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# *Staphylococcus aureus*
# of Unknown Origin

SYBREN LEKKERKERK

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**Dutch Methicillin-resistant *Staphylococcus aureus* of Unknown Origin**
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# Dutch Methicillin-resistant *Staphylococcus aureus* of Unknown Origin

## Nederlandse meticilline resistente *Staphylococcus aureus* van onbekende origine

Proefschrift

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# General introduction, aim and outline of this thesis

1

This thesis is on MRSA of Unknown Origin (MUO). MUO carriers are lacking risk factors as described in the national MRSA guideline. Without risk factors, MRSA carriers are not screened at hospital admission and go undetected. In general, MUO are detected by accident in a clinical culture or in a screening culture searching for a particular outbreak strain. Instead a genetically different strain is found. MUO will undermine our Search & Destroy (S&D) if left unattended. Important questions such as 'Which new risk factors describe MUO?', 'What is the genetic background of MUO?' and 'Is MUO a result of new transmission routes?' need answers. In this thesis we try to find these answers.

## CHANGING EPIDEMIOLOGY OF MRSA

During the last decade, the total number of MRSA reported to the Dutch MRSA surveillance has increased. (Figure 1) As has the number of MUO. (Figure 1) Furthermore, Dutch MRSA prevalence was 0.03% at hospital admission in 2000[1] and 0.11% in 2007[2]. Nethmap data from 2016 reported an 1.7% MRSA prevalence among reported clinical *S. aureus* isolates (including blood samples) collected by the Infectious Disease Surveillance and Information System for Antibiotic Resistance (ISIS-AR).[3] The low Dutch prevalence is – together with the Scandinavian countries - the exception to the European rule (Table 1). Currently, the number of reported MRSA is about 3000 MRSA isolates per year [4]. This is a low number, but has increased compared to the past, as this number was under 500 MRSA isolates per year before 2001.[5] (Figure 1) The current low MRSA numbers in The Netherlands and Scandinavia are most likely due to a combination of MRSA control measures and a prudent use of antibiotics.[6] In this regard, Scandinavia and The Netherlands are similar. For example, in their use of MRSA risk groups for MRSA screening. Although differences exist (Table 3).

Globally, the epidemiology of MRSA has changed as well. Due to or as a consequence of this, is that the classical division between hospital-acquired (HA-) and community-acquired (CA-) MRSA has gradually faded. The result was a surge of epidemiological terms in an attempt to describe the new MRSA situation: CA-MRSA (community-acquired, community-associated), HA-MRSA (first meaning hospital-acquired, and then hospital-associated or healthcare-associated), HCA-MRSA (healthcare-associated), HCA-CO-MRSA (healthcare-associated community onset), CO-MRSA (community-onset), HCA-HACO MRSA (healthcare-associated hospital-onset), HACO-MRSA (healthcare-associated hospital-onset).[7-9] The global change in epidemiology may be a driving factor for Dutch MUO. However, it is important to realize that MUO is not by default community MRSA (whether labelled as CA-MRSA, CO-MRSA or any of its many variants). The MUO label does not seek to impose a spatial division such as is the case with aforementioned terminology (CA-, CO-, HA-MRSA, etc.). MUO is simply the absence of known (Dutch) risk factors.

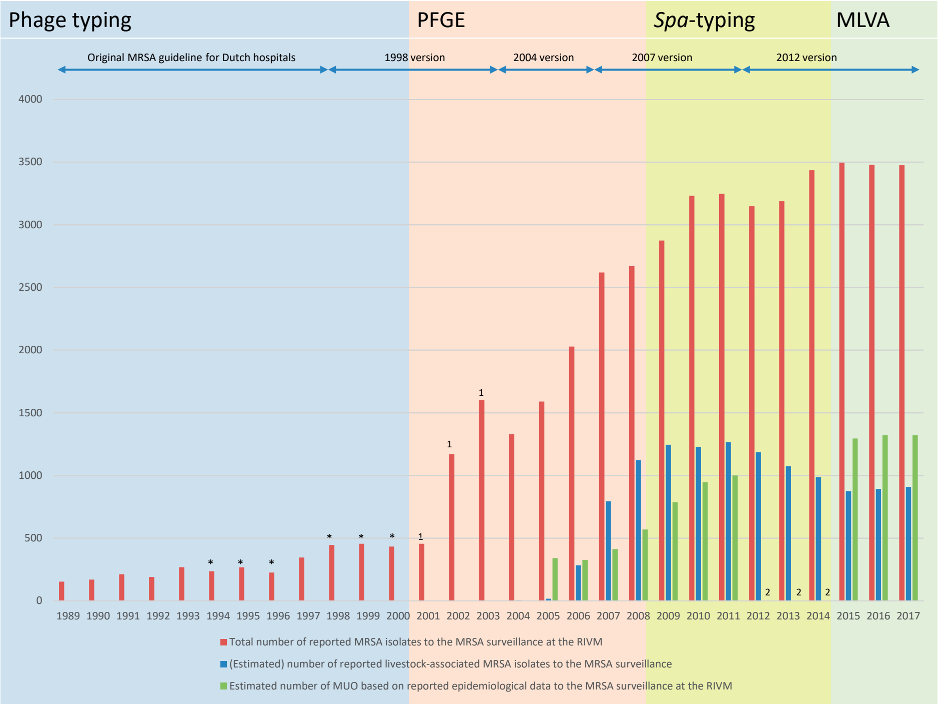


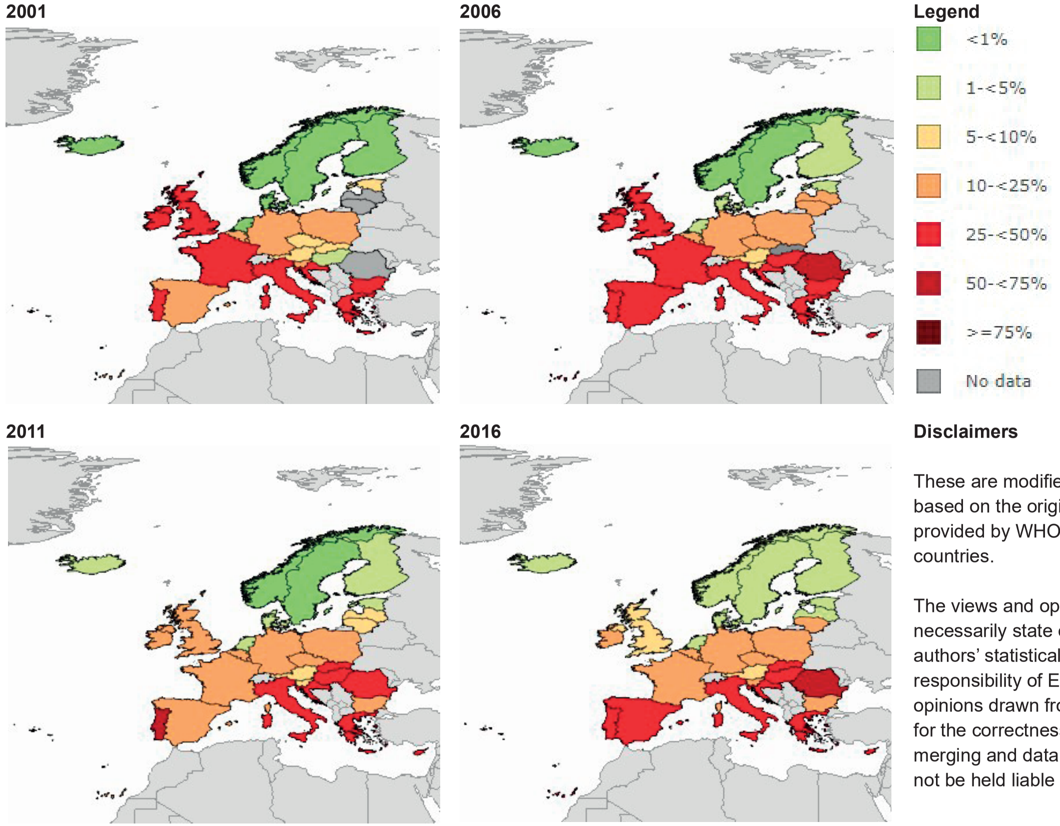
**Figure 1 – Reported total, MUO and live-stock associated MRSA isolates to the Dutch MRSA surveillance between 1989 and 2017**

* estimated number of MRSA isolates based on published figures (graphs [23-27]); [1] Only the number of MRSA isolates from patients were given, no health care workers. [2] No or minimal MRSA surveillance reports these years, thus no public data to show the number of MUO. This figure was based on the following public sources: [20-44]

## CHANGING DUTCH MRSA CONTROL

As epidemiology changes, so must MRSA control. Over the years the number of risk factors described in the Dutch MRSA guideline made by the former Dutch Working Party for Infection Prevention (WIP; 1981-2017) [10] has gradually increased. (Table 2) The original guideline at the start of the 1990's, listed three risk groups: MRSA positive patient, exposure through unprotected contact with a MRSA carrier, and a stay in a healthcare centre abroad. The last, from 2012 with a 2016 Rijksinstituut voor Volksgezondheid en Milieu (RIVM) addendum, lists ten major risk factors (MRSA positive patient, exposure through unprotected contact with a MRSA carrier [now including MRSA patients under follow-up after eradication therapy and household members of MRSA carriers], and a stay in a healthcare centre abroad, being a dialysis patient, contact with pigs[11], contact with veal calves, contact with broiler chickens, adoption and being an asylum seeker).[12, 13] If the targeted population described in the risk groups is sufficiently distinct of the overall population, then MRSA carriers can be successfully discerned.

**Table 1 – MRSA prevalence in Europe, 2001-2016**



**Disclaimers**

These are modified screen captures from the ECDC-website, based on the original dataset provided by ECDC based on data provided by WHO and Ministries of Health from the affected countries.

The views and opinions of the authors expressed herein do not necessarily state or reflect those of the ECDC. The accuracy of the authors' statistical analysis and the findings they report are not the responsibility of ECDC. ECDC is not responsible for conclusions or opinions drawn from the data provided. ECDC is not responsible for the correctness of the data and for data management, data merging and data collation after provision of the data. ECDC shall not be held liable for improper or incorrect use of the data.[45]

Methicillin Resistant *Staphylococcus aureus* (MRSA) isolates as proportion of total *Staphylococcus aureus isolates* (%).EARSS data 2001-2016, retrieved from https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc

**Table 2 – Dutch MRSA risk factors in Dutch MRSA guideline by WIP working group for infection prevention (1989-2018)**

| | | |
|---|---|---|
| **Original** | | • MRSA positive patient |
| | | • Exposure through unprotected contact with a MRSA carrier |
| | | • A stay in a hospital abroad |
| **1998** | Guideline now applies for patients AND healthcare workers | |
| **2004** | *Added* | • Being a dialysis patient |
| | | • Contact with pigs |
| | | • Contact with veal calves |
| | | • Family members of those who have contact with pigs/veal calves |
| | | • Adopted children with regular hospital contact |
| | | • Other |
| **2007** | *Removed* | • Family members of those who have contact with pigs/veal calves |
| **2012** | *Expanded* | • Exposure through unprotected contact with a MRSA carrier<br>o MRSA patients under follow-up eradication therapy<br>o Household members |
| | *Changed* | • A stay in a healthcare center abroad |
| | *Added* | Contact with broiler chickens |
| **2015** | *Added* | Being a refugee[1] |

Major changes to the Dutch MRSA guideline by the Working group for Infection prevention (WIP). [1] Risk factor added in the post-WIP era.

In S&D, risk adjustment aims to identify every potential MRSA carrier at hospital (and nursing home) admission by screening persons at risk for MRSA carriage (search). Patients screened positive for risk on MRSA are put into isolation and these contact precautions continue awaiting the results of the medical microbiological laboratory (preemptive isolation). MRSA carriers are kept in isolation and eventually treated to eradicate the MRSA (destroy) and their environment will be disinfected (destroy).[14]

To continue control of MRSA despite changing epidemiology, MRSA must be surveilled. Therefore, the MRSA surveillance at the RIVM [4], was started in 1989 at the request of the Geneeskundig hoofdinspectie (GHI; now known as Inspectie Gezondheidszorg en Jeugd / IGJ, and formerly known as the Inspectie voor de Gezondheidszorg / IGZ) [15]. The MRSA surveillance officially exists to gain insight as to why certain people are infected or colonized by MRSA, and to support the (then) independent Dutch WIP foundation in updating the Dutch national MRSA guideline by supplying it data.[16] The surveillance keeps track of total MRSA numbers, the genetic background (before: phage-type, PFGE-type, *spa*-type; now: MLVA complex; but also the presence of Panton-Valentine Leucocidin virulence factor [PVL]) and announces any epidemiological changes to MRSA in The Netherlands.[17]

To surveil MRSA, the RIVM maintains a database of MRSA isolates, with one isolate per unique person per year.[17] The first MRSA positive isolate of each detected MRSA carrier is send to the RIVM along with some epidemiological information (including the risk group the carrier belonged to). Unless there is an isolate from a clinical sample (e.g. from a wound) in the same period as carriage is identified, the RIVM prefers this isolate over

**Table 3 – MRSA Risk factors in national guidelines of low MRSA prevalence countries**

| | Denmark | Finland | Iceland | Netherlands | Norway | Sweden |
|---|---|---|---|---|---|---|
| Proven carrier of MRSA | X | X | X | X | X | X |
| Unprotected contact with MRSA carrier | X | X | X | X[T] | X | X |
| Inside the hospital (as part of contact tracing) | X | X | X | X | X | X |
| Outside the hospital (household members, partners, caretakers) | X | X | X | X | X | X |
| Stayed in a local care facility (unspecified) with an ongoing MRSA outbreak on the department | X | X | X | X[T] | X | X |
| Stayed in orphanage | . | X | X | . | . | X |
| **Related to higher MRSA prevalence among animals** | | | | | | |
| Contact with industrial live pigs, veal calves or broiler chickens | X[b] | X[b] | . | X | X[b] | X[b] |
| Regardless if professional contact or not | X[b] | X[b] | . | X | X[b] | X[b] |
| And/or lives on such a farm | X[b] | X[b] | . | X | X[b] | X[b] |
| **Related to higher MRSA prevalence abroad** | | | | | | |
| Adopted children from abroad living in this country | . | X | . | X | . | X |
| Foreign dialysis patients | . | . | X | X | X | X[S] |
| Local dialysis patients dialyzed abroad | . | . | X | X[T] | X | X[S] |
| HCW involved with patient-related activities in a foreign care facility | X | . | X | X[TT] | X | X[S] |
| HCW guided patients from a foreign to a local care facility without isolation precautions | . | . | . | X[T] | . | X[S] |
| Nursed in a foreign care facility | . | . | X | X[TT] | X | . |
| With risk factors (operation abroad, chronic infection or persistent skin lesions, presence of abscesses or furuncles at hospitalization in home country | . | . | . | X[p] | . | . |
| Refugee Camp | X | . | X | X | X | . |
| Staying abroad | . | . | . | . | . | . |
| With risk factors (operation abroad, chronic infection or persistent skin lesions, presence of abscesses or furuncles at hospitalization in home country | . | . | . | . | X | . |
| **Related to MRSA eradication therapy** | | | | | | |
| During MRSA eradication treatment | . | . | . | X | . | . |
| During follow-up after MRSA eradication therapy | . | . | . | X | . | . |

Table based on google translated online data from Scandinavia (Denmark[46, 47], Finland[47], Iceland[48], Norway[47, 49] and Sweden[47, 50-52]). Table data should be used with care as mistranslation or missing data in offline documents cannot be excluded.

[b] No broilers included; [p]Patients only; [S]in the last six months; [T]less than two months ago; [TT]more than twenty-four hours and less than two months ago

the one from a screenings sample.[4, 17] In 2004, a new category, “unknown”, was added to the request for epidemiological information, to get an idea of the number of MUO in The Netherlands. This addition has made it possible to measure the size of the MUO problem, and to study the MUO carriers for their underlying risk factors.

## THE SEARCH FOR MRSA OF UNKNOWN ORIGIN

The Dutch MRSA surveillance database at the RIVM was used to estimate the potential size of the MUO problem. A raw estimation was based on data from 2005, 2006 and 2007. The potential number of yearly reported MUO was estimated to be 23-27% of MRSA patient cases reported to the RIVM.[18] In the report form, accompanying the isolate, clinical microbiologists and infection prevention practitioners can do suggestions in an open text field on the possible source of the MUO carriage. Some suggestions were immigration, adoption and nursing homes. However, the reliability of the suggestions was questionable. Furthermore, the reliability of the questionnaires sent to the RIVM was unknown and not sufficient to come to an unequivocal classification and reliable count of the numbers of MRSA as MUO.[19] A thorough study of MRSA surveillance data on MUO was deemed necessary, leading to the national MRSA surveillance database at the RIVM becoming the primary source for the research described in this thesis.

## THESIS AIMS

The primary aim of this thesis is to map the magnitude of the presence of MUO, elucidate MUO’s risk factors and/or transmission routes. While the secondary aim is to find out whether MUO carriers carried different MRSA strains compared to MRSA strains of MRSA carriers with known risk factors (MKO).

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# Emergence of MRSA of unknown origin in the Netherlands

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# ABSTRACT

The Netherlands is known for its low methicillin-resistant Staphylococcus aureus (MRSA) prevalence. Yet MRSA with no link to established Dutch risk factors for acquisition, MRSA of unknown origin (MUO), has now emerged and hampers early detection and control by active screening upon hospital admittance. We assessed the magnitude of the problem and determined the differences between MUO and MRSA of known origin (MKO) for CC398 and non-CC398. National MRSA Surveillance data (2008–2009) were analysed for epidemiological determinants and genotypic characteristics (Panton–Valentine leukocidin, spa). A quarter (24%) of the 5545 MRSA isolates registered were MUO, i.e. not from defined risk groups. There are two genotypic MUO groups: CC398 MUO (352; 26%) and non-CC398 MUO (998; 74%). CC398 MUO needs further investigation because it could suggest spread, not by direct contact with livestock (pigs, veal calves), but through the community. Non-CC398 MUO is less likely to be from a nursing home than non-CC398 MKO (relative risk 0.55; 95% CI 0.42–0.72) and Panton–Valentine leukocidin positivity was more frequent in non-CC398 MUO than MKO (relative risk 1.19; 95% CI 1.11–1.29). Exact transmission routes and risk factors for non-CC398 as CC398 MUO remain undefined.

## INTRODUCTION

In the past 20 years, the Netherlands kept methicillin-resistant Staphylococcus aureus (MRSA) at bay through prudent use of antibiotics and a Search and Destroy policy. Part of Search and Destroy is active detection and isolation based on defined risk groups. For these reasons, MRSA prevalence in Dutch hospitals and community is still low [1,2]. The Dutch Working party on Infection Prevention developed a guideline on MRSA prevention. (Table 1) This guideline defines the national risk groups and the procedure of contact tracing around cases is described. The Dutch policy can therefore be seen as targeted surveillance on defined risk groups. However, MRSA was found in people who were not targeted by the Search and Destroy policy because they did not belong to the defined risk groups [3]. In the present study, these cases are defined as 'MRSA of unknown origin' (MUO). MUO can transmit, until detection, because preventive measures are not taken. To enable the targeting of control strategies for MUO, the magnitude of the problem was measured and the differences were determined between MUO and MRSA of known origin (MKO; comprising MRSA risk groups and contact tracing described in the targeted surveillance). Materials and Methods Data from the national MRSA surveillance database at the National Institute for Public Health and Environmental Protection (RIVM) between 1 January 2008 and 31 December 2009 were used. All MRSA strains sent to the RIVM by 68 Dutch laboratories, covering the whole country, are registered in this database. Of the cultures positive for MRSA taken from a single person, one, usually the first detected, strain is sent to the RIVM. A check on duplicates in the database further ensured one MRSA strain per person. At the RIVM the MRSA strains were confirmed by testing for the presence of the mecA gene and the coagulase gene. For all confirmed MRSA the *spa*-type, as described by Harmsen et al. [4], and the presence of the Panton–Valentine leucocidin gene (PVL-gene) were determined [5]. As there was no significant difference in the number and data of reported isolates and carriers between the 2 years, data were pooled to increase power. Based on *spa*-types we distinguished CC398 (livestock associated strains) and non-CC398 [6]. CC398 was checked by RIVM with multiple-locus variable number tandem repeat analysis (http://www.mlva.net/). CC398 was analysed as a separate group from non-CC398. Each strain was submitted with a form, with background information on hospital, demographic patient information, risk factors when applicable (Table 1), and other relevant epidemiological information. Laboratories were approached by the RIVM to complete their missing data as much as possible. Two defined groups, MUO and MKO, were classified based on the included information on defined risk factors requested. The absence of either defined risk factors or of risk factors found through contact tracing, led to a classification of MUO. Additional remarks were usually made on the form and/or the box for 'Unknown MRSA' was ticked. Isolates with no or incomplete additional epidemiological data (No data), which made classification impossible, were

**Table 1 – Dutch defined risk groups**

| Risk groups (Patients) | Numbers Patients (n=2538) [a] |
|---|---|
| Contact with roommates or carrier | |
| Single room shared with MRSA carrier | 89 (4%) |
| Contact tracing | 485 (19%) |
| Foreign | |
| Cared for in a foreign hospital | 342 (13%) |
| Foreign patients at a Dutch dialysis department | 1 (0.04%) |
| Adopted children: hospitalized or frequently visit the outdoor department | 62 (2%) |
| Dutch dialysis patients dialyzed abroad | 1 (0.04%) |
| Livestock | |
| Work related contact with alive pigs or veal calves | 1120 (44%) |
| Outbreak | |
| Patients from another Dutch hospital or nursing home, from a department or unit where there is a MRSA outbreak, which is not under control | 128 (5%) |
| MRSA Carrier | |
| Proven carrier | 119 (5%) |
| **Risk groups (Healthcare workers (HCW))** | **HCW (n=255) [a]** |
| Contact with roommates or carrier | |
| Unprotected contact without infection precautions | 165 (64%) |
| Protected contact with infection precautions | 19 (7%) |
| Contact tracing | 33 (13%) |
| Foreign | |
| Cared for in a foreign hospital | 2 (0.8%) |
| Worked < 2 months ago, but longer than 24 hours in a foreign hospital or nursing home | 10 (4%) |
| Worked (regularly) in an abroad hospital or escort patients from a foreign to a Dutch hospital | 7 (3%) |
| Livestock | |
| Work related contact with alive pigs or veal calves | 1 (0.4%) |
| MRSA Carrier | |
| Proven carrier | 5 (2%) |

MRSA, methicillin-resistant Staphylococcus aureus

[a] MRSA in the Netherlands in 2008-2009. A single carrier can have more than one risk.

not included in further descriptive and multivariate analysis. Finally, additional remarks on the form were categorized to gain insight into new sources and risk factors. The most prevalent *spa*-types were determined for the total amount of MRSA, CC398, non-CC398 MRSA, MUO and MKO. The *spa*-types were ranked with rank 1 being the most prevalent *spa*-type within the (sub)group; rank 2 the second most prevalent, etc. SAS statistical software (ENTERPRISE GUIDE version 4.2) was used for descriptive analysis, univariate analysis (Fisher's exact test) and multiple regression analysis (log-binomial regression model, proc GENMOD). A p value of <0.05 was considered significant. Goodness of fit was determined with the area under the curve of a receiver operating characteristic-curve (ROCR software). Relative risks (RR) with 95% CI were calculated.

## RESULTS

### General results

In 2 years, 5545 MRSA strains were sent to the reference laboratory and so were available for analysis: 2671 reported in 2008 and 2874 in 2009. From the 5545 MRSA, 3233 (58%) were non-CC398 and 2312 (42%) were CC398 (livestock-associated MRSA). The MUO and MKO proportions of these groups were determined (Table 2).

**Table 2 – MUO and MKO proportions among MRSA**

| | MUO (%) | MKO (%) | No Data* (%) | Total MRSA |
|---|---|---|---|---|
| **Non-CC398** | 998 (30.9) | 1407 (43.5) | 828 (25.6) | 3233 |
| **CC398** | 352 (15.2) | 1386 (59.9) | 574 (24.8) | 2312 |
| **Total MRSA** | 1350 (24.3) | 2793 (50.3) | 1402 (25.3) | 5545 |

MUO, Methicillin-resistant Staphylococcus aureus (MRSA) of unknown origin;
MKO, MRSA of Known Origin; CC398, Livestock associated MRSA (LA-MRSA);
Data are from The Netherlands over a two year period (2008-2009).
* Excluded from analysis.

### Molecular results

A total of 403 different *spa*-types were identified and 13 strains were not typeable. Five *spa*-types constituted 51% of the total MRSA, i.e. t011, t108, t008, t002 and t064. Among non-CC398, 364 different *spa*-types were identified, of which 210 were MUO and 209 were MKO. For CC398, there were 40 different *spa*-types, of which 17 *spa*-types were MUO and 26 were MKO (see also Tables 3 and 4). The *spa*-types t008 (ST8), t019 (ST30) and t044 (ST80) were more often found among non-CC398 MUO than among MKO (p <0.01) and type t034 (CC398) was more often found among CC398 MKO than CC398 MUO (p <0.01). Of all MRSA, 684 (12%) were PVL-positive. For non- CC398 MUO this was 461 (46%), for non-CC398 MKO it was 144 (10%) and for CC398 MKO it was 3 (0.2%) (see also Table 5). There were significantly more PVL-positive t008 (USA300) among non-CC398 MUO (106 events, 10.6% of total MUO), than among non-CC398 MKO (38 events, 1.7% of total MKO) (p <0.01). Comparing CC398 MUO with non-CC398 MUO Of the 998 non-CC398 MUO, 745 (75%) had added remarks on the form. Of the remarks, 101 (14%) were related to (health) care, 104 (14%) to foreigners (contact with a foreigner or being one), 95 (13%) to contact with a positive family member and no indica-

**Table 3 – Most prevalent *spa*-types in The Netherlands**

| | | | | | Non-CC398 | | | | | | CC398 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MRSA | | Non-CC398 | | MUO | | MKO | | CC398 | | MUO | | MKO | |
| | (n = 5545) | | (n = 3233) | | (n = 998) | | (n = 1407) | | (n = 2312) | | (n = 352) | | (n = 1386) | |
| **Rank** | **Spa** | **%** | **Spa** | **%** | **Spa** | **%** | **Spa** | **%** | **Spa** | **%** | **Spa** | **%** | **Spa** | **%** |
| 1 | t011 | 24 | t008 | 14 | t008 | 17 | t008 | 10 | t011 | 59 | t011 | 59 | t011 | 59 |
| 2 | t108 | 11 | t002 | 8 | t002 | 8 | t002 | 8 | t108 | 26 | t108 | 27 | t108 | 26 |
| 3 | t008 | 8 | t064 | 5 | t019 | 6 | t064 | 6 | t034 | 4 | t567 | 2 | t034 | 4 |
| 4 | t002 | 5 | t032 | 4 | t044 | 5 | t179 | 5 | t567 | 2 | t571 | 2 | t899 | 2 |
| 5 | t064 | 3 | t044 | 4 | t064 | 3 | t032 | 4 | t899 | 2 | t899 | 2 | t567 | 2 |

MUO, Methicillin resistant Staphylococcus aureus (MRSA) of unknown origin; MKO, MRSA of known origin; CC-398, Livestock associated MRSA. Data are from The Netherlands over a two year period (2008-2009). The five most prevalent *spa*-types are shown for the total amount of MRSA, Non-CC398 and CC-398 distribution. The latter two have a subdivision in MUO and MKO. Rank 1 means first most prevalent *spa*-type, rank 2 means second most prevalent *spa*-type, etc. Percentages are of group totals (mentioned with no.). In total, 403 different *spa*-types were typed (out of 5565 MRSA).

**Table 4 – Comparison of most prevalent *spa*-types in The Netherlands**

| | | | | | Non-CC398 | | | | | | CC398 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MRSA | | Non-CC398 | | MUO* | | MKO* | | CC398 | | MUO* | | MKO* | |
| | (n = 5545) | | (n = 3233) | | (n = 998) | | (n = 1407) | | (n = 2312) | | (n = 352) | | (n = 1386) | |
| ***Spa*-type** | **Rank** | **%** | **Rank** | **%** | **Rank** | **%** | **Rank** | **%** | **Rank** | **%** | **Rank** | **%** | **Rank** | **%** |
| t032 | 6 | 2 | 4 | 4 | 9 | 2 | 5 | 4 | - | - | - | - | - | - |
| t044 | 7 | 2 | 5 | 4 | 4 | 5 | 16 | 2 | - | - | - | - | - | - |
| t019 | 8 | 2 | 6 | 3 | 3 | 6 | 14 | 2 | - | - | - | - | - | - |
| t179 | 10 | 2 | 8 | 3 | 16 | 1 | 4 | 5 | - | - | - | - | - | - |
| t034 | 11 | 1 | - | - | - | - | - | - | 3 | 4 | 6 | 1 | 3 | 4 |
| t571 | 28 | 0.5 | - | - | - | - | - | - | 7 | 1 | 4 | 2 | 8 | 1 |

MUO, methicillin-resistant Staphylococcus aureus of unknown origin; MKO MRSA of known origin; CC-398: Livestock associated MRSA. *Spa*-types mentioned in table 2 as most prevalent for one group, but not found in a top 5 for one of the other groups in table 2, can be compared in this table for its prevalence in other groups. Rank 1 means first most prevalent *spa*-type, rank 2 means second most prevalent *spa*-type, etc. A dash means the *spa*-type was not present within the specific group. Data is from The Netherlands over a two year period (2008-2009).

tion for a possible source was obtained from 253 (34%). Of the 352 CC398 MUO, 300 had added remarks (85%). Fifty (17%) were attributed to a link with animals in general, of which 16 were through a positive relative; 28 (9%) were linked to a positive family member not involved with any animals, and 197 (66%) had no indication for a possible source. Pigs were the specifically mentioned animals for half of the animal related events (26; 52%), followed by bovids (9; 14%, seven cattle, one goat, one sheep), horses (6; 12%) and chickens (3; 6%).

## Epidemiological characteristics

The following determinants were positively associated with non-CC398 MUO after univariate analysis: age (≤20 years), being a male hospital patient, household (the location where the MRSA carrier resided at the time of detection), clinical isolates, three *spa*-types (t008, t019 and t044) and four Dutch provinces, (Table 5) whereas for CC398 MUO, these were age (≥65 years), patient, household, clinical isolates (but not blood) and three Dutch provinces (Table 5).

