Direct detection of nasal *Staphylococcus aureus* carriage via helicase-dependent isothermal amplification and chip hybridization

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**Abstract**

**Background:** The bacterium *Staphylococcus aureus* constitutes one of the most important causes of nosocomial infections. One out of every three individuals naturally carries *S. aureus* in their anterior nares, and nasal carriage is associated with a significantly higher infection rate in hospital settings. Nasal carriage can be either persistent or intermittent, and it is the persistent carriers who, as a group, are at the highest risk of infection and who have the highest nasal *S. aureus* cell counts. Prophylactic decolonization of *S. aureus* from patients’ noses is known to reduce the incidence of postsurgical infections, and there is a clear rationale for rapid identification of nasal *S. aureus* carriers among hospital patients.

**Findings:** A molecular diagnostic assay was developed which is based on helicase-dependent target amplification and amplicon detection by chip hybridization to a chip surface, producing a visible readout. Nasal swabs from 70 subjects were used to compare the molecular assay against culturing on “CHROMagar Staph aureus” agar plates. The overall relative sensitivity was 89%, and the relative specificity was 94%. The sensitivity rose to 100% when excluding low-count subjects (<100 *S. aureus* colony-forming units per swab).

**Conclusions:** This molecular assay is much faster than direct culture and has sensitivity that is appropriate for identification of high-count (>100 *S. aureus* colony-forming units per swab) nasal *S. aureus* carriers who are at greatest risk for nosocomial infections.

**Keywords:** *Staphylococcus aureus*, Nasal carriage, Molecular diagnostic, Helicase-dependent amplification

**Findings**

**Background**

Nosocomial bacterial infections are an important cause of morbidity and mortality, attributable for approximately 100,000 deaths annually in the United States [1]. The Gram-positive bacterial pathogen *Staphylococcus aureus* is causally involved in a significant fraction of these infections, with about 0.8% of all US hospital inpatients suffering from a *S. aureus* infection [2]. The anterior nares represent the primary ecological reservoir for *S. aureus* in humans, with one in three individuals being carriers [3]. Three different nasal carriage patterns have been discerned, with approximately 20% of the population being persistent carriers, 30-60% intermittent carriers, and the remainder being non-carriers [4]. The quantity of *S. aureus* colony-forming units (CFU) that can be recovered from swabs obtained from carriers’ noses varies widely, from single-digits to millions [5,6], and there is a strong association between high cell count and persistent carriage [7-9]. Based on various levels of evidence, it has been suggested that persistent carriers represent a separate group that is distinct from intermittent and non-carriers [8,10].

Colonization of the human nose by *S. aureus* represents a commensal relationship, and carriage is consequential to the healthy human host in every-day life. However, *S. aureus* nasal carriage translates into a three to four fold higher infection rate in hospital settings compared to non-carriers [11-14]. In one population of dialysis patients, persistent nasal *S. aureus* carriers carried a 3.4 times higher risk than intermittent carriers [13]. Prospective and retrospective studies revealed that
the majority of nosocomially infected nasal carriers suffer from a S. aureus strain that is clonally identical to the commensal strain carried in their nose, thereby strongly implicating an endogenous origin [11,14-16].

Topical intranasal mupirocin application provides an effective and safe option for S. aureus decolonization and, when used prophylactically, reduces the incidence of postsurgical infections [17]. The strongest evidence so far was provided by a double-blind, placebo-controlled, multicenter trial that used randomized patient groups from multiple hospitals in the Netherlands [16]. The results revealed a 2.4-fold reduction in risk of surgical-site S. aureus infections for the treatment group. The authors concluded that rapid identification of nasal S. aureus carriage at the time of hospital admission speeds decolonization and is a critical factor in reducing hospital-associated S. aureus infections. Due to the reported emergence of mupirocin resistance, it is prudent to restrict nasal mupirocin application only to patients who are likely to benefit from it [18]. This provides a strong incentive for hospital-based nasal S. aureus screening of patients who will be undergoing an invasive medical procedure, by using a molecular diagnostic test for rapid identification of nasal carrier status and initiation of S. aureus decolonization without delay.

