1 **Title**

2 What is the origin of Livestock-associated MRSA CC398 isolates from humans without

3 livestock contact: an epidemiological and genetic analysis.

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18 **Running head**

19 LA-MRSA CC398 from humans without livestock contact

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Abstract

Fifteen percent of all MRSA CC398 human carriers detected in The Netherlands had not been in direct contact with pigs or veal calves. To ensure low MRSA prevalence it is important to investigate the likely origin of these MRSA of unknown origin (MUO). Recently, it was shown that CC398 originating from humans and animals differ in the presence of specific mobile genetic elements (MGEs). We hypothesized that determining these specific MGEs in MUO isolates and comparing them with a set of CC398 isolated from various known origin, could provide clues to their origin. MUO CC398 isolates were compared to MRSA CC398 isolates obtained from humans with known risk factors, an MRSA CC398 outbreak isolate, LA-MRSA CC398 isolates from pigs, horses, chickens and veal calves, and five MSSA CC398 from known human origin. All strains were *spa*-typed and the presence or absence of, *scn*, *chp*, *φ3 int*, *φ6 int*, *φ7 int, rep7*, *rep27* and *cadDX* was determined by PCR. The MRSA CC398 in humans, MUO or MKO, resembled MRSA CC398 as found in pigs, and not MSSA CC398 as found in humans. The distinct human MSSA CC398 *spa*-type, t571, was not present among our MRSA CC398 strains, MRSA CC398 were tetracycline resistant and carried no *φ3* bacteriophage with *scn* and *chp*. We showed by simple PCR means that human MUO CC398 carriers carried MRSA from livestock origin, suggestive for indirect transmission. . Although the exact transmission route remains unknown, direct human-to-human transmission remains a possibility as well.

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Introduction

In The Netherlands, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) is low (1) and Dutch MRSA strains display a broad clonal diversity (2). One exception is the livestock-associated clone CC398, a major clonal reservoir in pigs and veal calves (3) and subsequently people with occupational exposure to animals. The reported number of MRSA CC398 has been around 40% of reported MRSA to the Dutch MRSA surveillance since 2008 (2, 4). However, only 78% of reported CC398 are found through screening of patients with direct (occupational) contact to pigs or veal calves at hospital admission (a risk factor introduced in 2006) (5).

The remaining MRSA CC398 carriers do not comply to the described risk factors in the Dutch MRSA guideline: contact with industrial, live pigs, veal calves or broiler chickens regardless whether this contact was occupational or not and/or the person lives on such a farm. Currently 15% (352/2312) of all Dutch and 15% (24/164) of all Danish MRSA CC398 carriers have not been in direct contact with pigs or veal calves (2, 4). In The Netherlands, these MRSA CC398 carriers are considered a MRSA of Unknown Origin (MUO) subgroup (MUO CC398). With MUO being any MRSA reported to the MRSA surveillance without known risk factors as defined in the Dutch MRSA guideline (4).

The reservoir or transmission route of MUO CC398 still remains unknown: possible transmission routes are direct animal-to-human transmission of animal sources not included as risk factor in the MRSA guideline (due to being unknown or a limited effect on the population as a whole), indirect animal-to-human transmission, through the environment e.g. by dust or air vehicle (6, 7), animal products such as meat (8), or human-to-human transmission (9). Hospital outbreaks of CC398 have been described illustrating the potential of human-to-human transmission by this clonal complex (10). Although the general thought is that long term colonization of CC398 in humans is rare, it was recently shown that CC398

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from animal origin *can* survive in a human nose, for at least 21 days, suggesting their ability to colonize humans (11). MUO CC398 is therefore an important topic, and the necessity to elucidate the origin of MRSA CC398 in humans without direct contact to pigs or veal calves (MUO CC398) is clear.

From genomic analyses on CC398 of different origins it can be concluded that the origin of CC398 is most likely human (12, 13). There are indications that methicillin-susceptible *Staphylococcus aureus* (MSSA) CC398 switched host in the past as result of human-animal interaction (12, 14), and that it adapted to animals by losing several Mobile Genetic Elements (MGEs) while gaining other MGEs, including resistance to tetracyclines and methicillin, before being reintroduced in humans as MRSA (3, 15).

