Bacterial fingerprints across Europe

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CHAPTER 4

Staphylococcus aureus spa-type t437: identification of the most dominant community-associated clone from Asia across Europe

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) belonging to the multilocus sequence type clonal complex 59 (MLST CC59) is the predominant community-associated MRSA clone in Asia. This clone, which is primarily linked with the *spa*-type t437, has so far only been reported in low numbers among large epidemiological studies in Europe. Nevertheless, the overall numbers identified in some Northern European reference laboratories have increased during the past decade. To determine whether the *S. aureus* t437 clone is present in other European countries, and to assess its genetic diversity across Europe, we analysed 147 *S. aureus* t437 isolates from 11 European countries collected over a period of 11 years using multiple locus variable number tandem repeat fingerprinting/analysis (MLVF/MLVA) and MLST. Additionally, 16 *S. aureus* t437 isolates from healthy carriers and patients from China were included. Most isolates were shown to be monophyletic with 98% of the isolates belonging to the single MLVA complex 621, to which nearly all included isolates from China also belonged. More importantly, all MLST typed isolates belonged to CC59. Our study implies that the European *S. aureus* t437 population represents a genetically tight cluster, irrespective of the year, country and site of isolation. This underpins the view that *S. aureus* CC59 has been introduced into several European countries, not being restricted to particular geographical regions or specific host environments. The European *S. aureus* t437 isolates thus bear the general hallmarks of a high-risk clone.

INTRODUCTION

The emergence and spread of human pathogens, such as *Staphylococcus aureus*, among hospital patients as well as in the community are threatening public health worldwide. The capability to acquire antibiotic resistance and a plethora of virulence factors make *S. aureus* formidably apt to cause disease in these different settings. This is underscored by the large number of different *S. aureus* types encountered in many hosts and environments [1-6]

Epidemiological and, more recently, molecular studies have shown that certain clones of *S. aureus* attain a geo-spatial predominance [1-6]. Importantly, various community-associated methicillin-resistant *S. aureus* (CA-MRSA) clones have evolved independently on different continents. Multilocus sequence type (MLST/ST) 80 is the predominant CA-MRSA clone in Europe, ST93 in Australia, ST30 in Oceania, ST8 in the United States of America (USA), and ST59 in Asia. Nevertheless, the exchange of clones between countries and continents has been observed [3,4,7-9], as can be expected from the current reach, volume and speed of travel [10].

The ST59 clone, which is the founder of the MLST clonal complex 59 (CC59), is one of the most frequent multidrug resistant CA-MRSA clones in Asia [8]. In 2007, Tristan and colleagues reported for the first time Asian *S. aureus* ST59 isolates in association with the *spa*-type t437 [11]. Subsequently, a large community and hospital study across Asia described the CC59 as the most prevalent CC, including ST59 and its variants ST1241 and ST338; moreover, ST59-MRSA-t437 was identified as the most prevalent clone between 2004 and 2006 [8]. In the study by Song *et al.*, the collected CA- and hospital-associated MRSA CC59 isolates from Asia were not only shown to spread rapidly between hospitals and the community in a bi-directional manner, but also across borders [8]. A similar picture emerged from other studies in Asia and Western Australia where ST59-MRSA-t437 was identified as a major clone [12-16]. In contrast, *S. aureus* CC59 isolates were incidentally reported in the USA [17] and in Europe [18-22].

Recently, a study from Belgium identified 4 (1%) ST338-t437 CA-MRSA isolates amongst 410 MRSA isolates collected between 2005 and 2009 [23]. Furthermore, a multicenter study performed in the 16 most populous European countries identified a total number of 22 (6%) *S. aureus* CC59 isolates with the *spa*-type t437 of which one (4.5%) was methicillin-sensitive *S. aureus* (MSSA) [24]. Intriguingly, Rolo and coworkers reported an increased frequency of the ST59 clone since 2007, and they concluded that this clone was most prevalent in Northern Europe (Finland, Sweden and Poland) [24]. This is in line with our own observations that the numbers of *S. aureus* t437 in Norway, Denmark and Poland are increasing. Specifically, in Norway *S. aureus* t437 is among the most commonly identified MRSA clones corresponding to ~2.5% of all MRSA isolates every year since 2008 (L. Marstein, personal communication). In Denmark, the numbers of *S. aureus* t437 isolates have gone up from zero before 2006, through 1-3 cases per year between 2006 and 2008, to 7-14 cases per year since 2007 (H. Westh, personal communication). Lastly, the ST338-t437 PVL-positive clone seems currently to be the most prevalent CA-MRSA clone in Poland (J. Empel, personal communication).

