Age- and gender-associated *Staphylococcus aureus spa* types found among nasal carriers in a general population. The Tromsø Staph and Skin Study.

Running title: Age- and gender-associated *spa* types

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ABSTRACT

Staphylococcus aureus nasal carriers risk autoinfection, however knowledge on factors making specific strains successful colonisers is limited. This study aimed to identify the most successful *S. aureus* clones in nasal carriers and compare their distribution among host groups. The population structure of *S. aureus* isolates from healthy adults was investigated by *spa* typing 1,981 isolates from persistent and intermittent nasal carriers attending a health survey. In the baseline screening (1,113 isolates), the most common *spa* types were t012 (8.4%), t084 (7.6%) and t065 (4.9%). Three large *spa* clonal complexes (*spa* CC012, *spa* CC065 and *spa* CC084) comprised 62.4% of the isolates. In multivariate models adjusted for age and smoking status, male sex was associated with higher risk of *spa* type t084 (Odds Ratio (OR), 1.72; 95% CI, 1.06-2.77), and lower risk of *spa* type t012 (OR, 0.60; 95% CI, 0.39-0.92) colonisation. The prevalence of *spa* type t012 decreased significantly with increasing age (p = 0.03), with a prevalence almost twice as high in the youngest group (age 30-44 years, prevalence = 11.1%) compared to the oldest group (age 60-87 years, prevalence = 5.6%). Also when studying the baseline isolates, *spa* type t084 had a twofold higher prevalence among intermittent carriers than among persistent carriers (10.6% versus 5.5%; p = 0.04). In summary, the two most prevalent *spa* types found in this study were significantly associated with age and/or gender. This may provide valuable clues to the multifactorial mechanisms, among them bacterial factors, involved in nasal colonisation with *S. aureus*.

INTRODUCTION

Staphylococcus aureus is a successful commensal colonising a large proportion of the human population and a serious pathogen potentially able to infect any tissue of the human body, causing life-threatening diseases, including sepsis, endocarditis, pneumonia and osteomyelitis. Globally, a large proportion of bloodstream infections (22%), ventilator-associated pneumonia (23%), and skin and soft tissue infections (39%) are caused by *S. aureus* (6). In Norway, *S. aureus* is the second most common cause of bloodstream infections, accounting for 13.9 % of the isolates, skin contaminants excluded (1).

Multiple sites of the human body can harbour *S. aureus* but the anterior nares is the main ecological niche (33). Within a healthy adult population, ~20% are persistent nasal carriers, ~30% intermittent carriers and ~50% non-carriers (8,13,15,35). Persistent nasal carriers have an increased risk of *S. aureus* infection compared with intermittent carriers and non-carriers (23). Higher levels of some antistaphylococcal antibodies were observed in persistent carriers than in others, and recently it was suggested that there are only two types of human nasal *S. aureus* carriers: persistent carriers and others (32).

Although the association between *S. aureus* nasal carriage and infection was reported already in 1931 (5), it was the more recent spread of community-acquired MRSA (methicillin resistant *S. aureus*) that caused *S. aureus* colonisation to be regarded as a major public health problem. The spread of MRSA limits treatment options in *S. aureus* infections and increases our need for prevention and alternative treatment strategies to reduce the burden of *S. aureus* disease.

spa typing is an established typing method for *S. aureus*, based on sequencing of a single polymorphic Variable Number Tandem Repeat (VNTR), namely the repeat region of the *S. aureus* protein A gene. Due to the clonal population structure of *S. aureus* (11), *spa* typing is regarded a highly discriminatory method that can be used for outbreak investigations as well as for assigning strains to phylogenetic lineages in population studies (16).

Little is known about factors making specific strains successful colonisers. The population structure of *S. aureus* of nonclinical origin has been thoroughly investigated in children (age 1-19 years) and elderly adults (>55 years) (19), however only smaller studies including a younger adult population have been performed (29), leaving a gap in our understanding of *S. aureus* diversity and population structure. We aimed to find the most successful *S. aureus* clones and compare their respective distribution in a population-based study, the Tromsø Staph and Skin Study, which included 4,026 healthy men and women aged 30-87 years. Male sex and younger age is positively associated with nasal *S. aureus* colonisation and carriage rates in this population (24).