Commercially available nasal screening tests generally use either polymerase chain reaction (PCR)-based or microbiological culture-based methods. PCR-based molecular diagnostic tests provide much faster turnaround relative to the lower cost culture-based methods, and the benefit of rapid turnaround has been well established [19]. The commercial PCR assay designs in use identify methicillin-resistant S. aureus (MRSA) [20-23], and they do so by targeting a mobile staphylococcal cassette chromosome (SCC) element referred to as SCCmec [24]. This assay design causes false positive rates that significantly impact MRSA screening efficacy [25-27], in particular in geographic regions where MRSA prevalence is low or on the decrease, and it can also cause false negatives [28,29]. We have developed an alternative molecular detection method that targets S. aureus-specific sequences in the thermonuclease (nuc) gene [30,31]. The assay system recapitulates the rapid turnaround time of PCR but at lowered cost, using isothermal amplification coupled to chip-based detection followed by digital camera capture of the chip image [32].

Direct culture

The head of swab A was detached from its stem, transferred into a tube containing 200 uL 10 mM Tris (pH 8.8), 10 mM NaCl (TN-Buffer), and vortexed for 30 seconds at maximum setting. This yielded a recoverable volume of approximately 160 uL nasal mucus suspension, and 100 uL thereof was directly plated onto a ‘BBL CHROMagar Staph aureus’ agar plate (Becton Dickinson) which was then incubated at 37°C for 18–20 hours. The samples that gave rise to mauve colonies on plates, indicative of S. aureus, were scored as positive by direct culture. The number of mauve colonies per plate was counted exactly (if <1000) or estimated (if >1000), and S. aureus CFU counts per swab was extrapolated by multiplying with a factor of 1.6.

Preparation of bacterial DNA from nasal swabs

The head of swab B was detached from its stem, placed into 500 uL TN-Buffer, and vortexed for 30 seconds at maximum setting. The sample was then centrifuged for 10 minutes at 14,000 x g at ambient temperature. The supernatant was discarded and the pellet re-suspended in 100 uL TN-Buffer containing 0.5 U/uL achromopeptidase (Wako Chemicals, Richmond, VA). The mixture was incubated for 15 minutes at 37°C, followed by 5 minutes at 98°C. Two 5 uL aliquots were used in replicate Helicase-dependent amplification reactions, and the rest was stored frozen at −20°C.

Asymmetric helicase-dependent amplification (HDA)

Amplification reactions were set up according to conditions as provided by the IsoAmp II Universal THDA Kit (BioHelix, Beverly, MA). For each individual HDA reaction, 5 uL of nasal mucus lysate was first mixed with 15 μL of Dilution Buffer, giving rise to concentrations of 20 mM Tris–HCl, pH 8.8, 10 mM KCl, 7.7 mM MgSO₄, 40 mM NaCl, 5 mg/mL BSA, and 0.02% Tween 20. This was then added to 20 uL 2x HDA-Mix (20 mM Tris–HCl, pH 8.8, 40 mM NaCl, 0.02% Triton X-100, 0.4x EvaGreen (Biotium, Hayward, CA), 6.8 mM dATP,
Chip preparation and hybridization

Crystalline silicon wafers were coated with the polymer amino functional T-structure poly-dimethylsiloxane (TSPS, United Chemical Technologies, Bristol, PA) and cured at 150°C for 24 hours. The TSPS coated wafer was further prepared by soaking in a 50 mg/L solution of poly (lys-phe) in 1x phosphate-buffered saline (PBS, pH 6) containing 2 M NaCl overnight at room temperature. Next, the poly (lys-phe) coated wafer was washed and soaked in 10 μM succimidyl-4-formyl benzoate (SFB, Sigma-Aldrich, St. Louis, MO) for 2 hours, the wafers were washed with 0.1% SDS, and these were processed as described above for direct culture and incubation at room temperature for 4 minutes. Chips were washed 3 times with WashBuffer-B before addition of 100 μL of Mbiter 3,3',5,5'-Tetramethylbenzidine (TMB, BioFX Laboratories, Eden Prairie, MN) to the chip surface and incubation at room temperature for 4 minutes. Chips were washed with water and methanol, air-dried, and then imaged.

Discrepancy resolution analyses

Nasal swab samples that gave rise to a negative result by direct culture but a positive result by the molecular assay were followed up by a repeat of the HDA and chip hybridization, using fresh aliquots of the original nasal swab lysates. Furthermore, HDA discordant positives were followed up by PCR amplification (Roche LightCycler 480) using the nuc gene amplification primers published by Brakstad and coworkers [31]. A product band of the correct size was verified by polyacrylamide gel electrophoresis. In addition, volunteers whose nasal swabs produced molecular assay discordant positives were asked to provide follow-up nasal Double Swabs, and these were processed as described above for direct culture as well as molecular assay analysis.