The log-binomial regression model, comprised four determinants (PVL, person, healthcare centre and source of specimen; Table 5), with an area under the curve of 0.81 (figure not shown) for non-CC398 and three determinants (source of specimen, age and provinces), with an area under the curve of 0.66 (figure not shown). There was no further significant effect when adding other determinants to the model. The strongest determinant associated with non-CC398 MUO was PVL positivity (RR 1.19; 95% CI 1.11–1.29). For CC398 MUO, this was age (20–65 years: RR 0.73; 95% CI 0.59–0.90). In the healthcare centre group, the nursing home had a lower risk for MUO in comparison with the other group (comprising revalidation centres and various other healthcare institutions) with an RR of 0.55 (95% CI 0.42–0.72). For nose, throat and perineum samples, there was a lower risk associated with MUO (RR 0.45; 95% CI 0.0–0.74). The risk for a healthcare worker to be associated with non-CC398 MUO rather than with non-CC398 MKO was greater in comparison with the risk for patients (Table 5).

## DISCUSSION

Of the 5545 MRSA isolates registered during 2008 and 2009, 24% were not found by targeted surveillance. The Netherlands has a CC398 MUO group (352; 26%) and a non-CC398 MUO group (998; 74%). The primary conclusion from the regression model was that PVL-positive MRSA was more frequent in non-CC398 MUO than MKO (RR 1.19; 95% CI 1.11–1.29) and that non-CC398 MUO was less likely to come from a nursing home than MKO (RR 0.55; 95% CI 0.42–0.72). Only a small portion of the CC398 MUO had a described link to animals and was not defined in the risk groups for MKO (50; 17%). Animals mentioned were bovids, horses and chickens. It remains unclear whether there was any relation of these MUO to livestock-related work. It is known that livestock-associated MRSA CC398 is not only found in pigs, but also in cattle, calf farmers, horses, horse personnel, poultry, slaughterhouse personnel and rats [7–12]. Remarks on the forms indicated that a specific link was not always found. CC398 MUO needs further investigation as it could suggest spread through the community not by direct contact with livestock. In 2009 Cuny et al. [13] concluded that the dissemination of MRSA CC398 (CC398) to non-exposed humans was infrequent and probably did not reach beyond

Table 5 – Epidemiological data on Non-CC398 and CC398 MRSA in The Netherlands

| | Non-CC398 | | | | | CC398 | | |
|---|---|---|---|---|---|---|---|---|
| | MUO | MKO[a] | Univariate analysis | Multiple regression | | MUO | MKO[a] | Univariate analysis |
| **Characteristics** | **(n=998)** | **(n=1407)** | **p-value** | **RR (95% CI)*** | **p-value** | **(n=352)** | **(n=1386)** | **p-value** |
| **Sex** | | | | | | | | |
| Male | 530 (53%) | 594 (42%) | < 0.01 | - | - | 219 (62%) | 917 (66%) | 0.16 |
| Female | 452 (45%) | 768 (55%) | < 0.01 | - | - | 133 (38%) | 469 (34%) | 0.16 |
| **Age** | | | | | | | | |
| ≤ 20 years | 161 (16%) | 173 (12%) | < 0.01 | - | - | 50 (14%) | 163 (12%) | 0.21 |
| 20 – 65 years | 543 (54%) | 769 (54%) | 0.94 | - | - | 227 (64%) | 1086 (78%) | < 0.01[e] |
| ≥ 65 years | 294 (29%) | 465 (33%) | 0.07 | - | - | 75 (21%) | 137 (10%) | < 0.01 |
| **Person** | | | | | | | | |
| Patient | 981 (98%) | 1188 (84%) | < 0.01 | - | - | 351 (99%) | 1350 (97%) | < 0.01 |
| Healthcare worker | 17 (2%) | 219 (16%) | < 0.01 | 3.21 (2.09-5.33) | < 0.01 | 1 (0.3%) | 36 (3%) | < 0.01 |
| **Healthcare center** | | | | | | | | |
| General hospital | 523 (52%) | 662 (47%) | 0.01 | 0.96 (0.86-1.12) | 0.58 | 218 (62%) | 1011 (73%) | < 0.01 |
| Academic hospital | 102 (10%) | 155 (11%) | 0.55 | 0.95 (0.81-1.13) | 0.58 | 23 (7%) | 59 (4%) | 0.07 |
| Categorical hospital | 6 (1%) | 4 (0.3%) | 0.34 | 0.98 (0.54-1.26) | 0.91 | 0 (0%) | 2 (0.1%) | 1.00 |
| Nursing home | 52 (5%) | 259 (18%) | < 0.01 | 0.55 (0.42-0.72) | < 0.01 | 2 (0.6%) | 17 (1%) | 0.29 |
| Unknown | 20 (2%) | 32 (2%) | 0.67 | 0.92 (0.66-1.17) | 0.56 | 8 (2%) | 14 (1%) | 0.06 |
| Household | 226 (23%) | 200 (14%) | < 0.01 | 1.08 (0.97-1.25) | 0.21 | 78 (22%) | 223 (16%) | 0.01 |
| Other[b] | 69 (7%) | 95 (7%) | 0.87 | - | - | 23 (7%) | 60 (4%) | 0.08 |

|  | Non-CC398 |  |  |  |  | CC398 |  |  |
|---|---|---|---|---|---|---|---|---|
|  | MUO | MKO[a] | Univariate analysis | Multiple regression |  | MUO | MKO[a] | Univariate analysis |
| Characteristics | (n=998) | (n=1407) | p-value | RR (95% CI)* | p-value | (n=352) | (n=1386) | p-value |
| **Source of Specimen**[c] |  |  |  |  |  |  |  |  |
| Nose, throat, perineum | 278 (28%) | 1083 (77%) | < 0.01 | 0.45 (0.0-0.74) | < 0.01 | 233 (66%) | 1229 (89%) | < 0.01 |
| Urine | 66 (7%) | 29 (2%) | < 0.01 | 1.31 (0.92-2.15) | 0.20 | 6 (2%) | 6 (0.4%) | 0.01 |
| Respiratory | 54 (5%) | 21 (1%) | < 0.01 | 1.32 (0.92-2.16) | 0.20 | 19 (5%) | 6 (0.4%) | < 0.01 |
| Skin and soft tissue | 392 (39%) | 144 (10%) | < 0.01 | 1.31 (0.95-2.13) | 0.18 | 42 (12%) | 27 (2%) | < 0.01 |
| Blood | 18 (2%) | 4 (0.3%) | < 0.01 | 1.44 (0.96-2.38) | 0.10 | 2 (0.6%) | 1 (0.1%) | 0.05 |
| Indwelling device[d] | 12 (1%) | 12 (1%) | 0.41 | - | - | 2 (0.6%) | 1 (0.1%) | 0.05 |
| Unknown | 59 (6%) | 85 (6%) | 0.93 | 0.8 (0.54-1.34) | 0.32 | 24 (7%) | 97 (7%) | 0.91 |
| Other | 119 (12%) | 29 (2%) | < 0.01 | 1.35 (0.97-2.20) | 0.14 | 24 (7%) | 19 (1%) | < 0.01 |
| **Typing** |  |  |  |  |  |  |  |  |
| PVL-positive | 317 (68%) | 144 (10%) | < 0.01 | 1.19 (1.11-1.29) | < 0.01 | 0 (0%) | 3 (0.2%) | 1.00 |

MUO, methicillin-resistant *Staphylococcus aureus* (MRSA) of unknown origin; MKO, MRSA of known origin; PVL, Panton–Valentine leukocidin; RR, relative risk. These data are from the Netherlands over a 2-year period (2008–2009). CC398 is the livestock-associated cluster in the Netherlands.

[a] As defined by the Dutch Working group of Infection Prevention. See also Table 1.

[b] Revalidation centre, prison, correctional facility, etc.

[c] Only one strain is sent to the reference laboratory. These are the counts of the sources of the strains sent. No information is available for whether other sources were positive as well. Therefore these numbers reflect the minimum.

[d] Catheters etc.

[e] The only significant factor from the multivariate analysis, for CC398 MUO, was age (20–65 years old: RR 0.73; 95% CI 0.59–0.90).

familial communities and a low human-to-human transmission was confirmed in several studies [14–16]. Surveillance will remain necessary to monitor livestock-associated MRSA evolution, its spread in the surrounding (innate) environment and to detect new risk factors or transmission routes. The possibility of increased incidence of livestock-associated MRSA, and subsequently of livestock-associated MRSA infections in the future, cannot be ruled out. Overall, there was more MUO in Dutch provinces without areas dedicated to intensive cattle breeding. Three *spa*-types in the non-CC398 MUO group, t008 (ST-8; North America, Europe and Southeast Asia [17,18]), t019 (ST-30; North America and Southeast Asia [17]) and t044 (ST-80; mainly found in Europe [19–21]), were found more often among MUO than among MKO (Tables 3 and 4). Addition of these three *spa*-types to the regression model of non-CC398 gave no significant effect in the presence of PVL. By definition, we do not know where MUO come from. MUO could be community-associated MRSA or comprise one or several new risk groups or reservoirs. A possible explanation for the PVL correlation with non-CC398 MUO might be found in the association of young age (children and young adults) with non-CC398 MUO (Table 5, univariate). The literature reports that children and young adults were a risk factor for community-associated MRSA infections [22]. The CANWARD study described a trend toward younger patient age for community-associated MRSA genotypes [23]. At first the univariate analysis in this study revealed a positive association with young age (≤20 years) as well, but its significant effect or trend was lost in the regression model. Another difference between the two studies is that this study defined MRSA MUO and MKO epidemiologically. Surprisingly the regression model of non-CC398 showed that it was less likely for (non-CC398) MUO to come from a nursing home (Table 5) than MKO. Dutch MRSA prevalence in nursing homes is still low (<1%) [24], in contrast to nursing homes in other parts of Europe (20%) and North America (18.8–35.7%) [25]. For nursing homes, the Working party on Infection Prevention also applies guidelines for general precautions and in particular to prevent MRSA. It is likely that, up to now, the Dutch nursing homes have not served as a source for MRSA and, as far as we can conclude from our data, nursing homes are not the source of MUO. Previous research has shown that spread of MRSA within households (not a risk group in the Working party on Infection Prevention ) was substantial [26]. Mollema et al. [26] showed that the transmission of MRSA from an index person to household contacts occurred in nearly half of the cases, and two-thirds of household contacts became MRSA positive. Yet in the regression model for non-CC398 the determinant household lost its significance. In our Search and Destroy policy, eradication is one of the cornerstones for keeping rates low [3,27]. If detected MRSA carriers were not offered eradication therapy, this would allow further spread, presumably in the household or through other close contacts. The early opportunity to eradicate MUO and to interrupt its transmission (according to the Search and Destroy policy) is missed, because MUO are not actively cultured for the presence of carriage [3,27]. Considering the amount of MUO, this would be at least

24% of the total MRSA in the Netherlands. It is important to realize that MUO are not targeted by the risk groups for active detection and isolation and go unnoticed until they are unexpectedly detected from a clinical sample. This explains the significantly higher MUO proportion found in clinical specimens, compared with MKO from persons who were actively screened. This gives a possible second explanation for the PVL correlation with non-CC398 MUO, but also suggests that the unexpected MUO found so far are the tip of an iceberg. Exact transmission routes and risk factors for MUO are, for now, obscure, although there is an indication that the community is a source of non-CC398 MUO. In addition, remarks on the forms for non-CC398 that are returned to the RIVM indicate having a foreign origin or having been abroad without having visited a hospital or having foreign relatives, which are all in line with studies reporting immigration as a risk factor [21,28]. Although cross-dissemination as a result of past foreign hospital visits, longer than 2 months before admission to a Dutch hospital, could also play a role [29]. The small proportion of CC398 MUO needs further research to see whether community spread indeed happens, despite the current dogma of no spread outside the risk population, because of person-to-person transmission or spread as a food-borne pathogen [30]. In conclusion, at least a quarter of the total Dutch MRSA is not from the defined risk groups. Studies on new sources and transmissions are urgently needed to possibly update the guidelines and to keep the MRSA prevalence low. Furthermore, Search and Destroy policy should be evaluated on their defined risk groups and the number of MUO. These are essential steps to take in order to cope with the dynamic nature of Staphylococcus aureus and its changing epidemiology.

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# What Is the Origin of Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* Clonal Complex 398 Isolates from Humans without Livestock Contact? An Epidemiological and Genetic Analysis

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## ABSTRACT

Fifteen percent of all methicillin-resistant Staphylococcus aureus (MRSA) clonal complex 398 (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 this MRSA of unknown origin (MUO). Recently, it was shown that CC398 strains 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 isolates of various known origin might provide clues to their origin. MUO CC398 isolates were compared to MRSA CC398 isolates obtained from humans with known risk factors, a MRSA CC398 outbreak isolate, livestock associated (LA) MRSA CC398 isolates from pigs, horses, chickens, and veal calves, and five methicillin-susceptible Staphylococcus aureus (MSSA) CC398 isolates of 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 PCRs. The MRSA CC398 in humans, MUO, or MRSA of known origin (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 was 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 of indirect transmission. Although the exact transmission route remains unknown, direct human-to-human transmission remains a possibility as well.

## INTRODUCTION

In The Netherlands, the prevalence of methicillin-resistant Staphylococcus aureus (MRSA) is low [1], and Dutch MRSA strains display broad clonal diversity [2]. One exception is the livestock-associated (LA) clone (clonal complex 398 [CC398]), a major clonal reservoir in pigs and veal calves [3] and subsequently in people with occupational exposure to animals. The reported number of MRSA CC398 strains has been around 40% of MRSA strains reported to the Dutch MRSA surveillance since 2008 [2, 4]. However, only 78% of reported CC398 strains 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 with the described risk factors in the Dutch MRSA guideline: industrial contact with live pigs, veal calves, or broiler chickens regardless of whether or not this contact was occupational and/or residence of the individual on such a farm. Currently 15% (352/2,312) 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 factors in the MRSA guideline (due to being unknown or having 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] or 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 strains in humans is rare, it was recently shown that CC398 strains 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 strains in humans without direct contact to pigs or veal calves (MUO CC398) is clear. From the genomic analyses on CC398 isolates 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 hosts in the past as a result of human-animal interactions [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 strains from humans in contact with animals differed from strains isolated from humans without contact with animals. The differences were seen in MGE located genes, e.g., φ3 int, chp, scn, rep27, φ7 int, and cadDX for humans and rep7 and φ6 int for pigs, in addition to genes encoding resistance to tetracycline and trimethoprim [14]. We therefore hypothesized that the presence of these MGE-encoding genes with resistance to tetracycline and trimethoprim-sulfamethoxazole might be used as a cheap and fast method to compare MUO CC398 isolates 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 of animal origin more closely than those of human origin, indicating that these MUO CC398 isolates most probably originated from livestock. (This work was presented in part as a poster at the International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), Lyon, France, 2012 [16].)

## 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 multilocus variable-number tandem-repeat analysis (MLVA) typing (http://www.mlva.net/). MLVA is the choice of the National Institute for

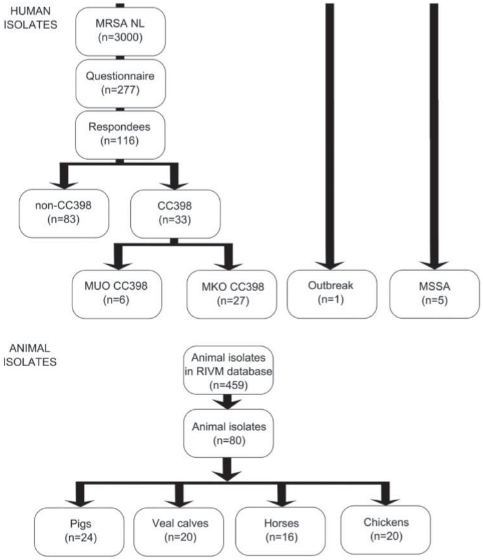


**Figure 1**

Flowcharts. (Top) Human isolates. MUO is MRSA without known risk factors as described by the Dutch national guideline. MKO is MRSA with known risk factors as described by the Dutch national guideline. MKO CC398 represents a pig or veal calf farmer, a person with direct contact to pigs and/or veal calves and/or who lives on a pig or veal calf farm, or a broiler chicken handler. (Bottom) Animal isolates. RIVM, the National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

Public Health and the Environment (RIVM), due to costs and also the agreement between the results for MLVA and multilocus sequence type (MLST). Only sequence types (STs) belonging to the same MLST clonal complex were grouped by MLVA. Furthermore, the *spa*-types associated with MLST clonal complexes show a remarkable agreement with those of the MLVA complexes [17]. The MLVA complexes were therefore named in accordance with the MLST complexes. The MLVA complex 398 is thus equal to the MLST clonal complex 398. The isolates were all from The Netherlands and from 2009, except for 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, 18]. All S. aureus CC398 isolates were stored at -80°C and grown on sheep blood agar plates (RBS) (Becton, Dickinson & Co., Belgium) at 37°C 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. [19]. The healthy veal calves were sampled at arrival on three Dutch farms [20]. The horse strains were nearly all (94%) samples from diseased horses that visited the Utrecht University equine clinic, the remaining 6% being from 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 [21]. *S. aureus* isolates were *spa*-typed by the RIVM according to the method of Harmsen et al. [22]. From the available livestock MRSA isolates (*n* = 459), the largest variability in *spa*-types was chosen (*n*=80) (Figure 2); whether an isolate was from screening or a clinical case was not a selection criterion. This resulted in a selection with both screening and clinical isolates.

## Bacterial strain 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 health care 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) and MKO (*n*=27) were from a previous study, in which an extended questionnaire was send to MRSA carriers. Five MSSA isolates were also of human origin. These isolates were previously described and isolated at the Erasmus MC in the period of 1998-1999 and 2002 [13, 18].

### Extended questionnaire study.

Around 3,000 MRSA strains have been 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 Working Party on Infection Prevention (WIP) guideline on MRSA [4], and also included further questions on risk factors as described in the literature, as found by a PubMed search up to 1 January 2010, using search keywords "MRSA" and "risk factor" (see supplementary table 1).

### *S. aureus* genotyping, detection of expression of β-hemolysin, and DNA isolation.

After an overnight culture on RBS plates, the hemolysin patterns were determined to detect the expression of β-hemolysin. No expression of β-hemolysin indicates the insertion of the *φ*3 bacteriophage into the bacterial genome, as *φ*3 inserts on the site that codes for β-hemolysin [23].DNA was isolated using a MagNA Pure system (Roche) according to the protocol supplied by the manufacturer.

### Mobile genetic elements.

The presence or absence of MGEs was determined by PCRs specific for *cadDX*, *φ3 int*, *scn*, *chp*, *rep7*, *rep27*, *φ*6 *int*, and *φ7 int* [14, 23, 24]. Primers for *φ*3 *int* (forward primer, TCC GGCTTCTTTGAAAATGT; reverse primer, CCGGAAAACCTACGA AGTCA; amplicon size, 220 to 323 bp; annealing temperature, 50°C) and *cadDX* (forward primer, TGATGTGATCTGTGTACATGAGGA; reverse primer, TGATGTGAAGTTGAAGCAACA; amplicon size, 207 bp; 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 supplementary tables 2 and 3).

### Antimicrobial susceptibility.

To determine the antimicrobial susceptibility of *S. aureus* strains, the standard disc diffusion method was applied using Oxoid antimicrobial susceptibility test discs (Thermo Fisher Scientific, Waltham, MA, USA) on Mueller-Hinton (MH) agar plates. The Clinical and Laboratory Standards Institute (CLSI) breakpoints were used for tetracycline (zone diameter breakpoints: susceptible [S], ≥19 mm; intermediate [I], 15 to 18mm; resistant [R], ≤14mm)and trimethoprimsulfamethoxazole (zone diameter breakpoints: S, ≥16 mm, I, 11 to 15 mm; R, ≤10 mm) [25].

### Statistical analysis.

Statistical analysis was performed with SAS Enterprise Guide software (version 4.2; SAS Institute, Inc., NC, 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 and between human epidemiological subgroups. Isolates were clustered transversally, using the Jaccard coefficient, on the presence of MGEs, β-hemolysin expression, and susceptibility to tetracycline and trimethoprim-sulfamethoxazole. The dendrogram was created based on unweighted-pair group method using average linkages (UPGMA) with Jaccard similarity.

## RESULTS

A total of 277 suspected MUO carriers from all of The Netherlands were approached by an extended questionnaire; 42% (116) responded and 33 were defined as CC398 carriers. Of these 33 CC398 strains, 6 were MUO (CC398), and these were 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,

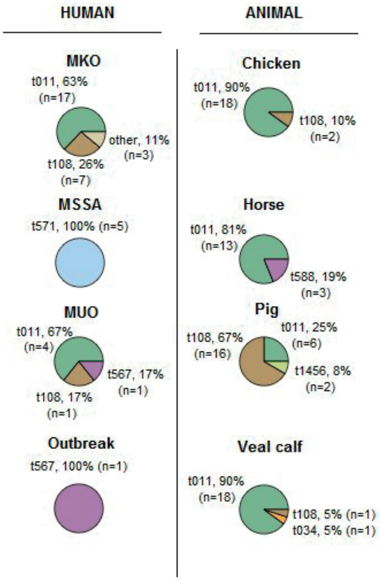


**Figure 2**

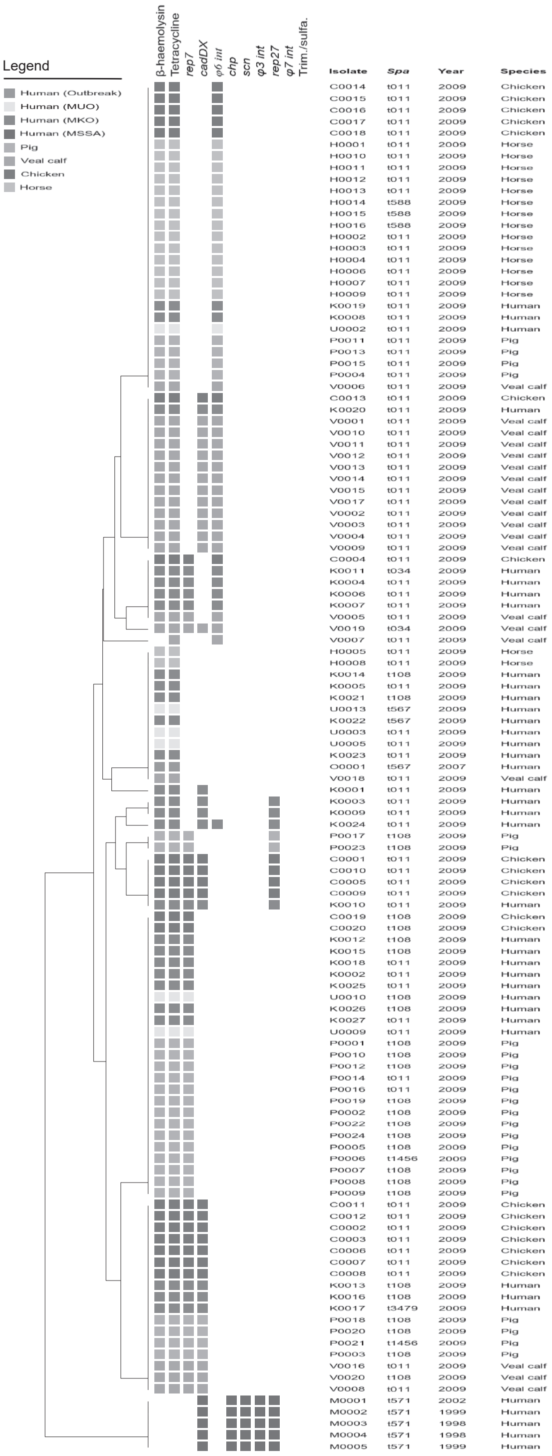
Selected *spa*-types for human and animal groups. MKO, MRSA of known origin (known risk factors described in the Dutch MRSA guideline); MSSA, methicillin-susceptible *Staphylococcus aureus*; MUO, MRSA of unknown origin (unknown risk factors not described in the Dutch MRSA guideline). The outbreak isolate was described by Wulf et al. [9].

where there is generally more pig farming. All MUO and MKO CC398 strains were distinctly different from human MSSA CC398 strains based not only on *spa*-type, but also on β-hemolysin expression, tetracycline resistance, and the lack of *φ3 int*, *scn*, and *chp* genes. The human MRSA CC398 strains resembled animal MRSA CC398 strains (Figure 3). The presence of *cadDX* and *rep27*, which are considered human-associated genetic markers as they are highly prevalent in human MSSA and significantly less prevalent in animal MRSA [14], were absent in the MUO strains and few in the 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: 16/20 (80%) for veal calves and 12/20 (60%) for chickens. *rep27* was absent in horse 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 were similar in their full susceptibility to trimethoprim-sulfamethoxazole. Also, there was no significant difference between the MUO and MKO strains in *rep7*, *rep27*, *φ*6 *int*, and *cadDX* content, resulting In MUO and MKO strains clustering together, despite some minor differences in MGE content.

When the combined data for MUO and MKO isolates were compared to data for the animal isolates, it was clear that the human isolates were less often *φ*6 *int* positive than the MRSA isolates from veal calves or horses ($P$ <0.01), more often *rep7* positive than the isolates from horses ($P$<0.01), more often *rep27* positive than the isolates from pigs ($P$ <0.01), and less often *cadDX* positive than the isolates from veal calves and chickens ($P$ <0.01). No significant differences between the MRSA isolates from the human subgroups (MUO, MKO, and outbreak) were found for *rep27*. Interestingly, the hospital outbreak strain lacked any previously mentioned human- or pig-associated markers (*rep7*, *rep27*, *φ*3, *φ6 int*, and *cadDX*) but displayed tetracycline resistance. MGE variation within a single *spa*-type was observed for human and animal isolates (Figure 3).

## DISCUSSION

The human MRSA CC398 isolates (MUO and MKO) in this study resembled animal MRSA CC398 isolates more than they resembled human MSSA CC398 isolates, because they were β-hemolysin producers and tetracycline resistant, had similar MGE patterns, and had *spa*-types similar to those found in animals. Furthermore, our MUO isolates almost always clustered together with MKO isolates in cluster analysis. The similarity between MUO CC398, MKO CC398, and animal MRSA CC398 strains suggests that these MUO CC398 strains belong to the same MRSA clade originating in animals and that these MUO CC398 strains are not part of the MSSA CC398 clade detected in humans. Stegger



| Isolate | *Spa* | Year | Species |
|---|---|---|---|
| C0014 | t011 | 2009 | Chicken |
| C0015 | t011 | 2009 | Chicken |
| C0016 | t011 | 2009 | Chicken |
| C0017 | t011 | 2009 | Chicken |
| C0018 | t011 | 2009 | Chicken |
| H0001 | t011 | 2009 | Horse |
| H0010 | t011 | 2009 | Horse |
| H0011 | t011 | 2009 | Horse |
| H0012 | t011 | 2009 | Horse |
| H0013 | t011 | 2009 | Horse |
| H0014 | t588 | 2009 | Horse |
| H0015 | t588 | 2009 | Horse |
| H0016 | t588 | 2009 | Horse |
| H0002 | t011 | 2009 | Horse |
| H0003 | t011 | 2009 | Horse |
| H0004 | t011 | 2009 | Horse |
| H0006 | t011 | 2009 | Horse |
| H0007 | t011 | 2009 | Horse |
| H0009 | t011 | 2009 | Horse |
| K0019 | t011 | 2009 | Human |
| K0008 | t011 | 2009 | Human |
| U0002 | t011 | 2009 | Human |
| P0011 | t011 | 2009 | Pig |
| P0013 | t011 | 2009 | Pig |
| P0015 | t011 | 2009 | Pig |
| P0004 | t011 | 2009 | Pig |
| V0006 | t011 | 2009 | Veal calf |
| C0013 | t011 | 2009 | Chicken |
| K0020 | t011 | 2009 | Human |
| V0001 | t011 | 2009 | Veal calf |
| V0010 | t011 | 2009 | Veal calf |
| V0011 | t011 | 2009 | Veal calf |
| V0012 | t011 | 2009 | Veal calf |
| V0013 | t011 | 2009 | Veal calf |
| V0014 | t011 | 2009 | Veal calf |
| V0015 | t011 | 2009 | Veal calf |
| V0017 | t011 | 2009 | Veal calf |
| V0002 | t011 | 2009 | Veal calf |
| V0003 | t011 | 2009 | Veal calf |
| V0004 | t011 | 2009 | Veal calf |
| V0009 | t011 | 2009 | Veal calf |
| C0004 | t011 | 2009 | Chicken |
| K0011 | t034 | 2009 | Human |
| K0004 | t011 | 2009 | Human |
| K0006 | t011 | 2009 | Human |
| K0007 | t011 | 2009 | Human |
| V0005 | t011 | 2009 | Veal calf |
| V0019 | t034 | 2009 | Veal calf |
| V0007 | t011 | 2009 | Veal calf |
| H0005 | t011 | 2009 | Horse |
| H0008 | t011 | 2009 | Horse |
| K0014 | t108 | 2009 | Human |
| K0005 | t011 | 2009 | Human |
| K0021 | t108 | 2009 | Human |
| U0013 | t567 | 2009 | Human |
| K0022 | t567 | 2009 | Human |
| U0003 | t011 | 2009 | Human |
| U0005 | t011 | 2009 | Human |
| K0023 | t011 | 2009 | Human |
| O0001 | t567 | 2007 | Human |
| V0018 | t011 | 2009 | Veal calf |
| K0001 | t011 | 2009 | Human |
| K0003 | t011 | 2009 | Human |
| K0009 | t011 | 2009 | Human |
| K0024 | t011 | 2009 | Human |
| P0017 | t108 | 2009 | Pig |
| P0023 | t108 | 2009 | Pig |
| C0001 | t011 | 2009 | Chicken |
| C0010 | t011 | 2009 | Chicken |
| C0005 | t011 | 2009 | Chicken |
| C0009 | t011 | 2009 | Chicken |
| K0010 | t011 | 2009 | Human |
| C0019 | t108 | 2009 | Chicken |
| C0020 | t108 | 2009 | Chicken |
| K0012 | t108 | 2009 | Human |
| K0015 | t108 | 2009 | Human |
| K0018 | t011 | 2009 | Human |
| K0002 | t011 | 2009 | Human |
| K0025 | t011 | 2009 | Human |
| U0010 | t108 | 2009 | Human |
| K0026 | t108 | 2009 | Human |
| K0027 | t011 | 2009 | Human |
| U0009 | t011 | 2009 | Human |
| P0001 | t108 | 2009 | Pig |
| P0010 | t108 | 2009 | Pig |
| P0012 | t108 | 2009 | Pig |
| P0014 | t011 | 2009 | Pig |
| P0016 | t011 | 2009 | Pig |
| P0019 | t108 | 2009 | Pig |
| P0002 | t108 | 2009 | Pig |
| P0022 | t108 | 2009 | Pig |
| P0024 | t108 | 2009 | Pig |
| P0005 | t108 | 2009 | Pig |
| P0006 | t1456 | 2009 | Pig |
| P0007 | t108 | 2009 | Pig |
| P0008 | t108 | 2009 | Pig |
| P0009 | t108 | 2009 | Pig |
| C0011 | t011 | 2009 | Chicken |
| C0012 | t011 | 2009 | Chicken |
| C0002 | t011 | 2009 | Chicken |
| C0003 | t011 | 2009 | Chicken |
| C0006 | t011 | 2009 | Chicken |
| C0007 | t011 | 2009 | Chicken |
| C0008 | t011 | 2009 | Chicken |
| K0013 | t108 | 2009 | Human |
| K0016 | t108 | 2009 | Human |
| K0017 | t3479 | 2009 | Human |
| P0018 | t108 | 2009 | Pig |
| P0020 | t108 | 2009 | Pig |
| P0021 | t1456 | 2009 | Pig |
| P0003 | t108 | 2009 | Pig |
| V0016 | t011 | 2009 | Veal calf |
| V0020 | t108 | 2009 | Veal calf |
| V0008 | t011 | 2009 | Veal calf |
| M0001 | t571 | 2002 | Human |
| M0002 | t571 | 1999 | Human |
| M0003 | t571 | 1998 | Human |
| M0004 | t571 | 1998 | Human |
| M0005 | t571 | 1999 | Human |

**Figure 3**

Results of β-hemolysin screening, PCR typing, and susceptibility testing. Isolates were clustered transversally, using the Jaccard coefficient, on the presence of MGEs, β-hemolysin expression, and susceptibility to tetracycline and trimethoprim-sulfamethoxazole. The dendrogram was created based on UPGMA with Jaccard similarity. The epidemiological subtypes in humans are MKO (MRSA of known origin: MRSA with known risk factors for acquisition) and MUO (MRSA of unknown origin: MRSA with unknown risk factors for acquisition). MUO 2007 are MUO isolates according to the 2007 guideline definition but no longer included under the 2012 guideline definition. MUO 2012 are MUO isolates according to the current guideline of December 2012. Outbreak represents an isolate involved from a MRSA CC398 outbreak in a Dutch hospital as described by Wulf et al. [9]. Mobile genetic elements: *chp* (gene encoding chemotaxis-inhibiting protein[CHIPS], which is found in the φ3 bacteriophage that contains the immune evasion complex [IEC] that *chp* is sometimes part of); *scn* (gene encoding staphylococcal complement inhibitor SCIN], which is found in the φ3 bacteriophage that contains the IEC that *scn* is sometimes part of); *φ3int* (integrase gene of bacteriophage 3); *φ6int* (integrase gene of bacteriophage 6); *φ7int* (integrase gene of bacteriophage 7);*rep7* (replication protein 7); *rep27* (replication protein 27); and *cadDX* (operon of gene *cadX* [cad operon regulatory protein], which encodes resistance against the heavy metal cadmium). Antimicrobial susceptibility: tetracycline susceptibility testing; Trim./sulfa., trimethoprim-sulfamethoxazole susceptibility testing.

et al. found two distinct phylogenetic clades based on single nucleotide polymorphisms (SNPs), revealing a basal human clade and a more derived livestock clade [26]. Although no whole-genome sequencing or SNP analysis was done, the outcome of our cheaper and quicker MGE-based method strongly suggests that these MUOCC398 strains 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 suggests spread of animal MRSA CC398 strains by means other than those currently described in the MRSA guideline.