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MATERIALS AND METHODS

Study design. The population-based Tromsø Staph and Skin Study is a cross-sectional study, performed as part of the sixth Tromsø Study in 2007-2008. Random samples of birth cohorts aged 30-87 years in the municipality of Tromsø were invited to participate in a health survey including clinical examinations, blood samples, nasal swab cultures, questionnaires and interviews; all procedures were performed by trained technicians (14). The participation rate was 66%. Nasal swab cultures were collected from 4,026 participants (2,285 women and 1,741 men) to assess *S. aureus* colonisation. To determine *S. aureus* carrier status, a second sample was taken from 2,997 participants (1,712 women and 1,285 men). The median time between baseline and the second screening was 28 days. In addition, all *S. aureus* positive bacteraemia samples collected from patients 30 years or older, living in Tromsø and diagnosed by the University hospital of North Norway (UNN) in 2007 and 2008 were included (n= 32).

***S. aureus* isolates.** Both vestibulum nasi were sampled by the same NaCl-moistened sterile rayon-tipped swab and placed in Amies charcoal transport medium (Copan, Murrieta, CA). All specimens were cultured within 3 days on blood agar (Oxoid, Cambridge, UK), chromID *S. aureus* agar plates (bioMérieux, Marcy l'Etoile, France) and chromID MRSA plates (bioMérieux), and were incubated for 48 hours at 37°C. If positive (green) colonies were found on the chromID plates, one colony was selected and confirmed as *S. aureus* by the Staphaurex Plus (Remel, Lenexa, KS) agglutination test, then frozen. Blood cultures were routinely analysed by BacT/ALERT (bioMérieux) and frozen at the Department of Microbiology and Infection Control, UNN.

Template for PCR. *S. aureus* isolates from frozen cultures (-70°C) in glycerol-containing medium were inoculated on blood agar (Oxoid) and incubated overnight at 37°C. 2-3 colonies were transferred to sterile 200 µl H2O and vortexed.

***spa* typing and BURP analysis.** The isolates were *spa* typed using primers spa-1113f and spa-1514r (31) with the following cycling conditions: 95°C 10', 35x [95°C 30'', 60°C 15'', 72°C 1'], 72°C 10', 4°C ∞. PCR products were sequenced on both strands by Macrogen Korea or Macrogen Europe. *spa* types were determined using Ridom StaphType software (Ridom GmbH, Würzburg, Germany) (12) and the Ridom SpaServer website (http://www.spaserver.ridom.de) that is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/). The BURP algorithm with default parameters (exclusion of *spa* types shorter than 5 repeats and clustering of *spa* types if cost is less or equal to 4) was applied (21). For isolates negative on *spa* PCR, the procedure was repeated, starting from retrieving the isolates from the freezer. Isolates twice negative on *spa* PCR were checked with coagulase test and the Staphaurex Plus (Remel) agglutination test. If both tests were positive the isolate was regarded as not typeable for *spa*, if not the isolate was excluded.

MLST and eBURST. Multilocus Sequence Typing (MLST) was performed on the first 176 consecutive baseline isolates from participants that had been sampled twice. The MLST analysis was performed as described previously (7). PCR products were sequenced on both strands by Macrogen Korea. Multilocus Sequence Types (STs) were assigned using BioNumerics software (version 6.0; Applied Maths, Sint-Martens-Latem, Belgium) and the *S. aureus* database at the MLST website (http://www.mlst.net). eBURST on the entire public MLST database (January 2011) was used to cluster STs into groups.