Volunteers whose nasal swabs gave rise to a positive result by direct culture but a negative result by the molecular assay were asked to provide a follow-up nasal swab a few weeks later. These follow-up swabs were processed by direct culturing as described above.

Results and discussion

Analytical assay performance

The molecular diagnostic assay described in this study is based on isothermal helicase-dependent amplification (HDA) [33] of *Staphylococcus aureus*-specific DNA sequences, derived from the thermonuclease gene *nuc*, followed by hybridization of the biotinylated amplification product to a *nuc*-specific capture probe.
immobilized on silicon chips. To define assay sensitivity, pooled nasal mucus was spiked with dilutions of *S. aureus* cells, and the chip could detect as few as 2 CFU per HDA reaction, which extrapolates to 40 CFU per swab (data not shown). This indicates that the protocol developed for this study efficiently enriched and lysed *S. aureus* cells present in nasal mucus, making the bacterial genomic DNA available for amplification. A panel of 21 *S. aureus* strains was then used to test reactivity of the nuc gene HDA primers and hybridization probe. All 21 strains gave rise to positive HDA and chip hybridization signals (Table 1). To verify that the assay detects only *Staphylococcus aureus*, a panel of prokaryotic and eukaryotic organisms was examined. No positive nuc amplicon signal was observed for the 8 other staphylococcal species or the 17 additional bacterial and eukaryotic organisms that were tested (Table 2). These results are consistent with previous studies establishing that the nuc gene contains *S. aureus*-specific sequences [30,31] and with a recently published study in which the nuc gene was present in 1781 of 1783 *S. aureus* isolates (99.9% sensitivity) [34].

**S. aureus-positive samples**

A total of 70 volunteers were enrolled for this nasal *S. aureus* detection study. Of these, 36 individuals were culture-positive on “CHROMagar Staph aureus” plates, with a range between 3 and more than 100,000 CFU per swab (Table 3). In comparison to direct culture, the molecular diagnostic assay had a relative sensitivity of 89% (Table 4). Using a cutoff of 100 *S. aureus* CFU per swab, the samples were stratified into “high-count” and “low-count” groups. This cutoff was selected originally based on data provided in Figure 2 from the article by Nouwen et al. [7], which indicated that swabs with more than 100 CFU are significantly more likely to originate from persistent carriers than from intermittent carriers. More recently published results revealed that 20/21 samples from persistent carriers were associated with nasal *S. aureus* loads of >100 CFU per swab, and 13/14 samples from non-persistent carriers were associated with loads of ≤100 CFU [9], further validating our selection of this cutoff value. Of the 36 nasal *S. aureus* carriers identified in this study, 29 were classified into the high-count group. All of these 29 individuals were positive by the molecular assay (Table 3), and for this subgroup the concordance between the molecular assay and direct culture was 100% (Table 5). Seven samples had fewer than 100 *S. aureus* CFU per swab (Table 3), four of which were negative by the molecular assay. This is not surprising since these samples contained *S. aureus* CFU numbers near or below the lower limit of detection. Two discrepant samples were

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**Table 1 Staphylococcus aureus strains tested for sensitivity of amplification primer set and detection probe**

| Organism | ATCC number | Other designations | Molecular assay |
|----------|-------------|--------------------|-----------------|
| *S. aureus* | 6538 | FDA 209 | positive |
| *S. aureus* | 14993 | PCI 1217 [21 J] | positive |
| *S. aureus* | 25923 | Seattle 1945 | positive |
| *S. aureus* | 33591 | 328 | positive |
| *S. aureus* | 33592 | 1063 | positive |
| *S. aureus* | 43300 | F-182 | positive |
| *S. aureus* | BAA-1720 | | positive |
| *S. aureus* | BAA-1749 | 96:308 | positive |
| *S. aureus* | BAA-1764 | 7031 | positive |
| *S. aureus* | BAA-1765 | 102-04 | positive |
| *S. aureus* | BAA-42 | HDE288 | positive |
| *S. aureus* | | | positive |
| *S. aureus* | | STAAC, 93062 | positive |
| *S. aureus* | | HDG2 | positive |
| *S. aureus* | | MA14 | positive |
| *S. aureus* | | MA15 | positive |
| *S. aureus* | | MA6 | positive |
| *S. aureus* | | MA8 | positive |
| *S. aureus* | | MSH7 | positive |
| *S. aureus* | | STAAW, 196620 | positive |
| *S. aureus* | | W15 | positive |

Isolated microbial colonies were suspended in TN-Buffer, lysed by incubation with achromopeptidase followed by boiling, and the equivalent of in the order of 10^6 cells was added to each HDA molecular assay reaction.
confirmed as nuc-negative (Table 3) by PCR analysis using previously published nuc gene primers [31].

