Prevalence of Staphylococcus aureus protein A (spa) mutants in the community and hospitals in Oxfordshire

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Abstract

Background: Staphylococcal protein A (spa) is an important virulence factor which enables Staphylococcus aureus to evade host immune responses. Genotypes known as “spa-types”, based on highly variable Xr region sequences of the spa-gene, are frequently used to classify strains. A weakness of current spa-typing primers is that rearrangements in the IgG-binding region of the gene cause 1-2% of strains to be designated as “non-typeable”.

Results: We developed an improved primer which enabled sequencing of all strains, containing any type of genetic rearrangement, in a large study among community carriers and hospital inpatients in Oxfordshire, UK (6110 isolates). We identified eight novel spa-gene variants, plus one previously described. Three of these rearrangements would be designated “non-typeable” using current spa-typing methods; they occurred in 1.8% (72/3905) asymptomatically carried and 0.6% (14/2205) inpatient S. aureus strains. Some individuals were simultaneously colonized by both formerly non-typeable and typeable strains; previously such patients would have been identified as carrying only currently typeable strains, underestimating mixed carriage prevalence and diversity. Formerly non-typeable strains were found in more spa-types associated with multilocus sequence type ST398 (35%), common among livestock, compared to other groups with any non-typeable strains (1-4%), suggesting particular spa-types may have been under-represented in previous human studies.

Conclusions: This improved method allows us to spa-type previously non-typeable strains with rearrangements in the spa-gene and to resolve cases of mixed colonization with deletions in one or more strains, thus accounting for hidden diversity of S. aureus in both community and hospital environments.

Keywords: Staphylococcus aureus, Spa-typing, Spa-gene, Deletions, Non-typeable isolates, Community and hospital strains

Background

Staphylococcus aureus is a commensal organism that colonizes nasal mucosa in 25-30% of the healthy human population [1-6] and is responsible for a wide range of human diseases including serious nosocomial infections. S. aureus encodes many virulence factors including the surface Ig-binding protein A (spa) whose function is to capture IgG molecules in the inverted orientation and therefore prevent phagocytosis of the bacterial cells by the host immune system [7-12].

Typing the highly variable Xr region of the spa-gene is one of the most common methods for genotyping S. aureus. Even if well-established genotyping methods like MLST are indispensable, spa-typing has major advantages due to its high discriminatory power, typing accuracy, speed, reproducibility and ease of interpretation. Spa-typing also facilitates communication and data comparison between national and international clinical laboratories [13]. However, one weakness of current spa-typing methods is that rearrangements in the in the IgG-binding region of the gene, where the forward spa-primer is located, lead to
1-2% of strains being designated “non-typeable”. Five non-
spa-typeable S. aureus clinical strains with rearrangements
in the IgG-binding domain of the spa-gene were first de-
scribed by Baum et al. in 2009 [14]. Although artificially
constructed spa-deficient S. aureus strains are used in la-
boratory experiments [15-18], only a few other studies
have reported variants isolated from human and cattle
with rearrangements in the spa-gene [19-24]. Missing par-
ticular variants that cannot be typed may affect inferences
about genotype associations. Whilst the prevalence of such
rearrangements can be directly estimated from the pro-
portion of non-typeable strains, detecting rearrangements
that do not affect spa-typing would require sequencing
the whole spa-gene; nevertheless such rearrangements
may still be informative with respect to population
structure.

Further complexity is introduced by the fact that most
studies type only one colony per sample, thus assuming
S. aureus colonization is by a single strain and likely sys-
tematically underestimating the number of spa-types per
individual. The presence of non-typeable S. aureus strains
with rearrangements in the spa-gene increases the number
of undetected circulating spa-types even further.

Here we therefore developed a new set of primers
to amplify the spa-gene from all formerly non-typeable
S. aureus samples regardless of the specific spa-gene re-
arrangement. We used our modified spa-typing protocol
to investigate the nature and proportion of strains with
rearrangements in the S. aureus spa-gene in two large
studies of community nasal carriers and inpatients, and
the potential impact of S. aureus protein A mutants on
epidemiological studies.

**Methods**

**Collection of isolates**

**Community samples**

Nasal swab samples were collected from consenting
individuals recruited from five General Practices (GP) in
Oxfordshire that were members of the Thames Valley
Primary Care Research Partnership, National Institute of
Health Research (approved by Oxfordshire Research
Ethics Committee B, reference 08/H0605/102, [25]). On
recruitment, a nasal swab was taken from each individ-
ual by a research nurse. Participants were trained in self-
swabbing and all participants who were culture-positive
for S. aureus (n = 360) on recruitment and one quarter
(n = 211) of initially culture-negative participants were
sent a self-swabbing kit after one month and then every
two months. Swabs in charcoal medium were returned
by mail and stored at 4°C before processing. During the
36 months of the study, S. aureus was isolated from at
least one swab of 442 individuals yielding 3905 samples
which were spa-typed and analyzed here.