Clustering comparison. Adjusted Rand and Wallace coefficients were calculated as described previously (3,10,25) for comparison of the two different typing methods. The Wallace's coefficient gives the probability that two isolates which are clustered together by one typing method, are clustered together by the other typing method. Isolates excluded from BURP clustering due to having less than 5 repeats, were placed in one single group, while singletons were assigned separately.

Statistical analyses. The SAS statistical software package (version 9.2) was used for statistical analyses. In analysis of the total study population, participants without any growth of bacteria in the nasal sample, and participants taking antibiotics with potential activity against *S. aureus* during the last 24 hours before swabbing, were excluded. Fisher's exact test was used to compare the prevalence of different *spa* types across age groups, genders and carrier states. The result was considered significant when a 2-sided P-value of less than 0.05 was obtained. Logistic regression models were used to study the association between *spa* types, gender and age, adjusting for smoking status (current daily smoker, yes/no).

Minimum spanning trees were generated by BioNumerics software (version 6.0; Applied Maths), using default settings.

Ethical considerations. The sixth Tromsø Study was approved by the regional committee of medical research ethics (REK) and followed the ethical standards of the Helsinki Declaration. A written consent was obtained from all participants.

RESULTS

***spa* typing revealed novelty and diversity.** In total, 1,981 isolates from the Tromsø Staph and Skin Study were included; 1,113 from baseline and 868 from the second screening. The isolates were assigned to 400 unique *spa* types according to the Ridom StaphType software. Thirteen isolates were not typed due to repeated negative *spa* PCR amplification or deviating repeat length (see below) and were designated not typeable (NT). Novel *spa* types, 91 in total, were identified. One new repeat was designated r359. Another new repeat with 25 bp length was also observed. No MRSA isolates were found.

The most common *spa* types at baseline were t012 (8.4 %), t084 (7.6 %) and t065 (4.9 %). A large proportion, 86.1 % (317 of 368) of the *spa* types were found in less than four individuals, and 65.5% (241 of 368) of the *spa* types were only found in single individuals, indicating large genetic diversity.

The 400 unique *spa* types grouped into 21 clusters and 16 singleton *spa* types by Ridom StaphType software (Figure 1). 35 *spa* types comprising 146 isolates were excluded from the BURP clustering due to having less than 5 repeats. Three *spa* clonal complexes comprised 62.4% of the *S. aureus* isolates at baseline; 28.3% of the isolates belonged to *spa* CC012, 18.2% to *spa* CC065 and 15.9% to *spa* CC084.

MLST confirmed novelty and diversity. MLST analysis of the 176 consecutive selected isolates revealed 49 unique STs, 23 of these were not previously recorded. Twenty-four new allele types were designated 209 (*arcC*), 276-281 (*aroE*), 252 (*glpF*), 156 (*gmk*), 203-208 and 210 (*pta*), 206-209 (*tpi*) and 210-213 (*yqiL*). New STs found in the study were submitted to

the MLST database. Thirty-three of the STs were only represented by one isolate, whereas 16 of the STs were represented by at least two isolates. The isolates were grouped into 16 different CCs, and four isolates were singletons. Sixty isolates were assigned to CC30 (34.1%), 44 to CC45 (25.0%) and 23 to CC15 (13.1%). The 176 MLST typed isolates displayed 105 unique *spa* types. One isolate was NT. BURP analysis grouped the isolates into 13 different *spa* CCs and one singleton. 15 isolates were excluded from the BURP clustering, including the NT isolate.

Adjusted Rand evaluation displayed a concordance between *spa* CCs (as defined by BURP clustering) and CCs (as defined by eBURST) of 0.76, while the Wallace coefficient was 0.90 for *spa* CC versus CC, indicating a 90% probability of two isolates belonging to the same *spa* CC also sharing CC (Figure 2). Considering *spa* type as the standard for comparison, the Wallace coefficient was 0.94 for *spa* type versus CC.