The exact mode of transmission remains unknown. An indirect route of transmission is the most likely mode of transmission for these MUO CC398 strains, taking into consideration where these MUO CC398 carriers live, their lack of contact with pigs and veal calves, and also their lack of contact with horses and chickens. Since living in areas with a high density of pigs [6] and private farm visits [27] were risk factors for livestock-associated MRSA carriage, modes of indirect transmission are most likely through contamination of areas in which people live and interact. Considering the survival of *S. aureus* bacteria in the environment and subsequent spread by air over large distances [7], transmission by air is a possibility [28], as well as transmission by vectors such as rodents [29]. 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 had had contact with a MRSA carrier (who or which MRSA type was unknown) 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 a risk, but contact in the community outside the household or hospital is not.

We know that a pig MRSA CC398 lacking *φ*3 can survive up until 21 days in a human nose [11], whereas *φ*3 is currently considered to be *the* marker for human host adaptation [12]. The successful outbreak isolate reported by Wulf et al. [10] lacked *φ*3 as well. Human host adaptation is explained by more than *φ*3 alone, or host adaptation might not have to be as extensive to facilitate transmission. In regard to the outbreak isolate, further research is 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 MGE-encoding genes, there were slight differences observed for *cadDX* and *rep7* between the MUO and MKO. Furthermore, *rep7*-positive isolates were as common in MKO as in animal strains, unlike MUO, which showed significantly less *rep7* than among pig strains ($P < 0.01$). *rep7* and *rep27* genes are typical of small plasmids carrying resistance genes, and *rep7* is reported to be associated with the tetracycline resistance gene, *tetK* [30]. 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 [31]. As for the 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 carriers with and without known risk factors, regardless of guideline changes. The number of MUO CC398 carriers in this study are few as 28% (33/116) of respondents were CC398 carriers, and only 21% (6/33) fit the MUO definition. However, the number of MUO CC398 isolates per year in The Netherlands is just over 5%, which means on average an additional 150 Dutch people with a MRSA CC398 strain while lacking the risk factors as described in the Dutch MRSA guideline [4]. We showed by simple PCR means that the MUO CC398 carriers in this study carry MRSA CC398 strains of livestock origin. This finding is suggestive of an indirect transmission route, possibly the environment (air or water) or through fomites, but we cannot rule out direct human-to-human transmission. Although, the reported numbers of MUO CC398 strains in The Netherlands are currently still small, the problem may increase, giving rise to more CC398 transmission and human host adaptation.

**Supplementary table 1 – Additional risk factors after literature search**

The following risk factors were added to the questionnaire in addition to the risk factors described in the WIP guideline on MRSA (2012).

- Activities deployed in relation to water (frequent beach, lake visits, frequent use of saunas or whirlpools)
- Animal contact (livestock species, household animals and other; which animals, where and how many animals)
- Antibiotic use (< or > 6 months)
- Broken skin barrier (eczema, tattooing, other)
- Comorbidity (CF, HIV, COPD, etc.)
- Crowding and close contact situations (households, material sharing such as bedding, pillows and towels)
- Drug abuse
- Ethnicity (Adoption, immigration)
- Hospital residence or visit
- Hospital residence or visit abroad (> 2 months ago)
- Long care term facility residence or visit
- Meat consumption (Vegetarian or not)
- Occupation (livestock related or not; specified which job and whether currently active or not)
- Sports (contact, group, individual; specified which sport)

**Supplementary table 2 – Primers for mobile genetic elements**

| Gene | # | Sequence | Base pairs |
|---|---|---|---|
| *chp* | 1 | TTTACTTTTGAACCGTTTCCTAC | 365 |
| | 2 | CGTCCTGAATTCTTAGTATGCATATTCATTAG | |
| *scn* | 1 | AGCACAAGCTTGCCAACATCG | 259 |
| | 2 | TTAATATTTACTTTTTAGTGC | |
| *φ3 int* | 1 | TCCGGCTTCTTTGAAAATGT | 220-323 |
| | 2 | CCGGAAAACCTACGAAGTCA | |
| *φ6 int* | 1 | CCT TGA ATT GAT GGC GAT TT | 202 |
| | 2 | TTG CTG GGG CTG TAG AAG TT | |
| *φ7 int* | 1 | TTCTGGCGCTTCCCTTTAAT | 509 |
| | 2 | AACACAGTCAAGCATACGCCT | |
| *rep7* | 1 | GCACTGCGGATAGAGCAATA | 168 |
| | 2 | GCAATTGTTTTTCGTTCGCT | |
| *rep27* | 1 | TTCGGTTGGATCAATTTCTTT | 619 |
| | 2 | TTTTGTCCTGTCTGTGCTTGG | |
| *cadDX* | 1 | TGATGTGATCTGTGTACATGAGGA | 207 |
| | 2 | TGATGTGAAGTTGAAGCAACA | |

Numbers 1 and 2 are forward and backward primer respectively. Base pair product size on the right of the table. Primers for *chp* and *scn* obtained from W. van Wamel. Primers for *φ6 int*, *φ7 int*, *rep7* and *rep27* from J. Lindsey and A. McCarthy. Primers for *φ3 int* and *cadDX* were made with primer3 software (http://frodo.wi.mit.edu/).

**Supplementary table 3 – PCR programs**

| Gene | PCR program | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T (ºC) | Time (s) | T (ºC) | Time (s) | T (ºC) | Time (s) | T (ºC) | Time (s) | T (ºC) | Time (s) |
| *chp* | 94 | 240 | 94 | 30 | | | | | | |
| | 1 cycle | | 30 cycles | | | | | | 1 cycle | |
| *scn* | 94 | 240 | 94 | 30 | 50 | 30 | 72 | 60 | 72 | 120 |
| | 1 cycle | | 30 cycles | | | | | | 1 cycle | |
| *φ3 int* | 94 | 300 | 94 | 15 | 50 | 30 | 72 | 60 | 72 | 120 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |
| *φ6 int* | 95 | 120 | 95 | 15 | 62 | 15 | 72 | 30 | 72 | 600 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |
| *φ7 int* | 95 | 120 | 95 | 15 | 62 | 15 | 72 | 30 | 72 | 600 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |
| *rep7* | 95 | 120 | 95 | 15 | 62 | 15 | 72 | 30 | 72 | 600 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |
| *rep27* | 95 | 120 | 95 | 15 | 60 | 15 | 72 | 30 | 72 | 600 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |
| *cadDX* | 94 | 300 | 92 | 30 | 60 | 15 | 72 | 30 | 72 | 600 |
| | 1 cycle | | 35 cycles | | | | | | 1 cycle | |

T is temperature in degrees Celsius. Time is in seconds. Cycles are the number of repeats of time and temperature instructions. The table is read from left to right.

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# Follow-up cultures for MRSA after eradication therapy: Are three culture-sets enough?

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## SUMMARY

Objectives: We compared the standard procedure of three MRSA follow-up culture-sets to six to determine the number of recurrences detected between the third and sixth follow-up culture-set, and studied possible risk factors for MRSA recurrence.

Methods: A retrospective carrier cohort (2005-2010) was studied. Data was collected on MRSA culture-sets, follow-up, risk factors and outcome (recurrences during follow-up). We compared outcome between three and six follow-up MRSA culture-sets, between HCWs and patients groups for complicated or uncomplicated carriers, and between nose-throat carriers and other carriers.

Results: Of 406 MRSA carriers, 179 had received eradication therapy and had a negative first follow-up MRSA culture-set. Between the third and sixth follow-up culture-set 54% (35/65) of total recurrences occurred. Over 88% of all recurrences were detected within two months. Combined nose and throat carriage (OR 25.5 (1.6-419.1)) and intravascular lines (OR 13.6 (1.2-156.2)) were risk factors for early recurrence.

Conclusions: We recommend five culture-sets till one year after successful eradication therapy with a distinction between those at risk for early recurrence and HCWs who require frequent culturing in the beginning and those not at risk for early recurrence. This recommendation is a balance between the need for swift detection of MRSA recurrence and the patients' burden.

## INTRODUCTION

The Netherlands is one of the few countries in the world with a low meticillin-resistant Staphylococcus aureus (MRSA) prevalence. This is most likely due to a rigourous control strategy, which includes decolonization treatment of MRSA carriers and their household members.[1] Decolonization treatment of MRSA carriers (MRSA eradication therapy) is a worldwide used strategy, but consensus and/or documentation on follow-up to determine MRSA clearance (successful MRSA decolonization after MRSA eradication therapy) varies per region or nation, or is even absent.[2] In The Netherlands, the current national guideline to prevent MRSA in healthcare centres (Working Party on Infection Prevention (WIP) guideline on MRSA[3]), recommends the use of three follow-up culture-sets to prove successful decolonization after eradication therapy. To the best of our knowledge, the only data reported on this issue, is a study from our research group in 2010 by Mollema et al., who studied 165 MRSA-positive individuals in a prospective observational follow-up study. This study concluded for a reliable assessment of successful MRSA eradication (>90%) at least five negative culture-sets would be required.[4] However the study did not address when the follow-up culture-sets should be collected after eradication therapy; nor the number of recurrences that would have been missed between the third and the sixth follow-up culture set; nor any risk factors for early or late recurrence. Although the Dutch national MRSA guideline suggests a minimum of three sets after eradication therapy is considered sufficient (with at least seven days between each set) at the Erasmus Medical Centre six culture-sets are routinely collected post MRSA eradication therapy. Therefore, we have data to compare the two approaches of three and six follow-up culture-sets.

In this study we determined the number of recurrences of MRSA detected between the third and sixth follow-up culture-sets after eradication therapy, and identified any risk factors associated with MRSA recurrence, and whether the risk factors were associated with early (first three follow-up culture-sets) or late (follow-up culture-sets four, five and six) MRSA recurrence.

## PATIENTS AND METHODS

### I - carriers and follow up

A retrospective cohort (2005-2010) of carriers (patients and healthcare workers (HCWs)) was studied. Carriers were retrospectively selected from reports on the MRSA carrier population that visited or worked at the Erasmus Medical Center, Rotterdam (Erasmus MC). All carriers, who had received MRSA eradication therapy and whose first culture-set after treatment was negative, were included in our routine follow-up procedure. Eradication therapy was described by Mollema et al. and derived from the Dutch guideline on the treatment of MRSA carriage.[1,4] A follow-up culture-set comprised a culture of the nose, throat, perineum and, if present, wounds. Specimen collection was performed by self-collection by the carriers after receiving written instructions, by their general practitioner or in the hospital. Exclusion criteria were loss to follow-up, or incomplete data or reports. (Figure 1) A complete follow-up period was defined as the time period between the negative results of the first follow-up culture-set after eradication therapy and the sixth follow-up culture-set, or earlier in case of MRSA recurrence. The major outcome of this study was either recurrence, defined as growth of MRSA with the same *spa*-type as before eradication therapy, at any screened site or successful eradication defined as no growth of MRSA at all screened sites at the end of a complete follow-up period. For each patient, data were collected on recurrence or successful eradication, number of culture-sets taken, follow-up period and data to analyse whether carriers were complicated or uncomplicated carriers, as defined by the guideline of the Dutch Working Party on Antibiotic Policy.[1] Furthermore, we analysed the outcome in defined subgroups, such as patients, health care workers, complicated or uncomplicated carriers and for combined nose throat carriers versus other

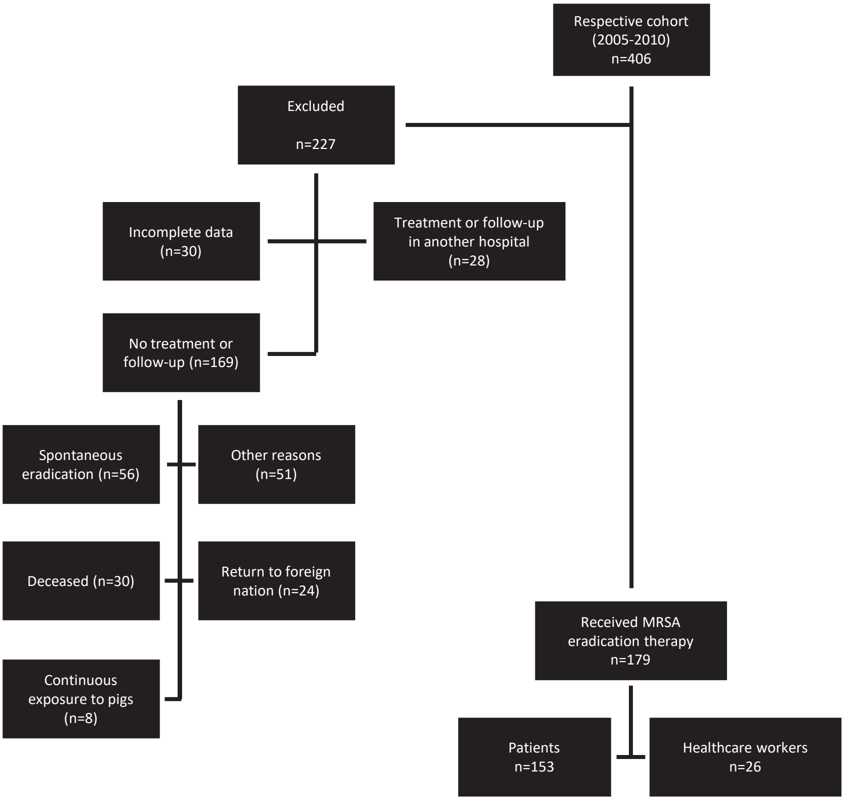


**Figure 1 – Flowchart**

carriers. Between 2005 and 2007 the microbiological method to detect MRSA was similar as described previously by Vos et al.[5] After 2007, an additional step was included. Swabs of newly suspected MRSA carriers that had been inoculated on phenol red mannitol enrichment broth were incubated for 24 h at 35°C, after which they were put in a PCR pool. If the PCR pool result was negative, this information was passed on to infection prevention team and the broth incubated for another 24 h, followed by subculture on a blood agar plate as usual. With a positive PCR pool result, PCR was redone for each individual isolate, followed by a subculture on blood agar plate for the positive isolate, as opposed to waiting another 24 h. Sampling was deemed improper if the number of colony forming units on the initial blood agar plate, incubated at the same time as the phenol enrichment broth, was under fifteen. Statistical analyses were performed with SAS Enterprise Guide 4.2 and included descriptive analyses, multiple regression and Kaplan Meier survival curves with Mantel-Cox test.

## II - risk factors for outcome

By using search terms in Pubmed (MRSA combined with one or more of the following search terms: drug effects, treatment failure, treatment outcome, anti-bacterial agents, therapeutic use, recurrence, risk factors, factors, determinants), risk factors for MRSA recurrence were extracted from the literature and selected for our analysis. Out of 56 original abstracts, ten remained based on relevance. One study was excluded because it described a risk factor (household infection) not obtainable from our hospital's electronic medical record system. Risk factors for recurrence were defined at start of eradication therapy and included sex, age,[6] complicated MRSA carriage,[6-8] presence of artificial devices,[8,9] presence of drains,[8] artificial ventilation,[10] presence of intravascular lines,[8] use of immunosuppressives[11] and presence of an underlying disease (presence of anatomical abnormality, auto-immune disease, cardiovascular disease, cerebrovascular disease, congenital disease, gastro-enteric disease, iatrogenic, infectious disease, neoplasm, psychiatric disease or traumatic events).[7-10,12-14] Furthermore, we added risk factors for acquisition of MRSA based on the Dutch (WIP) guideline on MRSA (MRSA of Known Origin/MRSA of Unknown Origin)[15] and risk factors described in literature: total duration of hospitalization,[10] Barthel index (Activities of Daily Living scale),[7] use of antibiotics (12 months prior to start eradication therapy),[10] drug abuse and the presence of Panton-Valentine leukocidin (PVL) in the carriage strain. Data on these risk factors were collected from the electronic medical record system. Risk factors with 50% or more missing values were omitted from statistical analysis. Statistical analysis was performed with SAS Enterprise Guide 4.2 using c2 and Fisher exact test. A p-value of <0.05 was considered significant. A multiple regression model was made, using all risk factors with p-values of <0.20.

## RESULTS

### I - carriers and follow up

Four hundred and six MRSA carriers were detected during the inclusion period of which 179 received eradication therapy and had a negative first follow-up culture-set (Figure 1), and were therefore included in the study (HCWs: n= 26, patients: n=153). The mean age of the study population was 32 years and 94 were male (52.5%; 94/179). Between the second and sixth set 36% (65/179) of participants showed a recurrence of MRSA carriage. Median period between the first negative culture-set (considered initially successful eradication therapy) and the occurrence of recurrence for the second, third, fourth, fifth or sixth culture-set, was 7, 14, 24, 38 and 49 days respectively (Table 1). Cumulative non MRSA recurrence at three sets was 0.88, and at six sets 0.71 (Figure 2). The difference (0.88-0.71) equalled 35 recurrences, which is 54% (35/65) of total recurrences during follow-up (Table 1, Figure 2). The median number of days to detect a recurrence was 24 (range 4-185 days; IQR 14-42). The first 2 months (61 days) of follow-up, 88% (57/65) of recurrences were detected. The remaining 12% was detected between 62 and 200 days (one at the second culture set, one at the third culture set, two at the fourth, three at the fifth culture set and one at the sixth culture set). All recurrences in HCWs were detected within 61 days. For all 153 patients, 55 showed recurrences with eight occurring after two months (15%; 8/55). Complicated carriers were 50% (13/26) of all HCWs and 83% (127/153) of all patients. There was insufficient data for 17 patients (two with recurrence) to conclude whether they were complicated or uncomplicated carriers (Figure 3). Recurrence in the subgroups was 1/9 (11%) for uncomplicated patient carriers, 52/127 (41%) for complicated patient carriers, 4/13 (31%) for uncomplicated HCWs and 6/13 (46%) for complicated HCWs. There were no significant differences for the patient, HCW and complicated carrier subgroups (log-rank, p = 0.3371, Wilcoxon p = 0.3343). The recur-

**Table 1 – Follow-up culture-sets after MRSA eradication therapy**

| | Days from the end of eradication therapy until the culture set was taken | | | | |
|---|---|---|---|---|---|
| **Culture set #** | **Range** | **Median** | **Mean** | **Number of recurrences [a]** | **MRSA negative persons left** |
| 1[b] | - | - | - | - | 179 |
| 2 | (4-128) | 7 | 22 | 11 | 168 |
| 3 | (8-77) | 14 | 21 | 19 | 149 |
| 4 | (16-200) | 24 | 40 | 16 | 133 |
| 5 | (27-185) | 38 | 70 | 8 | 125 |
| 6 | (36-117) | 49 | 60 | 11 | 114 |

[a] The number of MRSA recurrences during follow-up after MRSA eradication therapy.

[b] If positive at culture set #1, there has not been a successful eradication therapy in the first place. Therefore, all persons in this study were negative in the first culture set.

rence in the uncomplicated patient carrier subgroup was at 24 days. For combined nose throat carriers, recurrence was 17/31 (55%), in all other carriers recurrence was 38/116 (33%) (log-rank, p = 0.01. Wilcoxon p = 0.009). There was missing data for 27 carriers (26 HCWs and 1 patient for a total of 11 recurrences) on sites of carriage.

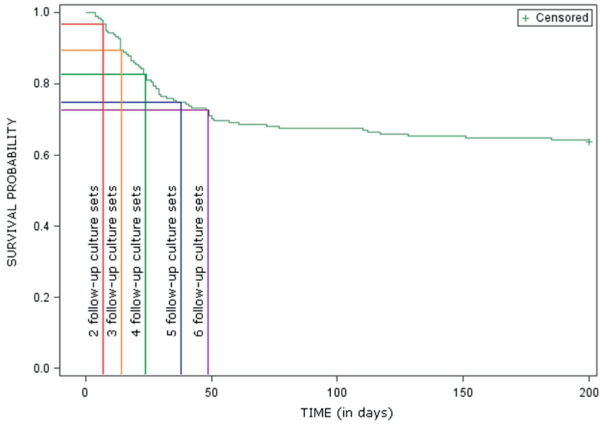


**Figure 2 – Follow-up culture-sets after MRSA eradication therapy and detection of MRSA recurrence.**
At day 0, all cases had completed their MRSA eradication therapy and had once tested negative for MRSA (first follow-up culture-set). The data was censored at 200 days, leaving a cumulative non MRSA recurrence of 0.64 for the remaining 114 cases (successfully decolonized as determined with six culture-sets). The median number of days at which the second, third, fourth, fifth or sixth culture-sets was taken, intersects with the non MRSA recurrence curve.

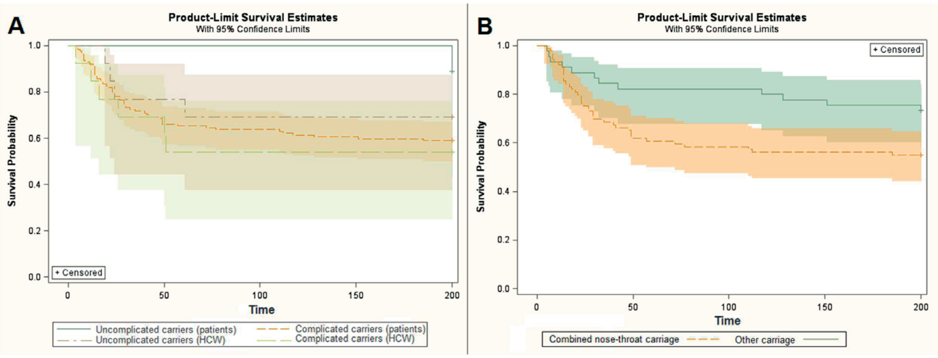


**Figure 3 – Subgroup analysis of HCWs, patients, complicated carriers and combined nose throat carriers.**
A: There was insufficient data for 17 patients to conclude whether they were complicated or uncomplicated carriers. Complicated/uncomplicated MRSA carriage definitions were according to the guideline of the Dutch Working Party on Antibiotic Policy.[1] There were no significant differences between the subgroups (log-rank, p = 0.3015, Wilcoxon p = 0.2543). B: There was a significant difference between combined nose throat carriers and other carriers (log-rank, p = 0.01, Wilcoxon p = 0.008).

### II - risk factors for outcome

For the 150 patients the average age was 31 years, 85 were male (56.7%; 86/150), and 31.3% were MUO (MRSA of Unknown Origin; no presence of described risk factors from the Dutch national guideline)[15] and 86% (129/150) were complicated MRSA carriers. Combined nose and throat carriage was a risk factor for early recurrence in multiple regression analysis (p = 0.02, OR 25.5 (1.6-419.1)), as was the presence of intravascular lines (p = 0.04, OR 13.6 (1.2-156.2)). Interestingly, ventilation at detection was a protective factor for recurrence itself in univariate and multiple regression (p = 0.02, OR 0.016 (0.03-0.75)), while age under 30 was a protective factor for early recurrence in multiple regression analysis (p = 0.04, OR 0.07 (0.01-0.85)). The backward elimination models for recurrence had a R2 of 0.11, with an area under the ROC curve (AUC) of 0.6170, and for early recurrence the R2 was 0.42, with an AUC of 0.8210. The univariate and multiple regression results for the analysis of risk factors are given in Table 2.