**Inpatient samples**

S. aureus isolates were obtained from samples collected
from the Intensive care Unit (ITU), Gerontology and
Trauma wards of the John Radcliffe hospital in Oxford
as a part of routine screening for inpatients for infection
control surveillance. For all three wards, nasal swabs
were collected from individuals at ward admission and
discharge as well as once a week during their stay within
the ward [26]. All swabs were taken by nurses, as de-
scribed above, and were processed by the routine la-
boratory at the John Radcliffe hospital, Oxford. In total,
S. aureus was isolated from 2205 samples from 1273 inpa-
tients (ITU: 1338 samples, 784 individuals; Gerontology:
134 samples, 72 individuals; Trauma: 733 samples, 417
individuals) which were spa-typed and analysed here.

**Isolation of S. aureus and DNA extraction**

Each nasal swab was placed in 5% NaCl enrichment
broth (E and O Laboratories) and incubated overnight
at 37°C. A loopful of enrichment broth was sub-cultured
onto SaSelect chromogenic agar (Bio-Rad) and incubated
at 37°C overnight. Pink/orange colonies regarded as S. aureus
were positively identified using a Prolex™ Staph Xtra
Latex Kit (Pro-Lab Diagnostics) and catalase, DNAs
and tube coagulase tests. Methicillin resistance was tested
on columbia agar with 5.0% salt (Oxoid) with BBL™
Sensi-Disc™ 1 μg Oxacillin discs (BD).

Mixed glycerol stocks of S. aureus cultures were prepared
by suspending several loopfuls of bacteria taken
by swiping across the SaSelect plate in 1.5 ml of saline
(E and O Laboratories) with 200 μl of 45% glycerol for
storage at −80°C. Taking a sweep across the plate rather
than picking a single colony for glycerol stocks allowed
us to maintain the genetic diversity of nasal strains in
the sample for later analyses.

Crude S. aureus DNA extracts (‘boilates’) used for spa-
typing were made from mixed glycerol stocks revived on
SaSelect plates. Using a 1 mm loop, a small amount of
bacteria was emulsified into 60 μl of Tris-EDTA (TE)
buffer (Sigma-Aldrich), then heated in a thermocycler at
99.9°C for 10 minutes and centrifuged at 13,200 × g for
2 minutes. 40 μl of supernatant was removed without
disturbing the pellet and stored at −20°C for use as a
PCR template.

**Spa-typing**

A staged spa-typing protocol was developed to enable
identification of multiple-strain colonization on a large-
scale [27]. The polymorphic X region of the protein A
gene (spa) was amplified with primers 1095 F: 5′-AGAC
GATCCCTCGGTGAC-3′ and 1517R: 5′-GCTTTTGCA
AATGTCAATTTACTG-3′ [28,29]. PCR reactions con-
sisted of 0.25 mM dNTPs (Qiagen), 0.5 U of GoTaq Flexi
DNA Polymerase (Promega), Colorless GoTaq Flexi
Buffer, 2.5 mM of magnesium chloride and 0.25 μM of primers in a volume of 10 μl. PCR conditions were 94°C for 2 min; 35 cycles each of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 5 min. PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter).

Samples were sequenced with the same primers as used in PCR. Sequencing reactions used BigDye v3.1 sequencing mix (Applied Biosystems) and were cycled using 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 2 min. Products were purified with Agencourt CleanSEQ beads (Beckman Coulter) and separated on an ABI 3730 DNA Analyzer (Applied Biosystems).

Chromatograms were analyzed using Ridom StaphType v2.0.3 software (Ridom GmbH). The relationships between spa-types were investigated using the BURP clustering algorithm [30] incorporated into Ridom StaphType.

Identification of rearrangements in spa-gene

A small proportion of isolates did not yield clean sequence traces with the original primers indicating the presence of rearrangements in the spa-gene. To identify possible rearrangements, primers spa-3 F: 5′-ATAGCGTGATTTTG CGGTT-3′ and spa-3R: 5′-CTAAATATAAATAATGTT GTCACTTGGA-3′ [14] were used to amplify the whole spa-gene. As some isolates failed to amplify even with this extended set of primers, an alternate forward primer, spaT3-F: 5′-CAACGCAATGGTTTCATCCA-3′ was used, binding upstream from 1095 F. Primer spaT3-F binds to a part of sequence encoding an IgG-binding domain of the spa-gene that is repeated five times in the gene (Figure 1). Due to presence of multiple binding sites for the spaT3-F primer within spa-gene, only the reverse primer (1517R) was used for sequencing.