Altogether, the combined literature data for Asia and Europe, and the apparently increasing numbers of *S. aureus* t437 isolates in Northern Europe formed the incentive to assess the presence of *S. aureus* t437 in other European countries and, more importantly, to determine their genetic relatedness. Clearly, the perceived risk for dissemination and establishment of a new community-associated *S. aureus* clone with a multidrug resistant phenotype in Europe would justify appropriate preventive infection control measures.

MATERIALS AND METHODS

Bacterial isolates

A total of 163 *S. aureus* isolates (147 from Europe and 16 from China) with the *spa*-type t437, collected between 2002 and 2012 from patients and healthy carriers in 11 different European countries and four different geographical locations in China (i.e. Hangzhou, Zhejiang Province; Hefei, Anhui Province; Harbin, Heilongjiang Province; and Beijing), were analysed in the present study. The epidemiological and molecular characteristics of the isolates, including origin, year of isolation, antibiotic phenotype and information on the patients are presented in Table 1 in the Supporting Information (available upon request). One isolate from the Czech Republic with an unrelated *spa*-type t442 was also included in the present study.

Antibiotic susceptibility and presence of *mecA* and the PVL locus

Antibiotic susceptibility for benzylpenicillin, chloramphenicol, ciprofloxacin, clindamycin (constitutive), erythromycin, fosfomycin, fusidic acid, gentamicin, kanamycin, linezolid, mupirocin, oxacillin, rifampicin, teicoplanin, tetracyclin, tobramycin, trimethoprim/sulfamethoxazole and vancomycin was determined with the VITEK 2 system (AST P633 card, bioMérieux, Marcy l'Etoil, France). The VITEK 2 minimum inhibitory concentration results were interpreted using the Advanced Expert System following EUCAST guidelines (www.eucast.org). The presence of the *mecA* and PVL-encoding genes (*lukF-PV*/*lukS-PV*) was determined by PCR [25].

Extraction of total DNA for typing

Total DNA for the MLVF typing was prepared as previously described [2]. The preparation of lysates for MLVA and *spa*-typing was performed as described by Schouls *et al.* [25].

spa-typing

spa-typing was performed according to the protocol as previously described [26]. The *spa*-types were assigned through the use of Ridom StaphType software version 2.2.1 (Ridom GmbH, Münster, Germany) and the SpaServer (http: //www.spaserver.ridom.de).

Multiple-locus variable number tandem repeat fingerprinting (MLVF)

MLVF was performed as described by Glasner *et al.* and Sabat *et al.* using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, United States) to separate PCR fragments [2,27]. For the analysis of electropherograms with Gel Compar II (Applied Maths, Kortrijk, Belgium), the position tolerance and optimization were set to 0.5% and 0.5%, respectively, and the dice formula was used to calculate the pairwise similarity coefficient. With the selected position tolerance, all Bioanalyzer runs for the control isolate M2 (clustering together at the bottom part of the MLVF dendrogram) were identical [27]. A dendrogram was created with the unweighted pair-group method using geometric averages (UPGMA).

Multiple-locus variable number tandem repeat analysis (MLVA)

MLVA was performed according to Schouls *et al.* [25]. Isolates that differed by one or more alleles were considered distinct types. Minimum spanning tree analysis of MLVA was performed using the BioNumerics software (Applied Maths, Kortrijk, Belgium) to group related MLVA types (MTs) into MLVA complexes (MCs). Such MCs encompass single locus variants as described by Schouls *et al.* [25]. A singleton was defined as an MT that was not grouped into an MC.

Multilocus sequence typing (MLST)

MLST was performed on representative *S. aureus* t437 isolates of each MT as described by Enright *et al.* [28]. In brief, the allelic profiles of each selected isolate were obtained by sequencing internal fragments of 7 housekeeping genes (*arc*C, *aro*E, *glp*F, *gmk*, *pta*, *tpi*, *yqi*L) and entering them on the

MLST homepage (http://www.saureus.mlst.net), where seven numbers depicting the allelic profile were assigned that defined a ST [28]. The allelic profiles of the *S. aureus* t437 isolates were compared by using the based upon related sequence types (BURST) [4].