The same *spa* types were repeatedly isolated from the nares of persistent carriers. An analysis of 846 baseline isolates from participants with a second culture revealed that 728 (86.1%) had two positive nasal cultures and thus were defined as persistent *S. aureus* nasal carriers. 118 of 846 (13.9%) had one positive sample, and were designated intermittent carriers. From the 728 persistent nasal carriers, 671 (92.2%) had the same *S. aureus spa* type in both samples. The most common *spa* types identified in the baseline sample from the 728 persistent nasal carriers were t012 (8.8%), t084 (5.6%) and t065 (5.2%).

***spa* types were associated with gender and age of carriers.** *spa* type t012 comprised 11.1%, 8.1% and 5.6% of the *S. aureus* isolates in the baseline screening in age tertiles 30-44 years, 45-59 years and 60-87 years, respectively, demonstrating a statistically significant decrease in

prevalence by increasing age of the colonised host (p = 0.03; Table 1). This age-dependent pattern in *spa* type t012 prevalence was even stronger when looking at the total population sampled in the baseline screening, with prevalence of 3.5%, 2.4% and 1.4% across the age tertiles (p = 0.002).

The prevalence of *spa* type t012 was almost identical between genders in the total population; 2.5% among males and 2.4% among females (Table 2). However, the general rate of *S. aureus* nasal colonisation and carriage is higher among men than women. Thus, *spa* type t012 demonstrated a significant gender association for the colonised subgroup in the baseline screening (n = 1,110); *spa* type t012 comprised 6.9% and 10.5% of the *S. aureus* isolates from colonised men and women, respectively (p = 0.03). For *spa* type t084 the corresponding frequencies were 9.1% in men and 5.6% in women (p = 0.03). In multivariate logistic regression models adjusted for age and smoking, male sex was associated with reduced risk of *spa* type t012 (OR, 0.60; 95% CI, 0.39-0.92) and increased risk of *spa* type t084 (OR, 1.72; 95% CI, 1.06-2.77) in the colonised population. For the total human study population, including non-carriers, significant gender differences were found for *spa* types t065 (p=0.03), t084 (p < 0.001) and t021 (p = 0.04), all positively associated with male sex (Table 2).

***spa* type t084 was associated with intermittent carriage.** Analyses of 846 baseline nasal isolates from participants with a second nasal swab culture, revealed that *spa* type t084 comprised 10.6% and 5.5% of the *S. aureus* subpopulation colonising intermittent and persistent carriers, respectively (p = 0.04). A total of 92.6% of *spa* type t012 and 76.9% of *spa* type t084 were from persistent carriers.

Most of the bacteraemia *spa* types coincided with carrier strain *spa* types. The 32 bacteraemia isolates displayed 23 different *spa* types, six of which were found more than once. Among these, the *spa* types t012, t084, t015, t002 and t021 were also found among the six most prominent *spa* types in carriers. However, *spa* type t024, only found in 0.9% of the colonisation isolates, was observed in three of the 32 bacteraemia isolates. In addition, five (21.7%) of the *spa* types found in bacteraemia isolates from Tromsø, were not found in any of the 1,981 carrier isolates from baseline and the second screening. BURP clustering of the bacteraemia *spa* types revealed that they all belonged to clusters found in the study of colonisation isolates.

DISCUSSION

The bacterial population from a large unselected collection of *S. aureus* isolates demonstrated both great diversity and clone dominance. As much as 86.1% of the *spa* types were found in less than four individuals, and 65.5% of the *spa* types were only observed in single individuals. This large diversity is consistent with previous findings, for both community- and clinical strains (20,29,31). Still, the three most successful strains comprised 21.0% and the three largest *spa* CCs (*spa* CC012, *spa* CC065 and *spa* CC084) 62.4% of the 1,113 *S. aureus* nasal isolates from healthy colonised individuals in the baseline screening. There was also a good correlation between *spa* types of the general population and the *S. aureus* blood culture isolates from the same time period, where 78.3% of the latter types were found in the general population. The remaining 21.7% may reflect other sources of infection than nasal carriage isolates, or could be explained by the large diversity of *spa* types in carriers. *S. aureus* carriers are at risk of autoinfection, and when developing *S. aureus* bacteraemia in a hospital setting, 80% or more were of endogenous origin (34,36). A recent study by Lamers *et al*. revealed a strong evolutionary relationship between clinical and nasal colonisation isolates (17), and Melles *et al*. provided evidence that virtually any *S. aureus* genotype carried by a human host can cause an invasive infection. There is controversy on the association between virulence and clonal lineages, but clusters with an overrepresentation of bacteraemia-isolates and skin disease were identified, indicating that some *S. aureus* clones are more virulent than others (19). As the most widespread clonal lineages among carriers are the ones most commonly found in blood cultures, one could speculate that the ability of the strain to evade the host's immune response, may also be beneficial when invading the host. Lindsay *et al*. suggested that the *S. aureus* genes necessary for invasive disease may be identical to the genes involved