To identify rearrangements in the spa-gene which do not affect standard spa-typing, a subset of 32 community samples and 67 bacteraemia samples were sequenced with both spa-3 F/spa-3R and spaT3-F/1517R sets of primers. 67 bacteraemia samples were randomly selected from previously existed collection of hospital invasive isolates.

Sequences were analysed using ProSeq v3.2 (http://dps.plants.ox.ac.uk/sequencing/proseq.htm).

Example sequences for each type of the spa-gene variant have been deposited in the GenBank under the accession numbers JX912490 to JX912498.

Statistical analyses

Fisher’s exact test, Chi square test and 5x2 exact test were used to compare categorical variables between groups. P values <0.05 were considered statistically significant.

Results and discussion

Identification of rearrangements in the spa-gene

Within two large longitudinal studies of S. aureus carriage in the community (3905 isolates) [25] and hospital (2205 isolates) [26] several non-typeable S. aureus strains were identified using standard spa-primers (1095 F/1517R) [14]. Isolates from both studies were spa-typed using a staged protocol, developed to resolve single- and multiple-strain colonization [27]. According to the protocol, spa-sequences were classified as follows: (i) clean sequence traces were interpreted as single strain colonisation, (ii) mixed sequence traces, characterised by distinct double peaks, were interpreted as putative multiple strain colonization, and (iii) unreadable sequence traces represented failed samples, which were retyped. Samples with mixed sequence traces were further resolved by isolating 12 individual colonies; if typing of individual colonies failed, strains were considered non-typeable with standard primers.

Sequence traces of non-typeable samples showed either complete lack of amplification, or mixed sequence traces from both DNA boilates of mixed glycerol stock and of 12 individual colonies. As previously shown [14], non-typeability of S. aureus strains can be attributed to

![Figure 1 Scheme of the spa-gene with annealing sites for the novel spaT3-F primer and standard primers. Notes: black arrows indicate five annealing sites for spaT3-F primer; grey arrow indicates annealing site for 1095 F standard primer; white arrow indicates annealing site for 1517R standard primer; figures represent distance between the beginning of spaT3-F primer and the beginning of Xr region. Spa-gene: s – signal sequence, E, D, A, B, C – IgG-binding domains, X – region which lacks IgG-binding activity and consists of repetitive region (Xr) and C-terminal region (Xc).](http://www.biomedcentral.com/1471-2180/14/63)
deletions in the spa-gene, explaining the lack of amplification in some of our samples. However the persistence of mixed sequence traces that could not be resolved by typing individual colonies indicated the presence of other types of spa-gene rearrangements.

To identify the nature of rearrangements in all our non-typeable strains we designed a new forward spaT3-F primer and combined it with reverse primer 1517R, used for routine spa-typing [29]. Primer spaT3-F has a binding site in each of the five IgG-binding domains of the spa-gene upstream of the repetitive Xr region (Figure 1) and resulted in up to five staged PCR products per sample, depending on the type of rearrangements in the IgG-binding region (Figure 2). Due to its multisite binding within the spa-gene, the spaT3-F primer could be used to type samples with deletions of up to four IgG-domains of the spa-gene and to detect and type samples with mixtures of S. aureus strains with and without deletions.

With the novel spaT3-F/1517R primer set we were able to type 100% of samples that could not be spa-typed using the standard current set of primers (denoted "formerly non-typeable").

In total, we found eight completely novel deletions/insertions in the IgG-binding region of the spa-gene plus one deletion that has been reported before [14], in 6110 community and inpatient S. aureus strains from Oxfordshire (Figure 3). We never observed the deletion of the whole or a part of the repetitive Xr region in S. aureus, in contrast to Baum et al who described partial or total deletions of the Xr region in three bacteraemia isolates [14]: our large study suggests this happens extremely rarely in carriage. One explanation for the difference may be that Baum et al. considered disease-causing isolates while most of our community and hospital isolates were carriage.