Individuals who carry low numbers of S. aureus cells are more likely to be intermittent rather than persistent nasal carriers [7-9]. Three volunteers whose nasal swab samples gave rise to discrepant results (#31, #33, #36, Table 3) were able to provide a follow-up swab a few weeks later. Two of the three follow-up swabs turned up negative on direct culture, classifying these two volunteers as intermittent S. aureus carriers (#33, #36, Table 3).

| Table 2 Species tested for specificity of amplification primer set and detection probe |
|-----------------------------------------------|-----------------|-----------------|
| Organism                     | Source of isolate | Characteristics | Molecular assay |
| S. schleiferi                | ATCC # 43808     | Coagulase-positive | negative        |
| S. capitis                   | ATCC # 35661     | Coagulase-negative | negative        |
| S. epidermidis               | ATCC # 12228     | Coagulase-negative | negative        |
| S. haemolyticus              | ATCC # 29970     | Coagulase-negative | negative        |
| S. hominis                   | ATCC # 700236    | Coagulase-negative | negative        |
| S. lugdunensis               | ATCC # 43809     | Coagulase-negative | negative        |
| S. saprophyticus             | ATCC # 15305     | Coagulase-negative | negative        |
| S. succinu                   | ATCC # 700337    | Coagulase-negative | negative        |
| Bacillus subtilis            | ATCC # 23859     | gram-positive    | negative        |
| Clostridium difficile        | ATCC # BAA-1382  | gram-positive    | negative        |
| Enterococcus faecalis        | ATCC # 700802    | gram-positive    | negative        |
| Enterococcus faecium         | ATCC # 51559     | gram-positive    | negative        |
| Micrococcus luteus           | ATCC # 10240     | gram-positive    | negative        |
| Mycobacterium abscessus      | ATCC # 19977     | gram-positive    | negative        |
| Streptococcus agalactiae     | ATCC # 13813     | gram-positive    | negative        |
| Streptococcus pneumoniae     | ATCC # 6308      | gram-positive    | negative        |
| Acinetobacter baumannii      | ATCC # 17978     | gram-negative    | negative        |
| Citrobacter freundii         | ATCC # 8090      | gram-negative    | negative        |
| Escherichia coli             | ATCC # 4157      | gram-negative    | negative        |
| Klebsiella pneumoniae        | ATCC # 13883     | gram-negative    | negative        |
| Neisseria gonorrhoeae        | ATCC # 53420     | gram-negative    | negative        |
| Pseudomonas putida           | ATCC # 47054     | gram-negative    | negative        |
| Candida albicans             | ATCC # 18804     | negative         | negative        |
| Saccharomyces cerevisiae     | Strain S288C     | negative         | negative        |
| Homo sapiens                 | Roche Cat. #11691112001 | negative |

Isolated microbial colonies were suspended in TN-Buffer (see Materials and Methods section), lysed by incubation with achromopeptidase and/or boiling, and the equivalent of in the order of $10^6$ cells (or 20 ng purified yeast DNA or 80 ng human DNA) was added to each HDA molecular assay reaction.

confirmed as nuc-negative (Table 3) by PCR analysis using previously published nuc gene primers [31].

| Table 3 Nasal swab samples giving rise to mauve colonies on “CHROMagar S. aureus” agar plates |
|-----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| #          | Initial Swab: CFU/Swab | Molecular assay | Follow-up PCR | Follow-up Swab: CFU/Swab |
| 1-27       | >100,000 to >2,000     | All Positive   |                |                  |
| 28         | 1152                   | Positive       |                |                  |
| 29         | 387                    | Positive       |                |                  |
| 30         | 38                     | Positive       |                |                  |
| 31         | 27                     | Negative       | ND             | 594              |
| 32         | 8                      | Positive       |                |                  |
| 33         | 6                      | Negative       | ND             | 0                |
| 34         | 5                      | Positive       |                |                  |
| 35         | 3                      | Negative       | Negative       | ND               |
| 36         | 3                      | Negative       | Negative       | 0                |