in nasal colonisation (18). Alternatively, *S. aureus* strains successfully colonising a host probe for host weaknesses and exploit these when given the opportunity (2).

As MRSA is not considered to be endemic in Norway, the absence of MRSA in this study was not unexpected. In 2007 and 2008, when the samples for this study were collected, the prevalence of MRSA in Norwegian *S. aureus* blood culture isolates was 0.2% and 0.7%, respectively, whereas the prevalence of MRSA among *S. aureus* wound specimens was 0.7% for both years (1). These numbers, however, do not represent a healthy population, and therefore cannot be directly compared to MRSA/MSSA colonisation rates in our study, including healthy persons only (i.e. not hospitalised or institutionalised).

The concordance between *spa* typing and MLST has been evaluated previously (10), concluding that *spa* typing has very good predictive power over clonal lineages defined by eBURST (Wallace coefficient = 0.94). A similar result was obtained by using our data, and we also found a good concordance between BURP and eBURST (Wallace coefficient = 0.90), indicating that the BURP-clusters were relevant entities for this investigation. The good concordance between BURP and MLST CCs gave confidence in our hypothesis of the clonal dispersion of our isolates, with *spa* CC012 corresponding to CC30, *spa* CC065 corresponding to CC45, and *spa* CC084 corresponding to CC15.

The Oxford study (11) looked at 179 isolates from colonised individuals and found distinct clonal lineages, with CC30 (33.5%), CC15 (11.7%), and CC45 (8.9%) as the major CCs. Melles *et al*., investigating a large group of children and elderly adults from the Netherlands, found that CC30 and CC45 contained almost half (47.3%) of all the nasal *S. aureus* isolates,

but CC15 was not prominent in this material (19). A Chinese study with 147 isolates from colonised children in kindergartens, found that CC121 was the most prominent (34.0%), while CC30 only accounted for 3.4% of the isolates, and CC45 was not present at all (9). In a study from Mali, CC15 and CC152 together comprised 52.3% of the nasal *S. aureus* isolates (27). CC30 is rarely observed at frequencies higher than approximately 30% in carriage samples, and Ruimy *et al*. suggest that 30% appears to be the approximate maximum frequency for any single CC within carriage samples, reflecting competition between lineages (26), which is also in line with our findings. If essentially any *S. aureus* strain is able to colonise the human host, the observed geographical divergence in CCs could be due to ethnic or sociodemographic differences in host susceptibility or the geographic distribution of *S. aureus* genotypes (30).

With a median time of 28 days between baseline and the second screening, 13.9% of the carriers eliminated colonisation, whereas 7.8% of the persistent carriers exhibited different *spa* types in the two samples. The presence of more than one *spa* type in nasal carriers has been described previously (4,29), suggesting that single colony sampling excludes the possibility to consider the influence of different co-colonisers. However, results from a recent study on nasal carriage indicated that strain replacement was more common than co-colonisation during a 9-month period (28).