McCarthy et al. showed that CC398 from humans in contact with animals, differed from strains isolated from humans without contact with animals. The difference was seen in MGE-located genes, e.g. *φ3 int*, *chp*, *scn*, *rep27*, *φ7 int* and *cadDX* for humans, and *rep7*, *φ6 int* for pigs, in addition to genes encoding resistance to tetracycline and trimethoprim (14). We therefore hypothesized that presence of these MGE-encoding genes, but also the resistance to tetracycline and trimethoprim/sulfamethoxazole, could be used as a cheap and fast method to compare MUO CC398 with isolates from humans (MSSA and MKO CC398) and animals (MRSA CC398) to predict the origin of the MUO CC398 in The Netherlands.

We showed that MUO and MKO isolates resembled CC398 isolates from animal origin more closely than CC398 isolated from human origin, indicating that these MUO CC398 most probably originated from livestock.

Materials and Methods

Bacterial strains and growth conditions

In total 119 isolates were included in the study (Figure 1). All isolates were predicted to have a CC398 background, based on MLVA typing. (http://www.mlva.net/) MLVA is the National Institute for Public Health and the Environment (RIVM) choice, due to costs, as well as there being an agreement between MLVA and MLST. Only STs belonging to the same MLST clonal complex were grouped by MLVA. Furthermore, *spa*-types show a remarkable agreement between the *spa*-types associated with MLST clonal complexes and the MLVA complexes (16). The MLVA complexes were therefore named in accordance to the MLST one. The MLVA complex 398 is thus equal to the MLST clonal complex 398. The isolates were all from The Netherlands and from 2009, except an outbreak strain from 2007 and five MSSA isolates from human origin, previously described and isolated at the Erasmus MC in the period of 1998-1999 and 2002 (13, 17). All CC398 *S.aureus* isolates were stored at -80°C and grown on sheep blood agar plates (RBS) (Becton, Dickinson & Co., Belgium) at 37ºC overnight.

Bacterial strain selection from animals

The 80 MRSA strains of animal origin included in this study were previously collected from livestock: pigs (n=24), veal calves (n=20), chickens (n=20) and horses (n=16). The pig isolates were from apparently healthy animals and originated from eight different farms across The Netherlands that were screened as part of a pilot for a later study by Broens *et al.* (18). The healthy veal calves were sampled at arrival on three Dutch farms (19). The horse strains were nearly all (94%) samples from diseased horses that visited the Utrecht University equine clinic, the remaining 6% being healthy horses. The chicken isolates were obtained from a study in six broiler slaughterhouses, where broilers from 40 flocks arriving at the slaughterhouses were sampled in the pharynx after stunning (20). *S. aureus* isolates were *spa*-typed by the RIVM according to Harmsen *et al.* (21). From the available livestock MRSA

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isolates (n=459) the largest variability in *spa*-types was chosen (n=80) (figure 2); whether an isolate from either screening or a clinical case, was not a selection criterion. This resulted in a selection with both screening and clinical isolates.

Bacterial strains selection from humans

The MRSA strains of human origin included an outbreak strain (n=1), MUO (n=6) and MKO (n=27). The outbreak strain reported in 2007 was chosen because it caused nine secondary cases (both patients and healthcare workers) in a single Dutch hospital after MRSA was cultured from a diabetic foot ulcer of a patient on a surgical ward (10). Both MUO (n=6) as MKO (n=27) were from a previous study, in which an extended questionnaire was send to MRSA carriers. Five MSSA isolates were also from human origin. These isolates were previously described and isolated at the Erasmus MC in the period of 1998-1999 and 2002 (13, 17).

Extended questionnaire study

Around 3000 MRSA are reported to the Dutch national MRSA surveillance by medical microbiological laboratories from The Netherlands with epidemiological data on applicable risk groups (2, 5). Potential MUO carriers reported to the surveillance, were approached by extended questionnaire. The questionnaire was set up to determine the known risk factors for MRSA, as described in the Dutch WIP guideline on MRSA (Supplementary Table 1)(4), as well as further questions on risk factors as described in the literature, which was searched in PubMed up till 01-01-2010, using search keywords 'MRSA' and 'risk factor' (Supplementary Table 2).