## DISCUSSION

Using six follow-up culture-sets, 88% of MRSA recurrences were identified within two months of follow up (after eradication therapy and one negative culture set). In contrast, using three culture-sets only 46% (30/65) of MRSA recurrences were identified. These results question the current recommendation by the Dutch national guideline, as pre-emptive declaration of successful decolonization may result in transmission within healthcare centres or in the community. To detect a recurrence, a test with a high negative predictive value (NPV) is required. Current PCR MRSA assays and chromogenic agars have very high NPV (between 99.1 and 99.5),[16] so the chance for a false negative outcome when screening for MRSA recurrence is small. A single swab at two months could detect the majority of the recurrences with a sufficient NPV. However, quick detection of a recurrence is desirable as it allows the carrier to readily receive repeat eradication therapy and prevents MRSA transmission. Especially in the household of the recurrent carrier, the chance of (re-)transmission of the strain to household members will be reduced. Also, in the healthcare setting, isolation measures would be continued thereby preventing transmission. The right balance between the number of culture-sets taken and the patient's burden needs to be found. We therefore recommend that carriers with risk factors for early recurrence are more frequently cultured at the start of follow-up, and we advise the same for HCWs because the duration of the follow-up has a strong impact on when they can start working again. In line with Mollema et al.[4] we recommend five culture-sets after the initial culture-set taken to confirm success of eradication therapy. Treated carriers at risk for early recurrence and HCWs should be cultured three times during the first month, then once more at two and six months after completion of MRSA

**Table 2 – Risk factors for recurrence after MRSA eradication therapy**

| | No recurrence (n=94) | Recurrence (n=56) | Univariate p-value [a] | OR (95%CI) | Early recurrence (n=29) | Late recurrence (n=27) | Univariate p-value [a] | OR (95%CI) |
|---|---|---|---|---|---|---|---|---|
| **Risk factors** | **n (%)** | **n (%)** | | | **n (%)** | **n (%)** | | |
| Sex | | | | | | | | |
| Male | 56 (60) | 29 (52) | 0.40 | - | 18 (61) | 11 (41) | 0.18[f] | - |
| Female | 38 (40) | 27 (48) | - | - | 11 (39) | 16 (59) | - | - |
| Age | | | | | | | | |
| ≤ 30 years | 48 (51) | 25 (45) | 0.50 | - | 10 (36) | 15 (56) | 0.18 [f] | 0.07 (0.01-0.85) |
| > 30 years | 46 (49) | 31 (55) | - | - | 19 (64) | 12 (44) | - | - |
| Complicated MRSA [e] | 76 (90) | 53 (98) | 0.09 | - | 29 (100) | 24 (96) | 0.46 | - |
| MRSA Carriage | | | | | | | | |
| N (Nose) | 11 (12) | 1 (2) | 0.03 | - | 0 (0) | 1 (4) | 0.48 | - |
| T (Throat) | 11 (12) | 7 (13) | 1.00 | - | 3 (10) | 4 (15) | 0.70 | - |
| P (Perineum) | 7 (7) | 2 (4) | 0.48 | - | 0 (0) | 2 (7) | 0.23 | - |
| W (Wound) | 6 (6) | 4 (7) | 1.00 | - | 3 (10) | 1 (4) | 0.61 | - |
| N, T | 14 (15) | 17 (30) | 0.04 | - | 12 (41) | 5 (19) | 0.08 [f] | 25.5 (1.6-419.1) |
| T, P | 0 (0) | 1 (2) | 0.37 | - | 1 (3) | 0 (0) | 1.00 | - |
| T, W | 0 (0) | 0 (0) | - | - | 0 (0) | 0 (0) | - | - |
| N, P | 2 (2) | 3 (5) | 0.36 | - | 2 (7) | 1 (4) | 1.00 | - |
| P, W | 0 (0) | 1 (2) | 0.37 | - | 0 (0) | 0 (0) | - | - |
| N, W | 2 (2) | 2 (4) | 0.63 | - | 2 (7) | 0 (0) | 0.49 | - |
| N, T, P | 14 (15) | 10 (18) | 0.65 | - | 3 (10) | 7 (26) | 0.17 | - |
| N, T, P, W | 4 (4) | 3 (5) | 1.00 | - | 2 (7) | 1 (4) | 1.00 | - |
| N, T, W | 6 (6) | 2 (4) | 0.71 | - | 1 (3) | 1 (4) | 1.00 | - |
| N, P, W | 3 (3) | 0 (0) | 0.29 | - | 0 (0) | 0 (0) | - | - |
| T, P, W | 1 (1) | 0 (0) | 1.00 | - | 0 (0) | 0 (0) | - | - |

**Table 2 – Risk factors for recurrence after MRSA eradication therapy (continued)**

| Risk factors | No recurrence (n=94) n (%) | Recurrence (n=56) n (%) | Univariate p-value [a] | OR (95%CI) | Early recurrence (n=29) n (%) | Late recurrence (n=27) n (%) | Univariate p-value [a] | OR (95%CI) |
|---|---|---|---|---|---|---|---|---|
| Presence of wounds | 36 (42) | 14 (25) | 0.05 | - | 10 (34) | 4 (15) | 0.13 | - |
| Artificial devices | 38 (40) | 10 (18) | <0.01 | - | 7 (24) | 3 (11) | 0.30 | - |
| Drains | 27 (33) | 7 (16) | 0.06 | - | 5 (26) | 2 (8) | 0.21 | - |
| Intravascular lines | 28 (34) | 8 (20) | 0.14 | - | 6 (35) | 2 (9) | 0.05 [f] | 13.6 (1.2-156.2) |
| Ventilation | 25 (29) | 3 (7) | < 0.01 [f] | 0.16 (0.03-0.75) | 2 (11) | 1 (4) | 0.56 | - |
| Other | 14 (17) | 2 (6) | 0.15 | - | 2 (12) | 0 (0) | 0.48 | - |
| Hospitalization before therapy | 33 (35) | 15 (27) | 0.37 | - | 10 (34) | 5 (19) | 0.23 | - |
| Hosp. Duration | | | | | | | | |
| ≤ 1 week | 10 (30) | 4 (27) | 1.00 | - | 3 (30) | 1 (20) | 1.00 | - |
| >1 week | - | - | - | - | 7 (70) | 4 (80) | - | - |
| Underlying disease[c] | 66 (84) | 36 (84) | 1.00 | - | 20 (91) | 16 (76) | 0.24 | - |
| Antibiotic use [b] | 20 (29) | 10 (27) | 1.00 | - | 5 (33) | 5 (23) | 0.71 | - |
| Imm.supp. use | 13 (16) | 6 (15) | 1.00 | - | 2 (12) | 4 (18) | 0.68 | - |
| MUO [d] | 32 (34) | 15 (27) | 0.37 | - | 8 (28) | 7 (26) | 1.00 | - |
| PVL-positive | 18 (23) | 9 (18) | 0.66 | - | 3 (13) | 6 (23) | 0.47 | - |

[a] 2x2 tables with Fisher Exact test.

[b] In the 12 months prior to MRSA eradication therapy

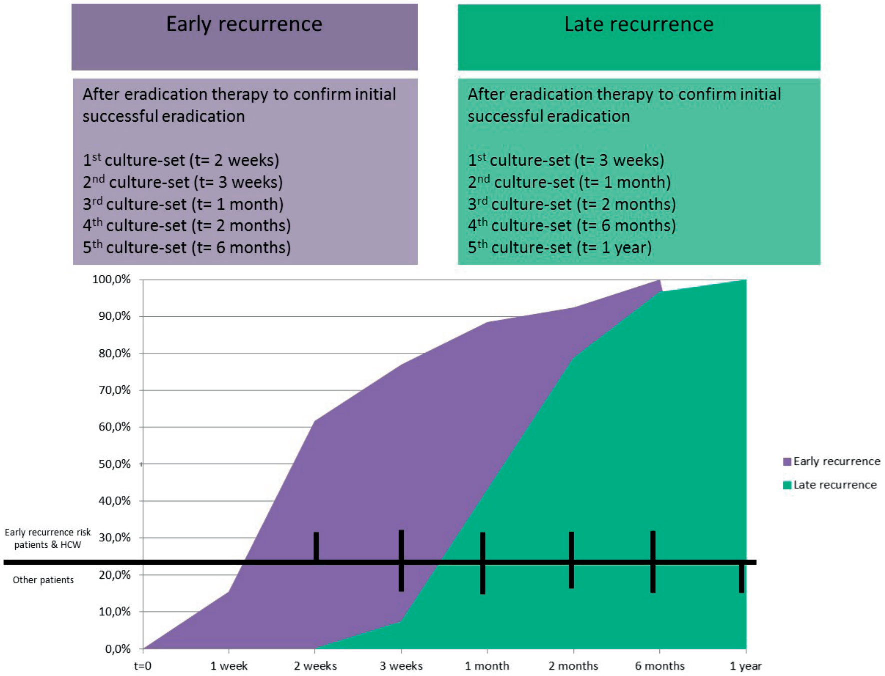
[c] Presence of anatomical abnormality, auto-immune disease, cardiovascular disease, cerebrovascular disease, congenital disease, gastroenterestic disease, iatrogenic, infectious disease, neoplasm, psychiatric disease or traumatic events.

[d] MRSA without described risk factors in the Dutch WIP guideline. (MRSA of Unknown Origin)

[e] Complicated MRSA defined according to Dutch SWAB guideline.

[f] The backward elimination model for early recurrence with the following risk factors: male sex, age under 30, combined nose and throat carriage, presence of intravascular lines. AUC: 0.8210, $R^2$max 0.42. Risk and protective factors remaining significant: age under 30 years p=0.04, OR 0.07 (0.01-0.85), presence of intravascular lines p=0.04, OR 13.6 (1.2-156.2) and combined nose and throat carriage p=0.02, OR 25.5 (1.6-419.1).

The backward elimination model for recurrence had the following risk factors: complicated MRSA, combined nose and throat carriage, the presence of any wounds, the presence of drains, the presence of intravascular lines, having been ventilated, other applied artificial devices. AUC 0.6170, $R^2$max 0.11. Protective factor remaining significant: having been ventilated p=0.02, OR 0.16 (0.03-0.75) eradication therapy. For all others we recommend two culture-sets during the first month, followed by a culture-sets at 2 and 6 months, and finally the last culture-set at 1 year after eradication therapy ended (Figure 4).



| Early recurrence | Late recurrence |
|---|---|
| After eradication therapy to confirm initial successful eradication | After eradication therapy to confirm initial successful eradication |
| 1st culture-set (t= 2 weeks)<br>2nd culture-set (t= 3 weeks)<br>3rd culture-set (t= 1 month)<br>4th culture-set (t= 2 months)<br>5th culture-set (t= 6 months) | 1st culture-set (t= 3 weeks)<br>2nd culture-set (t= 1 month)<br>3rd culture-set (t= 2 months)<br>4th culture-set (t= 6 months)<br>5th culture-set (t= 1 year) |

**Figure 4 – Recommendations for follow-up culture-sets.**
HCWs: Healthcare workers. HCWs follow-up is similar to early recurrence risk patients for fast recovery of personnel.

As risk factors for recurrence, we found colonization of the nose in combination with the throat, as well as presence of intravascular lines. The high risk on recurrence in nose-throat carriers cannot be explained by treatment failure due to the sole use of topical antibiotics, since MRSA eradication therapy for complicated carriers consisted of two oral antibiotics in addition to nasal ointment of mupirocin and chlorhexidin wash for skin and hair. Ventilation was protective for recurrence, however, we did not understand this finding. Possibly this is a confounder for other, unknown determinants, or it is related to the amount of antibiotics ventilated patients receive. We found that ventilated patient carriers received more antibiotics ($p < 0.01$), had more drains ($p < 0.01$) and artificial devices ($p < 0.01$), but both ventilated as non-ventilated carriers were similar in age, combined nose throat carriage or complicated carriage composition. Limitations of this study were its retrospective design, the use of data from a single hospital, and the omission of risk factors due to missing data. Furthermore, in our patient population 86% were complicated carriers, due to colonization on other sites than the nose only and/or the presence of active skin lesions or medical artificial devices. This is higher than the 54% reported before in a national study.[7] Our data showed that a regime of three follow-up cultures was not sufficient, and would have missed 54% (35/65) of total recurrences, which is in line with the previously recommended minimum of five culture-sets by Mollema et al.[4] Since culture-sets were not always taken with equal time intervals, we calculated the median number of days that each culture-set was collected from start of follow up, using the median number of days to determine the number of recurrences for each culture-set.

Although small group sizes were inevitable due to the low prevalence in The Netherlands (0.11% at hospital admission[17]), current group sizes are the result of six years of patient data (2005-2010). To our knowledge, these data are the only available data worldwide specifically addressing the question on the required number of culture-sets during follow-up after eradication therapy to establish success of eradication. Our recommendation is a five culture-set scheme up till one year after successful eradication therapy with a distinction between those at risk for early recurrence and HCWs who require more frequent culturing in the beginning and those not at risk for early recurrence. The reason to still culture at half or one year is to catch the last remnants of recurring MRSA after two months as cost-effective as possible. This recommendation is a balance between the need for swift detection of recurrence and the patients' burden. Nevertheless, it remains most important that household members of the carrier are also screened and if necessary treated to prevent recurrence due to household transmission.[18]

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# 5

# Seafarers: a new risk group for methicillin-resistant *Staphylococcus aureus* (MRSA)

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**Eurosurveillance, October 2013**

We applaud the successful implementation of meticillin-resistant *Staphylococcus aureus* (MRSA) screening programme on the German side of the Dutch–German border region (EUREGIO) [1]. The described strategy is based on the risk-based admission screening approach [1]. From our experience with 'search and destroy' (S and D) in the Netherlands, we learned that it is essential to evaluate and timely enlarge risk categories when epidemiology changes to prevent MRSA of unknown origin (MUO), i.e. not fitting any defined risk factors [2]. Herewith, we report our recent finding of a previously unrecognised risk group for MRSA: seafarers. In 2010, we noticed that clinical cultures with unexpected MRSA in the Harbour Hospital (Port of Rotterdam, the Netherlands) were mainly from seafarers. Although the Harbour Hospital is especially equipped for seafarers, this patient population only accounts for 1.2% of all admissions [3]. Since seafarers are not considered an MRSA risk group, all seafarer patients were screened (nose and throat) at the Emergency Department of the Harbour Hospital in a six month prospective surveillance (2011). Perineum and wounds were screened additionally, if active infection or skin lesions were present. Detection of MRSA was performed as previously described [4], and *spa*-typing was done at the reference laboratory of the National Institute for Public Health and the Environment (RIVM) [5]. Furthermore, since the hospital is visited by a large number of seafarers, a case–control study was designed to identify risk factors for MRSA carriage among seafarers, in order to identify specific risk factors within this putative new risk group. Cases were defined as seafarers with a positive MRSA culture of any site, whereas controls had a negative MRSA culture. Data on demographics, medical history, laboratory and naval parameters were collected retrospectively. Data were then analysed by univariate (chisquared, Fisher's exact test) and multivariate analysis (logistic regression model).

In the study period 124 seafarers (men, 22–51 years of age) were included. Four seafarer patients had an unknown MRSA status and were excluded. MRSA prevalence among seafarers was 5.8% (95% confidence interval (CI): 4.6–7.1) and the incidence rate was 24.8/1,000 seafarer population. Seven MRSA-positive seafarers were identified as cases, leaving 113 MRSA negative seafarers as controls. Of seven MRSA carriers, four had wounds, of which three were cultured MRSA positive as well. Furthermore, of seven MRSA strains, five had similar *spa*-repeat successions: t019 (twice),

t122, t975 and t4557. The remaining two were t4845 and t9231. Nationality was only known for 32 seafarers.

Twenty-five of them were of Asian origin, with 18 seafarers from the Philippines. Of MRSA carriers, nationality was known in two cases: the Philippines and India. Severe missing data for many proposed variables forced us to exclude many variables, as registration of seafarers was basic due to swift departure and communication difficulties. The remaining risk factors are listed in the Table. Presence of wounds or abscesses was the only significant risk factor ($p<0.01$) in both univariate

analysis and multivariate regression analysis (odds ratio (OR): 40.8 (95% CI: 5.9–278.3)). The multivariate regression model was based on forward selection with 'presence of wounds or abscesses', 'detection of pus' and 'C-reactive protein (CRP)' (area under the receiver operator characteristic criterion (AUC): 0.75, $R^2$max: 0.4603). The positive predictive value for finding an MRSA carrier when a seafarer had a wound was 44% whereas the negative predictive value (MRSA carrier when a seafarer has no wound) was 2%. The MRSA carriage did not influence the duration of hospitalization of the seafarers ($p=0.36$). In our limited sample of 124 seafarers, the 5.8% MRSA prevalence detected is 52-times higher than the normal prevalence in the Netherlands (0.11% at hospital admission) [4], and the presence of wounds or abscesses gave a 40-times higher risk of being MRSA positive. Some bias might play a role due to our screening procedure. However, this choice was made due to communication difficulties and cultural differences. Given the high prevalence rate of carriage among seafarers, we recommend that all seafarers should be screened for MRSA in the Netherlands, regardless of wounds or underlying disease, and to apply pre-emptive isolation while awaiting test results, and should be taken into consideration as risk group by other European nations. Further studies are necessary to understand the impact of global transmission of MRSA clones by seafarers.

**Table 1 – Risk factors for meticillin-resistant *Staphylococcus aureus* (MRSA) in seafarers, Rotterdam, the Netherlands**

| Risk factor | MRSA positive (N=7) n(%) | MRSA negative (N=113) n(%) | P-value | Odds Ratio (95% CI) |
|---|---|---|---|---|
| Male sex | 7 (100) | 112 (99) | - | - |
| Specialist involved with patient | | | | |
| Internal medicine | 2 (29) | 21 (19) | 0.61 | - |
| Surgery | 5 (71) | 59 (52) | 0.45 | - |
| Physical examination | | | | |
| Presence of wounds or abscesses | 4 (57) | 5 (4) | < 0.01 | 40.8<br>(5.9-278.3) |
| Pus detected | 2 (28) | 2 (2) | 0.01 | 26.7<br>(2.9-241.3) |

CI: confidence interval; OR: odds ratio.
Presence of wounds or abscesses was the only risk factor ($p<0.01$) in univariate analysis and in the multiple regression model with an OR of 40.8 (95%CI: 5.9–278.3). The multiple regression model was based on forward selection with 'presence of wounds or abscesses', 'detection of pus' and 'C-reactive protein (CRP)' (area under the receiver operator characteristic criterion (AUC): 0.75, $R^2$max: 0.4603).

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# Newly identified risk factors for MRSA carriage in The Netherlands

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6

## ABSTRACT

### Objectives

To elucidate new risk factors for MRSA carriers without known risk factors (MRSA of unknown origin; MUO). These MUO carriers are neither pre-emptively screened nor isolated as normally dictated by the Dutch Search & Destroy policy, thus resulting in policy failure.

### Methods

We performed a prospective case control study to determine risk factors for MUO acquisition/carriage (Dutch Trial Register: NTR2041). Cases were MUO carriers reported by participating medical microbiological laboratories to the RIVM from September 1st 2011 until September 1st 2013. Controls were randomly selected from the community during this period.

### Results

Significant risk factors for MUO in logistic multivariate analysis were antibiotic use in the last twelve months, aOR 8.1 (5.6−11.7), screened as contact in a contact tracing but not detected as a MRSA carrier at that time, aOR 4.3 (2.1−8.8), having at least one foreign parent, aOR 2.4 (1.4−3.9) and receiving ambulatory care, aOR 2.3 (1.4−3.7). Our found risk factors explained 83% of the MUO carriage.

### Conclusions

Identifying new risk factors for MRSA carriers remains crucial for countries that apply a targeted screening approach as a Search and Destroy policy or as vertical infection prevention measure.

## INTRODUCTION

In The Netherlands MRSA prevalence is low, measured at 0.12% at hospital admission in 2005−2007[1] and 0.8% in Dutch outpatients in the Dutch-German border region in 2012.[2] Among *S.aureus* blood-cultures the MRSA prevalence was 1.0% (24/2,386).[3] To keep MRSA prevalence low, prudent use of antibiotics is instigated and a Search and Destroy policy (S&D) is in place. S&D consists of screening of defined risk patients (Table 1) at hospital admission, and by pre-emptive isolation of them pending the screening results.[4] Colonized patients and healthcare workers are treated with strict treatment regimens to eradicate the carriage of MRSA. [4] One of the revisions on MRSA risk groups, was due to the discovery of livestock-associated MRSA with sequence type 398 (LA-MRSA ST398) in The Netherlands.[5] The risk groups for S&D were subsequently updated with pig and veal calf farmers. In 2016, LA-MRSA accounted for 26% (892/3,478) of MRSA isolates.[3] Apart from the discovery of LA-MRSA, it appeared that the proportion of reported MRSA without known risk factors, thus not defined as risk patients, became substantial. In 2008−2009, 25% (1350/5545) of all MRSA were reported as MRSA without known risk factors [6] In 2016, this has increased to 38% (810/2,121). [3] The MRSA without known risk factors were named MRSA of Unknown Origin or MUO. MUO are per definition unexpected and are mostly detected in clinical samples. However, in screening samples on MRSA, MUO can be detected as well. This is the case when the found MRSA genotype does not match the MRSA genotype of the index person.[6] We started a nation-wide study to explore the risks and causes of MUO, so the defined risk groups in S&D policy can be updated and unnoticed dissemination of MRSA in healthcare settings and the community can be stopped. In this paper, we report the results from our prospective case control study to determine the risk factors for carriage of MUO.

## METHODS

### MRSA surveillance

In the Netherlands, all MRSA are detected either through active surveillance screening or in a clinical sample and are mandatorily sent to the Dutch National Institute for Public Health and the Environment (RIVM). Yearly, around 3,000 isolates are submitted to the RIVM.[7] Along with the isolate, risk factors for MRSA carriage, as defined in the MRSA guideline by the Dutch Working party on Infection Prevention (WIP), are reported to the RIVM by standard questionnaire.[8, 9] Any person detected with MRSA and reported with one or more risk factors as described in this WIP guideline is defined as MRSA of Known Origin (MKO). Any person detected with MRSA lacking these risk factors, is defined as MRSA of Unknown Origin (MUO).

Table 1 – Risk categories in the Dutch WIP guideline on MRSA

| January 2007 (updated: March 2008) [8] | | December 2012 [7] | |
|---|---|---|---|
| **Patients** | **Healthcare workers** | **Patients** | **Healthcare workers** |
| **Category 1** | **Category 1** | **Category 1** | **Category 1** |
| - Proven carrier status of MRSA | - Proven carrier status of MRSA | - Proven carrier status of MRSA<br>- Follow-up after MRSA eradication therapy (3 follow-up culture-sets) | - Proven carrier status of MRSA<br>- Follow-up after MRSA eradication therapy (3 follow-up culture-sets)<br>- Undergoes MRSA eradication treatment |
| **Category 2** | **Category 2** | **Category 2** | **Category 2** |
| - Nursed < 2 months > 24h in a foreign hospital<br>- Nursed < 2 months < 24h in a foreign hospital with the following risk factors at arrival in a Dutch hospital: operation, infection, catheter or drains present<br>- Patient from department (hospital or nursing home) with an ongoing MRSA outbreak<br>- Adopted children regularly hospitalized or visiting the hospital<br>- Foreign patient at dialysis unit<br>- Share a room with unexpected MRSA carrier<br>- After MRSA eradication therapy but before follow-up culture-sets are taken<br>- Contact with live pigs or veal calves | - Unprotected contact with MRSA carrier<br>- Hospitalized < 2 months ago in a foreign hospital, were operated abroad, received a drain or catheter, were intubated, have skin lesions or possible infectious sources such as abscesses or furuncles. | - < 2 months ago unprotected contact with MRSA carrier inside (as part of contact tracing) or outside (household members, partners, caretakers of MRSA carriers) the hospital<br>- Nursed < 2 months < 24h in a foreign care facility with at least one of the following risk factors: operation abroad, chronic infection or persistent skin lesions, presence of abscesses or furuncles at hospitalization in the NL.<br>- Foreign dialysis patients<br>- Stayed < 2months ago in a Dutch care facility (unspecified) with an ongoing MRSA outbreak on the department<br>- Adopted children from abroad living in the Netherlands<br>- Contact with industrial, live pigs, veal calves or broiler chickens regardless whether this contact was professional or not, and/or lives on such a farm. | - Nursed < 2 months > 24h in a foreign care facility<br>- Nursed < 2 months < 24h in a foreign care facility with at least one of the following risk factors: operation abroad, chronic infection or persistent skin lesions, presence of abscesses or furuncles at hospitalization in the Netherlands. |

**Table 1 – Risk categories in the Dutch WIP guideline on MRSA (continued)**

| January 2007 (updated: March 2008) [8] | | December 2012 [7] | |
|---|---|---|---|
| **Patients** | **Healthcare workers** | **Patients** | **Healthcare workers** |
| **Category 3** | **Category 3** | **Category 3** | **Category 3** |
| - Dutch dialysis patients dialyzed abroad<br>- First year after MRSA eradication therapy with MRSA negative follow-up culture-sets<br>- Nursed > 2 months in foreign hospital with persistent skin infections or risk factors | - Protected contact with MRSA carriers<br>- < 2 months ago worked abroad >24h in a hospital or nursing home<br>- First year after MRSA eradication therapy with MRSA negative follow-up culture-sets | - Unprotected contact with MRSA positive HCW < 2 months ago<br>- Dutch dialysis patients dialyzed abroad < 2 months ago<br>- Nursed > 2 months in foreign hospital with persistent skin infections or risk factors<br>- First year of follow-up after MRSA eradication therapy and the first three negative follow-up culture-sets<br>- Persistent exposure with a negative MRSA test less than three months ago. | - Persistent exposure with a negative MRSA test less than three months ago.<br>- Unprotected contact with MRSA positive patient < 2 months ago inside or outside the hospital<br>- < 2 months ago > 24h patient-related activities in a foreign care facility<br>- Guided patients < 2 months ago from a foreign to a Dutch care facility without isolation precautions<br>- Carrier with uncomplicated MRSA who was negative before the start of MRSA eradication therapy |
| **Category 4** | **Category 4** | **Category 4** | **Category 4** |
| - Nursed > 2 months ago in a foreign hospital without persistent skin lesions or risk factors<br>- Stayed < 24h in a foreign hospital without risk factors or operations<br>- On a department with MRSA where adequate precautions were taken<br>- Negative follow-up culture-sets a year after MRSA eradication therapy ended | - Successful MRSA eradication therapy > 1 year ago. Follow-up culture-sets remained MRSA negative<br>- Negative follow-up culture-set after protected contact with MRSA carrier | - None of the above categories applies | - None of the above categories applies |

HCW: Healthcare worker. For the trawling and case control questionnaires, the risk categories of the 2007-2008 WIP guideline were used. In 2015, the RIVM added as risk factor a refugee visiting a Dutch hospital who had been in a refugee camp less than two months before (category 2/3).

## Trawling study

A trawling questionnaire was forwarded by Dutch Medical Microbiological Laboratories (MML) in 2010 to all MUO carriers reported to the RIVM in 2009. The retrospective trawling questionnaire was set-up to learn which risk factors could be involved with MUO to narrow and specify the number and kind of questions in the case control questionnaire, as well to choose the best control group for the case control study. To prevent recall bias, the maximum timespan for events in the past that had to be recalled by trawling study participants was limited to two years. To confirm that these MUO carriers were not misclassified MKO carriers, the questionnaire included questions on the described risk factors for MRSA in the Dutch WIP guideline on MRSA (Table 1). Furthermore, the questionnaire included questions on occupations, sports, leisure, social habits and lifestyles, and risk factors in other populations described in the literature. (PubMed at 01-01-2010, search keywords `MRSA' and `risk factor'). Excluded cases were non-responders, the deceased, potential cases that lacked an address or were misclassified as MKO for various reasons. Included cases were all MUO carriers not misclassified as MKO upon return of the questionnaire. Questionnaire data was analysed as described under `statistics', and the results were interpreted to update the questionnaire and define the best controls.

## Case control study

*Study population*

The study population consisted of patients detected with MRSA but without known risk factors (MUO) and population controls. The sample site and frequency of detection was not taken into account. In the Netherlands, persons detected with MRSA are all included in the national MRSA database, regardless of sample site, infection or indication for sampling. To determine the risk factors for MUO, we approached cases and controls with questionnaires (Supplementary list 1). Case control study participants who answered ≥95% questions of the total of 43 questions and of whom informed consent was obtained, were rewarded with 25 euros and enrolled as case or control. To detect an odds ratio (OR) of 2 or higher with 80% power and an alpha of 0.05, we aimed to enrol 500 cases and 1,000 controls (1:2 case-control ratio), based on an estimated 700 MUO reported to the MRSA surveillance in two years (on a total of ±3,000 MRSA carriers reported per year).

*Case definition*

Potential cases were MRSA carriers, reported by the participating MML (medical microbiologist or infection prevention personnel) as MUO to the RIVM for the MRSA surveillance from September 1st, 2011 until September 1st, 2013. Before sending the questionnaire, assumed MUO cases were checked on the following exclusion criteria: death, lack of address or misclassification (of a MKO as a MUO). Upon return of the questionnaire, we checked once more for misclassification. True MUO were included as case.

### *Control definition*

Selecting the right controls was based on the results from the trawling study (see results below). The best control group was considered to be unmatched community controls. This choice was based on the fact that the MUO were selected from the RIVM database, which contained not only MRSA carriers detected at hospitals, but also those detected by general practitioners or in long-term care facilities. Furthermore, MUO carriers were shown to be a diverse group of carriers. The controls were randomly chosen from 60 Dutch municipalities from all over of The Netherlands. These 60 municipalities were a national representative subset of all 415 municipalities in The Netherlands, and contained large, middle and small municipalities.

### *Inclusion procedure*

Every two weeks a printout was made in Microsoft Excel of the newly reported MUO to the RIVM. Questionnaires for MUO carriers were sent to participating MML (Supplementary table 1). The MML checked the carriers on exclusion criteria before forwarding the questionnaires to the MUO carriers. After three weeks, non-responders were once again approached by questionnaire or by telephone. Returned questionnaires of both cases and controls were checked to exclude any MKO carriers misclassified as MUO. Misclassifications were not included as cases. Controls were sent a questionnaire by mail. After three weeks non-responders were approached once more by mail or if possible by telephone. This study was approved by the medical ethical committee at the Erasmus MC and registered in the Dutch Trial Register under NTR2041. Written informed consent was requested from both cases as controls, before participating in this study by questionnaire. Data were aggregated and anonymized before analysis.

### *Statistics*

Questionnaire data were analysed with SAS Enterprise Guide (version 4.2 by SAS Institute Inc., North Carolina, USA), using descriptive, univariate (2x2 tables and Fisher's Exact test) and multivariate analysis (multiple regression logit model, backward elimination with significance level of 0.05 to stay in the model, with and without dummy variables).

# RESULTS

## Trawling study

Of the reported 794 MUO to the MRSA surveillance, only 277 fulfilled inclusion criteria and could be approached by questionnaire through participating MML (Figure 1). Of these 37% (104/277) responded and all age groups were present. Of the 104 returned questionnaires, 22 were MKO, and thus misclassified as MUO and the remaining 82 were MUO.

Fifty-two percent (43/82) of MUO carriers were male and none of the MUO were healthcare workers. Sixty-six percent (54/82) of MUO were patients detected in the hospital. However the other MUO carriers were detected by general practitioners or community-based healthcare institutions other than hospitals.

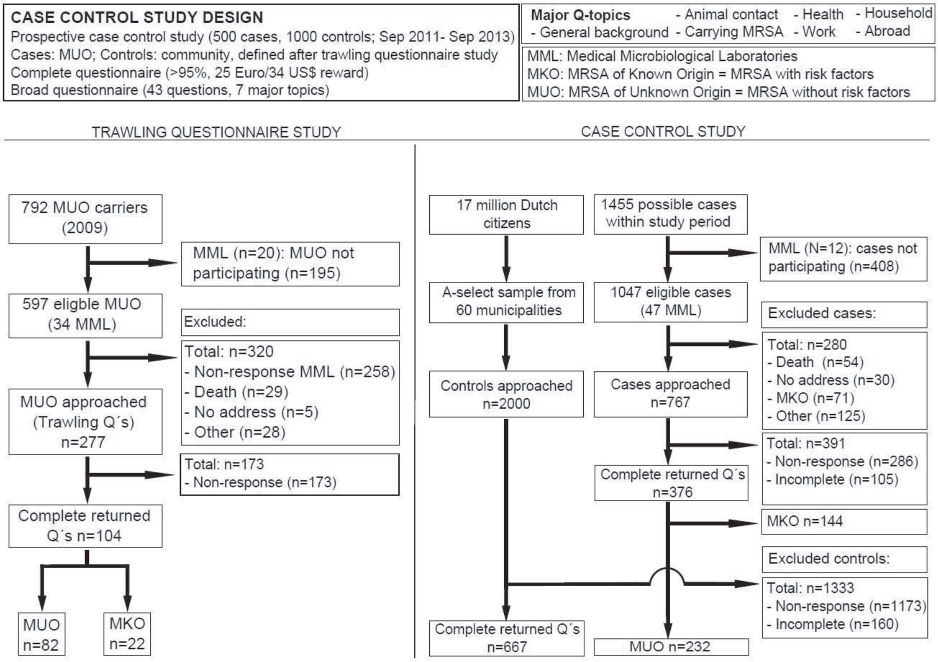

**Figure 1 – Flowcharts for trawling and case control studies.**

## Case control study

Between September 1st 2011 and September 1st 2013 1,455 MUO were reported to the RIVM and 767 MUO were approached by questionnaire (Figure 1). The response rate among cases was 49% (376/767), that of controls 33% (667/2,000). Of the 376 returned questionnaires, 38% (144/376) of cases turned out to be MKO, thus misclassified as MUO, leaving 232 cases for analyses. None of these 232 cases were healthcare workers.