Interestingly, an association between intermittent carriage and *spa* type t084, was found in our study. In *vivo* abundance of bacteria in terms of colony forming units could be an important factor in successful colonisation as it has been demonstrated that this depends on the bacterial genotype. Sakwinska *et al*. (29) found a lower CFU for MLST CC15 (including *spa* type t084) than for CC30 including t012. However, the CFU value for CC45 was marginally lower

than for CC15, indicating that the same effect should have been observed for this lineage as well, which was not the case. Thus, the unique association between t084 and intermittent carriage may be an interesting clue in the search for colonisation factors.

Associations between *S. aureus* genotype and host attributes such as gender and age have been searched for but, to our knowledge, without success. The intriguing gender and age preferences among *spa* types found in this work suggest host-microbe match where both phenotypes are relevant for successful colonisation. Bacterial factors prevalent among isolates with a specific *spa* type may contribute to adhesion or immune evasion in some hosts but not in others. Persistent nasal carriers inoculated with a mixture of different *S. aureus* strains have been demonstrated to select for their original resident strain, indicating the importance of a good match between host- and bacterial factors (22).

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Table 1. Distribution of the six most common *spa* types by age tertiles. *S. aureus* isolated from nasal samples in the screening. The Tromsø Staph and Skin Study.

spa type	Total numbers (n) of *spa* type			Prevalence (%) in the Total population, n = 3897[a]				Prevalence (%) in the Colonised population, n = 1110[b]			
	30-44	45-59	60-87	30- 44 n = 1323	45-59 n = 1230	60-87 n = 1344	p[c]	30-44 n = 414	45-59 n = 358	60-87 n = 338	p[c]
t012	46	29	19	3.48	2.36	1.41	0.002	11.11	8.10	5.62	0.03
t065	20	16	19	1.51	1.30	1.41	0.90	4.83	4.47	5.62	0.77
t084	34	27	23	2.57	2.20	1.71	0.31	8.21	7.54	6.80	0.77
t002	14	11	5	1.06	0.89	0.37	0.11	3.38	3.07	1.48	0.24
t021	14	13	15	1.06	1.06	1.12	0.99	3.38	3.63	4.44	0.74
t015	13	12	13	0.98	0.98	0.97	0.999	3.14	3.35	3.85	0.86

[a]Inclusion criteria: growth of bacteria in nasal sample; not taking antibiotics within the last 24 hours.

[b]Inclusion criteria: *S. aureus* isolated and *spa* typed.

[c]Fisher's exact test.

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Table 2. Distribution of the six most common *spa* types by gender. *S. aureus* isolated from nasal samples in the screening. The Tromsø Staph and Skin Study.

spa type	Number isolates (n)		Prevalence (%) in the Total population, n = 3897[a]			Prevalence (%) in the Colonised population, n = 1110[b]		
	Male	Female	Male n = 1710	Female n = 2187	p[c]	Male n = 613	Female n = 497	p[c]
t012	42	52	2.46	2.38	0.87	6.85	10.46	0.03
t065	32	23	1.87	1.05	0.03	5.22	4.63	0.65
t084	56	28	3.27	1.28	<0.001	9.14	5.63	0.03
t002	18	12	1.05	0.55	0.07	2.94	2.41	0.59
t021	25	17	1.46	0.78	0.04	4.08	3.42	0.57
t015	19	19	1.11	0.87	0.44	3.10	3.82	0.51

[a]Inclusion criteria: growth of bacteria in nasal sample; not taking antibiotics within the last 24 hours.

[b]Inclusion criteria: *S. aureus* isolated and *spa* typed.

[c]Fisher's exact test.