RESULTS

Collection of *S. aureus* t437 isolates from Europe and China

To explore the presence and genetic relatedness of *S. aureus* t437 across Europe, a convenience sample of 147 isolates with the *spa*-type t437 was established at the Medical Microbiology Department at the University Medical Center Groningen (UMCG, The Netherlands). This was achieved through the identification of *S. aureus spa*-type t437 isolates by inspection of the RIDOM *spa* server database (http://www.spaserver.ridom.de). In addition, representatives from all Staphylococcal Expert and Reference Laboratories were approached and asked for *S. aureus spa*-type t437 isolates. All *S. aureus* isolates were then sent to the UMCG, stored and subsequently propagated for subsequent molecular analyses. The *spa*-type t437 is currently ranked 24th on the RIDOM *spa* server with a frequency of 0.63% and a total number of 1878 isolates (June 2014). The collected *S. aureus* t437 isolates were from 11 European countries, namely the Netherlands (n=64), Scotland (n=27), Norway (n=20), Germany (n=13), Denmark (n=10), Spain (n=3), Poland (n=3), France (n=3), Sweden (n=2), Czech Republic (n=1) and Hungary (n=1). Sixteen *S. aureus* isolates with the *spa*-type t437 from patients and healthy carriers from China that were available during the time of investigation at our institute were added to the collection. One isolate from the Czech Republic was originally submitted as *S. aureus spa*-type t437 but, during the course of investigation, it was determined to have the unrelated *spa*-type t442. Seventy-eight (47.9%) *S. aureus* t437 isolates were sampled from abscesses and skin infections, and 56 (34.3%) were isolated from blood or the nose, ear or throat (Supporting Information Table 1, available upon request). For the remaining 29 (17.8%) *S. aureus* t437 isolates the source of isolation is unknown.

Antibiotic resistance and proportion of *pvl* and *mecA*

Antibiotic susceptibility testing showed that the antibiotic profiles of the 143 (87.7%) MRSA t437 isolates were very similar to those of the 20 (12.3%) MSSA t437 isolates (Table 1). Consistent distinctions in the resistance of the investigated MRSA t437 and MSSA t437 isolates were only observed for oxacillin and chloramphenicol. The increased chloramphenicol resistance of the collected MRSA t437 isolates is in accordance with results from Asian CA-MRSA studies [7,15]. The majority of the investigated t437 isolates were resistant to clindamycin (constitutive), erythromycin, kanamycin and penicillin, and all t437 isolates were susceptible to fosfomycin, fusidic acid, linezolid, mupirocin, rifampicin, teicoplanin and vancomycin (Table 1). No clear association between countries of origin or sample source with a certain antibiotic resistance profile could be detected. In accordance with the observed oxacillin resistance profiles, 88% of the isolates tested positive for *mecA* (143 isolates). Furthermore, 82% (134 isolates) tested positive for the Panton-Valentine leukocidin (PVL) locus [3]. The majority of *S. aureus* t437 isolates were thus both *mecA*- and PVL-positive (127 isolates, 78%), as previously reported for Asian CC59 isolates [12,15]. Fifteen isolates were *mecA*-positive and PVL-negative, while 21 isolates were *mecA*-negative of which 8 were PVL-positive and 13 PVL-negative.

Table 1. Antibiotic resistance profiles of the 143 MRSA t437 and 20 MSSA t437 isolates.

Antibiotic	MRSA	MSSA
	No. (resistance rate %)	No. (resistance rate %)
Ciprofloxacin	2 (1.4)	2 (10)
Chloramphenicol	93 (65)	3 (15)
Clindamycin (constitutive)	118 (82.5)	17 (85)
Erythromycin	121 (84.6)	17 (85)
Fosfomycin	0 (0)	0 (0)
Fusidic Acid	0 (0)	0 (0)
Gentamicin	3 (2.1)	2 (10)
Kanamycin	120 (83.9)	16 (80)
Linezolid	0 (0)	0 (0)
Mupirocin	0 (0)	0 (0)
Oxacillin	143 (100)	0 (0)
Penicillin	143 (100)	18 (90)
Rifampicin	0 (0)	0 (0)
Teicoplanin	0 (0)	0 (0)
Tetracyclin	102 (71.3)	11 (55)
Tobramycin	3 (2.1)	2 (10)
Trimethoprim/sulfamethoxazole	1 (0.7)	5 (25)
Vancomycin	0 (0)	0 (0)

*For details on the antibiotic resistances of the study isolates, please see Table 1 in the Supporting Information (available upon request).