Comparing 232 cases with 667 controls, some risk factors, such as hospitalization of a household member, chronic disease, and carriage of ST398 MRSA without professional contact with pigs/veal calves or other farm animals, were significant in univariate analysis, but not in the final regression model (Table 2). Significant factors in the multivariate logistic regression model were antibiotic use in the last twelve months aOR 8.1 (95%CI 5.6−11.7), screened as contact in a contact tracing but not detected as a MRSA carrier at the time aOR 4.3 (95%CI 2.1−8.8), having at least one foreign parent aOR 2.4 (95%CI 1.4−3.9), and receiving ambulatory care aOR 2.3 (95%CI 1.4−3.7). The most frequently used antibiotics by cases and controls were β-Lactam-antibiotics. There was no significant difference (p = 0.9) between cases and controls for β-Lactam-antibiotics use in general, although there was a significant (OR 5.7; 95%CI 1.4−23.1) difference for the use of Amoxicillin/clavulanic acid (13.2% among cases (7/53) versus 2.6% (3/116) among controls). Among ambulatory care use, home care was the most common, and MUO carriers were significantly more exposed (OR 3.3; 95%CI 1.5−6.9) to it. Eighty-three percent of all MUO could be explained by the found independent risk factors in multiple regression model. (Table 3).

**Table 2 – Multivariate logistic regression for MUO risk factors**

| Risk factor | MUO (n=232) | Controls (n=667) | p-value | aOR[a] | 95%CI |
|---|---|---|---|---|---|
| ST398 but without professional contact with pigs/ veal calves or other farm animals* | 18 | 0 | n.s. | 1.8 | (0.6-5.2) |
| Hospitalization within household[b] | 84 | 157 | n.s. | 1.1 | (0.7-1.7) |
| Chronic disease[2] | 85 | 135 | n.s. | 1.4 | (0.9-2.0) |
| Antibiotic use in last 12 months | 150 | 139 | < 0.01 | 8.1 | (5.6-11.7) |
| Screened as part of a contact tracing but not found to be a MRSA carrier at the time | 24 | 23 | < 0.01 | 4.3 | (2.1-8.8) |
| At least one foreign parent | 48 | 71 | < 0.01 | 2.4 | (1.4-3.9) |
| Ambulatory care received | 55 | 66 | < 0.01 | 2.2 | (1.4-3.7) |

aOR: adjusted Odds Ratios; CI: Confidence Interval;

* before 2012 only professional contact to pigs/veal calves was a risk factor. After 2012 any contact to pigs, veal calves and broiler chickens became a risk factor. But presence on a farm is not a risk factor per se, unless at the farm they have pigs, veal calves or broiler chickens.

[a] Logistic regression model with backward elimination containing the following factors: no professional contact with pigs/veal calves or other farm animals, antibiotic use in the preceding 12 months, chronic disease, not detected with contact tracing, at least one foreign parent, hospitalization within the household and ambulatory care. The $R^2$max of the model was 0.29, while the AUC was 0.79.

[b] These factors were univariate significant, as well as possible confounders for receiving ambulatory care.

**Table 3 – Risk factors for MUO**

| Risk factors | MUO (%; n=232) |
|---|---|
| All cases with the risk factor: | |
| Antibiotic use in the last 12 months | 150 (64.7) |
| Screened as part of a contact tracing but not found to be a MRSA carrier at the time | 24 (10.3) |
| At least one foreign parent | 48 (20.7) |
| Ambulatory care received | 55 (23.7) |
| | |
| Number of cases that only have this one risk factor | |
| Antibiotic use in the last 12 months | 52 (22.4) |
| Screened as part of a contact tracing but not found to be a MRSA carrier at the time | 5 (2.2) |
| At least one foreign parent | 8 (3.4) |
| Ambulatory care received | 6 (2.6) |
| | |
| Number of cases that only have one risk factor | 71 (30.6) |
| Number of cases with a combination of 2 or more of the above risk factors | 121 (52.2) |
| Total cases of MUO explained by these risk factors | 192 (82.8) |
| Remaining unexplained MUO | 40 (17.2) |

MUO carriers had a single risk factor in 30.6% (71/232) and had in 52.2% multiple risk factors. Among those MUO with a single significant risk factor, antibiotic use in last twelve months accounted for 22.4%, at least one foreign parent for 3.4%, ambulatory care received for 2.6%, and screened as part of a contact tracing but not found to be a MRSA carrier at the time for 2.2%.

## DISCUSSION

We identified the following independent risk factors for MUO: antibiotic use in the preceding 12 months, receiving ambulatory care, and being screened for MRSA in contact tracing but not having been detected at the time. Travelling abroad was not a risk factor, although we found a significant association with having a foreign parent. In literature, antibiotic use in the last twelve months has been described before as risk factor for the general population; as a risk factor for MRSA carriage in children [10, 11], and within households where carriers were present [12]. Also, a systematic review showed a association between antibiotic exposure to quinolones, glycopeptides, cefalosporins and beta-lactams and an increased risk of MRSA isolation in adults.[13, 14] These former findings were confirmed by our study, as we found a significant difference in amoxicillin/clavulanic acid use between cases and controls. Greater use of amoxicillin/clavulanic acid may be due to more infections among MUO carriers compared to the controls. We cannot rule out this possibility as we did not measure the number of infections among MUO, since submission of an infection isolate to the MRSA database is preferred but not obliged. The Netherlands has the lowest use of all antibiotics in the European Union. In 2013, 2015 and 2017, it was 10.8, 10.7 and 10.4 defined daily doses/1000 inhabitants/day respectively.[3, 15]

Interestingly, having been screened as part of a contact tracing in the past, but not detected at that time, was a significant risk factor for MUO carriage. This is an important risk factor for countries with S&D policy, as this policy aims to identify all people at risk, including contacts. Explanations for this risk factor could be a too low sensitivity of MRSA culture, missing sampling sites, or when sampling occurs too early after exposure, and is not follow-upped with repeated sampling. For this reason it is also recommended to sample healthcare workers on start of their next duty instead of immediately after unprotected contact with a MRSA carrier. The current guideline indicates one set of samples from nose, throat, rectum and wounds when present. The guidelines assumes a sampling frequency of one set to be sufficient. There are no indications in the guideline on the timing of sampling of contacts after exposure when tracing MRSA contacts. Indeed, in our previous study we showed that carriage is not always detectable in each sample moment when sampling after MRSA eradication therapy to monitor MRSA recurrence.[16] Further studies on reliability of contact tracing should be conducted, especially in regards to the number and sample sites of cultures when screening for contacts. Having at least one foreign parent, was also one of the significant risk factors. Possibly, an immigration background from countries with a higher MRSA prevalence may result in a higher exposure to MRSA by visiting or close contact within the family. Especially those countries with higher levels of CA-MRSA. This is in line with findings from Denmark which showed 40% of affected individuals CA-MRSA infections with certain CA-MRSA clones had a positive family history related to foreign regions where such clones were predominant. [17] Furthermore, the MRSA prevalence among actively screened asylum seekers (refugees/immigrants) in The Netherlands was 9.7% (87/898). [18] Similar to findings in Germany, but much higher than the prevalence in the general population at hospital admission.[18, 19] In 2015, the RIVM added as risk factor a refugee visiting a Dutch hospital who had been in a refugee camp less than two months before (category 2/3).[20] It is possible that a limited understanding of the Dutch language prevented refugees from participating. If this is the case then our aOR for having at least one foreign parent, is an underestimation. Our national MRSA database currently contains *spa*, MLVA and PVL data. But this typing data, along with scarce epidemiological data, is currently not sufficient to link MUO to outbreaks abroad or transmission or sources occurring outside a Dutch health care centre. Furthermore, in this study we did not analyse the typing data, including PVL. As we did so in a previous study, and learned that PVL positive MRSA isolates were significantly larger among MUO than among MKO.[6]

Ambulant or home care exposure are scarcely published in literature as risk factor for MRSA carriage, as only ambulatory care facilities in Germany were described and designated as a reservoir for dissemination.[21] Theoretically, transmission of MRSA through ambulatory care could be possible, thus creating new MUO carriers. This finding necessitates further investigation in the future in The Netherlands, as ambulatory care facilities are becoming more important in a population with a growing segment of the elderly. The use of arbitrary cut-offs in risk factor definitions could theoretically result in MUO. However, after multivariate analysis we found no significant risk factors related to arbitrary cut-offs in risk factor definitions, such as `less than two months ago' in case of a visit to a hospital abroad.

We could not confirm poultry consumption or scuba gear sharing as risk factors as found in the study by van Reijen et al.[22] Possible explanations for the difference could be due to design, different selection and inclusion criteria of cases and controls, difference in questioning, and the number and selection of participating MML. Continuous analyses of MUO and its risk factors in the future will be necessary, not only to measure the effect of new policy implementation, but also to elucidate differences in outcome between studies. Two risk factors published in recent years, fine air particles for Cystic Fibrosis (CF) patients and livestock-density for livestock-associated MRSA, were not included in our case control questionnaire.[23, 24] However, we think the impact of the absence of these risk factors in the questionnaire is minimal.

The use of standardized questionnaires, representative nationwide participation and community controls were study strengths which allows us to generalize results for all MUO in The Netherlands. Due to low national MRSA prevalence at hospital entry,[1] the odds of including MRSA carriers among the controls were very low. The confines of the MRSA surveillance database, the lack of exact data on infection/carriage, the necessity to contact and possible recall bias were limitations of our study. We aimed to enrol 500 cases and 1,000 controls based on an estimation of 700 MUO per two years to detect odds ratios of two or higher with 80% power. Inclusion of MUO was more difficult due to misclassification of some MUO and a lower willingness to participate in the study than expected. We therefore ended up including 232 cases and 667 controls, which was still enough to detect OR of 2 or higher with an 80% power and an alpha of 0.05, due to a larger number of controls per case. In the trawling questionnaire study, as well as in the case control study, we found there was misclassification of MKO as MUO, inflating the number of MUO in the MRSA surveillance. In the future, more effort is needed to detect the presence of risk factor before classifying a carrier as MUO, and thus registration of MKO or MUO in the RIVM database should be improved to reduce the number of misclassified MUO. Currently, 38% of total reported MRSA are reported as MUO[3, 7], underlining its significance. Even after correction for misclassification, MUO is estimated to be a fourth of total MRSA reported to the surveillance each year.

Some of the newly defined risk factors, such as antibiotic use, can be common (Table 2) and would have low specificity when included into S&D risk groups. Other risk factors, such as being part of contact tracing in the past, could result in changes to the national guideline in regards to sampling frequency and timing. To determine the probability of MRSA carriage more precisely in the future, the known risk factors (current ones in the WIP guideline and from this study) should be analysed by creating risk tables or an algorithm. The presence of a single or combined risk factors could thus lead to targeted action such as screening or screening in combination with isolation on admission. Such a probability analysis could be subject of a next study.

For countries that apply a S&D approach as vertical infection prevention approach, MUO identification and elucidation is important. In a targeted screening approach as in S&D, persons at risk for MRSA carriage are identified (targeted) by means of risk factors. Monitoring MRSA is necessary to evaluate the effect of policy adjustments and any epidemiological changes that may give rise to new risk factors. Antibiotic use in the preceding 12 months, receiving ambulatory care and having at least one foreign parent, are common risk factors with limited practicality, but could still prove useful when combined to determine the probability of MRSA carriage risk based on a risk table and algorithm. In conclusion, risk factors for MUO were mainly healthcare related despite MUO carriers not always being hospital-associated. Our new risk factors elucidated 83% of MUO, bringing us a step closer to preventing MUO from undermining successful S&D policy.[25]

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## SUPPLEMENTARY LIST 1
## CASE CONTROL QUESTIONNAIRE

0. Date of filling out the questionnaire

**General**

1. What is your sex?
2. What is your birthdate?
3. What is your postal code? (4 out of 6 characters)
4. a. What is your country of birth?
   b. If you were born outside the Netherlands, were you adopted?
5. In which country were your parents born?
6. a. Did you move the last two years?
   b. If yes, what was your previous postal code? (4 out of 6 characters)
7. What is your sexual preference?
8. In your household, are you the one who regularly cooks?
9. Do you wash your hands when preparing the food?
10. a. Are you vegetarian?
    b. If no, do you eat raw meat?
11. Do you visit one of the following locations? (Swimming pool, beach/sea, river/lake, sauna, whirlpool, pedicure, beauty salon)
12. Did you have a tattoo or piercing in the last twelve months?

**Household**

13. How many people are in your household?
14. Which of the following objects do you regularly share among your household members? (towels, wash cloths, bedsheets, shaving razors, toothbrushes, combs, creams, other, none)
15. Was someone in your household hospitalized the last year?

**MRSA**

16. a. Have you ever been MRSA positive?
    b. In the last twelve months?
    c. Do you know the exact period?
    d. Who detected the MRSA? (Hospital, long term care facility, general practitioner, other)
17. a. Have you ever been treated against MRSA with an 'eradication therapy'?
    b. Did this get rid of the MRSA?
    c. During eradication therapy, were your household members screened?
    d. Were they also treated?

**General health**

18. a. Do you sport?
    b. What type of sport? (team, individual, contact, with animals)
19. Do you smoke?
20. a. Do you dialyze?
    b. If so, have you been dialyzed abroad?
21. a. Do you know if you have/had contact with a MRSA carrier?
    b. If so, where did you have contact with this MRSA carrier?
    c. If elsewhere, where exactly?
    d. What was your relation compared to this MRSA carrier?
    e. Do you have close contact with certain people? (inside the household, outside or with people from inside and outside the household)
22. a. Have you ever been contacted in regards to contact tracing for another MRSA carrier?
    b. Were you then found to be MRSA positive?
23. a. Did you use antibiotics in the last twelve months?
    b. If so, how often and when?
    c. If so, do you remember the names?
24. a. Do you have a skin disease diagnosed by a doctor?
    b. If so, is this eczema?
25. a. Do you have a chronic disease?
    b. If so, to which group does your chronic disease belong? (lungs, cardiac/heart, auto-immune, other metabolic, diabetes, chronic infection, congenital, other, don't know)
    c. Do you happen to know the name of your chronic disease?
    d. Do you use immunosuppressive medications?
    e. If so, do you remember the names of these medications?
26. a. Did you receive ambulant care the last two years?
    b. If so, how often?
    c. If so, which kind? (homecare, general practitioner, mental healthcare, obstetric

    care, maternity, supervised living, physiotherapy, dispensary, other)

27. Did you visit an outpatient department of a Dutch hospital in the last twelve months?
28. Were you admitted to a Dutch hospital or nursing home in the last twelve months?
29. Were you care for on a department in a Dutch hospital or nursing home in the last twelve months where there was a known MRSA outbreak?
30. Did you come into contact with a MRSA carrier in a Dutch hospital or nursing home?

**Work**

31. Which situation applies to you? (Employed, retired, standing entrepreneur, student, jobless, going to school, volunteer job, <4 years old)
32. In which sector do/did you work? (Healthcare, animals, shipping, other)
33. In which sector does/did your partner work? (healthcare, animals, shipping, other)
34. In which sector does/did your household members work? (healthcare, animals, shipping, other)

***Following questions only for those working in healthcare***

35. a. Have you come into contact with a MRSA carrier?
    b. Did you take infection prevention precautions?
    c. Was MRSA detected at you after you had contact with a MRSA carrier?
36. Did you accompany patients in the last twelve months when they were transferred from a hospital abroad to one in the Netherlands? (Or the other way around)

**Animal Contact**

37. a. Did you have contact with pigs or veal calves outside your work?
    b. If yes, with pigs, veal calves or both?
    c. Where did you come into contact with these animals?
38. a. Did you partner or one of your household members come into contact with pigs or veal calves outside work?
    b. If yes, where did they come into contact with these animals?
39. a. Do you keep pets or farm animals?
    b. If so, which ones?
40. Do you ride or take care of horses?
41. Do you visit farms or petting zoos?

**Abroad**

42. a. Have you been abroad the previous year?
    b. If so, please name the countries you travelled to in your last five trips abroad within a maximum timespan of two years. And whether or not you visited a hospital then.
    c. If you visit a hospital abroad in the last two years, what was the reason? (Visit a patient, small surgery, outpatient clinic visit, admitted to hospital as patient, for work, other)
    d. Did you become MRSA positive after your visit to an abroad hospital in the last two years?
    e. Did you visit a Dutch hospital after your abroad hospital visit?
    f. Have you *ever* been in an abroad hospital?
43. a. Did a household or family member become MRSA positive after an abroad hospital visit? (in the last two years)
    b. Has a household or family member been to a Dutch hospital after visit an abroad hospital? (in the last two years)
    c. Has a household or family member *ever* been to an abroad hospital?

# Combining high resolution typing by cgMLST with epidemiological data improves the identification of the origin of MRSA with previously unknown origin

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## ABSTRACT

Introduction: The Netherlands has a growing population carrying methicillin resistant *Staphylococcus aureus* of Unknown Origin (MUO, i.e. carriers without know risk factors). Earlier findings from a case control study on MUO, drove us to identify links of MUO with MRSA of Known Origin (MKO, i.e. carriers with known risk factors).

Methods: We used core genome multi-locus sequence typing (cgMLST) combined with epidemiological data on a set of 106 ST8 MRSA (54 MUO and 52 MKO) from three regions in the Netherlands, to identify the origin of MUO and link them to known clusters of MRSA.

Results: We successfully identified the origin of 26% (14/54) of MUO by clustering them genetically and epidemiologically to other carriers of MRSA. We were able to identify regional Dutch clusters within a group of ST8 MRSA isolates, as well as detect two MRSA clusters imported from Taiwan and Aruba. Furthermore, we found that MUO isolates that were cultured in two different medical microbiological laboratories within the Rotterdam region belonged to the same genetic cluster.

Discussion: Through the combined effort of cgMLST and epidemiological data, we identified the origin of 26% (14/54) of MUO by successfully linking MUO to other known carriers of MRSA. The sources for two MUO clusters were MUO carriers which had their MRSA from abroad and who had not visited foreign health care centers. At least at regional level, cgMLST should be combined with epidemiological data to identify the sources of MRSA of previously unknown origin.

## INTRODUCTION

Traditionally, The Netherlands has low MRSA prevalence rates (0.11% 2007). However, a growing population of MRSA carriers are being detected without known risk factors as defined by our national MRSA guideline.[1] These known risk factors are used to screen at hospital admission for MRSA carriage and carriers without defined risk factors are mostly detected accidentally, for example from clinical cultures, i.e. from samples not taken with the purpose to screen for MRSA. [2]

The Dutch Health Inspectorate has urged all hospitals to submit MRSA isolates and supporting epidemiological data to National Institute for Health and the Environment (RIVM) as part of the Dutch national MRSA database. [3] As a result, a large collection of MRSA isolates and accompanying epidemiological data is available. The number of MRSA carriers without risk factors, defined as MRSA of Unknown Origin (MUO), increased in 2016 to 38% (810/2,121) of total reported MRSA isolates [4] from 31% (1000/3,247) in 2011 and 27% (786/2,969) in 2009.[1, 5] This is a significant increase of the proportion of MUO. ($p<0.01$ for 2009-2011, 2011-2016 and 2009-2016)

The last few years, highly discriminatory techniques such as whole-genome sequencing (WGS) have become popular to use in explaining and managing outbreaks. [6-9] Epidemiological studies using WGS focused on special communities like hospitals [9-12], or other relatively small communities such as long term care facilities[13], or households.[14] However, such techniques can also be used on a national level to explore transmission routes, in health care centres and in the community, and to explore new reservoirs or risk groups. The use of WGS techniques will increase our ability to infer if MRSA isolates are truly MUO or that they can be genotypically linked to MRSA isolates of known origin (MKO) and/or regional MRSA clusters.

To identify the origin of MRSA in MUO, we previously performed a case control study to detect new epidemiological risk factors present in these MUO and successfully correlated MUO with newly identified risk factors.[15] Compared to controls, 10% of MUO carriers correlated with having been a contact of a MRSA carrier in the past. (aOR 4.3)[15] At the time of contact and first screening, these MRSA carriers were not detected as MRSA positive. When MRSA was detected at a later stage, usually in a clinical sample, there was no recognition of a known epidemiological link and therefore such MRSA carriers were labelled as MUO.

This previous finding drove us to identify links of MUO with MKO. As sequence type 8 (ST8) was the most prevalent sequence type among MUO and MKO[1] in Dutch MRSA isolates, we explored whether ST8 MUO isolates could be linked to well-defined ST8 MKO isolates and clusters through the use of core-genome multi locus sequence typing (cgMLST).

We hypothesized that we could link MUO to other MKO- MRSA isolates and define new clusters based on genetic and epidemiological data, and thereby identify new plausible reservoirs or sources of MRSA.

## METHODS

### General definitions

MUO are defined as persons in whom MRSA was detected, but at time of detection without known risk factors as described in the Dutch MRSA guideline by the Dutch Working group of Infection Prevention (WIP).[2] MKO are defined as persons in whom MRSA was detected and who had known risk factors according to the same Dutch MRSA guideline.[1]

*Spa*-type association to ST8 was determined by use of the *spa*-typing website (http://www.spaserver.ridom.de) that is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/).

Epidemiological information was defined as any data which could identify a transmission between MRSA carriers, i.e. data related to described risk factors in the Dutch MRSA guideline, any data obtained from the national case control questionnaire[15], such as jobs, abroad visits, etc., and any additional data collected by infection control practitioners on transmission routes, such as shared family members, stays on wards, etc.

### Definitions for inclusion

Isolates were collected from two groups. Group 1 was defined as all MRSA isolates from MRSA carriers who had participated in a former large national case control study between 2011 and 2013[15], and whose *spa*-types were associated with ST8. We had already collected extensive epidemiological background information on these MRSA carriers[15], and ST8 is the most common ST among MUO (14% of MUO in the Netherlands).[1] Of the 232 MUO cases in the national case control study, 30 were ST8 isolates. These were from one of three regions based on proximity to our hospital: the Rotterdam region (25 km radius), the larger Randstad city region (60 km radius), and the eastern region of The Netherlands (150 km east of Rotterdam).

Group 2 was defined as all MRSA isolates from the Erasmus Medical Centre Rotterdam from 2008 until 2013 with *spa*-types associated with ST8 (MUO or MKO)(Flowchart 1), and whose epidemiology was known in detail to our local infection prevention unit. Group 2 isolates were from three subgroups: hospital outbreak clusters, household clusters and non-clustered isolates. (Table 1).

All included isolates 43% (28/64) were Panton-Valentine leukocidin (PVL) positive and had been stored at -80°C prior to the study.

**Table 1 – Overview of included ST8 MRSA isolates**

| Groups | Categories | | MUO (n=54) | MKO (n=52) | Total isolates (n=106) |
|---|---|---|---|---|---|
| Group 1 | National isolates | Rotterdam region | 10 | 2 | 12 |
| | | Randstad region | 8 | 2 | 10 |
| | | Eastern region | 7 | 1 | 8 |
| | | **Total** | | | **30** |
| Group 2 | Non-clustered isolates | Hospital isolates | 14 | 13 | 27 |
| | Clustered isolates | Household isolates | 5 | 16 | 21 |
| | | Hospital isolates | 10 | 18 | 28 |
| | | **Total** | | | **49** |

MUO: MRSA of unknown origin, MRSA without known risk factors; MKO: MRSA of known origin, MRSA with known risk factors. ST: Sequence Type; typed by multi-locus sequence typing (MLST).

Non-clustered isolates were isolates that could not be linked to any clusters by *spa*-typing and epidemiology, and which could be either MUO or MKO depending on the presence of any known risk factors at the time of detection.

## Definitions for outcome

MRSA clusters were defined as two or more MRSA isolates clustered together based on core genome multi-locus sequence typing (cgMLST) if the difference in core genome genes between the two isolates was less than 10 genes. Epidemiological data of the new clusters were rechecked after cgMLST analyses to explain new links between MUO and MKO.

## DNA isolation

MRSA strains were grown overnight at 37°C on Tryptic soya agar (TSA) and chromosomal DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer.

## Whole genome sequencing

Isolates were transported under constant -20°C conditions from The Netherlands to Scotland, where the isolates were whole-genome sequenced at Edinburgh Genomics (University of Edinburgh, Scotland). DNA samples were quality assessed on arrival and then prepared for independent barcoded genomic DNA sequencing libraries. Library preparation took place with Nextera XT (Illumina, San Diego, USA). The libraries were pooled into two independent libraries of 96 samples each and sequenced for 100 base paired end in a HiSeq 2500 (Illuimina, San Diego, USA) with at least 25 times coverage.

### Multi-locus sequence typing

With the sequence data, Multi-Locus Sequence Typing (MLST) was performed using SeqSphere software version 3.5.0 (Ridom GmbH, Münster, Germany) for confirmation that the selected MRSA isolates were indeed ST8. Isolates not ST8 were excluded from further analysis.

### Core genome multi-locus sequence typing

With the sequence data, core genome multi-locus sequence typing (cgMLST) was performed using an available cgMLST scheme in the SeqSphere software version 3.5.0 (Ridom GmbH, Münster, Germany). The results were imported in BioNumerics version 7.6.2 to be able to perform further comparative analysis (Applied Math, Sint-Martens-Latem, Belgium).

## RESULTS

### Isolate selection and characteristics

Thirty-one presumed ST8 isolates were included from our national case control database. One was excluded after MLST confirmation check as it was not a ST8. The remaining thirty ST8 isolates formed group 1. (Table 1). Of these, 83% (25/30) were defined at time of detection as MUO, the rest as MKO.

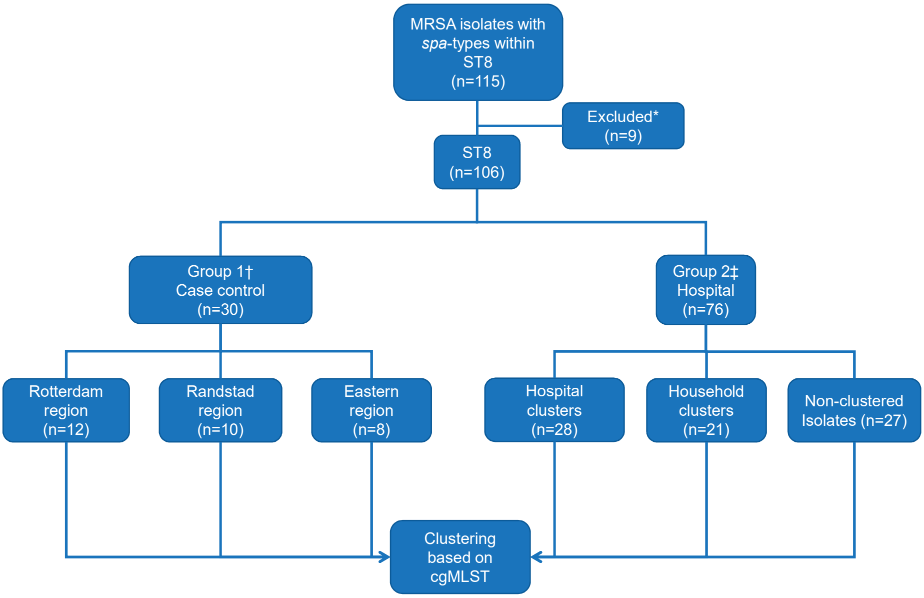
For group 2, 84 isolates presumed to be ST8 based on *spa*-typing, were selected from our hospital out of 191 isolates (43.9% 84/191). After MLST, eight isolates were excluded and 76 ST8 isolates remained. Twenty-eight isolates were from hospital clusters, 21 from household clusters and 27 were non-clustered isolates (Flowchart 1, Table 1). Of these, 38% (29/76) were MUO, the others were MKO. (Table 1)

### cgMLST clustering (group 1 and 2)

Core genome MLST of all 106 isolates from group 1 and group 2 revealed the presence of 16 genetic clusters when using a similarity cut-off of ten genes (Figure 1) . The number of isolates per cluster ranged from two to 12 isolates with a genetic variance from one to 15 genes (median 3 genes; average 4.4 genes). In three clusters (B, P and N), we found that the maximum genetic distance in the cluster was larger than the set cut-off (10 genes) between two isolates.

On average, isolates detected over a two year period formed clusters, and in one case (B) this was a period of three years.

One cluster (L) was formed by merging two previous clusters and one large outbreak cluster was split in half to form two new ones (cluster M and N). (Table 2) One cluster from group 2 was de-clustered (n=2; Figure 1) after cgMLST.



**Flowchart 1**

* Excluded due to not having sequence type 8, checked with cgMLST; †Well-defined MRSA isolates from the case control study; ‡MRSA isolates from the Erasmus Medical Centre Rotterdam.

ST: Sequence Type; cgMLST: core-genome multi locus sequence typing; *spa*-typing: staphylococcal protein A typing; Rotterdam region: the Rotterdam region (25 km radius); Randstad region: the larger Randstad city region (60 km radius); Eastern region: the eastern region of The Netherlands (150 km east of Rotterdam).

A third (C, D, F, G and J) of the cgMLST clusters were group 1 clusters. These were all intraregional clusters from either the Eastern or Randstad region. (Table 2) Of the isolates in group one, 43% (13/30) was clustered.

## Combining epidemiology and cgMLST

Fifty-four MUO isolates were subjected to cgMLST. Of these MUO isolates, 25 clustered with other isolates in the minimum spanning tree. The other 29 (53%; 29/54) remained non-clustered. Fourteen of the 25 clustered MUO isolates (56% 14/25) could be epidemiologically explained with the epidemiological data at hand. (Table 2)

Three clusters (D, G and J) from group 1 were found to be household clusters. For one cluster (D) no source or possible transmission event could be uncovered. Cluster G MUO were found to be MRSA positive in the past and underwent eradication treatment repeatedly. We speculate that either the eradication failed or the follow-up was too short. In cluster J, a hospital stay abroad of more than two months ago possibly resulted in introduction of MRSA into the household.