As expected, non-typeable samples that did not amplify with the standard set of primers had one of two types of deletions affecting the binding site for the original forward primer. Deletion E (174 bp) was previously described by Baum et al. in a clinical S. aureus strain [14]. Deletion G (63 bp) is a novel deletion always paired with insertion B (63 bp) (Figure 3). Non-typeable samples with persistent mixed sequence traces revealed the presence of the insertion C2 (174 bp) (Figure 3). This insertion contains

![Figure 2 Amplification of spa-locus with novel primers spaT3-F/1517R from the samples with rearrangements in the spa-gene.](image)

A control sample does not have any rearrangements in the spa-gene and shows five PCR bands according to five annealing sites of the spaT3-F primer, including a faint band in the region of 500 bp. DelH shows one thick band most likely consisted of two merged bands from the two spaT3-F annealing sites that had been brought close together by the deletion. InsC2 has a bright band (600 bp) most likely consisted of two PCR products due to insertion of additional spaT3-F annealing site. The rest of the samples display the number of bands according to the types of rearrangements (Figure 3). Amplification of these samples with the standard spa-typing primers 1095 F/1517R will give no bands for the samples with delE and delG, which affect the position of 1095 F standard primer. For the rest of the sample PCR will generate a single band (double band for the insC2) located at a variable position on the ladder depending on the number of repeats within Xr region of each sample.
additional binding sites for the spaT3-F and original spa-forward primer, producing two PCR products and distinct double peaks in sequence traces when sequenced with the original spa-forward primer. Sequencing from the reverse primer (1517R) produced clean sequence traces without double peaks.

Surprisingly, in some samples that did not amplify with the standard primer set we found rearrangements represented by deletion A (357 bp) and deletion D/insertion A (174 bp/10 bp) that do not affect the position of the standard forward primer. To investigate the presence of deletions that do not affect spa-typing and therefore can remain unnoticed, we sequenced the whole spa-gene from 32 community carriage and 67 bacteraemia isolates chosen at random from the previously spa-typed collection. We found four novel deletions, deletion D (174 bp) in both bacteraemia and community strains, deletion L (183 bp) only in community strains, deletion H (705 bp) and deletion I/insertion C1 (531 bp/174 bp) only in bacteraemia isolates (Figure 3). The largest deletions of three to four IgG-binding domains were found only in Staphylococcus aureus bacteraemia strains.

Therefore, the presence of different types of deletions and insertions in the spa-gene, identified by spaT3-F/1517R primers, demonstrates that S. aureus colonization/infection is highly complex. People may have a single strain without rearrangements, with deletions that do not affect spa-typing, or with rearrangements that do affect spa-typing. Alternatively, they may carry multiple strains without deletions in any strain, with ‘hidden’ deletions that do not affect spa-typing in one or more strains, or with rearrangements that do affect spa-typing in one or more strains.

Prevalence of spa-gene rearrangements in community and hospital strains

Spa-typing of 3905 community S. aureus isolates and 2205 hospital isolates using the staged spa-typing protocol showed that 1.8% (n = 72) of samples from 1.8% community carriers and 0.6% (n = 14) of samples from 0.7% inpatients were formerly non-typeable (Table 1). Significantly more strains from individuals in the community were formerly non-typeable compared with hospital inpatients (p < 0.0001), and there was also a trend towards more individuals carrying formerly non-typeable strains in the community than hospital (p = 0.053). One explanation may be because more longitudinal samples were collected per individual for the community compared with the inpatient study (maximum 20 versus 4 samples respectively), providing more opportunity to encounter non-typeable strains in individuals in the community study.

The proportion of S. aureus strains with ‘hidden’ deletions in the IgG-binding region of the spa-gene that do not affect spa-typing was estimated using spaT3-F/1517R primers on a random subset of previously typed samples. These hidden spa-gene deletions were found in 11% (6-19%) of S. aureus strains from 11% (6-19%) of individuals (Table 2).
Thus up to 13% of *S. aureus* carriers could, at some point, be colonized with a strain that has deletions/insertions in the IgG-binding region of the *spa*-gene, 2% carrying completely ‘non-typeable’ strains.

**Spa**-gene rearrangements lead mixed *S. aureus* colonization in humans to be underestimated

The staged *spa*-typing protocol allowed us to detect the simultaneous presence of two or more strains in 11% of *S. aureus* carriers. However, the presence of deletions that affect *spa*-typing in one or more strains within the mixture complicates the typing process and leads to underestimation of the prevalence of multiple colonization and number of strains involved.

The problem is illustrated using nine samples obtained over 14 months from one community nasal carrier AE (Table 3). *Spa*-typing with the standard primer sets (1095 F/1517R) demonstrated that AE only ever carried one strain, which was type t230 at most time points, the *spa* type t008 on one occasion and one non-typeable strain. Re-typing the same DNA extractions with our alternative novel primers (spaT3-F/1517R) showed that all samples had mixed sequence traces, apart from the former non-typeable strain that had deletion E associated with *spa*-type t012. Therefore, 12 single colonies were isolated for each sample and re-typed with alternative primers. This identified five *spa*-types carried by AE at various time points, and mixed strain colonization by two-three *spa*-types on four occasions, including two strains with deletion E. We were unable to resolve all samples by typing 12 individual colonies, even though they showed presence of mixed sequence traces (time points 4, 10, 12 and 14), which could be explained by a low frequency of one of the colonizing strains.