S. aureus genotyping, detection of expression of β–haemolysin and DNA isolation

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After overnight culture on RBS plates, haemolysin patterns were determined to detect expression of β–haemolysin. No expression of β–haemolysin indicates the insertion of the *φ3* bacteriophage into the bacterial genome, as *φ3* inserts on the site that codes for β–haemolysin (22). DNA was isolated, using a MagNA Pure (Roche) according to the protocol supplied by the Manufacturer.

Mobile Genetic Elements

The presence or absence of MGEs was determined by PCR's specific for: *cadDX, φ3 int*, *scn*, *chp*, *rep7*, *rep27* , *φ6 int* and *φ7 int* (14, 22, 23). Primers for *φ3 int* (Forward primer: TCCGGCTTCTTTGAAAATGT, Reverse primer: CCGGAAAACCTACGAAGTCA, amplicon size 220-323bp, annealing temperature 50°C.) and *cadDX* (Forward primer: TGATGTGATCTGTGTACATGAGGA, Reverse primer: TGATGTGAAGTTGAAGCAACA, amplicon size: 207bp, annealing temperature 60°C) were designed with primer3 software (http://frodo.wi.mit.edu/). All amplified PCR products were visualized by agarose gel (1.2%) electrophoresis. (See also Supplementary Table 3 and 4.)

Antimicrobial susceptibility

To determine antimicrobial susceptibility of *S. aureus* strains, standard disc diffusion method was applied using Oxoid™ antimicrobial susceptibility test discs (Thermo Fisher Scientific, Waltham USA) on MH-agar plates. The Clinical and Laboratory Standards Institute (CLSI) breakpoints were used for tetracycline (zone diameter breakpoints: S ≥19 mm, I 15-18 mm, R ≤14 mm.) and trimethoprim/sulfamethoxazole (zone diameter breakpoints: S ≥16 mm, I 11-15 mm, R ≤ 10 mm) (24).

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Statistical analysis

Statistical analysis was performed with SAS Enterprise Guide software (version 4.2 by SAS Institute Inc., North Carolina, USA) using 2x2 tables and Fisher's exact test. P-values of <0.01 were considered significant to correct for multiple testing. A comparison was made between animal and human hosts, as well as between human epidemiological subgroups. Isolates were clustered transversally, using the Jaccard coefficient, on MGE presence, β–haemolysin expression and susceptibility for tetracycline and trimethoprim/sulfamethoxazole. The dendrogram was created based on UPGMA with Jaccard similarity.

Results

Two hundred and seventy-seven suspected MUO carriers from all of The Netherlands were approached by an extended questionnaire, of which 42% (116) responded and 33 were defined as CC398 carriers. Of these 33 CC398, 6 were MUO (CC398) and confirmed to have had no contact with pigs, veal calves or (broiler) chickens in the year before questioning. The MUO CC398 carriers were found to reside in the Dutch province 'Noord Brabant' where there is generally more pig farming.

All MUO and MKO CC398 strains were distinctly different from human MSSA CC398 not only in *spa*-type, but also based on β-haemolysin expression, tetracycline resistance, lacking *φ3 int*, *scn* and *chp* genes. The human MRSA CC398 strains resemble animal MRSA CC398 strains (Figure 3). The presence of *cadDX* and *rep27*, considered human-associated genetic markers, as they were highly prevalent in human MSSA and significantly less prevalent in animal MRSA (14), were absent in MUO and few in MKO strains: *cadDX* (0/6 for MUO and 9/27 for MKO) and *rep27* (0/6 for MUO and 4/27 for MKO). In horse and pig isolates, *cadDX* was almost absent, while in veal calf and chicken isolates *cadDX* was found frequently: veal calves (16/20; 80%) and chickens (12/20; 60%). *Rep27* was absent in horse

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and veal calf isolates, and only incidentally found in chickens and pigs: 8% (2/24) for pigs and 20% (4/20) for chickens. All 119 isolates of both MRSA and MSSA isolates were similar in full susceptibility for trimethoprim/sulfamethoxazole. Also, there was no significant difference between MUO and MKO in *rep7*, *rep27*, *φ6 int*, and *cadDX* content, resulting in MUO and MKO clustering together, despite some minor differences in MGE content.