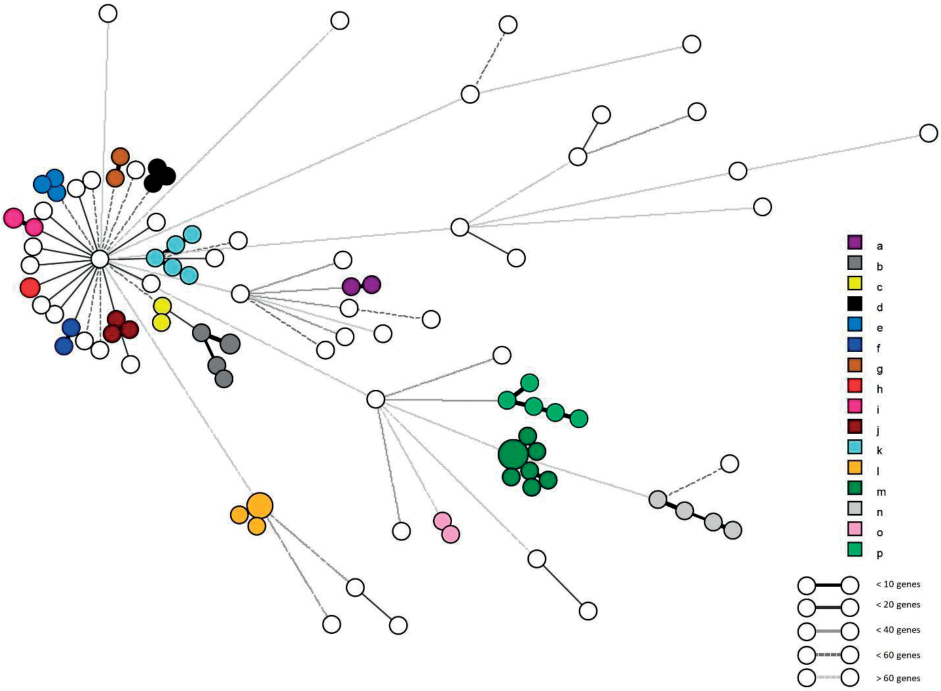


**Figure 1 – New clusters in the core-genome multi-locus sequence typing tree**
Core-genome multi-locus sequence typing (cgMLST) of 106 isolates based on 1641 of 1861 common genes. Circles are coloured if the isolate was part of one of the 16 new clusters (A to P; see also table 2) based on cgMLST and epidemiology. White circles are not part of any cluster. Lines between circles are the distance in genes.

The MUO in cluster K was resolved: it was the result of close contact between two children (age of 10) from separate families. Cluster L was formed out of two, initially considered separate, MUO outbreak clusters becoming one. Thereby identifying many MUO as part of one outbreak, except for the initial index carrier who remained an MUO. In cluster M, one MUO was solved: a carrier detected half a year post-outbreak, was clustered into outbreak cluster M.

For two clusters (B and I) epidemiology could provide a plausible explanation that had been previously missed: the first was a cluster of import-MRSA isolates from Taiwan (cluster B). The MRSA isolates were from persons who in retrospect had been admitted to the same Taiwanese hospital. The isolates in cluster I (2 genes differences within the cluster) were two single introductions of Aruban MRSA in the Rotterdam region. Although there were no obvious links between these two persons involved, they both originated from the island of Aruba. (Table 2) and were detected by two different laboratories in the Rotterdam region.

For 11 MUO isolates (44%; 11/25) no conclusion could be drawn as to the initial source or transmission route of the MUO.

**Table 2 – New clusters by cgMLST and epidemiology**

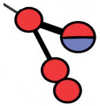
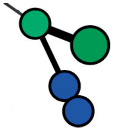
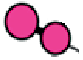
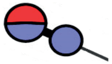
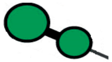
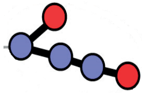
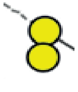
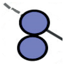
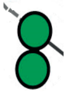
| | | | | New cluster data | | | |
|---|---|---|---|---|---|---|---|
| | | | | Distance (genes) | | MUO resolved | Epidemiology |
| New clustering | | MUO/MKO | By sampling year | Av. | Max. | | |
| | **Import MRSA (group 1 and 2)** | | | | | | |
| B |  |  |  | 5 | 15 | 1 | Taiwan cluster. A series of adopted children from Taiwan clustered together. Initially thought to be unrelated individuals from Taiwan, where they have more CA-MRSA, after cgMLST the children were discovered to be adopted through an organization and hospital in Taipei. In The Netherlands, these children were part of different families and detected as MRSA carriers over a three year period. |
| I |  |  |  | 1 | 4 | 2 | MUO carrier (student) with furunculosis. Linked by cgMLST to a four years older carrier. According to the epidemiological information, both were born on the tropical island of Aruba, but are from different families and detected in different medical microbiological laboratories within the same region. |
| | **Non-clustered isolates linked to cluster (group 2)** | | | | | | |
| H |  |  |  | 0 | 0 | 0 | MUO clinical isolate from a patient with a knee infection and partner (MKO contact). Despite not linked by *spa*-typing initially (but still ST8), assumed to be a hospital cluster, now confirmed with cgMLST. Source of index-MUO remains unknown. |
| P |  |  |  | 6 | 10 | 0 | Cluster of a patient with her mother and partner. The other two are patients of which one was identified before as part of the cluster, and the other not until after cgMLST. Source of index-MUO remains unknown. |
| | **Group 1 clustering** | | | | | | |
| C |  |  |  | - | 2 | 0 | Two MUO from the Randstad region. Both were isolated by the same medical microbiological laboratory, but considered unrelated. No common source was found despite questioning both cases with extensive questionnaires. |

Table 2 – New clusters by cgMLST and epidemiology (continued)

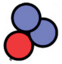
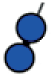
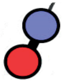
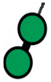
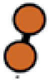
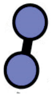
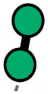
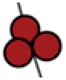
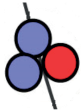
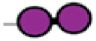
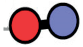
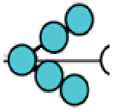
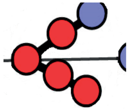
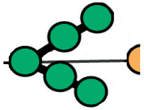
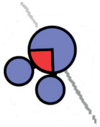
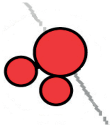
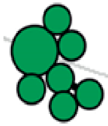
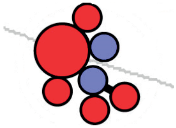
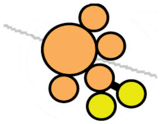
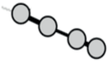
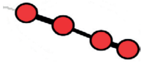
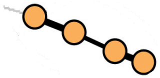
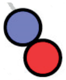
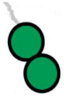
|  |  |  | New cluster data |  |  |  |
|---|---|---|---|---|---|---|
|  |  |  | Distance (genes) |  | MUO resolved | Epidemiology |
| New clustering | MUO/MKO | By sampling year | Av. | Max. |  |  |
| D  |  |  | 1 | 1 | 1 | Two MUO in the Eastern region, later found to form a household cluster together with a third household contact (MKO). No source or transmission event could be identified despite questioning with extensive questionnaires. |
| F  |  |  | - | 4 | 0 | MUO carrier and the household contact (MKO). Possibly MRSA acquisition by MUO carrier after a holiday in Spain. |
| G  |  |  | - | 2 | 2 | Considered to be two single MUO cases; a household cluster of brother and sister in the Eastern region. Extended questionnaire revealed past eradication attempts may not have been adequate. |
| J  |  |  | 1 | 2 | 2 | Two MUO carriers with their household contact (MKO) in the Randstad region of the Netherlands. A hospital stay abroad for more than two months ago possibly resulted in introduction of MRSA into the household. |
| **Group 2 clustering** |  |  |  |  |  |  |
| A  |  |  | - | 4 | 0 | Two siblings, one with a MUO boil, the other the household contact (MKO). Considered a cluster before, and now confirmed with cgMLST. |
| E  |  |  | 1 | 2 | - | Household cluster of known MRSA carriers (MKO). Considered a cluster before, and now confirmed with cgMLST. |
| K  |  |  | 4 | 7 | 1 | Household cluster of two adults with two children known to be MRSA positive (MKO). With one MUO contact, not part of contact tracing, that was a friend of one of the children in this cluster. (Both 10 years old.) Now confirmed by cgMLST as part of the cluster. |

**Table 2 – New clusters by cgMLST and epidemiology (continued)**

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
|  |  |  | **New cluster data** |  |  |  |
|  |  |  | **Distance (genes)** |  | **MUO resolved** | **Epidemiology** |
| **New clustering** | **MUO/MKO** | **By sampling year** | **Av.** | **Max.** |  |  |
| L  |  |  | 1 | 2 | 4 | Two outbreak clusters of unexpected clinical MRSA isolates (MUO) including a contact (MKO), becoming one cluster after cgMLST. |
| M  |  |  | 1 | 4 | 1 | Hospital outbreak cluster that started with one MUO carrier (child). Index had five contacts (two employees, three children). The mother of the index case was MRSA positive. As well as both parents of another child that became positive outside contact tracing with identical *spa*-type and a stay on the same department. Yet another child was detected half a year later (MUO). |
| N  |  |  | 9 | 11 | - | Household cluster consisting of a mother, grandmother and two children. The *spa*-type was different, but the isolate was ST8, with strong epidemiological links to cluster M: one of the children was detected in the contact tracing for this cluster and stayed on the same department during hospital stay as the index of cluster M. |
| O  |  |  | - | 1 | 0 | MUO hospital patient and his wife (MKO, household contact). Considered a cluster before, and now confirmed with cgMLST. Source of the index-MUO remains unclear. |

New cluster: clustering based on cgMLST and epidemiology; Av: average gene difference between isolates within one cluster; Max: maximum gene difference between isolates within one cluster; Epidemiology: epidemiological background. MRSA of Unknown Origin (MRSA without known risk factors; MUO) in the MUO/MKO column are coloured light blue, MRSA of Known Origin (MRSA with known risk factors; MKO) in the same column are red coloured. In the year column, the circles are coloured on their year of isolation : red is 2008, light orange is 2009, yellow is 2010, light green is 2011, dark green is 2012, and blue is 2013. If a circle has two colours or the circle is larger, the gene difference between the two isolates is 0 (isolates are identical to each other in this analysis).

## CONCLUSIONS

We clustered 46% (25/54) MUO genetically and successfully linked 56% (14/25) of these MUO epidemiologically to other known carriers of MRSA within three Dutch regions. Importantly, ST8 isolates in the Netherlands were found to represent multiple, distinct clusters. We were able to distinguish regional Dutch ST8 clusters from each other, as well as detect import-MRSA clusters from Taiwan and Aruba. In large case-control studies, visits abroad have not shown to be an independent risk factor for MUO acquisition [15, 16]. However, in our case control study, having one or two foreign parents turned out to be a risk factor. [15] Together with the detected import-MRSA clusters, our data suggests that import of MRSA from higher MRSA prevalence countries may have played a role (Table 2). Most importantly, we found that some MUO isolates that clustered together within the Rotterdam region were cultured in different medical microbiological laboratories (MML) and the cluster remained undetected as the findings were not shared between laboratories since there was no necessity for it, such as during outbreaks. Yet, such intraregional cgMLST clusters confirm that MRSA control should be a regional effort and that cgMLST typing results should always be reported and shared regional.

Furthermore, Cluster B showed that isolates sampled three years apart from each other still clustered. It is therefore likely that by performing cgMLST on a large number of isolates obtained over a large time period increases the chance to cluster and identify the source of MUO and non-clustered isolates in general. This study focused only on the most common sequence type ST8. Despite this, we found previous unknown links, new clusters and identified sources for MUO in all three regions examined. It is likely that in case of rarer sequence types, common epidemiological links are easier to find, making the identification of MUO easier.

Retrospective epidemiological data was used for this study. We had to rely on extensive epidemiological information from our earlier case control study that included many detailed epidemiological data compared to the standard epidemiological information collected by infection control practitioners. An increase in the number of identified MUO is expected if we had been able to actively (re)quest the MRSA carriers whose isolates clustered after applying cgMLST.

To identify sources for MUO in the future, epidemiological data are essential, as it the typing efficacy. It is therefore of utmost importance that these data are shared regionally between MML. We found that we were able to differentiate well between regional clusters for ST8 isolates. Which was previously impossible with *spa*-typing.

Despite our retrospective approach, we were able to explain 56% of the clustered MUO in this study. Ideally, if isolates are clustered over a larger period of time, are shared between MML intraregional, and if infection control practitioners can return to MRSA carriers to (re)question them directly after cgMLST clustering; then we have a greater chance of further identifying the origins and transmission routes of MUO.

In conclusion, by combining results of cgMLST and epidemiological data, we were able to identify the origin of MUO clustering together with other MRSA isolates in one of three Dutch regions and successfully linked 26% of MUO genetically and epidemiologically to other carriers of MRSA. Import-MRSA played an important role in most of the identified MUO, as well as close contact between primary school children from different families, possible roles for failed eradication treatment and a need to share cgMLST and epidemiology across MML to cluster MRSA.

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# Summary of main findings

# Conclusions, recommendations and future perspectives

# 8

## SUMMARY OF MAIN FINDINGS

In this thesis we studied MRSA of Unknown Origin (MUO). MUO are MRSA without known risk factors as defined in the risk categories of the Dutch Workinggroup for Infection Prevention (WIP) guideline for MRSA.[1] As a consequence, MUO carriers are not recognized by the so called triage taken at hospital admission. MUO carriers remain undetected and thus, during admission, they can spread MRSA in the hospital. We studied MUO strains and carriers to find new risk factors and transmission routes, so the MRSA guideline can be updated and future MRSA transmission reduced. Below a summary of the main findings is given, followed by its conclusions and the recommendations to reduce the number of MUO.

In **chapter 1** we assessed the magnitude of the MUO problem and determined the differences between MUO and MRSA of known origin (MKO). We found that a quarter (24%) of 5545 registered MRSA isolates (2009-2010) known at the national reference laboratory (RIVM) were MUO. Based on typing, MUO isolates could be divided in two main groups: CC398 MUO (26%; 352/1350) and non-CC398 MUO (74%; 998/1350).

We hypothesised that MUO could have been acquired in the community setting. Therefore, we checked all MUO isolates for the presence of Panton-Valentine Leukocidin (PVL), as the presence of this virulence factor promotes the spread of MRSA in a community setting.[2] Of all MRSA isolates, 12% (684/5554) were PVL positive. Of all MUO isolates 46% (461/998) were PVL positive. Within the group of non-CC398 types, PVL positivity was more frequently encountered in MUO compared to MKO (relative risk 1.19; 95% CI 1.11–1.29). Moreover, community associated *spa*-types such as *spa*-types t008 (ST8), t019 (ST30) and t044 (ST80) were significantly ($p<0.01$) more present among MUO compared to MKO. PVL-positive *spa*-type t008 was found significantly more often among MUO than MKO (10.6% vs. 1.7%, $p<0.01$). We hypothesised that the presence of PVL in these *spa*-types gave an evolutionary advantage in the community but the found difference could also be explained by overrepresentation: MUO are often clinical isolates detected by sampling clinical sites although exact figures on this are not known, as medical microbiological laboratories (MML) send the MRSA as soon as the MRSA carrier is detected. If a MRSA infection occurs later, than this is not known at the RIVM. (**Chapter 1**).

CC398 MRSA positive individuals are known to acquire their CC398 MRSA due to exposure to livestock (pigs, veal calves, broilers). However, we identified persons with CC398 MUO, who lacked direct contact with livestock. Based on literature, it is assumed that human-to-human transmission of CC398 MRSA is less likely, because CC398 MRSA is 72% less likely and 6 times less transmittable than non-CC398 MRSA [3, 4], although sporadic nosocomial transmission events and outbreaks were described.[5, 6] In regards to the origin (animal or human) and transmission routes (direct or indirect) of MUO CC398, all options are open. Therefore, CC398 MUO was further explored in **chapter 2** to study whether the CC398 MUO strains were different from the CC398 MKO strains, and as such could more easily transmit from human-to-human than CC398 MKO strains, directly transmitted from livestock to humans. For now, there are two CC398 variants: one of animal and one of human origin. Besides their differences in transmission, they also differ in presence or absence of certain mobile genetic elements (MGEs). [7]

By polymerase chain reaction (PCR) we determined whether MGEs - *scn*, *chp*, *φ3 int*, *φ6 int*, *φ7 int*, *rep7*, *rep27*, and *cadDX* - were present in MUO isolates and by comparing them with a set of CC398 isolates of various known origin (humans, pigs, horses, chickens, and veal calves), we studied the origin of CC398 MUO. We found that the distinct human CC398 MSSA *spa*-type, t571, was not present among the Dutch CC398 MRSA strains from humans and pigs that we studied. Furthermore, the CC398 MUO were tetracycline resistant and carried no φ3 bacteriophage with *scn* and *chp*.

In short, the CC398 MRSA in this study, MUO or MKO, were found to resemble CC398 MRSA as found in pigs and not CC398 MSSA as described as human pathogens. We concluded that human CC398 MUO carriers carried MRSA of livestock origin, despite MUO carriers not having an epidemiological link with pigs and pig farming. This finding is worrisome, as it suggests other transmission routes than direct exposition to livestock.

**In chapter 3** we studied the necessary number of follow-up culture-sets in a retrospective MRSA carrier cohort (2005-2010) to declare a successful MRSA eradication therapy and a non-carriage status. We compared the then standard procedure of three MRSA follow-up culture-sets to six culture-sets, to determine when to be cultured and how many culture-sets are necessary during a time-period of one year. We found that between the third and sixth follow-up culture-set 54% (35/65) of total recurrences occurred. Over 88% of all recurrences were detected within two months. Combined nose and throat carriage OR 25.5 (1.6-419.1)) and intravascular lines (OR 13.6 (1.2-156.2)) were found to be risk factors for early recurrence. An early recurrence is defined as a recurrence during the first three follow-up culture-sets as opposed to the last three culture-sets.

Many seafarers visit the port city of Rotterdam. They are a difficult to contact group due to language and cultural barriers and their short stay before shipping off again. Because of above reasons, we could not include them in our MUO case control study. Therefore, we studied the seafarers separately from the MUO case control study (as described in **chapter 5**) and determined the MRSA prevalence of seafarers in the Netherlands upon hospital admission in (**chapter 4**). We detected a 5.8% (7/124) MRSA prevalence. This was a MRSA prevalence 52-times higher than the normal prevalence in the Netherlands (0.11% at hospital admission).[8] Furthermore, we found that the presence of wounds or abscesses gave seafarers a 40-times higher risk of being MRSA positive. This finding resulted in addition of seafarers as new risk group for MRSA in the Harbour hospital and the Erasmus University Medical Center in Rotterdam.

In **chapter 5** we described a prospective case control study to determine risk factors for MUO acquisition/carriage with the aim to find new risk factors for MRSA, and add these to the risk categories of the Dutch MRSA guideline by the (successor of) the Dutch Workinggroup for Infectionprevention.

Between September 1st of 2011 and September 1st of 2013, MUO cases were included in a case control study. Cases were all MUO carriers reported to the RIVM in this period, with randomly selected controls from the community during the same period. Cases and controls were approached by mail and were asked to fill in a questionnaire which included the known risk factors as described in the WIP guideline, to be able to filter the MKO misclassified as MUO. The questionnaire also contained questions on health, behaviour, profession and (sport) activities that could be possible MUO risk factors. These questions were chosen based on a literature study and an earlier pilot study (trawing study).

One of the case control study outcomes was that 38% (144/376) of MUO carriers were misclassified and actually MKO. These misclassified carriers were excluded from further analyses. We found the following significant risk factors for MUO in logistic multivariate analysis: antibiotic use in the last twelve months, aOR 8.1 (5.6-11.7), screened as contact in a contact tracing but not detected as a MRSA carrier at the time, aOR 4.3 (2.1-8.8), having at least one foreign parent, aOR 2.4 (1.4-3.9) and receiving ambulatory care, aOR 2.3 (1.4-3.7). These risk factors explained 83% of the MUO cases.

We hypothesised that part of the MUO carriers were initially missed as MRSA carrier, only to be later detected and subsequently categorized as MUO. This meant that some MUO carriers had unidentified links to other MRSA carriers. Therefore, in **chapter 6**, we studied genetic links between MUO strains and other MRSA (MUO or MKO) strains, together with some crude epidemiology data of the carriers.

Therefore, we included MUO carriers from the case control. We then selected well-defined hospital and household clusters, and compared them with randomly selected, well-defined regional and national isolates. All isolates had the same genetic background (ST8) and were clustered with core genome multi-locus sequence typing (cgMLST). Our goal was to discern or link MUO isolates to known MRSA clusters (MKO) or single MRSA isolates (MUO or MKO). In this study, we elucidated 28% (15/54) of MUO by linking them to known MRSA clusters or isolates through cgMLST and epidemiology. Moreover, we were able to link isolates from the same region but different MML to each other. This resulted in the formation of new regional clusters that previously had gone unnoticed. These isolates were considered MUO and not part of a cluster. Also, the use of cgMLST resulted in reclassification of old clusters previously based on epidemiology and *spa*-typing.

Different typing methods, such as cgMLST, will generate different outcomes and thus change the presence and/or the extent of outbreaks and therefore how many people need to be screened. Those not screened at the time of the outbreak, and detected later in time, will be defined as MUO. CgMLST has a higher discriminatory power, so we were able to discern within and between t008 MRSA clusters as based on *spa*-typing. Furthermore, we successfully discerned between Dutch MRSA t008 clusters and import-MRSA t008 clusters from Aruba and Taiwan, which was previously not possible with *spa*-typing.

## CONCLUSIONS, RECOMMENDATIONS AND FUTURE PERSPECTIVES

In Nethmap 2018, the RIVM reported that 38% (1,066/2,792) of the reported MRSA isolates to the MRSA surveillance in 2017 were MRSA of Unknown Origin (MUO).[9] Based on our findings in the case control study, 38% (144/376 returned questionnaires) of the MUO cases in the MRSA surveillance were actually misclassified MKO. Assuming a stable amount of misclassification every year this would mean that, in 2017, 23% (661/2792) of the reported MRSA to the surveillance were MUO. (In absolute numbers, this is 405 “MUO” less than originally reported) If we calculate the same for 2009, we had 786 MUO from 2874 reported MRSA originally, which becomes 17% (487/2874) after correction for misclassification. Which is an estimated increase of 35.7% of MUO over a period of 8 years (2009-2017). Despite misclassification we are still confronted with a large group of MUO. It remains important to understand MUO’s origin. In this thesis we studied MUO and learned that they are diverse and fall apart in several subgroups, both genetically as epidemiologically.

### MUO CC398

An important subgroup are the MUO CC398 isolates (352/2312 MRSA CC398; 15% as described in **Chapter 1**). These are MUO isolates from the livestock associated cluster 3398, whose carriers had no direct occupational contact to livestock. (As discussed in **Chapter 2**.) It is important because The Netherlands, like Denmark, is a low MRSA prevalence country with a relative large livestock-associated MRSA reservoir. Also, it is currently assumed that the tetracyclin-resistant livestock-associated MRSA (LA-MRSA) does not significantly transmit from human to human and acquisition is based on exposure through (occupational) contact.[3] The limited human-to-human transmission fortunately limits the impact of these LA-MRSA strains. The existence of MUO CC398 however implies acquisition despite no direct contact. Thus we hypothesised if MUO CC398 isolates belong to a subgroup of this cluster that is solely isolated from humans. McCarthy et al. showed that CC398 strains from humans in contact with animals differed from strains isolated from humans without contact with animals in mobile genetic element (MGE) content.[7] However, we found MUO CC398 to be similar to MKO CC398 and not part of the subgroup of the CC398 solely isolated from humans. (**Chapter 2**) This means that the human host adapted MRSA CC398 is less likely the source of MUO CC398. A more likely hypothesis for MUO CC398 existence could be indirect transmission, for example by air, rodents, or indirect by exposure of persons in the community to livestock exposed CC398. [10-14] Further studies on MUO CC398 are necessary to determine if indirect exposure indeed plays an important role in the transmission of MUO CC398 . We also recommend to analyse the reported number of MUO CC398, and report the aggregated data nationally and to the MML. Presence of MGEs like *Φ3* (*ΦSa3*) in MUO CC398 can be easily monitored, as was shown by a study by van Rijen et al. who screened for MUO CC398 with *Φ3* and *tetM* allowing the quick distinction between CC398 of animal-, and human origin.[15]

## Import-MRSA and seafarers

A proportion of MUO is directly related to (an exposure to) higher MRSA prevalence abroad: so called import-MRSA. For example, seafarers (**Chapter 4**), having a foreign parent (**Chapter 5**) or import-MRSA clusters through travellers visiting friends and relatives or through immigration (Aruba, Taiwan) (**Chapter 6**) are all factors explaining part of MUO carriers. Interestingly, abroad visits, travelling abroad, and abroad hospital or healthcare visits longer than two months ago were not significant risk factors in the case control (**Chapter 5**). The question remains whether the arbitrary cut-off of two months for hospital visit abroad is not too strict and not too general, as the arbitrary cut-off could potentially label some MRSA as MUO who would otherwise be classified and treated as MKO. (**Chapter 5**)

Furthermore, we recommend to add seafarers as risk factor for MRSA carriage. Given the high prevalence rate of carriage among seafarers, we recommend that all seafarers should be classified as WIP category 2, and thus screened for MRSA when they are present in the Netherlands, regardless of wounds or underlying disease; and to apply pre-emptive isolation while awaiting test results. Since 2010, seafarers have been included as such in the triage for admission to the Erasmus MC (and the former Harbour hospital). The global impact on transmission of MRSA by seafarers is currently unknown. (**Chapter 4**)

## Case control risk factors: intersection between healthcare and community

The results of the MUO case control suggest that a large portion of MUO cases are still healthcare or disease related as we found risk factors such as antibiotic use and ambulatory care. We also found that MUO carriers had been screened before in contact tracing but had not been detected as MRSA carrier at that time. It seems MUO carriers have regular contact with healthcare, but not necessarily the hospital. Most likely they acquired their MRSA somewhere in the “fringes of healthcare”, outside the hospital where the community and healthcare world overlap. Examples can be ambulatory care or the use of antibiotics in nursing homes or at general practitioners. (**Chapter 5**) Adding these more general risk factors to the risk categories of the Dutch MRSA guideline of the WIP will possibly decrease the number of MUO, but will also generate high costs of screening and also a higher burden if we also choose to pre-emptive isolate the additionally screened patients as well, because of the larger number of people who qualify for having one of these risk factors. The total number of people needed to be screened to find one MUO with new, general risk factors such as ambulatory care use or use of antibiotics, could become quite high and therefore be too expensive and too labour intensive. Still, our general risk factors explained 83% of the MUO cases in the case control. A different approach using a risk algorithm is therefore desirable, (vide infra). For the remaining 17% of MUO cases no explanation was found.

For case control studies, the choice for the controls is difficult but important. As stated above, we found general risk factors that explained 83% of MUO. However, the outcome of a case control study depends highly on the choice of the control; matched or not. Our study of MUO's characteristics (**chapter 1**) and the initial trawling questionnaire (**chapter 5**) revealed a very diverse group of carriers, a diversity better paralleled by choosing a community control group. Cases, i.e. MUO carriers were not just MRSA carriers reported by hospitals, but also those reported by general practitioners (GP) or long-term care facilities (LTCF). If we would select controls only from patients admitted or treated at the hospital or also from the GP or LTCF, then each option would result in a different control population. We also considered matching on having an (suspected) infection, as MUO are often discovered by accident – for example from clinical isolates. However, this was not possible as it was not certain whether reported cases had an infection or not. The registration of the presence of infection was not reliable. If the submitted case isolate was not an isolate of an infectious site, then infection elsewhere or later could not be ruled out. Furthermore, our case control was set up to find unknown risk factors without a-prior bias. Matching would introduce bias and decrease the change on finding risk factors. Therefore, we chose unmatched controls selected from the community, reducing bias as much as possible while maximizing the chance to find new risk factors within the MUO population. Given the known prevalence of MRSA in the community (between 0 and 0.11%[8]) we assumed the chance of including a MRSA carrier as a control too low to screen for.

To identify patients at risk for MRSA carriage, an algorithm is necessary that computes the a-prior risk for MRSA carriage using the current WIP risk factors and our newly defined case control risk factors. Unfortunately, some of the new risk factors are too general and/or too frequent, to use them as a yes/no answer in normal triage for admission. In future, an algorithm should be developed in which factors are weighted in a specified population, so a weighted risk can be calculated from more than one factor. The outcome of this algorithm should then lead to measures to prevent transmission of MRSA. Ideally, a national uniformity in this algorithm and performance should be aimed for.

Finally, to enhance feasibility and completeness, an interactive chatbot could be programmed with the questions to be used by patients to be admitted or treated. The inputted answers can be loaded into an algorithm to calculate the a-prior risk of MRSA carriage. Chatbot technology is already in use today and used by companies with many clients, such as KLM.[16]

## MRSA recurrence after MRSA eradication therapy

MRSA recurrence is missed when using only three follow-up culture-sets after MRSA eradication therapy. These MRSA recurrences can later be detected as MUO. We showed that MRSA recurrences are missed in 54% (35/65 recurrences) when using three follow-up culture-sets in comparison to six follow-up culture-sets. Using our results, the then WIP guideline was changed on the timing of culture-sets after MRSA eradication therapy (**Chapter 3**). We recommend five culture-sets within a period of one year after eradication therapy. In the 6 months after eradication therapy, we recommend to frequently culture carriers whose MRSA was eradicated and who are at risk for early recurrence. Persons at risk for early recurrence are those with combined nose and throat carriage (OR 25.5 (1.6–419.1)) or those who have intravascular lines (OR 13.6 (1.2–156.2)). Persons with late recurrence, could have their follow-up culture-sets with more time in between each set, with a final culture-set one year after their eradication therapy. Persons at risk for early recurrence are detected sooner, and their recurrence happens within six months. These persons would then not have to wait a year for their final follow-up culture-set. This recommendation is a balance between the need for swift detection of MRSA recurrence, the patients' burden, and lowering the number of missed MRSA recurrences after successful MRSA eradication therapy.