MLVF

MLVF analysis identified 37 different banding patterns among the 163 *S. aureus* t437 isolates as shown in Figure 1. Sixteen patterns were represented by two or more isolates (142 isolates in total). The remaining 21 patterns each consisted of a single isolate. Application of previously published cut-off values of 64%, 67% or 75% led to 2, 2 and 9 clusters, respectively [2,27]. The two lower cut-off values joined 161 and 154 isolates into one major cluster respectively, indicating a high genomic relatedness of these isolates. Even without the application of a cut-off value resulting in MLVF clusters, the relatedness of all *S. aureus* t437 isolates can be inferred by inspection from the highly similar MLVF banding patterns (see also [2,27]). Slight differences in the band sizes indicate loss or gain of repeats in the respective variable number of tandem repeats (VNTRs). As expected, the isolate with the unrelated *spa*-type t442 displayed a different MLVF pattern with only 34% similarity to the closest related isolate in the MLVF dendrogram (Figure 1). The MLVF patterns did not unveil any epidemiological signal, such as the country of origin, year of isolation or source. Thus isolates from different countries, even from China, appeared randomly distributed over the MLVF dendrogram. Notably, MLVF is a highly discriminatory but non-portable PCR-based DNA fingerprinting method that utilizes size differences in five coding regions (*sdrCDE, clfA*, *clfB*, *sspA* and *spa*) containing VNTRs. In contrast, the subsequently implemented MLVA method for DNA typing is suitable for inter-laboratory comparisons and allows the determination of clonal relationships between isolates. To this end, MLVA targets ten non-coding loci, which are sequenced and for which the exact number of repeat units is measured. This approach is therefore more precise than the visualization of MLVF results on agarose gels or microfluidic chips.