Samples are sorted by CFU/Swab. The 29 samples giving rise to >100 mauve colonies per plate were classified as "high-count" and are indicated in bold. CFU: Colony Forming Units. ND: Not Done.
Table 4: Comparison of the molecular assay results to the direct culture method

|                   | Direct culture | Molecular Assay |
|-------------------|----------------|-----------------|
|                   | Positive       | Negative        |
| Molecular Assay   |                |                 |
| Positive          | 32             | 2               |
| Negative          | 4              | 32              |
| Total             | 36             | 34              |
| Sensitivity:      | 89%            |                 |
| Specificity:      | 94%            |                 |

S. aureus-negative samples

Of the 70 volunteers, 34 were culture-negative. Two of these 34 samples gave rise to a positive result by the molecular assay, which translates into a relative specificity of 94% (Table 4). Both samples remained positive upon repeated molecular analysis. Follow-up Double Swabs were subsequently obtained from the two volunteers. Swabs from both individuals were again molecular assay-positive, and individual I was again culture-negative while individual II yielded 3 CFU, indicating that individual II was a low-count carrier. The samples from both volunteers were confirmed as nuc-positive by PCR analysis [31].

Resolution of the discrepancies between the identification of S. aureus by nuc gene amplification versus direct microbiological culture will require further analyses. While the microbiological method chosen for this study, direct culturing on “CHROMagar Staph aureus” chromogenic medium, is characterized by its simplicity as well as excellent specificity, the sensitivity of this method is not 100%. Among 310 S. aureus-positive clinical specimens, CHROMagar Staph aureus was 95.5% sensitive [35]. Therefore, one would expect one or two of the 34 negative samples in the present study to be falsely negative. Alternatively, it is conceivable that swabs obtained from certain low-count individuals may contain mostly dead cells that would go undetected by culture, since this method relies upon the presence of live cells. Discrepancy resolution might be achieved by the amplification and sequencing of the rpoB gene which allows for accurate differentiation of staphylococcal isolates at the species and subspecies level [36]. However, since the nasal environment is known to contain a mixture of multiple bacterial species [37], sequencing of amplification products obtained from nasal swab lysates would require sequence analysis of large numbers of clones.

Prevalence of S. aureus nasal carriage

The fraction of volunteers in this study who were positive for nasal carriage of S. aureus (36-38/70; 51-54%) is higher than the 32% prevalence that was reported by the large (>10,000 subjects) US National Health and Nutrition Examination Survey conducted between 2001 and 2002 [3]. Notably, nasal S. aureus carriage clusters in families [38], and the higher prevalence observed in the present study could be explained by the fact that the volunteer group did not represent a random population sample but contained a significant number of biological relatives. Furthermore, the volunteer group for this study contained more males than females, and the S. aureus nasal carriage prevalence is higher among males than females [3]. In addition, the dry climate in Utah, where this study was conducted, is associated with higher rates of nose bleeding which is correlated with nasal S. aureus carriage [39] and appears to be directly mediated by the presence of hemoglobin [40].

Conclusions

The molecular diagnostic assay described in this study combines helicase-dependent isothermal amplification of a S. aureus species-specific DNA sequence out of nasal swab lysate with chip-based detection by hybridization and an eye-visible readout. In the present study involving nasal swabs from 70 volunteers, this molecular assay showed 100% sensitivity in identifying those individuals who are high-count nasal carriers of S. aureus (>100 CFU per swab). It is these individuals who have increased risk of infection after invasive procedures in hospitals, and therefore need to be rapidly identified and de-colonized prior to an invasive procedure. The assay concept presented here lends itself to incorporation into an automated molecular diagnostic platform for rapid identification of nasal S. aureus carriers in hospital settings.

Updated commercial PCR strategies can incorporate mecA gene amplification to mitigate the problem of false positive results in so-called “empty cassette” strains that result from incomplete SCCmec cassette excision (Arbefeville SS et al 2012. J Clin Micro 49:2996-2999).

Competing interests

All authors were employed by the company Great Basin Corporation.

Authors’ contributions

GCF wrote the manuscript, participated in the design of the study, acquisition of data, and directed the analysis and interpretation of the data. DM was responsible for data acquisition and participated in the analysis and interpretation of the data. RDJ originated the assay concept and critically revised the manuscript. BJH participated in the design and conception of the study, and critically revised the manuscript. All authors read and approved the final manuscript.
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