## Misclassification of MKO as MUO

We encountered a high number of misclassified MKO isolates that were listed in the national MRSA database as MUO. Analysis of the case control study showed 38% of all MUO isolates in the MRSA surveillance database were actually MKO. Misclassification can be attributed to early uploading accompanying epidemiological data to the RIVM together with sending the MRSA strain by medical microbiological laboratories (MML) to the RIVM. This early uploaded data, especially before the typing results are known, will lead to less details on possible MRSA risk factors (**Chapter 5**). It is not always possible to ascertain the source of acquisition before the typing result is available. Typing results should be used to elucidate sources and transmission routes. When sources happen to be the healthcare centre, then the MRSA carrier can be defined as a MKO. The solution to this problem is a two-step procedure: first, the strain is sent and then, after getting back and analysing the typing results, the MML is asked for the final epidemiological decision whether we are dealing with a MKO or a MUO. This decision will depend on the (assumed) sources and risk factors of the carrier. Ideally the typing results are returned to the medical professionals in the shortest turn-around-time to support contact tracing efforts and help them define MRSA sources. The latter should then be reported back to the MRSA surveillance, further improving the reliability of the epidemiological data.

Misclassification is due to the quality of the epidemiological data. This quality is based on the time, effort and possibility to acquire the data. Currently, there is no standard procedure when contacting and questioning MRSA carriers or contact tracing and who should do that. So the inputted effort will vary per person as will the quality. A procedure for questioning and contacting MRSA carriers is therefore recommended. Misclassification can also happen through limited contact tracing in MRSA outbreaks (**Chapter 5 and 6**). Too limited contact tracing will miss MRSA carriers (and thus MKO) who can be detected later or elsewhere as MUO. This could explain the found risk factor in the case control study: having been screened in the past before for MRSA carriage, but found to be MRSA negative at that time.

## Remaining MUO and the necessity to cooperate and type

There is a remaining portion of MUO that cannot be explained. In our case control study, this was 17%. Taking the Nethmap 2018 data, this would mean that out of the 661 MUO in 2017 (23% of total reported MRSA to the surveillance), 17% (112/661) would remain without explanation. That would be 4% (112/2792) of total MRSA reported to the MRSA surveillance in 2017. (Figure 1) This remaining group consists of various MUO cases in which we could not identify risk factors in our study. A proportion of these MUO may still be elucidated if genomic information from MRSA isolates across healthcare institutions or within a ZorgRegio or even nationally are combined. We have shown that two MMLs in the Rotterdam region each discovered a MUO independent of each other, without knowing these two were genetically linked, until they were linked through our cgMLST data **(Chapter 6)**. We also show in **chapter 6** that we can discern between clonal t008 MRSA clusters with the higher discriminatory power using cgMLST. Application of cgMLST data by MML in contact tracing could prove crucial to elucidate the last remaining 17% of MUO cases in The Netherlands.

Preferably these data should also be shared within the regional network of healthcare institutions to detect intraregional (outbreak) clusters (such as described in **Chapter 6**). The current Antibiotic Resistance Care Network or ZorgRegio ABR could take up this task and find a way to share data on MRSA strains, typing results and carriers. Ideally, healthcare institutions within the ZorgRegio should be informed on MRSA carriers in the region, and they could be asked to make agreements on sharing information and equalizing policies, taking into account the privacy laws. Sharing information on MRSA carriers and MRSA strains between institutes, especially if the patient is not directly transferred, is

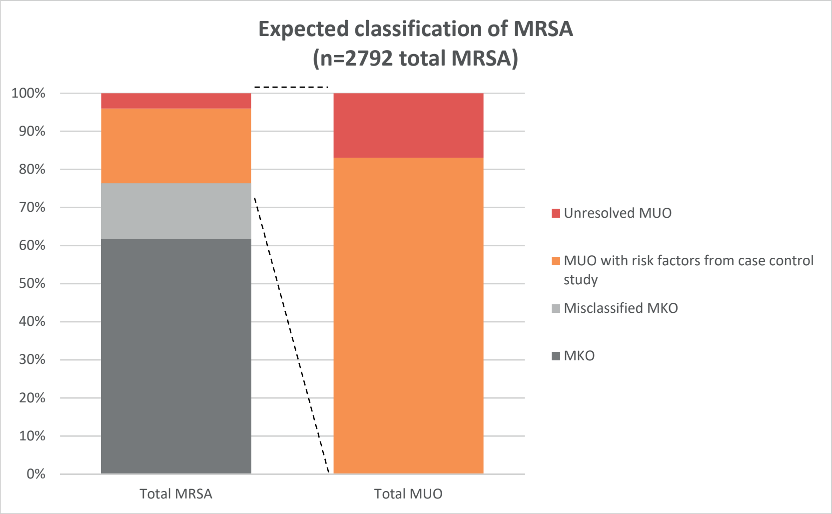

**Figure 1 – Dutch MRSA and MUO, relative contributions**
Data for MUO were generated by using results of the MUO case control study applied to the Nethmap 2018 MRSA data (n=2792). Data for MKO were generated by using MRSA surveillance data of 2008-2011 and extrapolating the average percentage to the given numbers in the Nethmap 2018 data. Definition of misclassified: MRSA isolates indicated as MUO by the MMLs in the questionnaire sent to RIVM but defined in our case control study as MKO.

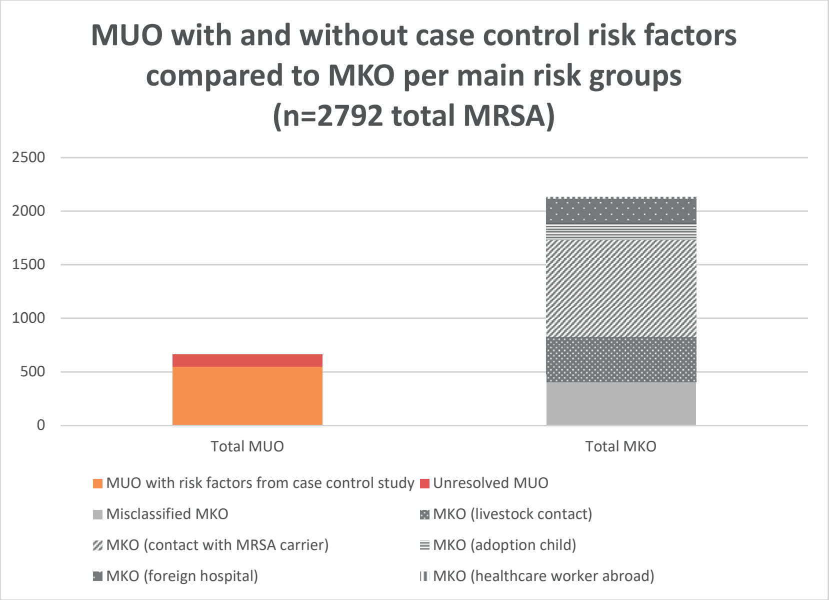

**Figure 2 – MUO compared to MKO in absolute numbers**
Data for MUO were generated by using results of the MUO case control study applied to the Nethmap 2018 MRSA data (n=2792). Data for MKO were generated by using MRSA surveillance data of 2008-2011 and extrapolating the average percentage to the given numbers in the Nethmap 2018 data. Definition of misclassified: MRSA isolates indicated as MUO by the MMLs in the questionnaire sent to RIVM but defined in our case control study as MKO.

highly desirable from an infection prevention point of view, but may right now be a bridge too far.

In summary, we make the following recommendations: to conduct further studies on MUO CC398, to monitor MUO CC398 in surveillance and feedback to national and MML level, to add seafarers as MRSA risk group to the Dutch MRSA guideline, as well as classify seafarers as a WIP category 2, to make a risk algorithm, in which a weighted a-prior risk is calculated based on multiple risk factors, to culture post-MRSA eradication therapy with five follow-up culture-sets, four of which in the first two months (the fourth in 2 months) after MRSA eradication therapy and the latter after half a year or year depending on the presence of risk factors or being a healthcare worker. Other recommendations are to reduce misclassification through more intensive collaboration between RIVM which provides typing, and the MML that provide the epidemiological data, to share at least the typing data within the ZorgRegio and if possible also on a supraregional level so that arbitrary boundaries between adherence areas of MML do not limit our ability to discover MRSA clusters and to continue to monitor the number of MUO as 17% of Dutch MUO are without risk factor or explanation.

Continuous control of MUO is essential to keep MRSA prevalence low in The Netherlands!

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# Nederlandse samenvatting

# Nederlandse conclusies, aanbevelingen en toekomstperspectieven

# 9

## NEDERLANDSE SAMENVATTING

In dit proefschrift hebben we MRSA van onbekende origine (MRSA of Unknown Origin, MUO) bestudeerd. MUO zijn MRSA zonder bekende risicofactoren zoals gedefinieerd in de risicocategorieën van de Nederlandse MRSA richtlijn van de werkgroep voor infectiepreventie (WIP). [1] Als gevolg hiervan worden MUO dragers niet herkend tijdens triage bij ziekenhuisopname. De MUO dragers blijven dus onontdekt tijdens opname en kunnen op die manier een verspreidingsbron van MRSA in het ziekenhuis worden. Wij hebben MUO dragers en hun stammen bestudeerd om nieuwe risicofactoren en transmissieroutes te vinden, zodat de MRSA richtlijn kan worden aangescherpt, en zo de kans op MRSA transmissie in de toekomst te verlagen. Hieronder volgt een samenvatting van de belangrijkste bevindingen van dit proefschrift, gevolgd door de conclusies en aanbevelingen om het aantal MUO te verminderen.

In **hoofdstuk 1** hebben we de omvang van het MUO probleem en de verschillen tussen MUO en MRSA van bekende oorsprong (MRSA of known origin, MKO) onderzocht. In een kwart (24%) van de 5545 bij het nationale referentielaboratorium (RIVM) gedurende 2009 en 2010 geregistreerde MRSA isolaten, bleek het om een MUO te gaan. Op basis van genetische typering konden de MUO isolaten onderverdeeld worden in MUO CC398 (26%; 352/1350) en MUO niet-CC398 (74%; 998/1350).

We veronderstelden dat MUO verworven zou kunnen zijn in de "community". Daarom hebben we alle MUO isolaten gecontroleerd op de aanwezigheid van Panton-Valentine Leukocidin (PVL), omdat de aanwezigheid van deze virulentiefactor de verspreiding van MRSA in een "community" bevordert.[2] Van alle MRSA isolaten was 12% (684/5554) PVL-positief. Van alle MUO isolaten was 46% (461/998) PVL-positief. Binnen de groep van niet-CC398-typen werd vaker PVL-aangetroffen in MUO stammen (68%; 317/998) dan in MKO stammen (10%; 144/1407) (relatief risico 1,19, 95% CI 1,11-1,29). Bovendien waren "community"-gerelateerde *spa*-types zoals t008 (ST8), t019 (ST30) en t044 (ST80) significant meer aanwezig ($p<0.01$) onder MUO isolaten dan onder MKO isolaten. Het PVL-positieve *spa*-type t008 werd significant vaker gevonden onder MUO isolaten dan onder MKO isolaten (10,6% versus 1,7%, $p <0{,}01$). We veronderstellen dat de aanwezigheid van PVL in deze *spa*-typen een evolutionair voordeel geeft in de "community", maar het gevonden verschil kan ook worden verklaard door oververtegenwoordiging: MUO zijn vaak klinische isolaten die worden gedetecteerd door klinische locaties te bemonsteren. Exacte getallen hierover ontbreken, aangezien medisch microbiologische laboratoria meestal de MRSA stam insturen zodra de MRSA drager is gedetecteerd. Als later dan infectie met MRSA ontstaat, dan is dit niet bekend bij het RIVM. (**Hoofdstuk 1**).

MRSA CC398-positieve personen krijgen hun MRSA CC398 als gevolg van blootstelling aan vee (varkens, vleeskalveren, vleeskuikens). Echter, wij hebben personen geïdentificeerd met MUO CC398, die geen direct contact hebben gehad met vee. Uit de literatuur blijkt dat transmissie van MRSA CC398 van mens tot mens niet zo waarschijnlijk is, omdat MRSA CC398 72% minder waarschijnlijk en 6 keer minder overdraagbaar zijn dan MRSA niet-CC398 [3, 4], hoewel er wel sporadisch nosocomiale transmissie en uitbraken zijn beschreven.[5, 6] In het kader van de origine (dier of mens) en transmissieroutes (direct of indirect) van de MUO CC398 zijn dus nog alle opties open. De MUO CC398 hebben wij daarom verder bestudeerd in **hoofdstuk 2** waarbij de vraag was of MUO CC398 stammen anders waren dan MKO CC398 stammen en gemakkelijker van mens-tot-mens overgedragen konden worden dan MKO CC398 stammen, welke voornamelijk overgedragen worden van vee op mensen. Vooralsnog blijken er namelijk twee CC398 varianten te bestaan : één van dierlijk-, en één van menselijk afkomst. Naast de verschillen in hun manier van verspreiden zijn er ook verschillen in de aan- en afwezigheid van mobiele genetische elementen (MGE's).[7]

We bepaalden met behulp van polymerasekettingreacties (PCR) of de MGE's: *scn*, *chp*, *φ3 int*, *φ6 int*, *φ7 int*, *rep7*, *rep27* en *cadDX* – aanwezig waren in MUO-isolaten en door deze te vergelijken met een set CC398-isolaten van verschillende doch bekende oorsprong (mensen, varkens, paarden, kippen en vleeskalveren), hebben we de oorsprong van MUO CC398 onderzocht. We vonden dat het onderscheidende humane MSSA CC398 *spa*-type t571 niet aanwezig was onder de Nederlandse MRSA CC398 stammen van mensen en vee. Daarnaast waren de MUO CC398 tetracycline resistent en was de φ3 bacteriofaag met *scn* en *chp* afwezig.

Samenvattend, de MRSA CC398 in deze studie, MUO of MKO, lijken het meest op de MRSA CC398 afkomstig van vee en niet op de CC398 stammen reeds beschreven als menselijk pathogeen. We concludeerden dat humane MUO CC398 dragers MRSA van dierlijke oorsprong droegen ondanks dat deze MUO dragers geen epidemiologisch verband met bijvoorbeeld varkens en varkenshouderij hadden. Deze bevinding is zorgwekkend, omdat het andere transmissieroutes suggereert dan directe blootstelling aan vee.

In **hoofdstuk 3** bestudeerden we in een retrospectief MRSA-dragerscohort (2005-2010) het benodigde aantal follow-up kweeksets om een succesvolle MRSA eradicatie therapie en subsequent niet-dragerschapstatus vast te stellen. We vergeleken de toenmalige standaardprocedure van drie MRSA follow-up kweeksets met zes kweeksets om te bepalen wanneer er moet worden gekweekt, en hoeveel kweeksets er nodig zijn gedurende een periode van een jaar. We vonden dat tussen de derde en de zesde follow-up kweekset 54% (35/65) van het totaal aantal recidieven plaats vond. Meer dan 88% van alle recidieven werd binnen twee maanden gedetecteerd. Gecombineerde neus- en keel dragerschap OR 25,5 (1,6-419,1)) en intravasculaire lijnen (OR 13.6 (1.2-156.2)) bleken risicofactoren te zijn voor vroeg recidiveren. Een vroeg recidief wordt gedefinieerd als een recidief tijdens de eerste drie follow-up kweeksets in tegenstelling tot de laatste drie kweeksets, bij het in totaal doen van zes kweeksets.

Veel zeevarenden bezoeken de havenstad Rotterdam. Ze zijn een moeilijk te contacteren groep vanwege taal- en culturele barrières en hun korte verblijf in Rotterdam. Vanwege bovenstaande redenen konden de zeevarenden niet opgenomen worden in een case control studie (zoals beschreven in **hoofdstuk 5**), maar bepaalden we de MRSA prevalentie van zeevarenden in het Havenziekenhuis na ziekenhuisopname (**hoofdstuk 4**). We ontdekten een MRSA-prevalentie van 5,8% (7/124) onder zeevarenden. Dit betrof een MRSA prevalentie die 52 keer hoger was dan de normale prevalentie in Nederland (0,11% bij ziekenhuisopname).[8] Bovendien bleken zeevaarders met wonden of abcessen 40-maal vaker MRSA-positief te zijn dan zeevaarders zonder. Deze bevinding heeft geleid tot het opnemen van zeevarenden als een nieuwe risicogroep voor MRSA dragerschap voor het Havenziekenhuis en het ErasmusMC in Rotterdam.

In **hoofdstuk 5** beschrijven we de prospectieve case control studie waarin risicofactoren voor MUO verwerving/dragerschap zijn onderzocht met als doel om nieuwe risicofactoren voor MRSA dragerschap te vinden, en deze toe te voegen aan de risicocategorieën van de Nederlandse MRSA richtlijn van de (opvolger van de) werkgroep infectiepreventie.

Wij hebben een case control studie uitgevoerd, waarin MUO die gedetecteerd waren tussen 1 september 2011 en 1 september 2013, zijn geïncludeerd. Cases waren alle MUO dragers gerapporteerd aan het RIVM in deze periode, met als controles willekeurig geselecteerde personen uit de samenleving in dezelfde periode. Cases en controles werden per post benaderd en werden gevraagd om een vragenlijst in te vullen met de bekende risicofactoren zoals beschreven in de WIP-richtlijn, om zo de eventuele MKO eruit te filteren die onterecht als MUO geclassificeerd waren. De vragenlijst bevatte daarnaast ook vragen over gezondheid, gedrag, beroep en (sport) activiteiten die mogelijk risicofactoren zouden kunnen zijn voor het oplopen van een MUO stam. Deze vragen waren gekozen op basis van literatuur studie en een eerdere pilot studie (trawling studie).

Een van de uitkomsten van deze case control studie was dat 38% (144/376) van de MUO dragers verkeerd waren geclassificeerd en feitelijk MKO waren. Deze fout geclassificeerde dragers werden daarom uitgesloten van verdere analyses. Middels logistische multivariaat analyse kwamen de volgende significante risicofactoren voor MUO dragerschap naar voren: antibioticagebruik in de laatste twaalf maanden, aOR 8.1 (5.6-11.7), eerder gescreend als contact in een contactonderzoek maar toen niet gedetecteerd als een MRSA-drager, aOR 4.3 (2.1-8.8), het hebben van tenminste één buitenlandse ouder, aOR 2.4 (1.4-3.9) en het ontvangen van ambulante zorg, aOR 2.3 (1.4-3.7). Deze risicofactoren verklaarden 83% (192/232) van de MUO cases.

We veronderstelden dat een deel van de MUO dragers aanvankelijk als MRSA drager werd gemist, om later alsnog te worden gedetecteerd en vervolgens te worden gecategoriseerd als een MUO. Als dit zo is, dan zou dit betekenen dat sommige MUO dragers niet-geïdentificeerde links naar andere MRSA dragers hebben. Daarom is in **hoofdstuk 6** het moleculair-genetische verband bestudeerd tussen MUO stammen en andere MRSA stammen (MUO of MKO), samen met enkele epidemiologische gegevens van de dragers wat het mogelijk maakt om MUO dragers te koppelen aan andere MRSA dragers (MUO of MKO).

Hiertoe hebben we MUO dragers uit de case control geïncludeerd. We hebben vervolgens goed gedefinieerde ziekenhuis- en huishoudclusters geselecteerd en deze vergeleken met willekeurig geselecteerde, goed gedefinieerde regionale en nationale isolaten. Alle isolaten hadden dezelfde genetische achtergrond (ST8) en waren geclusterd met multi-locus sequentie-typering van het kerngenoom (cgMLST). Ons doel was om MUO isolaten te onderscheiden van of te koppelen aan bekende MRSA-clusters (MKO) of andere niet-geclusterde MRSA isolaten (MUO of MKO). In deze studie konden we 28% (15/54) van de MUO isolaten verklaren door ze te koppelen aan bekende MRSA clusters of isolaten via cgMLST en epidemiologie. Bovendien waren we in staat isolaten uit dezelfde regio, maar gedetecteerd door verschillende MML, aan elkaar te koppelen. Hierdoor werden nieuwe regionale clusters aangetoond die voorheen onopgemerkt waren gebleven. Deze isolaten werden eerder als MUO beschouwd en waren voor het insturende MML toendertijd geen deel van een cluster. Ook resulteerde het gebruik van cgMLST in herindeling van oude clusters, die voorheen waren gebaseerd op epidemiologie en *spa*-typering.

Verschillende typeringsmethoden, zoals cgMLST, zullen verschillende uitkomsten genereren en kunnen als zodanig de aanwezigheid en / of de omvang van uitbraken veranderen, en daarmee de hoeveelheid mensen die nog gescreend moet worden. Degenen die niet zijn gescreend op het moment van de uitbraak en die later alsnog worden gedetecteerd, worden gedefinieerd als MUO dragers. CgMLST heeft een hoger onderscheidend vermogen dan *spa*-typering, dus we konden onderscheid maken binnen en tussen t008 MRSA clusters (voorheen geclusterd op basis van *spa*-typering). Verder hebben we met succes onderscheid kunnen maken tussen Nederlandse MRSA t008 clusters en import-MRSA t008 clusters uit Aruba en Taiwan, wat voorheen niet mogelijk was met *spa*-typering.

## CONCLUSIES, AANBEVELINGEN EN TOEKOMSTPERSPECTIEVEN

In 2018 rapporteerde het RIVM dat 38% (1.066 / 2.792) van de gemelde MRSA isolaten aan de MRSA-surveillance in 2017 een MRSA van onbekende origine (MUO) was.[9] Onze bevindingen van de case control studie, tonen aan dat 38% (144/376 teruggezonden vragenlijsten) van de MUO isolaten uit de MRSA surveillance verkeerd geclassificeerde MKO waren. Uitgaande van een stabiel aantal misclassificaties per jaar zou dit betekenen dat, in 2017, 23% (661/2792) van de gemelde MRSA isolaten aan de surveillance daadwerkelijk MUO was. (In absolute aantallen betekent dit 405 “MUO” minder dan oorspronkelijk gerapporteerd.) Als we hetzelfde berekenen voor 2009, hadden we oorspronkelijk 786 MUO uit 2874 gerapporteerde MRSA, waar na correctie voor misclassificatie nog 17% (487/2874) overblijft. Over een periode van 8 jaar (2009-2017) geeft dat een geschatte toename van het aantal MUO met 35,7%. Ondanks misclassificatie van MUO, blijft er nog steeds een grote groep van MUO over. Het is dus nog steeds belangrijk antwoorden te vinden op de vraag waar ze vandaan komen. In dit proefschrift zijn we op zoek gegaan naar MUO en we hebben gevonden dat MUO geen homogene groep zijn en uit elkaar vallen in verschillende subgroepen, zowel genetisch als epidemiologisch.

## MUO CC398

Een belangrijke subgroep van de MUO isolaten zijn de MUO CC398 (352/2312 MRSA CC398; 15% zoals beschreven in **hoofdstuk 1**). Dit zijn MUO isolaten uit het vee-geassocieerde cluster 398, wiens dragers geen directe beroepscontact met vee hadden. (Zoals besproken in **hoofdstuk 2**.) Dit is een belangrijk gegeven, omdat Nederland een land is met een lage MRSA prevalentie, maar met een relatief groot vee-geassocieerd MRSA-reservoir, net als in Denemarken. Ook wordt er van uitgegaan dat de tetracycline resistente vee-geassocieerde MRSA CC398 (LA-MRSA) niet significant van mens tot mens overgaat en dat de transmissie gebaseerd is op blootstelling via (beroeps) contact.[4] De beperkte van-mens-tot-mens transmissie limiteert gelukkig tot op heden de impact van LA-MRSA-stammen op de gezondheidszorg. Het bestaan van MUO CC398 impliceert echter acquisitie, zonder (direct) contact met vee. Daarom was onze vraagstelling of MUO CC398 mogelijk tot een subgroep van dit genetische cluster behoren, die allen in mensen gevonden wordt. McCarthy et al. toonden aan dat CC398-stammen van mensen in contact met dieren verschilden van CC398-stammen geïsoleerd van mensen zonder contact met dieren qua gehalte van mobiele genetische elementen (MGE).[7] Wij vonden echter dat de MUO CC398 stammen vergelijkbaar waren met de MKO CC398 stammen en dus niet tot het menselijk cluster behoren (**hoofdstuk 2**). Dit betekent dat aan de mens aangepaste MRSA CC398 waarschijnlijk niet de oorsprong zijn van MUO CC398. Een waarschijnlijkere hypothese voor het bestaan van MUO CC398 zou indirecte overdracht kunnen zijn, bijvoorbeeld via de lucht, knaagdieren, of indirect door blootstelling van personen in de gemeenschap die op hun beurt wel waren blootgesteld aan LA-MRSA.[10-14] Verder onderzoek is noodzakelijk om vast te stellen of indirecte overdracht inderdaad een rol speelt in de verspreiding van MUO CC398. Ook bevelen wij aan om gerapporteerde MUO CC398 te analyseren en de geaggregeerde data terug te rapporteren op zowel nationaal niveau als per insturend MML. De aanwezigheid van MGE als *Φ3* (*ΦSa3*) in MUO CC398 zou makkelijk gemonitord kunnen worden, zoals eerder is uitgevoerd door van Rijen et al., die voor MUO CC398 screenden aan de hand van *Φ3* en *tetM* waardoor een snelle rubricering tussen CC398 van dierlijke-, of menselijk oorsprong mogelijk wordt.[15]

## Import-MRSA en zeevarenden

Een deel van de MUO is direct gerelateerd aan (blootstelling aan) hogere MRSA-prevalentie in het buitenland: de zogenaamde import-MRSA. Bijvoorbeeld zeevarenden (**hoofdstuk 4**), het hebben van een buitenlandse ouder (**hoofdstuk 5**) of import-MRSA-clusters via reizigers die vrienden en familieleden bezoeken of via immigratie (Aruba, Taiwan) (**hoofdstuk 6**). Dit zijn allemaal factoren die een deel van de MUO dragers verklaren. Interessant is dat bezoeken aan en reizen naar het buitenland en het bezoeken van een ziekenhuis of gezondheidsinstelling in het buitenland langer dan twee maanden geleden, geen significante risicofactoren waren in de case control studie. (**hoofdstuk 5**). Het blijft de vraag of het arbitraire afkappunt van twee maanden voor een ziekenhuisbezoek in het buitenland niet te strikt en niet te algemeen is, aangezien arbitrair afkappen het duiden van een MRSA als MUO of als MKO mede bepaalt. (**Hoofdstuk 5**)

Ons advies is wel om zeevarenden toe te voegen als risicogroep voor MRSA dragerschap. Gezien de hoge prevalentie van dragerschap onder zeevarenden raden we aan dat alle zeevarenden worden ingedeeld als WIP-categorie 2 en dus gescreend worden op MRSA wanneer zij aanwezig zijn in Nederland, ongeacht of zij wonden of onderliggende ziekte hebben; en dat preventieve isolatie wordt toegepast in afwachting van de testresultaten. Sinds 2010 zijn zeevarenden opgenomen in de triage bij opname van het Erasmus MC (en het voormalige Havenziekenhuis). Over de wereldwijde impact van overdracht van MRSA door zeevarenden is momenteel niets bekend (**hoofdstuk 4**).

## Case control risicofactoren: intersectie gezondheidszorg en gemeenschap

De resultaten van de MUO case control geven aan dat een groot deel van de MUO cases gezondheidszorg of ziekte gerelateerd zijn, omdat we risicofactoren hebben gevonden zoals antibioticagebruik en ambulante zorg. We toonden aan dat MUO dragers in een eerdere periode gescreend waren tijdens een contactonderzoek, maar op dat moment niet als MRSA-drager waren gedetecteerd. Het lijkt erop dat MUO dragers regelmatig contact hebben met de gezondheidszorg, maar niet noodzakelijkerwijs met een ziekenhuis. Hoogstwaarschijnlijk hebben ze hun MRSA tijdens of door het ontvangen van gezondheidszorg verworven, mogelijk buiten het ziekenhuis waar de openbare en institutionele gezondheidszorg elkaar raken. Voorbeelden hiervan zijn ambulante zorg of het gebruik van antibiotica in verpleeghuizen of de huisartsenzorg. (**Hoofdstuk 5**) Het toevoegen van de aangetoonde, meer algemene, risicofactoren aan de risicocategorieën van de Nederlandse MRSA richtlijn van de WIP zal mogelijk het aantal MUO kunnen verminderen. Echter, het zal ook tot hoge kosten van screening leiden en een hogere werklast genereren, zeker als we er voor kiezen om de extra gescreende patiënten ook pre-emptief te isoleren, vanwege de verwachte hoge aantallen die daar dan voor in aanmerking komen. Met andere woorden, het totale aantal mensen dat gescreend moet worden om één MUO drager te vinden met de nieuwe, algemene risicofactoren zoals het gebruik van ambulante zorg of het gebruik van antibiotica, kan door toevoeging van deze gevonden risicofactoren te hoog worden en daardoor te duur en te arbeidsintensief. Toch verklaarden onze algemene risicofactoren 83% van de MUO cases in de case control. Een andere aanpak middels risico algoritme is daarom gewenst, (vide infra). Voor de overige 17% van de MUO cases werd geen verklaring gevonden.

Voor case control studies is de keuze van de controles moeilijk maar belangrijk. Zoals hierboven vermeld, vonden we algemene risicofactoren die 83% van MUO cases verklaarden. De uitkomst van een case control studie hangt echter in hoge mate af van de keuze van de controle; gematched of niet. Onze studie naar de kenmerken van MUO (**hoofdstuk 1**) en de initiële "trawling" vragenlijst (**hoofdstuk 5**) gaven een zeer diverse groep van dragers weer, een diversiteit die beter gerepresenteerd werd door te kiezen voor een controlegroep vanuit de gemeenschap ("community"). Cases, d.w.z. MUO dragers, waren niet alleen MRSA dragers gerapporteerd door ziekenhuizen, maar ook MRSA dragers gerapporteerd door huisartsen (HA) of langdurige zorgfaciliteiten (LDZF). Indien we de controles zouden matchen op opname of behandeling in het ziekenhuis, of op gedetecteerd zijn bij de huisarts dan wel in de Verpleeg- en verzorgingstehuis (VVT) sector, dan zouden er verschillende controle populaties nodig zijn. Ook hebben we overwogen om controles te matchen op het hebben van een (vermoedelijke) infectie, omdat MUO vaak worden ontdekt als bijkomstigheid wanneer een kweek wordt aangevraagd voor de detectie van een infectie. Dit was echter niet mogelijk omdat het niet zeker was of gerapporteerde gevallen daadwerkelijk een infectie hadden of niet. De registratie van de aanwezigheid van infectie is namelijk niet betrouwbaar. Als het ingediende isolaat van de case niet een isolaat van een infectie locatie was, dan sloot dit geen infectie elders of later uit. Bovendien was onze case control opgezet om onbekende risicofactoren te vinden zonder vooroordelen. Matching zou vooroordelen introduceren en de kans op het vinden van risicofactoren verminderen. Daarom kozen we voor niet-gematchte controles vanuit de bevolking, waardoor vooroordelen zoveel mogelijk werden beperkt en de kans werd gemaximaliseerd om nieuwe risicofactoren binnen de MUO populatie te vinden. Gezien de bekende prevalentie van MRSA in de gemeenschap (tussen 0 en 0,11%[8]) beschouwden we de kans om een MRSA drager als controle in onze studie op te nemen dusdanig laag dat er niet op gescreend hoefde te worden.