The limitations of the conventional *spa*-typing protocol make it impossible to identify and type *S. aureus* strains with rearrangements in the *spa*-gene in individuals with

Table 1 *S. aureus* isolates with and without different types of rearrangement in the *spa*-gene in community and inpatient samples: formerly non-typeable isolates

| Group | Community1 | Hospital2 |
|-------|------------|-----------|
|       | Isolates   | Isolates  | |
|       | no. | %  | no. | %  | no. | %  |
| Total | 3,905 | 100% | 442 | 100% | 2,205 | 100% |
| Pure without deletions/insertions or with hidden deletions | 3,647 | 93.4% | 334 | 75.6% | 2,055 | 93.2% |
| Mixed with or without deletions and/or rearrangements | 258 | 6.6% | 108 | 24.4% | 150 | 6.8% |
| Formerly non-typeable: i.e. pure with rearrangements affecting standard *spa*-typing | 72 | 1.8% (from total) | 8 | 1.8% (from total) | 14 | 0.6% (from total) |
| | 27.9% (from 12 picks) | 7.4% (from 12 picks) | 9.3% (from 12 picks) | 7.3% (from 12 picks) |

1 – nasal swabs collected from individuals recruited in 5 GP practices in Oxfordshire.
2 – nasal swabs from individuals admitted to the adult ITU, Gerontology and Trauma wards of the Oxford University Hospitals NHS Trust.
3 – indicates where all samples from an individual were pure using our *spa*-typing protocol (i.e. were without deletions/insertions or only with hidden deletions) versus any sample did not fall into this category.
4 – subjected to 12 single colony picks, i.e. 12 sub-colonies analysed from each sample.

Note: Hidden deletions were found in 16% (5/32) of *S. aureus* strains from 16 (5/31) individuals in the community and in 9% (6/67) strains from 9% (6/66) hospital in patients with bacteraemia (p = 0.33); pooled data are therefore presented.

Table 3 *Spa*-typing of *S. aureus* strains from a single individual AE with two sets of primers: standard primers 1095 F/1517R and novel primers spaT3-F/1517R

| Time points, months | DNA prep (mixed boilate) | 12 single colony picks
|---------------------|--------------------------|--------------------------|
|                     | 1095 F/1517R | spaT3-F/1517R | spaT3-F/1517R |
| AE-0                | t230         | MST          | t230/t012     |
| AE-1                | non-typable  | t012         | t012          |
| AE-2                | t230         | MST          | t230/t012     |
| AE-4                | t230         | MST          | t230          |
| AE-6                | t230         | MST          | t230/t571     |
| AE-8                | t008         | MST          | t008/t012/t571|
| AE-10               | t230         | MST          | t230          |
| AE-12               | t230         | MST          | t230          |
| AE-14               | t230         | MST          | t230          |

1 mixed sequence traces; 2 *spa*-types in bold have delE and could not be typed with standard primers; *spa*-repeats: t230: 08-16-02-16-34.

Table 2 *S. aureus* isolates with and without different types of rearrangement in the *spa*-gene in community and inpatient samples: isolates with hidden deletions

| Group | Isolates | Individuals |
|-------|----------|-------------|
|       | no. | %  | no. | %  |
| Total strains without deletions/insertions or with only hidden deletions investigated | 99 | 100% | 97 | 100% |
| Hidden deletions found | 11 | 11% | 11 | 11% |

Note: Hidden deletions were found in 16% (5/32) of *S. aureus* strains from 16 (5/31) individuals in the community and in 9% (6/67) strains from 9% (6/66) hospital in patients with bacteraemia (p = 0.33); pooled data are therefore presented.
multiple strain colonization. The staged spa-typing protocol allows us to resolve cases of mixed strain colonization with deletions in one or more strains. Even 12 single colony picks could not always identify the presence of low-frequency strains with deletions, illustrating the even greater challenge of estimating the proportion of non-typeable strains within mixed colonization. Thus diversity in colonizing and infecting strains is inevitably underestimated.

**Inpatients’ strains can acquire deletions in the spa-gene**

We also found that *S. aureus* strains can acquire deletions in the *spa*-gene during inpatients’ hospital admission. Such acquisition of deletions was never observed for longitudinal carriage strains from individuals in the community, with those deletions observed being present from the first time the strain carrying the deletion was identified (Additional file 1: Table S1).

