinarians vary from 12.5% to 45% in various international studies [8, 21].

In order to assess how widespread the dissemination of this unusual type of MRSA in the general population was, we screened individuals that would have had the highest risk of carriage when MRSA CC398 would have been spread into the community. This study aimed and succeeded to determine if MRSA CC398 has spread from the farms into the rest of the community in areas with an extremely high density of pig farms. The results of this study are shown in chapter 6. Using a random mailing in 3 selected municipalities in the Netherlands, adult persons were asked to fill in a questionnaire and to take a nasal swab. In total, complete information was obtained on 583 persons. Of

the 534 persons without livestock-contact only one person (0.2%; 95% CI <0.01–1.2) tested positive for MRSA. In contrast, thirteen (26.5%; 95% CI 16.1–40.4) of the 49 persons with livestock-contact (either work at or live on a livestock farm) tested positive for MRSA. All recovered MRSA strains had *spa*-types that belong to the known livestock-associated clone CC398 [22].

Origin and epidemiology of the CC398 lineage

Apart from the occurrence of livestock-associated MRSA CC398 among humans, there is a second epidemiological event (i.e. the emergence of MSSA CC398 infections) for which an epidemiological link to livestock is lacking. Microarrays and whole-genome sequencing approaches applied to a large number of CC398 isolates distinguished two clades within the CC398 lineage [23–25]: the classical livestock-associated clade, isolates of which have long been responsible for frequent colonization, and rare infections among farmers and veterinarians [26]; and a human clade. This human clade is comprised of two subpopulations: the ancestral human subpopulation, and the emerging human-adapted non-LA CC398 subpopulation that has recently and increasingly been causing invasive infections worldwide in humans living in animal-free environments [24, 27, 28], and that readily colonize and spread between humans [23]. Price et al. suggested that livestock-associated MRSA CC398 was derived from a human MSSA CC398 lineage that only recently acquired *mec*A on repeated occasions when it disseminated into animal populations. The human-to-livestock jump was accompanied by the loss of immune evasion cluster (IEC) genes and the acquisition of tetracycline resistance due to *tet*(M).

The two clades of the CC398 lineage are characterized by different prophages. Livestock-associated CC398 isolates commonly carry φ2 and φ6 [25], or a φAvβ prophage [24]. By contrast, isolates belonging to the human clade contain β-converting φ3 prophage variants that encode and express two human-specific virulence genes (*chp* and *scn*) [22, 23, 25]. There is now evidence that the emerging subpopulation differs from the ancestral human subpopulation by additional prophage features (φ1, φ2, φ5 or φ7) relevant to its epidemiology [23, 25]. More recently, the φMR11-like helper prophage has been described, that may facilitate the expression of the φ3 prophage virulence genes *chp* and *scn* [29].

This human-adapted CC398 subclone is now increasingly identified in hospitals. Also, a strong increase over time in the prevalence of *S. aureus* CC398 in bloodstream

infection was observed in a recent study [27]. Seventeen of the 18 bloodstream infection isolates were methicillin-susceptible and none had the common pig-borne *spa*-types t011, t034 or t108. The mode of acquisition of the *S. aureus* CC398 isolates by the patients remains unclear. Moreover, all *S. aureus* CC398 bloodstream infections were diagnosed in patients lacking livestock exposure. These invasive infections are in general associated with MSSA CC398 *spa*-type t571 [16, 27, 30]. These results were confirmed by our study (chapter 7). In total, 612 consecutive episodes of *S. aureus* bloodstream infections (BSI) diagnosed before and during the emergence of CC398 were included. Three strains (2 MSSA and 1 MRSA) that were isolated from bacteremia patients between 2010–2011 were positive in a CC398 specific PCR. There was a marked increase in prevalence of *S. aureus* CC398 BSI isolated between 2010–2011 compared to the combined collections that were isolated between 1996–1998 and 2002–2005 (3/157, 1.9% *vs.* 0/455, 0.0%; $p = 0.017$). We conclude that *S. aureus* CC398 might be an increasing cause of invasive staphylococcal disease. Our results are in line with other findings that certain *S. aureus* CC398 isolates, especially *spa*-type t571, can cause invasive infections in humans.