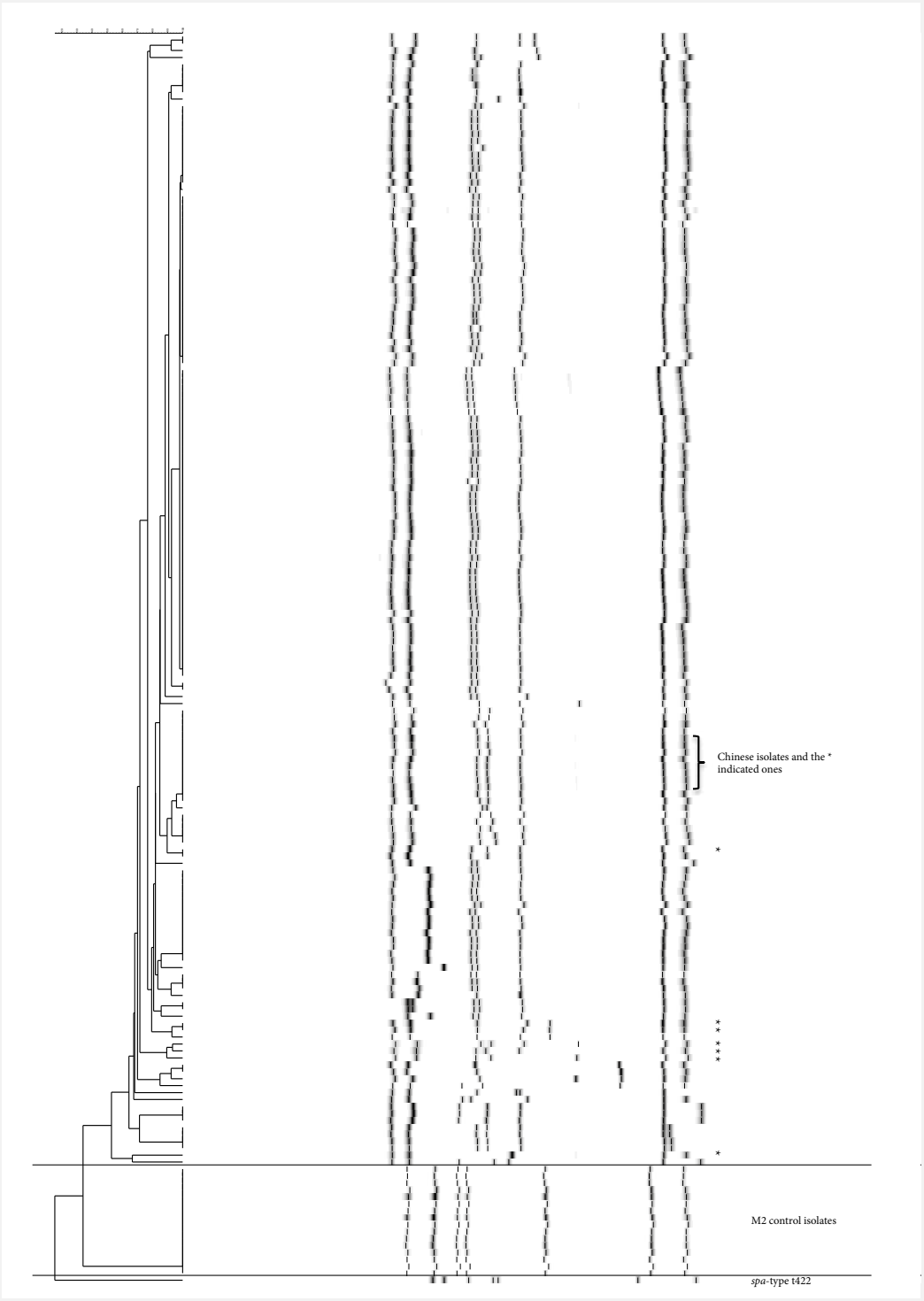

Figure 1. MLVF dendrogram of the 163 investigated *S. aureus* t437 isolates generated by the UPGMA algorithm. In addition to the study isolates, one isolate with a different *spa*-type (t442), MLVA type and MLST, and 16 controls (designated M2) were included in the analysis. Details on the different isolates from top to bottom are provided in the same order in Table 1 in the Supporting Information (available upon request). *, lanes corresponding to Chinese t437 isolates.

MLVA

The 163 *S. aureus* t437 isolates produced 13 different MTs, namely MT621, MT1035, MT1297, MT1831, MT1870, MT1875, MT2075, MT2322, MT3560, MT4124, MT4125, MT4126 and MT4183 (Figure 2A and Supporting Information Table 1, available upon request). The MT621 was clearly the most predominant type in the present collection comprising 133 (82%) isolates and, more importantly, 159 (98%) *S. aureus* isolates belonged to the same MC0621, comprising nine different MTs (Figure 2A). The remaining four isolates were MLVA singletons (MCnone). Generally, six MTs were shared by two or more isolates (156 isolates in total), whereas seven MTs were represented by single isolates. The included t422 isolate from the Czech Republic belonged to the unrelated MT165 and MC5.

MLST

A total of 46 representative *S. aureus* t437 isolates comprising one MT each were selected for MLST analysis (Figure 2B). This showed that 39 (85%) of these isolates belonged to the CC59. ST59 was the predominant ST with 26 (57%) isolates, and the two single-locus variants ST87 and ST338 were identified 2 (4%) and 11 (24%) times, respectively. The remaining seven isolates, which were all from China, belonged to ST2147 differing from ST59 by three alleles. The included isolate with the *spa*-type t422 belonged to the unrelated ST5. The relationship between the MLST and MLVA data is depicted in Figure 2B. The MLST minimum spanning tree shows that ST59, the founder of CC59, is composed of ten different MTs.

DISCUSSION

The present study underpins the view that the *S. aureus* ST59 clone and other STs of CC59 with the *spa*-type t437, which are commonly encountered in Asia, are present in several European countries. More importantly, by implementing three different highly discriminatory molecular typing tools, namely MLVF, MLVA and MLST, we demonstrate a high degree of molecular similarity in the studied *S. aureus* t437 isolates that were collected over a period of 10 years from 11 different European countries. This shows that this specific *S. aureus* clone is not restricted to particular geographical regions or specific host environments, and that *S. aureus* t437 in Europe belongs to a very tight molecular cluster of *S. aureus* isolates. The European *S. aureus* t437 isolates thus bear the general hallmarks of a high-risk clone.