Among six hospital patients with deletions that affect *spa*-typing, three individuals (BA, BB and BF) already carried the strain with the deletion when they were admitted. The three other patients (BC, BD and BE) acquired a deletion during their hospital stay on the background of a *spa*-type carried at admission (Table 4). There were eight, four and six days between the last swab without and the first swab with the acquired deletion for BC, BD and BE respectively. All three patients acquiring deletions during hospital admission either had long-term illnesses and/or had taken several antibiotics (BC: teicoplanin; BD: doxycycline; BD: ciprofloxacin, vancomycin, erythromycin, gentamicin, tetracycline).

The repetitive nature of the *spa*-gene makes it unstable and highly prone to internal rearrangements, which in bacteria occur via either RecA-dependent or RecA-independent recombination [31-33]. These rearrangements might have positive or negative effects as protein A is an important virulence factor that plays a central role in *S. aureus* defence against the host immune response. There is new evidence that the antibiotic ciprofloxacin increases the intrachromosomal DNA recombination rate in *Escherichia coli* [34]. Other antibiotics might potentially have similar effects, yet undiscovered. Taking into account that the three inpatients who acquired deletions during their stay at the hospital had been taking specific antibiotics for a long time or a wide range of antibiotics for a short period, including ciprofloxacin, it is possible that antibiotic pressure might be one factor that drives genetic rearrangements in the *S. aureus* protein A gene. However, we also cannot exclude the possibility that these deletions may have been present already at low frequency, and undetected, before increasing to become the majority variant (rather than being acquired de novo). Nevertheless this scenario also would support antibiotics playing a role in emergence of deletions to detectable levels.

In the community, most individuals colonized by *S. aureus* strains carry them without displaying any symptoms. However, when some of them became invasive, the change of habitat, for example on a background of antibiotic pressure, might promote acquisition of rearrangements in the *spa*-gene that might be advantageous in new environment even if they lead to loss or change of protein function. As shown previously, progress of infectious disease is typically associated with acquisition of mutations or rearrangements in the pathogen, many of which lead to loss of function in a number of genes [35].

Deletions appear to be over-represented in clonal lineages related to livestock

In total, we found 20 *spa*-types from 33 individuals associated with nine types of rearrangements in the *spa*-gene (Additional file 2: Table S2). All types of deletions were associated with a mixture of related and unrelated *spa*-types, only insertion C2 was associated with a group of 3 closely related *spa*-types: t021, t012 and t10173. The 20 *spa*-types with rearrangements were clustered into five groups of closely-related variants and five non-related singletons (Table 5).

Any clusters of related *spa*-types with a higher prevalence of rearrangements affecting *spa*-typing (delE, delG-insB or insC2) would be likely to be underrepresented, or even missing, in the majority of studies based on routine *spa*-typing protocols. To test this hypothesis, we compared the proportion of individuals with and without rearrangements affecting *spa*-typing in the four groups of *spa*-types and the singleton that we found had one or more rearrangements affecting *spa*-typing (5 × 2 exact test, Table 5). In group 1 35% of strains were

Table 4 Individuals who acquired a deletion in the *S. aureus* *spa*-gene during their hospital admission

| Individual ID | Date swab taken | Results | Spa type | Rearrangements |
|---------------|-----------------|---------|----------|----------------|
| BC            | 30/01/2011      | MSSA    | t298     | delG-insB      |
| BC            | 08/02/2011      | MSSA    | t298     | delG-insB      |
| BD            | 14/04/2011      | MSSA    | t571     | delG-insB      |
| BD            | 19/04/2011      | MSSA    | t571     | delG-insB      |
| BD            | 26/04/2011      | MSSA    | t571     | delG-insB      |
| BE-a | 20/06/2011 | MSSA | t179 | delE |
| BE-g | 20/06/2011 | MSSA | t179 | delE |
| BE-n | 20/06/2011 | MSSA | t179/t078 | delE |
| BE-th | 20/06/2011 | MSSA | t179/t078 | delE |
| BE | 05/07/2011 | MSSA | t179/t078 | delE |
| BE | 12/07/2011 | MSSA | t179/t078 | delE |
| BE | 20/07/2011 | MSSA | t179/t078 | delE |

1-4body sites swabs: a – axilla, g – groin, n – nose, th – throat; all other swabs are nasal swabs; *spa*-types in bold acquired deletion that affects binding site for standard forward *spa*-typing primer.
affected by these rearrangements, a significantly higher proportion compared with the 1-4% in other groups or the singleton t571 (p < 0.0001). Therefore, spa-type t571 and its closely related variants such as t3085 may well be underrepresented in most S. aureus studies based on spa-typing, as they could not be typed with the standard set of primers when common deletions are present.