Dynamics of MRSA CC398 carriage

There is still debate about the dynamics of MRSA CC398 carriage in persons with regular contact to livestock. Question is whether an MRSA CC398 positive person is truly colonized or merely inhaled MRSA contaminated dust during work, without being truly colonized. This is an important distinction, because contamination with MRSA CC398 is less likely thought to lead to infection, and can probably easily be eliminated by a period of non-exposure. Cuny et al. [21] found an extreme high MRSA CC398 nasal colonization of 45% among livestock veterinarians. Carriage may be prolonged, as shown in a recent report from Germany, where the majority of pig farmers (59%) did not lose their MRSA CC398 carriage after the holidays [31]. Furthermore, when volunteers were actively colonized with methicillin-susceptible *S. aureus* (MSSA) CC398, they often carried it for prolonged periods [32]. We conducted a two-year prospective cohort study with 137 veterinarians that mainly work with pigs and veal calves (chapter 8). This study demonstrated a mean MRSA CC398 prevalence of 44% (range 42–46%). In total, 88 veterinarians (65%) carried MRSA CC398 at least once. Thirty-two veterinarians (23%) had MRSA-positive test results throughout the entire study period and 18 of those (56%, 13% of all veterinarians) had five identical MLVA types and can therefore be considered as true persistent MRSA CC398 carriers. On the

other hand, there are several studies that stated that MRSA CC398 is not a good colonizer in humans. A study among field workers with short-term occupational exposure to pigs and veal calves suggested a high rate of transient contamination, without substantial persistent colonization [33]. Another study showed that MRSA prevalence among veal calf farmers was strongly reduced (58%) after absence of animal contact [10]. The reasons for these discordant findings are unclear and require further investigation.

We conclude that regular livestock contact can indeed lead to true persistent colonization with MRSA CC398. Veterinarians that mainly work with pigs and veal calves frequently carried the same strain for prolonged periods.

Human-to-human transmission of MRSA CC398

Human-to-human transmission is the main determinant for the spread through a human population. The magnitude of the public health threat depends mainly on this characteristic, in combination with strain virulence. Several recent studies have shown that MRSA CC398 was 4 to 6-fold less transmissible compared with HA-MRSA strains in a hospital-setting [34–36], limiting its impact on public health and justifying modified control measures. At present, the human-to-human transmissibility of MRSA CC398 in a community setting is still unclear. In addition, studying the human-to-human transmissibility of MRSA CC398 is hampered by the fact that the reservoir of MRSA CC398 is restricted to humans having direct contact with livestock, and that the majority of individuals working in this sector live on the farms together with their families. The household members mostly have direct animal contact themselves. Therefore, livestock veterinarians are an excellent group for studying human-to-human transmissibility of MRSA CC398 since their household members do not have direct contact with pigs or veal calves themselves. We conducted a prospective cohort study in Dutch livestock veterinarians and their household members (chapter 9). Our study demonstrates a relatively high mean prevalence of MRSA CC398 colonization among household members of 4.0%. In total, 36 household members (9.3%), originating from 28 families (20.4%), harbored MRSA CC398 at least once during the one-year study period. These data confirm the results from a previous study performed in Germany in which an MRSA CC398 prevalence of 9.0% among household members of veterinarians was reported [21]. In our study, the prevalence of MRSA CC398 carriage among household members was shown to be highly dependent on the carrier state of the veterinarian.

In addition, to compare transmissibility of MRSA CC398 strains with other MRSA isolates, a cross-sectional survey was performed in MRSA-positive hospital-based patients and their household members. The prevalence of MRSA among household members was significantly higher for control patients carrying MRSA non-MC398 strains than for veterinarians carrying MRSA CC398 (PRR 6.0; 95% CI 2.4–15.5). These data suggest that MRSA CC398 spreads less easily from humans with professional livestock contact to their household members than other MRSA isolates in a community setting.

Considering the extensive reservoir in animals and people who work with livestock, the occurrence of MRSA CC398 in people who are not directly involved in farming is strikingly low. One of the first studies that examined the role of living in a livestock-dense region as a risk factor did not find it to be a risk factor (chapter 6). This cross-sectional survey found that only 0.2% of adult individuals without livestock contact were positive for MRSA CC398. Meanwhile, there are a few recent studies indicating that MRSA CC398 already have spread into the general population. The ways in which MRSA CC398 can be transmitted to humans without livestock contact are direct contact MRSA-positive individuals, environmental contamination [37–39], and eating or handling contaminated meat [40]. A recent study by Lekkerkerk et al. [41] found that MRSA with no link to established Dutch risk factors for acquisition, so-called MRSA of unknown origin (MUO), has now emerged. National MRSA surveillance data (2008–2009) were analyzed for epidemiological determinants and genotypic characteristics. A quarter (24%) of the 5545 MRSA isolates registered were MUO, i.e. not from defined risk groups. Two distinct genotypic MUO groups were distinguished: MUO CC398 (352; 26%) and MUO non-CC398 (998; 74%), which suggests spread of MUO CC398, not by direct contact with livestock (pigs, veal calves), but through other risk factors.