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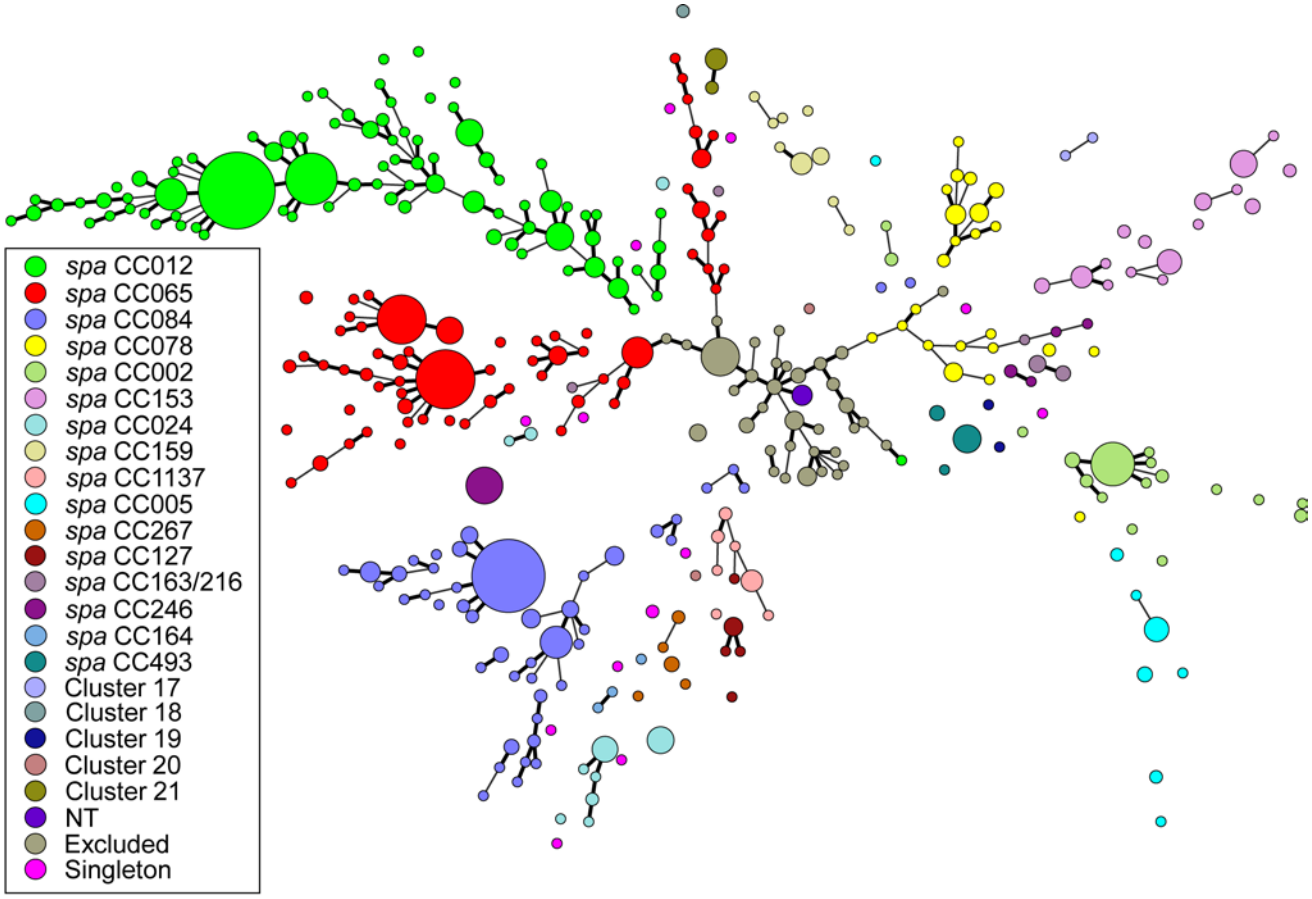

Figure 1. Minimum Spanning Tree (MST) analysis of 1,113 *S. aureus* nasal isolates from the baseline screening, based on *spa* types. Each circle represents a *spa* type, and the size of the circle corresponds to the number of isolates. Colours indicate *spa* CC, as defined by BURP clustering of the 400 *spa* types assigned from the 1,981 isolates collected in the baseline and the second screening.