Om patiënten te kunnen identificeren met een verhoogd risico op MRSA dragerschap, is een algoritme noodzakelijk dat het a-priori risico voor MRSA dragerschap berekent met behulp van de bestaande en de nieuwe risicofactoren. Helaas zijn een aantal nieuwe risicofactoren te algemeen en/of veel voorkomend om ze als een ja/nee-antwoord in de normale triage te includeren. In de toekomst zal daarom een algoritme ontwikkeld moeten worden waarin risicofactoren worden gewogen in een specifieke populatie, zodat een gewogen risico kan worden berekend op basis van meer dan één risicofactor. De uitkomst van dit algoritme moet dan leiden tot het instellen van eventuele maatregelen om overdracht van MRSA te voorkomen. Idealiter moet gestreefd worden naar nationale uniformiteit in dit algoritme en de uitvoering daarvan.

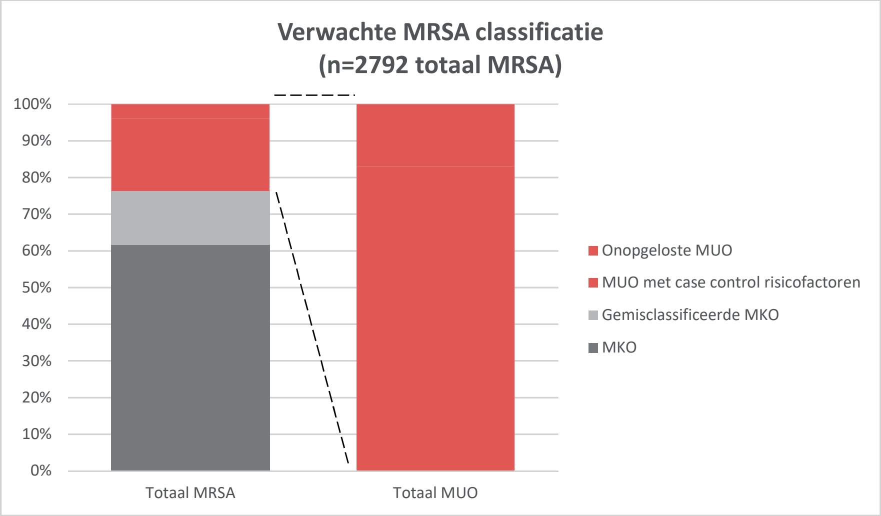
Om de haalbaarheid en volledigheid te verbeteren, zou een interactieve chatbot kunnen worden geprogrammeerd met vragen die door patiënten kunnen worden ingevuld wanneer zij worden opgenomen of behandeld. De ingevoerde antwoorden kunnen in een algoritme worden geladen om het a-priori risico van MRSA dragerschap te berekenen. Chatbot technologie is al mogelijk en wordt gebruikt door bedrijven met veel klanten, zoals KLM.[16]

## MRSA recidivering na MRSA eradicatie therapie

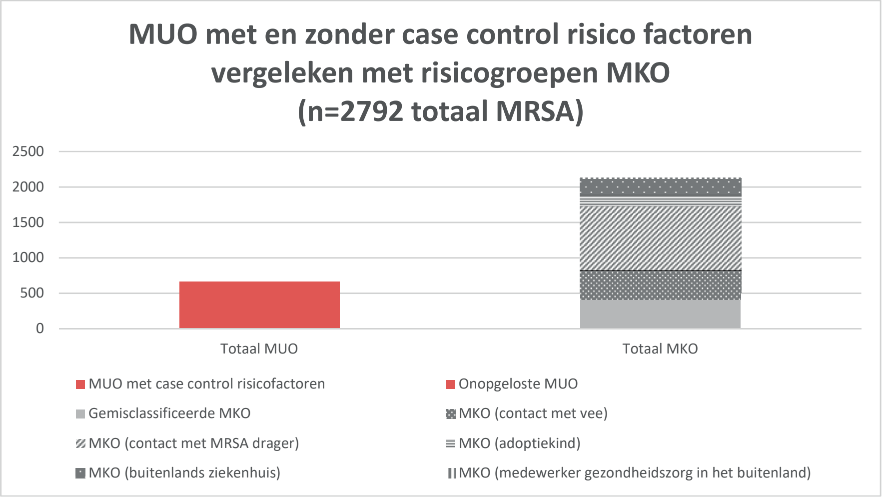
MRSA recidieven worden gemist wanneer slechts drie follow-up kweeksets worden gebruikt na MRSA eradicatie therapie. Tevens zullen deze MRSA recidieven dan later gedetecteerd kunnen worden als MUO. We hebben aangetoond dat MRSA recidieven worden gemist in 54% (35/65 recidieven) bij het gebruik van drie follow-up kweeksets in vergelijking met zes follow-up kweeksets. Op basis van onze resultaten werd de toenmalige WIP-richtlijn gewijzigd op de timing van kweeksets na MRSA eradicatie therapie (**Hoofdstuk 3**). We bevelen vijf kweeksets aan binnen een jaar na de eradicatie therapie. In de zes maanden na de eradicatie therapie, raden we aan om behandelde dragers die het risico lopen op een vroeg recidief regelmatig te kweken. Personen met een verhoogde kans op een vroeg recidief, zijn de neus en keel dragers (OR 25.5 (1.6-419.1)) of degenen die intravasculaire lijnen hebben (OR 13.6 (1.2-156.2)). Personen die laat recidiveren, kunnen hun vervolgkweken met meer tijd ertussen afnemen en zouden één jaar na hun eradicatie therapie hun laatste controle kweek moeten krijgen. Personen met een vroeg recidief, wil je sneller vangen en recidiveren binnen een half jaar. Deze personen zouden dan niet tot een jaar na hun eradicatie therapie hoeven te wachten op de laatste controle kweek. Deze aanbeveling is een balans tussen de noodzaak van snelle detectie van een MRSA recidief, de belasting van de patiënt en het verlagen van het aantal gemiste MRSA-recidieven na succesvolle MRSA eradicatie therapie.

## Misclassificatie van MKO als MUO

We kwamen een groot aantal misgeclassificeerde MKO isolaten van dragers tegen die in de nationale MRSA database als MUO geregistreerd stonden. Analyse van de case control studie toonde aan dat 38% van alle gerapporteerde MUO isolaten in de MRSA surveillance database in feite een MKO was. Misclassificatie kan worden verklaard door het te vroeg invullen van bijbehorende epidemiologische gegevens aan het RIVM, die tezamen met de MRSA stam door de MML aan het RIVM worden opgestuurd. Te vroeg aanleveren van gegevens, met name voordat de typering bekend is, geeft onvolledige informatie (**hoofdstuk 5**). Het is namelijk niet altijd mogelijk om de bron vast te stellen



**Figuur 1 – Nederlandse MRSA en MUO, relatieve bijdragen**
Data voor MUO werden gegenereerd met behulp van resultaten van de MUO case control studie en geëxtrapoleerd naar de Nethmap 2018 MRSA data (n = 2792). Data voor MKO werden gegenereerd door het gebruik van MRSA surveillance data van 2008-2011 en door het extrapoleren van het gemiddelde percentage naar de gegeven getallen in de Nethmap 2018 data. De definitie van gemisclassificeerd: MRSA isolaten aangegeven als MUO door de MML's in de vragenlijst verzonden naar het RIVM maar gedefinieerd in onze case control studie als MKO.



**Figuur 2 – MUO vergeleken met MKO in absolute aantallen**
Data voor MUO werden gegenereerd met behulp van resultaten van de MUO case control studie en geëxtrapoleerd naar de Nethmap 2018 MRSA data (n = 2792). Data voor MKO werden gegenereerd door het gebruik van MRSA surveillance data van 2008-2011 en door het extrapoleren van het gemiddelde percentage naar de gegeven getallen in de Nethmap 2018 data. De definitie van gemisclassificeerd: MRSA isolaten aangegeven als MUO door de MML's in de vragenlijst verzonden naar het RIVM, maar gedefinieerd in onze case control studie als MKO.

voordat het typeringsresultaat beschikbaar is. Typeringsresultaten zijn er voor om bronnen en transmissieroutes te verklaren. Wanneer de bronnen vanuit de zorg komen, kan de MRSA drager worden gedefinieerd als een MKO. De oplossing voor dit probleem is een tweestapsprocedure: eerst wordt de stam verzonden en vervolgens, na het terugkrijgen en analyseren van de typeringsresultaten, wordt de MML gevraagd voor de uiteindelijke epidemiologische beslissing of er sprake is van een MKO of een MUO. Deze beslissing zal afhangen van de (veronderstelde) bronnen en risicofactoren van de drager. Idealiter worden de typeringsresultaten binnen de kortste doorlooptijd teruggestuurd naar de medische professionals om contact onderzoek inspanningen te ondersteunen en te helpen met het definiëren van MRSA-bronnen. Dit laatste moet vervolgens terug gerapporteerd worden aan de MRSA surveillance, waardoor de betrouwbaarheid van de epidemiologische gegevens verder wordt verbeterd.

Misclassificatie is te wijten aan de kwaliteit van de epidemiologische gegevens. Deze kwaliteit is gebaseerd op de tijd, moeite en mogelijkheid om de gegevens te verkrijgen. Momenteel is er geen standaardprocedure bij het contacteren en bevragen van MRSA dragers, voor het uitvoeren van het contact onderzoek en ook niet wie dat dan zou moeten doen. De inspanning die geleverd wordt, zal dus per persoon verschillen, en daarmee de kwaliteit. Een procedure voor ondervraging en voor het contact opnemen met MRSA dragers is daarom aan te bevelen. Er kan namelijk ook sprake zijn van misclassificatie door een te beperkt contact onderzoek uit te voeren bij MRSA uitbraken (**hoofdstuk 5 en 6**). Een te klein contact onderzoek mist MRSA dragers (en dus MKO) welke later ter plaatse of elders als MUO kunnen worden gedetecteerd. Dit zou de gevonden risicofactor in de case control studie kunnen verklaren: eerder in het verleden gescreend voor MRSA-dragerschap, maar op dat moment MRSA negatief bevonden.

## Onverklaarde MUO en de noodzaak tot samenwerking en typering

Het deel van de MUO cases dat niet kon worden verklaard was 17%. Met de Nethmap 2018 data zou dit betekenen dat van de 661 gerapporteerde MUO isolaten in 2017 (23% van de totale gemelde MRSA voor de surveillance), 17% (112/661) zonder verklaring zou blijven. Dat zou 4% (112/2792) van de totale MRSA zijn die in 2017 aan de MRSA-surveillance werd gerapporteerd. (Figuur 1) Deze resterende groep bestaat uit verschillende MUO cases waarbij we in onze studie geen risicofactoren konden identificeren. Een deel van deze MUO cases kan wellicht nog worden opgehelderd als genoom data van MRSA isolaten tussen zorginstellingen of binnen een ZorgRegio worden gecombineerd, al dan niet op nationaal niveau. We hebben aangetoond dat twee MML’s in de regio Rotterdam onafhankelijk van elkaar een MUO ontdekten, zonder te weten dat deze twee genetisch aan elkaar verwant waren, door cgMLST-resultaten aan elkaar te koppelen (**Hoofdstuk 6**). We laten in **hoofdstuk 6** ook zien dat we onderscheid konden maken tussen klonale t008 MRSA clusters door gebruik te maken van cgMLST. Het toepassen van cgMLST data om MML te helpen bij het traceren van contactpersonen zou van cruciaal belang kunnen zijn om de laatste 17% van MUO in Nederland op te helderen.

Bij voorkeur worden deze gegevens ook gedeeld op het niveau van het regionale netwerk van zorginstellingen om intraregionale (uitbraak) clusters te detecteren (zoals beschreven in **hoofdstuk 6**). Het huidige antibioticaresistentiezorgnetwerk of ZorgRegio ABR kan deze taak op zich nemen en een manier te vinden om gegevens over MRSA stammen, typeringen en dragers te delen. Idealiter zouden zorginstellingen binnen de zorgregio geïnformeerd moeten worden over MRSA dragers in de regio. Binnen de ZorgRegio's kunnen zorginstellingen gevraagd worden afspraken te maken over het delen van deze informatie en het gelijktrekken van beleid, rekening houdend met de privacywetgeving. Het delen van informatie over MRSA dragers en MRSA stammen tussen instituten, met name als de patiënt niet direct wordt overgeplaatst, is zeer wenselijk vanuit het oogpunt van infectiepreventie, maar is mogelijk op dit moment nog een brug te ver.

Kort samengevat doen wij de volgende aanbevelingen: het doen van verdere studies naar MUO CC398, het monitoren van MUO CC398 in de surveillance en de terugkoppeling daarvan op nationaal en MML niveau, het toevoegen van zeevarenden als MRSA risicogroep aan de geldende richtlijn, alsook indelen als WIP-categorie 2, het maken van een risico algoritme, waarin een gewogen a-priori risico wordt berekend op basis van meerdere risicofactoren, het doen van vijf follow-up kweeksets waarvan vier in de eerste twee maanden (de vierde op 2 maanden) na MRSA eradicatie therapie en de laatste follow-up kweekset na een half jaar of een jaar, afhankelijk van de aanwezigheid van risicofactoren of het zijn van een medewerker in de gezondheidszorg. Andere aanbevelingen zijn het reduceren van de misclassificatie door intensievere samenwerking tussen RIVM waar getypeerd wordt en de MML die de epidemiologische gegevens aanleveren, het delen van minstens de typeringsdata binnen de ZorgRegio en zo mogelijk ook supraregionaal, zodat arbitraire grenzen tussen adherentiegebieden van MML niet langer het vinden van clusters in de weg staat, en het vervolgen van de aantallen MUO aangezien bij 17% van de MUO vooralsnog geen risicofactor dan wel verklaring gevonden is. Blijvende controle op MUO is essentieel om de prevalentie van MRSA in ons land laag te houden!

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Publications
PhD portfolio
Curriculum vitae
Dankwoord

# 10

# PUBLICATIONS

## This thesis

*Combining high resolution typing by cgMLST with epidemiological data improves the identification of the origin of MRSA with previously unknown origin*
**Lekkerkerk WS**, Witteveen S, van Wamel WJB, Fitzgerald JR, Bosch T, Schouls LM, Vos MC.
*Submitted*

*Newly identified risk factors for MRSA carriage in The Netherlands.*
**Lekkerkerk WSN**, Haenen A, van der Sande MAB, Leenstra T, de Greeff S, Timen A, Tjon-A-Tsien A, Richardus JH, van de Sande-Bruinsma N, Vos MC.
PLoS One. 2017 Nov 30;12(11):e0188502. doi: 10.1371/journal.pone.0188502. eCollection 2017.
PMID: 29190731 Free PMC Article

*What Is the Origin of Livestock-Associated Methicillin-Resistant Staphylococcus aureus Clonal Complex 398 Isolates from Humans without Livestock Contact? An Epidemiological and Genetic Analysis.*
**Lekkerkerk WS**, van Wamel WJ, Snijders SV, Willems RJ, van Duijkeren E, Broens EM, Wagenaar JA, Lindsay JA, Vos MC.
J Clin Microbiol. 2015 Jun;53(6):1836-41. doi: 10.1128/JCM.02702-14. Epub 2015 Mar 25.
PMID: 25809975 Free PMC Article

*Follow-up cultures for MRSA after eradication therapy: are three culture-sets enough?*
**Lekkerkerk WS**, Uljee M, Prkić A, Maas BD, Severin JA, Vos MC.
J Infect. 2015 May;70(5):491-8. doi: 10.1016/j.jinf.2015.01.006. Epub 2015 Jan 15.
PMID: 25597821

*Letter to the editor: seafarers: a new risk group for meticillin-resistant Staphylococcus aureus (MRSA).*
**Lekkerkerk WS**, van Genderen PJ, Severin JA, Peper JP, Storm EF, Vos MC.
Euro Surveill. 2013 Oct 24;18(43). pii: 20618. No abstract available.
PMID: 24176620 Free Article

*Emergence of MRSA of unknown origin in the Netherlands.*

**Lekkerkerk WS**, van de Sande-Bruinsma N, van der Sande MA, Tjon-A-Tsien A, Groenheide A, Haenen A, Timen A, van den Broek PJ, van Wamel WJ, de Neeling AJ, Richardus JH, Verbrugh HA, Vos MC.

Clin Microbiol Infect. 2012 Jul;18(7):656-61. doi: 10.1111/j.1469-0691.2011.03662.x. Epub 2011 Oct 3.

PMID: 21967090 Free Article

## Other

*Good performance of the SpectraCellRA system for typing of methicillin-resistant Staphylococcus aureus isolates.*

te Witt R, Vaessen N, Melles DC, **Lekkerkerk WS**, van der Zwaan EA, Zandijk WH, Severin JA, Vos MC.

J Clin Microbiol. 2013 May;51(5):1434-8. doi: 10.1128/JCM.02101-12. Epub 2013 Feb 20.

PMID: 23426926 Free PMC Article

# PHD PORTFOLIO

| | |
|---|---|
| **Name PhD student** | Wouter Sybren Niels Lekkerkerk |
| **ErasmusMC department** | Medical microbiology and infectious diseases |
| **PhD period** | 2009 - 2019 |
| **Promotor** | Prof. Dr. M.C. Vos |
| **Co-promoter** | Dr. W.J.B. van Wamel |

| | **Year** |
|---|---|
| **Courses** | |
| Safety Standards for Diagnostic and Research Laboratories Training, Family Health International | 2009 |
| Case Control studies, NIHES | 2011 |
| Principles of Research in Medicine, NIHES | 2011 |
| Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen, CBO | 2012 |
| Masterclass Scientific Integrity | 2012 |
| ICM - Zakelijke correspondentie | 2013 |
| Gezondheidsrecht, Desiderius school | 2015 |
| Klinische mycologie, Radboud Universitair Medisch Centrum Nijmegen | 2015 |
| Medische parasitologische diagnostiek, Leids Universitair Medisch Centrum | 2016 |
| Communicatie, Desiderius school | 2016 |
| Medische ethiek, Desiderius school | 2017 |
| Samenwerking, Desiderius school | 2017 |
| Openbare Gezondheidszorg, Rijksinstituut voor Volksgezondheid en Milieu | 2017 |
| Phenotypic interpretation of the antimicrobial susceptibility determination, Molmed | 2017 |
| Infectiepreventie, Amphia Academy Infectious Disease Foundation | 2018 |
| Ziekenhuismanagement, NVMM en Academie voor Medisch Specialisten | 2018 |
| Ziekenhuismanagement, Desiderius school | 2019 |
| **Oral and poster presentations** | |
| ISSSI 2010 (Bath, UK) - *Dutch MRSA of Unknown Origin* (poster) | 2010 |
| Scientific Autumn Meeting NVMM (Amsterdam, The Netherlands) – *Dutch MRSA of Unknown Origin* (invited speaker and poster) | 2010 |
| Boerhaave nascholing & WIP congress (Leiden, The Netherlands) – *MRSA van onbekende origine* (invited speaker) | 2011 |
| VHIG congress (Almelo, The Netherlands) – *MUO in het oranje schemergebied* (Invited speaker) | 2012 |
| ISSSI 2012 (Lyon, France) - *Three is not enough; or why follow-up needs more cultures* (poster), *Search & Destroy policy evaluated* (poster), *Mobile genetic elements of ST398 MRSA in humans and four animal species: a comparison* (poster) | 2012 |
| ECCMID 2013 (Berlin, Germany) - *High prevalence of MRSA in seafarers in The Netherlands* (poster) | 2013 |
| Scientific Spring Meeting NVMM (Papendal, The Netherlands) - *Transmission by not isolated MRSA carriers still an issue despite good Search & Destroy* (poster) | 2013 |

| | Year |
|---|---|
| ICAAC 2014 (Washington DC, USA) - *Linking USA300 CA-MRSA to Dutch Hospital Outbreaks* (poster) | 2014 |
| ISSSI 2014 (Chicago, USA) – *New risk factors revealed for MRSA in The Netherlands: A case-control study* (invited speaker), *Unknown risk factors revealed for MRSA in The Netherlands; a case-control stud*y (poster) | 2014 |
| ISSSI 2016 (Seoul, South Korea) – *Epidemiology of MRSA of Unknown Origin (MUO)* (poster) | 2016 |
| **Teaching** | |
| Supervision of second year medical students, as part of their medical training during various practical ‘VO-onderwijs’ and clinical case sessions. | 2013-2019 |
| Supervision of third year medical students, as part of their medical and scientific training | 2012 |
| **Other** | |
| President of Young European Federalists, The Hague | 2018-2019 |
| Member of the 1st NVAMM sailing committee | 2017 |
| Founder and member of the FeestCIE MMIZ, ErasmusMC | 2013-2016 |
| Member of the FeestCIE, EPI, RIVM | 2010-2013 |
| Member of the Research Day committee, MMIZ, ErasmusMC | 2012 |

## CURRICULUM VITAE

Sybren Lekkerkerk was born on September the 6th, 1985 in Gouda, The Netherlands. He grew up in the quaint little city of Haastrecht, tucked away between the polders and the rivers Vlist and Hollandsche IJssel. In 2003, he graduated from the Coornhert Gymnasium in Gouda and started his medical training at the Erasmus University Medical Center in Rotterdam the same year. In 2009, he finished his medical doctorate (MD) and obtained the title of doctorandus under supervision of dr. H.F.L. Wertheim and J.L. Nouwen; but not before travelling to Vietnam for an internship at the Oxford Clinical Research Unit in Hà Nội (Hanoi). Just before graduating, he started his PhD-research at the department of medical microbiology of the Erasmus University Medical Center on *MRSA of Unknown Origin*, under supervision of Prof. dr. H.A. Verbrugh (now em. prof. dr.) and Prof. dr. M.C. Vos. Between 2009 and 2014 he regularly worked at the department of epidemiology and surveillance of the RIVM as part of his PhD-research and communted daily back and forth between either Lelystad and Bilthoven or Rotterdam. In 2014, he started as clinical resident of medical microbiology under supervision of Prof. dr. H.A. Verbrugh (briefly), Dr. A.G. Vonk, Dr. R.W. Vreede, and Dr. L.C. Smeets at the departments of medical microbiology of the Erasmus University Medical Center in Rotterdam and of the Reinier de Graaf Gasthuis, locatie Reinier Haga Medisch Diagnostisch Centrum (RHMDC) in Delft, respectively. At the time of writing, Sybren is living in Hoogvliet Rotterdam together with his wife and his daughter, and works as clinical microbiologist in the Maasstad Hospital.

# DANKWOORD

Lang, lang geleden in een voorportaal van een kantoor hier ver, ver vandaan (gesloopt), besloot ik heel naïef dat ik wilde promoveren. Het liefst in vier jaar. Het werd iets langer. Het is – cliché alert - zonder twijfel de grootste uitdaging die ik mezelf heb aangedaan. Maar als u[1] dit leest, betekent het dat mijn boekje nu toch een feit is.

Mijn woestijntocht naar een ongrijpbare horizon lijkt daarmee ten einde.[2]

Reflecterend op deze tocht, realiseer ik me dat ik onderweg velerlei mensen ben tegengekomen. Sommigen slechts als passanten of figuranten, anderen om verder mee te lopen, deels of tot het eind. Zoals beeldhouwers interacteren met een stuk steen en zo een beeld creëren, vormen, zo deden deze mensen dat, al dan niet bewust, met mij. Ik ben ze dus veel dank verschuldigd.

Echter, na tien jaar is de lijst te lang om iedereen bij naam te noemen en persoonlijk te bedanken. (Wellicht ten overvloede omdat u het uiteraard al gelezen had, maar een deel van de mensen die ik dankbaar ben, staan genoemd op het eind van hoofdstuk 6 en zullen niet herhaald worden.) Staat u niet specifiek met naam genoemd in het dankwoord? Bent u toch benieuwd hoe ik onze samenwerking vond? Vraag het me gerust!

Wie een betekenisvol dankwoord wil schrijven, kan overigens putten uit de clichés van voorgangers. In de voorbereiding, kwamen een aantal gouwe ouwen voorbij, die ik u niet wil onthouden: In het dankwoord is alles al een keer gezegd en geschreven. Het proefschrift kwam tot stand door inzet en wilskracht. Met dank aan veel verschillende, samenwerkende mensen. Het dankwoord is een van de moeilijkst te schrijven doch meest gelezen onderdeel van het proefschrift. Pieken, dalen, uitdaging, etc. etc. Bedankt, wat hadden we een toffe tijd. Fijn dat je me pipeteerslaaf was. Ik hoop dat ik die ene niet vergeten ben. Ik hou van je. We zien elkaar weer spoedig, echt waar! (Maar niet heus.)

Was op het eind, in retrospectie, de weg er naartoe belangrijker dan het behalen van het einddoel zelf? Mijns inziens is vooral de combinatie, een weg met een eind, het meest waardevol. Hiervan is het dankwoord een onderdeel.

Overigens, als u van klassieke dankwoorden zonder lange introducties houdt, dan had u deze pagina over kunnen slaan. Helaas, volgende keer beter.

[1] Als u geen probleem heeft met tutoyeren, mag u ook je of jij lezen.

[2] Soundtrack: John Frusciante’s Central van het album The Empyrean

Beste Prof. dr. M.C. Vos, beste Greet, van alle mensen die ik de afgelopen tien jaar tegen kwam, heb ik veruit het meest van jou geleerd. In alle eerlijkheid is de gehele backbone van mijn professionele standaard gevormd door onze interactie. Hoewel ik veel van allerlei supervisoren geleerd heb, ben jij figuurlijk de smid geweest die het zwaard tien jaar geleden in het vuur van de smidse stak en het heeft gevormd door het eindeloos te bewerken met hamer. Dat was een zware taak, maar een rol die je desondanks vervulde ondanks alles. Dat heeft mij heel veel opgeleverd voor het proefschrift, als toekomstig arts-microbioloog, en als mens. Heel veel dank daarvoor!

Beste dr. Vonk, beste Alieke, hoewel ik als AIOS een afscheidscadeau van je voorganger was, hoop ik dat mijn lange aanwezigheid mee viel. Jij leidt mensen op, niet alleen maar AIOS. Die instelling heeft me veel gebracht, ook tijdens de diepe dalen gedurende het promotie traject. Daarvoor mijn eeuwige dank.

Beste dr. Van Wamel, beste Willem, ik weet dat je niet van *die artsen* houdt. Maar ik hoop dat ik op je lijstje sta van artsen die ok zijn. Het werk in het lab en het daaruit voortvloeiende MUO CC398 deel van het proefschrift blijft mijn favoriete onderdeel. Ik heb genoten van onze discussies en uitwisselingen van gedachten. Je deur stond altijd open om geduldig te luisteren of te discussiëren, ook toen je niet mijn officiële co-promotor was. Bedankt voor alles!

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Beste dr. Vreede en dr. van Trijp, beste Rolf en Marijke, zonder jullie was er helemaal geen boekje gekomen. Na alle voor en tegens tegen elkaar afgewogen te hebben, besloot ik de stekker eruit te trekken die middag in Delft. Op dat moment hebben jullie niet getwijfeld en op me ingepraat. Waarvoor mijn dank! (Toch wel.)

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Beste prof. dr. Richardus, prof. dr. Wertheim en prof. dr. Kluytmans, dank jullie wel voor het plaats nemen in de leescommissie van mijn proefschrift. Ik waardeer dit zeer.

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Beste oud kamergenoten, ik weet serieus niet meer met hoeveel jullie nu uiteindelijk zijn. Zowel de duur van mijn promotietraject als de introductie van flexwerken heeft de teller dusdanig opgedreven dat ik de tel ben kwijtgeraakt. Ik hoop dat jullie me een prettige kamergenoot vonden waarop jullie met plezier terugkijken. In ieder geval: dank jullie wel voor jullie tijd, aandacht, gedeelde muzieksmaak (indien van toepassing) en uiteraard gezelligheid. Sommigen van jullie waren prettig gestoord, en ik kijk daar met plezier op terug.

Lieve ex-mede-AIOS (in het bijzonder maar zeker niet uitsluitend: Martijn, Khoa, Maurits, George, Tien, Dimard, Angga, Carla, Marrit, Maris, Annemiek, Kara en Ga-Lai), het werken met jullie was elke dag een feest. Dank jullie wel voor jullie collegialiteit en vriendschap tijdens en na het werk!

Maike, dank voor je *don't-ask-don't-tell* policy rondom mijn promotietraject. Ik realiseer me dat ik broodnodig dat ene e-mailtje nog moet beantwoorden. Maar dat kan ik dan eindelijk zonder schuldgevoel over sociaal leven doen. Dank ook voor de fijne gesprekken en lunchwandelingen in mijn RIVM periode!

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Paul, Willem Jan, Suus en Annemieke: dank voor de lange, goede vriendschap bij goed en slecht weer. Binnenkort maar weer eens afspreken?

Lieve mam, je bent een power-vrouw door weer en wind! Je maakt me trots en ik houd van je! Dank je wel voor alle steun die je altijd hebt gegeven!

Lieve, lieve Liên, chồng yêu vợ rất nhiều. Dank je wel dat je bent wie je bent. Moge er samen nog vele mooie momenten komen.

Oh mijn kleine Evelien, je maakt het leven fantastisch.

Eindelijk klaar.
En nu maar hopen dat ik die ene niet vergeten ben.