# 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.

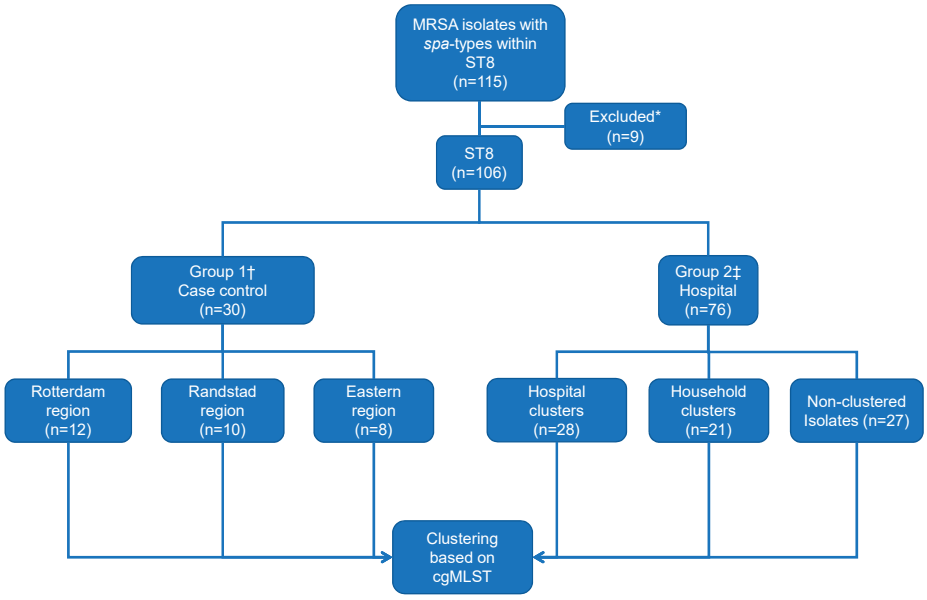
For group 2, 84 isolates presumed to be ST8 based on *spa*-typing, were selected from our hospital out of 191 isolates (43.9% 84/191). After MLST, eight isolates were excluded and 76 ST8 isolates remained. Twenty-eight isolates were from hospital clusters, 21 from household clusters and 27 were non-clustered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

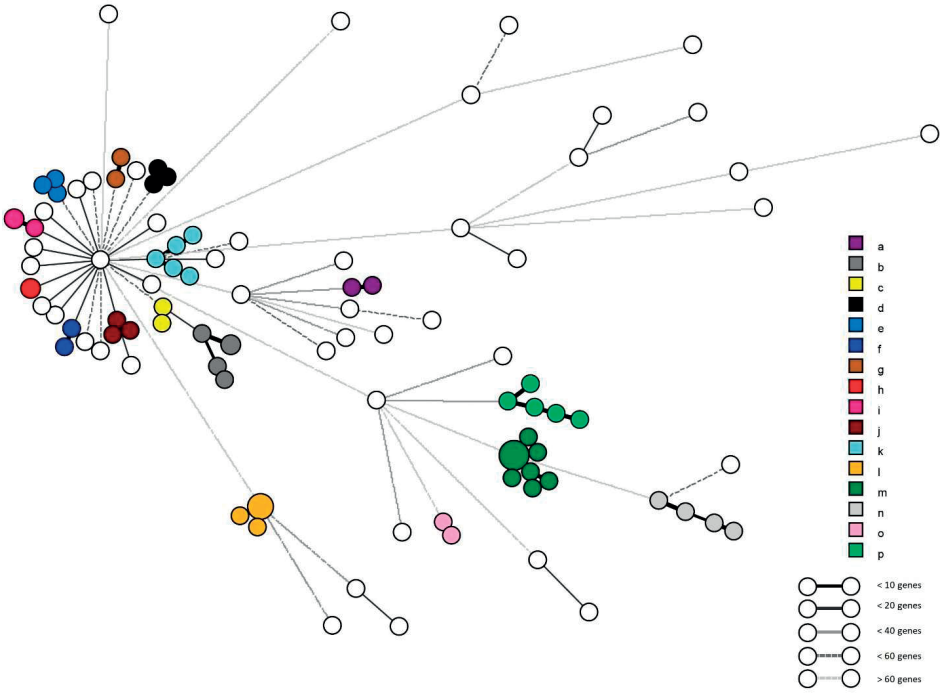



**Figure 1 – New clusters in the core-genome multi-locus sequence typing tree**
Core-genome multi-locus sequence typing (cgMLST) of 106 isolates based on 1641 of 1861 common genes. Circles are coloured if the isolate was part of one of the 16 new clusters (A to P; see also table 2) based on cgMLST and 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**

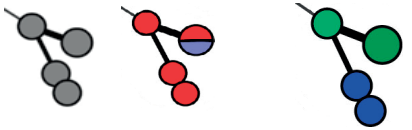
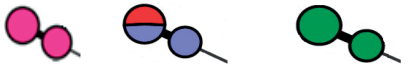
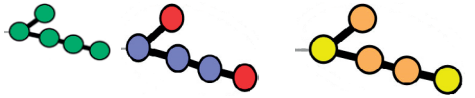
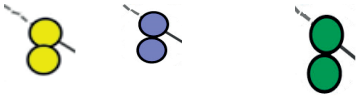
| | | | New cluster data | | | |
|---|---|---|---|---|---|---|
| | | | Distance (genes) | | MUO resolved | Epidemiology |
| New clustering | MUO/MKO | By sampling year | Av. | Max. | | |
| **Import MRSA (group 1 and 2)** | | | | | | |
| B |  | | 5 | 15 | 1 | Taiwan cluster. A series of adopted children 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)**

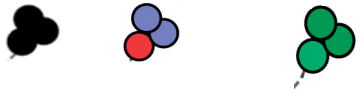
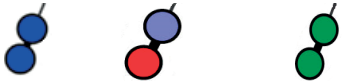
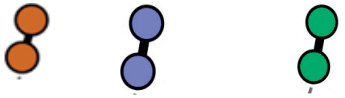
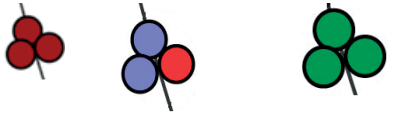
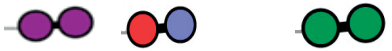
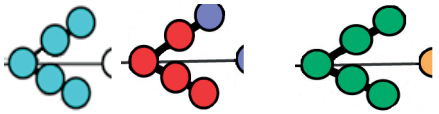
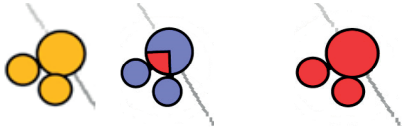
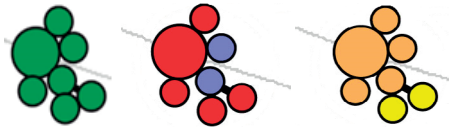
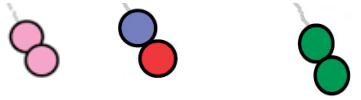
| | | | New cluster data | | | |
|---|---|---|---|---|---|---|
| | | | Distance (genes) | | MUO resolved | Epidemiology |
| New clustering | MUO/MKO | By sampling year | Av. | Max. | | |
| D |  | | 1 | 1 | 1 | Two MUO in the Eastern region, later found to form a household cluster together with a third 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 car-

riers 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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