At least 35 (21.5%) of the patients from whom the currently investigated European *S. aureus* t437 isolates have been collected are immigrants or adopted children, or had travelled to countries outside Europe, Asia in particular (Supporting Information Table 1; available upon request; note that for 104 isolates no such information could be retrieved,). Such patients may have introduced the CC59 clone in Europe and subsequently transmitted it to other European citizens, including their family members, as can be inferred from the analysis of the isolates from the Netherlands and Denmark (Supporting Information Table 1, available upon request). This view is also supported by the high genetic relatedness of the European isolates with the 16 Chinese *S. aureus* t437 isolates included in our study as shown by MLVF and MLVA. Although seven Chinese isolates belonged to ST2147 (a triple-locus variant of ST59) and only one isolate belonged to ST59, the Chinese isolates clustered among all other *S. aureus* t437 isolates in the MLVF dendrogram (Figure 1 and Supporting Information Table 1, available upon request), and 15 of these isolates belonged to MC0621 which includes 98% of the 163 investigated *S. aureus* t437 isolates. Notably, in most Asian studies only MRSA isolates were collected and typed. It is therefore conceivable that MSSA with the *spa*-type t437 belonging to CC59 has thus far been overlooked in Asia. In contrast, the present European *S. aureus* t437 isolates also include MSSA isolates. This observation is in line with the findings reported by Rolo *et al.* [24]. Otherwise, the antibiotic resistance profiles of the presently investigated European *S. aureus* t437 isolates were very similar to those described for Asian CC59 isolates, but not to those of other CA *S. aureus* clones from Europe [3,8,14,29,30]. Lastly, the frequency of the PVL-encoding genes (82%) in the present *S. aureus* t437 sample is in accordance with the reported numbers in the Asian studies [12,15,31].