When the combined data of MUO and MKO were compared to animal isolates, it was clear that human isolates were less often *φ6 int* positive than MRSA from veal calves or horse isolates ($p=<0.01$), more often *rep7* positive than horse isolates ($p<0.01$), more *rep27* positive than pig isolates ($p<0.01$) and less *cadDX* positive than isolates from veal calves and chickens ($p<0.01$). No significant differences between MRSA isolates from human subgroups (MUO, MKO, outbreak) were found for *rep27*. Interestingly, the hospital outbreak strain lacked any previously mentioned human or pig-associated markers (*rep7*, *rep27*, *φ3, φ6 int*, and *cadDX*), but displayed tetracycline resistance. MGE variation within a single *spa*-type was observed for human as well as animal isolates (Figure 3).

Discussion

Human MRSA CC398 isolates (MUO and MKO) in this study resembled animal MRSA CC398 more than they resembled human MSSA CC398, because they were β-haemolysin producers, tetracycline resistant, had similar MGE patterns, and had *spa*-types similar to those found in animals. Furthermore, our MUO in cluster analysis almost always clustered together with MKO. The similarity between MUO CC398, MKO CC398 and animal MRSA CC398 suggest that these MUO CC398 belong to the same MRSA clade originating in animals, and that these MUO CC398 are not part of the MSSA CC398 clade detected in humans. Stegger *et al.* found two distinct phylogenetic clades based on single-nucleotide polymorphisms (SNPs), revealing a basal human clade and a more derived livestock clade (25). Although no whole-

genome sequencing or SNP-analysis was done, the outcome of our cheaper and quicker MGE-based method strongly suggests that these MUO CC398 belong to the livestock clade with MKO CC398 and MRSA CC398 from animals, and not to the MSSA CC398 clade found in humans. The lack of risk factors in our MUO CC398 carriers suggest spread of animal MRSA CC398 by other means than currently described in the MRSA guideline.

The exact mode of transmission remains unanswered. An indirect route of transmission would be the most likely mode of transmission for MUO CC398, taking into consideration where the MUO CC398 carriers live, their lack of contact with pigs and veal calves, but also their lack of contact with horses and chickens. Since, living in high-density pig areas (6), as well as private farm visits (26), was a risk factor for livestock-associated MRSA carriage, modes of indirect transmission are most likely through area contamination in which people live and interact. Considering *S. aureus* survival in the environment and subsequent spread by air over large distances (7), transmission by air is a possibility (27), as well as transmission by vectors such as rodents (28). Nevertheless, transmission by human-to-human contact cannot be ruled out: of the six MUO CC398 carriers investigated by extended questionnaire in this study, one MUO CC398 carrier stated to have had contact with an MRSA carrier (unknown who or which MRSA type) outside the family or household, while another had visited a farm without contact to animals. Neither is currently considered an at risk event. In the Dutch guideline MRSA positive household members are considered as a risk, but contact outside the household or hospital, in the community, is not.

We know that a CC398 pig MRSA, lacking *φ3*, can survive up till 21 days in a human nose (11), whereas *φ3* is currently considered to be *the* marker for human host adaptation (12). The successful outbreak isolate reported by Wulf et al (10), lacked *φ3* as well. Human host adaptation is explained by more than *φ3* alone or host adaptation might not have to be as extensive to facilitate transmission. In regards to the outbreak isolate, further research is

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necessary to determine what makes this outbreak isolate so different and successful compared to other human MRSA isolates.