Transmission of MRSA CC398 within the healthcare setting has been observed in several studies. The first documented hospital-associated outbreak of MRSA CC398 occurred in the Netherlands [42]. The authors found five patients with MRSA CC398 colonization and/or infection. All strains belonged to CC398 and were *spa*-type t567. Likewise, an outbreak of MRSA CC398 in a nursing home in the Netherlands is described in chapter 10. Seven residents and two healthcare workers with MRSA CC398 were identified. The MRSA strain responsible for this outbreak was *spa*-type t011, which belong to CC398. The most likely source had been living on a pig farm until recently, before he moved to the nursing home. He reported regular visits to his son at the pig farm.

However, MRSA CC398 isolates are hard to discriminate when using current molecular typing techniques, such as *spa*-typing, MLST and MLVA [43]. Although the PFGE using *Cfr*9I provides a much better differentiation of CC398 isolates this method is laborious and yields data that are not easily electronically exchanged [44]. This hampers studies that investigate possible transmission events and outbreaks caused by this MRSA clade. The whole-genome mapping (WGM) described in chapter 11 provides a typing method with high discriminatory power that appears to be suitable to identify MRSA CC398 transmission events. The discriminatory power of WGM was illustrated by the ability to type and differentiate MRSA CC398 isolates obtained from epidemiologically unrelated veterinarians frequently visiting livestock farms for which *spa*- and MLVA-typing failed to provide clear distinction. Furthermore, WGM was performed to assess whether this technique was suitable to identify transmission events of MRSA CC398 in a community setting (i.e. transmission from veterinarians to their household members). Indeed, we obtained virtually identical WGMs of the isolates obtained from livestock veterinarians and their household members. We conclude that WGM now enables us to identify transmission events of MRSA CC398 which would be impossible using *spa*-typing or MLVA and with much more uncertainty when using PFGE. We are currently conducting WGM of isolates obtained from a larger number of veterinarians and their household members to study MRSA CC398 transmission among this group in further detail.

Virulence of MRSA CC398

The capacity of livestock-associated MRSA CC398 to cause infections in humans has been demonstrated, ranging from relatively minor or localized infections [34, 45–47], as well as more serious or invasive infections [30, chapter 6]. Despite the diverse array of infection types reported, it has been suggested that MRSA CC398 is less virulent than other human MRSA strains. Nevertheless, the exact morbidity associated with MRSA CC398 carriage in relatively healthy individuals is largely unknown. Considering the huge spread of MRSA CC398 among persons with livestock contact, there is a need to monitor the occurrence of infections caused by MRSA CC398 in the community.

Our prospective cohort study demonstrates that persistent MRSA CC398 as well as persistent *S. aureus* carriers had significantly more skin and soft tissue infections (SSTIs) than *S. aureus* non-carriers during a two-year study period (chapter 12). Moreover, carriage of MRSA CC398 and MSSA at a given sampling moment were found to be associated with a higher chance of developing a SSTI in the next 4 months

as compared with *S. aureus* non-carriers. Our data confirm the results of several studies that MRSA CC398 carriage mainly causes moderate to severe SSTIs [30, 45, 46, 48]. Another recent study demonstrated that MRSA CC398 was not less pathogenic for humans than *S. aureus* in general [49].

We found that the risk of having SSTIs during the study period among MRSA CC398 and MSSA carriers was comparable, indicating that the SSTIs were caused by *S. aureus* carriage alone and not by the fact that these strains were methicillin-resistant. Noteworthy, chapter 8 describes an extremely high mean prevalence of *S. aureus* carriage (72%) among livestock veterinarians, which is high compared to the general population [50]. Therefore, livestock veterinarians are more likely to develop a SSTI compared to the general population, and *S. aureus* carriage (MRSA CC398 and MSSA) can now been seen as an occupational hazard.

Public health threat by MRSA CC398

The previous paragraphs have shown that a public health threat may arise from livestock-associated MRSA CC398, which needs to be controlled. At present, in healthcare settings MRSA CC398 appears to be under control. The impact on public health of MRSA CC398 appears to be low at the moment, illustrated by the limited spread into the community and the minimal amount of serious invasive infections caused by MRSA CC398, as shown in chapters 6 and 7. While prevalences in livestock farming probably have increased close to a saturation point, MRSA CC398 seldom seems to cause serious problems in hospitals. Nonetheless, experts worry that the rapid evolution of MRSA CC398 may result in gaining new characteristics in the near future [23, 24], since MRSA CC398 has proven to be able to exchange many mobile genetic elements between strains, suggesting that this clade can rapidly adapt to changes [25, 51]. Also, a study based on whole-genome sequencing provided evidence that the clade of MRSA CC398 originated in humans, and lost some immune evasion genes when it entered the livestock population, while acquiring resistance genes. This creates worries about a possible reverse event (acquisition of virulence factors while maintaining resistance traits) from the immense reservoir that has been created in livestock. Indeed, there is now evidence from a recent study that livestock-associated MRSA CC398 is now readapting to humans [29]. In this study, hybridization of genomic DNA with microarrays revealed no livestock-associated prophages remnants in the genomes of the ancestral human subpopulation. In contrast, they also demonstrate that the human-adapted isolates and the livestock-associated isolates share some

prophage elements, suggesting an animal origin of the newly described φMR11-like helper prophage. However, further investigations are required to confirm these findings.