Interestingly, spa-type t571 belongs to clonal lineage ST398 that contains MRSA and MSSA strains common among livestock. Spa-type t571 is closely related to type t011 and t034, all most commonly associated with pigs [36-40]. These spa-types have been found less commonly in dogs, cats and horses, and occasionally in cats and poultry [41,42]. Large-scale screening of pigs [36] showed that 60% of them carried t034, 14% t1255 and t011 and t034, all most commonly associated with pigs among livestock.

### Table 5 Spa-types and groups in which deletions/insertions in the spa-gene were observed

| Spa-types | Spa-repeats | Individuals with deletions, no. (column %) | Hidden deletions not affecting spa-typing (no.) | Deletions/insertions affecting spa-typing (no.) | Individuals with deletions affecting spa-typing/total individuals with this spa-type |
|-----------|-------------|-------------------------------------------|-----------------------------------------------|------------------------------------------------|---------------------------------------------------------------------------------|
| Group 1   |             |                                           |                                               |                                                |                                                                                  |
| t571      | 08–16–02–25–02–25----------34-25       | 7 (21%)                                    |                                               | delG-insB(5); delE(1)                      | 7/20 (35%)*                                                                     |
| t3085     | 08–16–02–25–02–25–34-25–34-25         | 6 (18%)                                    | delE(1)                                       | 6/19 (32%)                                  |
| Group 2   |             |                                           |                                               |                                                |                                                                                  |
| t021      | 15–12–16–02–16----------02–25–17–24    | 4 (12%)                                    | delE(1)                                       | 4/1 (100%)                                  |
| t508      | 15–12–16–02----------17–24             | 3 (9%)                                     | delE(1)                                       | 1/5 (20%)                                   |
| t10173    | 15–12–02–16----------25–17–25–02–25–24 | 1 (3%)                                     | insC2(1)                                      | 1/1 (100%)                                  |
| t012      | 15–12–16–02–16----------02–25–17–24    | 3 (9%)                                     | delE(1); insC2(1)                             | 2/123 (2%)                                  |
| t6803     | 15–12–16–02–16----------02–25–17–24–17 | 1 (3%)                                     | delD-insA(1)                                  | 0/2 (0%)                                    |
| Group 3   |             |                                           |                                               |                                                |                                                                                  |
| t084      | 07–23–12–34–34–12–12–23–02–12–23       | 3 (9%)                                     |                                               |                                              |
| t085      | 07–23–12–34–34–12–23–02–12–23          | 3 (9%)                                     |                                               |                                              |
| Group 4   |             |                                           |                                               |                                                |                                                                                  |
| t280      | 04----------20–17–12–12----------17    | 4 (12%)                                    |                                               | delG-insB(1)                                 | 4/1 (25%)                                                                      |
| t227      | 04----------12–12–17                    | 4 (12%)                                    |                                               | delE(1)                                      | 0/3 (0%)                                                                       |
| t078      | 04–21–12–41–20–17–12–17–17–17–17–17–17 | 1 (3%)                                     | delE(1)                                       | 1/26 (4%)                                   |
| t216      | 04----------20–17–20–17----------31–16–34 | 1 (3%)                                     | delG-insB(1)                                  | 1/41 (2%)                                   |
| Group 5   |             |                                           |                                               |                                                |                                                                                  |
| t032      | 26–23–23–13–23–31–29–17–29–17–25–17–25–26–28 | 2 (6%)                                     | delD(1); delA(1)                              | 1/79 (1%)                                  |
| t223      | 26–23–13–13–23–05–17–17–25–25–16–28    | 3 (9%)                                     |                                               | 0/13 (0%)                                   |
| Singletons|             |                                           |                                               |                                                |                                                                                  |
| t213      | 07–23–12–21–24–33–22–17                | 7 (21%)                                    |                                               |                                              |
| t6792     | 08–16–02–16–17–13–17–13–17–16–34       | 3 (9%)                                     | delD(3)                                       |                                              |
| t6417     | 14–44–13–12–17–13–12–17–17–23–13       | 3 (9%)                                     | delD(1)                                       |                                              |
| t530      | 11–19–12–21–17–34–24–34–16             | 3 (9%)                                     | delE(1)                                       | 1/3 (33%)*                                  |
| t7960     | 299–25–17–17–16–16–16–16–16–16–16–16–16 | 1 (3%)                                     | del-insC1(1)                                  |                                              |
| Total     |             |                                           |                                               |                                                | 33 (100%)                                                                       |

* P < 0.00001 comparing four groups of spa-types and the singleton with rearrangements affecting spa-typing (5 x 2 Fisher's exact test).