Despite no significant difference between MUO and MKO for genes encoded by MGEs, there were slight differences observed for *cadDX* and *rep7* between MUO and MKO. Furthermore, *rep7* positive isolates were as common in MKO as in animals, unlike MUO which showed significantly less *rep7* than among pig strains ($p<0.01$). *rep7* and *rep27* genes are typical of small plasmids encoding resistance genes, and *rep7* is reported to be associated with the tetracycline resistance gene, *tetK* (29). We also observed MGE variation within single *spa*-types within humans or animals, as can best be seen in t011 and t108, fitting the known relative stability of MGE (30).

As for limitations of this study, we do not know whether our isolates were obtained from persistent MRSA carriers or transient carriers (contaminated humans). Follow-up data are important to better understand host adaptation, but since this was a retrospective study, carrier data over time was unfortunately not available. This study's strength is the questionnaire that allowed discrimination between MRSA CC398 with and without known risk factors, regardless of guideline changes. The number of MUO CC398 are few in this study as 28% (33/116) of respondents was a CC398 carrier, and only 21% (6/33) fitted the MUO definition. However, the number of MUO CC398 per year is just over 5% for The Netherlands, which means on average an additional 150 Dutch people with a MRSA CC398 while lacking risk factors as described in the Dutch MRSA guideline (2012).We showed by simple PCR means that MUO CC398 carriers in this study carry MRSA from CC398 livestock origin. This finding is suggestive for an indirect transmission route, possibly the environment (air, water) or through fomites, but we cannot rule out direct human-to-human transmission. Although, the reported numbers of MUO CC398 in The Netherlands are currently still small, the problem may increase, giving rise to more CC398 transmission and human host adaptation.

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Figures and tables

Figure 1 – Flowchart

MUO: MRSA without known risk factors as described by the Dutch national guideline .

MKO: MRSA with known risk factors as described by the Dutch national guideline. A CC398 MKO is a pig-, veal calf, farmer or a person with direct contact to pigs and/or veal calves, or living on a pig or veal calf farm, or a broiler chicken handler. RIVM, National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

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402 **Figure 2 – Selected *spa*-types for human and animal groups**

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404 MKO: MRSA of Known Origin (known risk factors described in Dutch MRSA guideline),

405 MSSA: Methicillin susceptible *Staphylococcus aureus*, MUO: MRSA of Unknown Origin

406 (unknown risk factors not described in Dutch MRSA guideline 2012), Outbreak isolate

407 described by Wulf et al. Euro Surveill. 2008 Feb 28;13(9).

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Figure 3 – Results of β-haemolysin screening, PCR typing and susceptibility testing

Isolates were clustered transversally, using the Jaccard coefficient, on MGE presence, β–haemolysin expression and susceptibility for tetracycline and trimethoprim/sulfamethoxazole. The dendogram was created based on UPGMA with Jaccard similarity. Epidemiological subtypes in humans: MKO (MRSA of Known Origin; MRSA with known risk factors for acquisition), MUO (MRSA of Unknown Origin: MRSA with unknown risk factors for acquisition). "MUO 2007" were MUO according to the 2007 guideline definition, but no longer under the 2012 guideline definition. "MUO 2012" are MUO according to the current guideline of December 2012. Outbreak (An isolate involved from a MRSA CC398 outbreak in a Dutch hospital: described by Wulf et al. Euro Surveill. 2008.), Mobile genetic elements: *chp* (Gene encoding chemotaxis-inhibiting protein (CHIPS). This gene is found in the φ3-bacteriophage that contains the Immune Evasion Complex (IEC) of which *chp* is sometimes part of), *scn* (Gene encoding Staphylococcal complement inhibitor (SCIN). This gene is found in the φ3-bacteriophage that contains the Immune Evasion Complex (IEC) of which *scn* is sometimes part of), *Φ3 int* (Integrase gene of bacteriophage 3), *Φ6 int* (Integrase gene of bacteriophage 6), *Φ7 int* (Integrase gene of bacteriophage 7), *rep7* (Replication protein 7), *rep27* (Replication protein 27), *cadDX* (Operon of gene *cadX* (cad operon regulatory protein), which encodes resistance against the heavy metal cadmium), Antimicrobial susceptibility: Tetracycline (Tetracycline susceptibility testing), Trim./sulfa. (Trimethoprim/Sulfamethoxazole susceptibility testing).