Future research

The two-year prospective cohort study described in chapters 8, 9 and 12 contains valuable epidemiology information. MRSA as well as MSSA isolates have been collected from veterinarians and their household members that had no livestock contact, and further molecular characterization with sophisticated typing methods can give more insight into the exact mechanisms of persistent carriage of MRSA CC398, higher transmissibility and virulence factors. We are currently monitoring a large number of MRSA and MSSA CC398 isolates using whole-genome sequencing to assess the risk for the emergence of a sustainable community reservoir for livestock-associated *S. aureus* CC398.
In addition, we have identified several veterinarians that never were colonized with *S. aureus* during the two-year study period despite an extremely high exposure to MRSA CC398 and MSSA in pig and veal calf stables. These individuals seem to be "immune" to colonization. Determination of the genetic characteristics and the microbiome of the nares of these non-carriers may result in targets for new drugs or vaccines to prevent colonization and subsequent infection.

To evaluate whether the MRSA CC398 prevalence in people without livestock contact is rising in an area with a high density of pig farms a cross-sectional survey can be performed once again after five years.

Conclusions

The research in this thesis gained more insight into the detection, dynamics, transmissibility and virulence of MRSA CC398 in veterinarians with livestock contact, and contributed to a better understanding of the possible public health threat. The following main conclusions can be drawn from this thesis: (1) Oxoid *Brilliance*™ MRSA Agar, Bio-Rad MRSA*Select*™ medium and chromID® MRSA medium are all sensitive method for the screening of MRSA CC398 in combination with broth enrichment, but positive results require confirmation. Our studies showed a substantial increase in

sensitivity following enrichment compared with direct cultures. Therefore, broth enrichment are indispensable for reliable MRSA detection. (2) Regular livestock contact can indeed lead to true persistent colonization with MRSA CC398. Veterinarians that mainly work with pigs and veal calves frequently carried the same strain for prolonged periods. (3) A relatively high mean prevalence of MRSA CC398 colonization of 4.0% was found among household members. In addition, prevalence of MRSA CC398 carriage among household members was shown to be highly dependent on the carrier state of the veterinarian. Also, MRSA CC398 spread less easily from humans with professional livestock contact to their household members than other MRSA isolates in a community setting (RR 6.0; 95% CI 2.4–15.5). (4) The cross-sectional survey we performed in areas with an extremely high pig-density found that only 0.2% of adult individuals without livestock contact were positive for MRSA CC398, which is comparable to the carriage level of the general population. (5) Whole-genome mapping enables discrimination of MRSA CC398 and identification of transmission events, which would be impossible using *spa*-typing or MLVA and with much more uncertainty when using PFGE. (6) Veterinarians with persistent MRSA CC398 and persistent *S. aureus* carriers had significantly more skin and soft tissue infections than *S. aureus* non-carriers during a two-year study period.

Recommendations

The Dutch search and destroy policy was revised in December 2012, and all household members of confirmed MRSA patients have to be screened for MRSA on hospital admission. Nonetheless, household members of livestock veterinarians are not yet screened upon admission to a hospital. However, we showed that they have a relatively high MRSA carriage in comparison to the Dutch general population [50], independent of the MRSA state of the veterinarian. Consequently, we advocate that household members of all livestock veterinarians should also be screened for the presence of MRSA carriage upon hospital admission.

From the public health perspective, continuous surveillance of the virulence potential, antimicrobial resistance profiles and human colonization capacity of MRSA CC398 is strongly recommended. Simultaneous adaptation of CC398 to humans and animals would represent a considerable threat to public health, because of the huge CC398 reservoir in livestock, combined with the fact that this lineage seems to acquire foreign DNA quite easily.

When MRSA CC398 eventually will readapt to humans and can successfully spread from human to human it will constitute a significant public health problem in the near future. This will necessitate major adaptations of the existing guidelines for control in the community and in healthcare institutions. It is questionable if the current control strategy in the Netherlands can be maintained in this scenario. At least the associated costs will increase significantly.

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