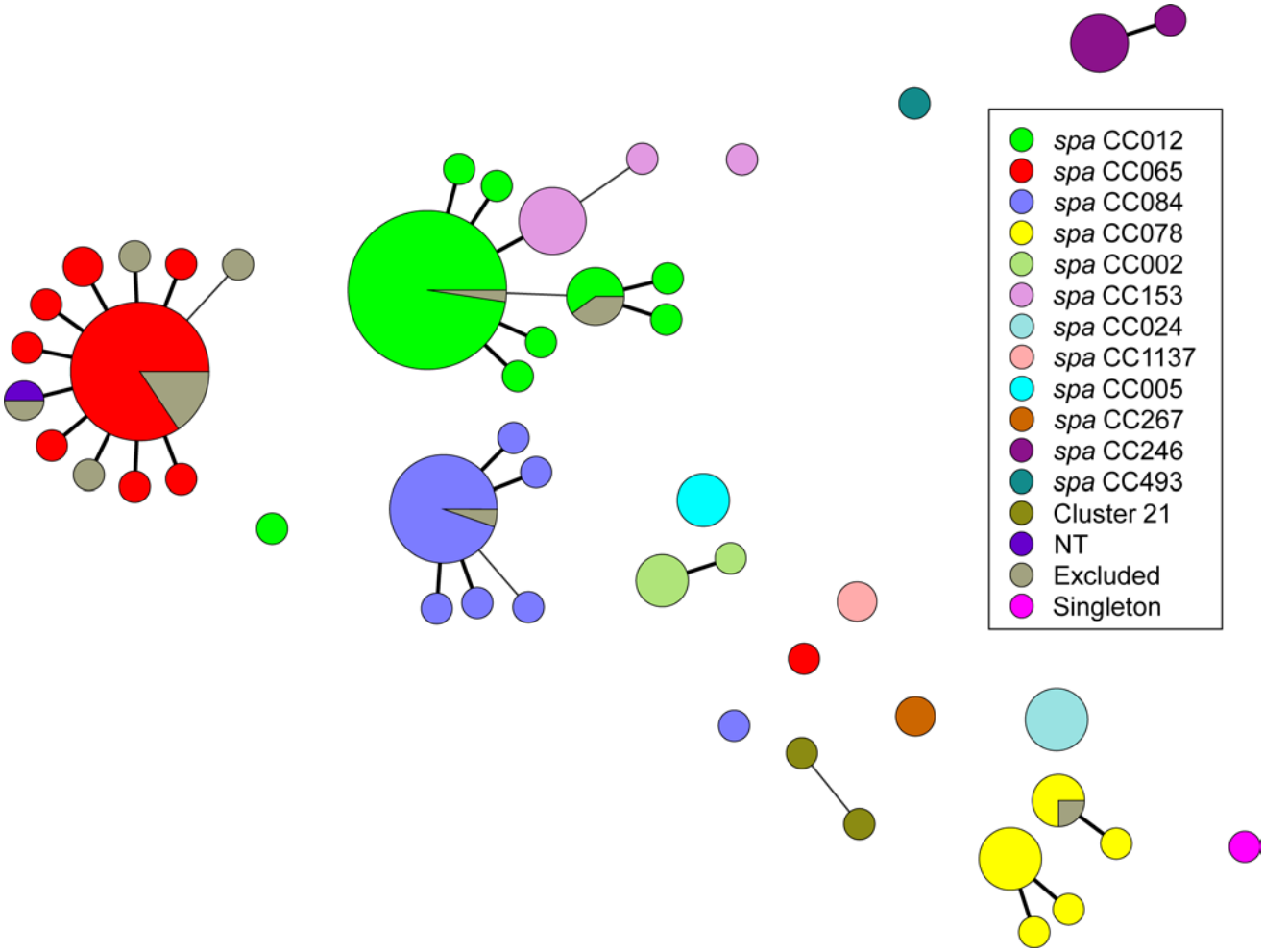

Figure 2. MST based on MLST typing of 176 consecutive isolates. Thick lines indicate single-locus variants, thin lines indicate double-locus variants. Colours indicate *spa* CC as defined by BURP-clustering.