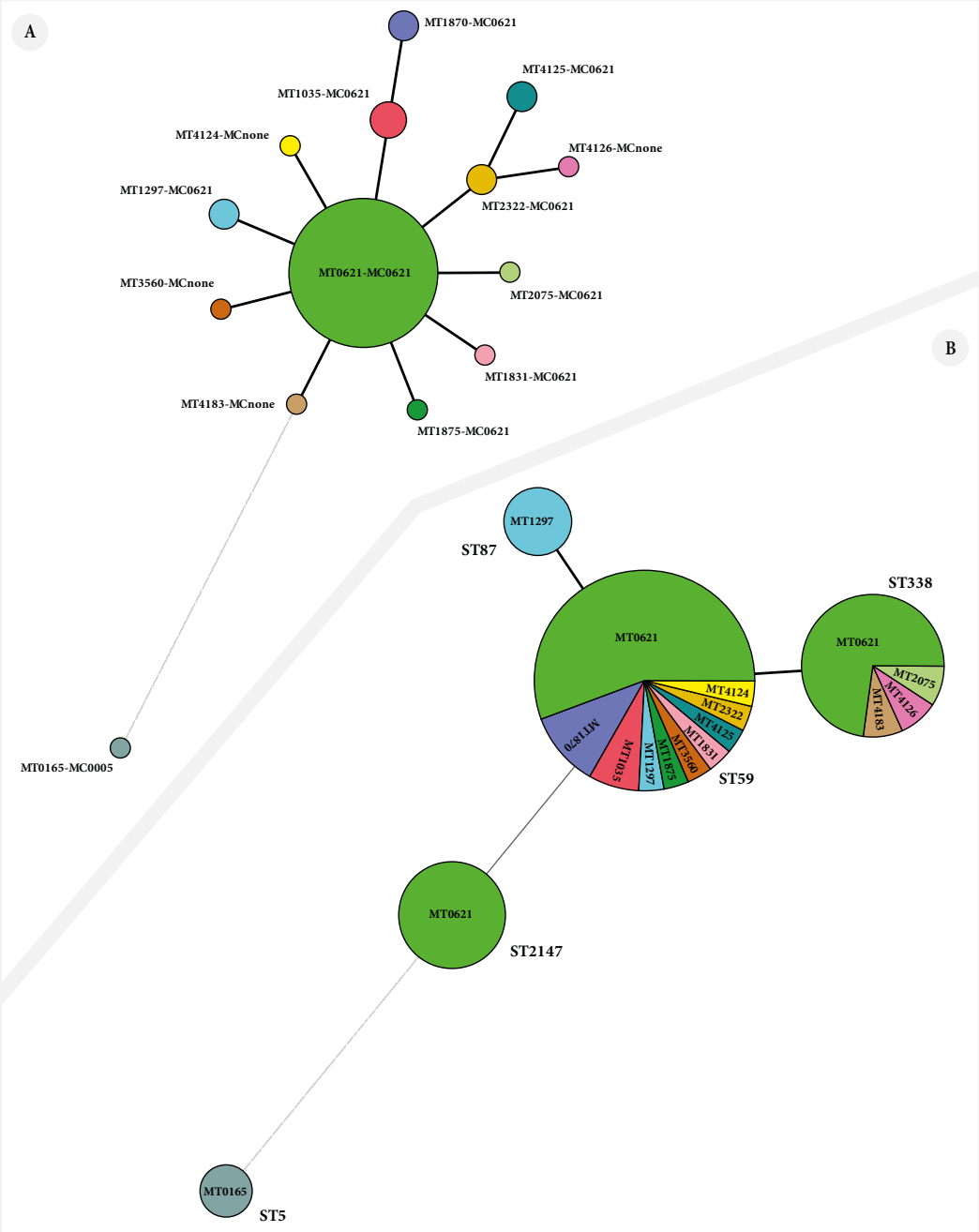

Figure 2. (A) Minimum spanning tree of the 163 *S. aureus* t437 isolates typed by MLVA. Clustering of MLVA profiles was performed with a categorical coefficient. In the minimum spanning tree, the MLVA types are displayed as circles labelled with different colours. The size of each circle indicates the number of isolates of the particular MLVA type. MLVA complexes were assigned if two neighbouring types did not differ in more than one VNTR locus. MLVA types and complexes are indicated in characters e.g. 621 denotes MLVA type 621, and MC0621 denotes MLVA complex 621. The three MTs 1870, 4125 and 4126 are double-locus variants of MT0621 and do not belong to any MC (MCnone). The t442 isolate from the Czech Republic is also included in the minimum spanning tree, showing that it belongs to the unrelated MT165. **(B)** MLST analysis of 46 representative *S. aureus* t437 isolates with BURST identifies one MLST CC59, which includes ST59 (26), ST338 (10) and ST87 (2). The two other identified STs, ST2147 (7) and ST5 (one isolate from the Czech Republic with *spa*-type t442) are singletons. The colours within each ST circle indicate the different MTs belonging to the respective ST.

In conclusion, we have combined MLVF, MLVA and MLST to obtain a high-resolution snapshot of the *S. aureus* t437 population in Europe based on retrospectively collected isolates that had already been *spa*-typed. Since *spa*-typing or any of the three other molecular typing methods implemented in the present study are not yet performed on a daily routine basis in most local laboratories and hospitals, we consider it likely that the CC59 clone has so far remained under-detected in Europe. This is a cause for concern in view of the predominance of *S. aureus* CC59 in Asia and its clinical repercussions, the multi-drug resistant soft tissue and skin infections in particular, even though the prevalence of CC59 in Europe is probably still relatively low. Where possible, the further dissemination of this potentially high-risk clone should therefore be prevented, for example through active screening of patients with staphylococcal infections who have a history of travel in Asia.

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TRANSPARENCY DECLARATIONS

The authors declare no conflicts of interest.

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AUTHOR'S CONTRIBUTIONS

C. Glasner, H. Grundmann and J. M. van Dijl: designed the study and wrote the manuscript. C. Glasner and G. Pluister: performed laboratory investigations. G. Pluister, H. T. Westh, J. P. Arends, J. Empel, E. Giles, F. Laurent, F. Layer, L. Marstein, A. Matussek, A. Mellmann, M. Perez-Vasquez, E. Ungvári, X. Yan and H. Zemlickova: performed epidemiological investigations, provided the study isolates, provided feedback, contributed with comments and reviewed the manuscript.

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‘Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world.’
Albert Einstein (1879 – 1955)