21* repeat differing in one base from repeats 12 and 20.
12* repeat differing in two bases from repeats 20.
41* repeat differing in one base from repeat 17.
31* repeat differing in two bases from repeat 16.
16* repeat differing in one base from repeat 17.
34* repeat differing in two bases from repeats 12 and 20.
05* repeat differing in one base from repeat 29.
working with pigs [36,37]. Veterinary personnel and pet owners are also more likely to carry these animal-related types [43]. Recent studies have also reported the emergence of livestock-associated MRSA clones of \textit{S. aureus} ST398 causing bacteraemias in humans, supporting animal-independent transmission of such strains between humans [44,45].

It is unclear why ST398 \textit{S. aureus} strains commonly found in livestock frequently develop deletions in the IgG-binding part of protein A gene after transmission to humans. One possible explanation is that this might be a part of \textit{S. aureus} strain adaptation to a different immune background where protein A plays a major role [7,8,12]. Another explanation might be that the livestock associated strains have more rearrangements in the \textit{spa}-gene prior the transmission to humans due to high level of antibiotic exposure in food-animal production [46–48]. Nevertheless, our findings highlight the potential for these strains to have been substantially under-represented in epidemiological studies to date, and for strains formerly non-typeable using standard methods to be a source of bias.

Conclusions

\textit{Spa}-typing is a common technique used to classify \textit{S. aureus} strains in clinical practice (eg outbreak management) and research. Rearrangements in the IgG-binding region of the \textit{spa}-gene make strains “non-typeable” with commonly used primers. Using a novel primer, we typed 100% of samples and identified eight novel \textit{spa}-gene variants, plus one previously described; three of these rearrangements cause strains to be designated as “non-typeable” using current \textit{spa}-typing methods.

\textit{Spa}-typing of 6110 \textit{S. aureus} isolates showed that 1.8% of samples from 1.8% community carriers and 0.6% of samples from 0.7% inpatients were formerly non-typeable. We also found evidence of mixed colonization with strains with and without gene rearrangements, and estimated that up to 13% of carriers are colonized with “hidden” \textit{S. aureus} with deletions/insertions in the IgG-binding region at some point. Using standard primers therefore underestimates \textit{spa}-type diversity. We also found evidence of inpatients acquiring spa-gene deletions de novo during a hospital admission, suggesting that antibiotic pressure might be one factor driving genetic rearrangements in the \textit{S. aureus} protein A gene. Finally, we found that deletions formerly causing strains to be designated as “non-typeable” were over-represented in clonal lineages related to livestock, indicating that these may well be have been underrepresented in most \textit{S. aureus} studies. This new improved spa-typing protocol therefore enables previously overlooked \textit{S. aureus} strains to be typed and therefore contribute to our understanding of diversity, carriage and transmission of \textit{S. aureus} strains in community and hospitals.

Additional files

Additional file 1: Table S1. Swab data for individuals with rearrangements in the \textit{spa}-gene.

Additional file 2: Table S2. Association between rearrangements in the \textit{spa}-gene and \textit{spa}-types.

Competing interests

All authors declare that they have no competing interests.

Authors’ contributions

AAV carried out laboratory experiments, participated in the analysis of data and writing of the manuscript, RF contributed to the collection and processing of samples for the study, RRM contributed to the design of the sample collection and sample database development, KK supervised recruitment of participants, as well as collection and processing of samples for the study, HG recruited participants for the study, DHW contributed in the design of the study and laboratory experiments, RB participated in the design of the study, DWC participated in the design of the study and contributed in the drafting of the manuscript, ASW performed statistical analysis and participated in the writing of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to thank Dr. Teresa Street for discussion of the laboratory results, Dr. Kate Dingle for the comments on the manuscript, Ms. Alison Vaughan and Mr. David Griffiths for their assistance in the laboratory. This study was supported by the Oxford NIHR Biomedical Research Centre and the UKCRC Modernising Medical Microbiology Consortium, with the latter funded under the UKCRC Translational Infection Research Initiative supported by Medical Research Council, Biotechnology and Biological Sciences Research Council and the National Institute for Health Research on behalf of the Department of Health (Grant G0800778) and The Wellcome Trust (Grant 086746/Z/08/Z).

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Received: 11 December 2013 Accepted: 4 March 2014

Published: 12 March 2014

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doi:10.1186/1471-2180-14-63
Cite this article as: Votintseva et al: Prevalence of Staphylococcus aureus protein A (spa) mutants in the community and hospitals in Oxfordshire. BMC Microbiology 2014 14:63.