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Genetic assessment of *Staphylococcus aureus* in an underreported locality: Ambulatory care clinic

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ABSTRACT

Background: Staphylococcus aureus has strong association with anthropogenic environments. This association has not been well supported by use of genetic tools. The aim of this study was to phylogenetically relate numerous isolates from three environments — NCBI samples from hospitals, a community, and a previously unexplored healthcare environment: an ambulatory care clinic (ACC).
Methods: This study incorporated hospital samples from NCBI, a community database from the University of Central Florida (UCF), and newly added samples taken from employees of an ambulatory care clinic located at UCF. Samples were collected from nasal swabs of employees, and positive samples were cultured, extracted, and sequenced at seven MLST loci and one virulence locus (spa). MLST sequences were used in eBURST and TCS population structure analyses and all sequences were incorporated into a phylogenetic reconstruction of relationships.
Results: A total of 185 samples were incorporated in this study (15 NCBI sequences from hospital infections, 29 from the ACC, and 141 from the community). In both phylogenetic and population genetics analyses, samples proved to be panmixic, with samples not segregating monophyletically based on sample origin.
Conclusion: Samples isolated from ambulatory care clinics are not significantly differentiated from either community or hospital samples at the representative loci chosen. These results strengthen previous conclusions that *S. aureus* may exhibit high genetic similarity across anthropogenic environments.

Introduction

Staphylococcus aureus is a widespread human pathogen of high concern to human health globally [1]. This pathogen is most often associated with healthcare settings [2]. In addition, *S. aureus* is commonly found community sampling, where asymptomatic carriage is associated with eventual infection [3]. *S. aureus* literature delineates strains into one of two categories: hospital-acquired (HA) strains, and strains that are community acquired (CA) [4,5,6]. Phenotypic variation in pathogenesis factors of strains and the symptoms of resulting infections are suggestive of such categories [7]. Despite a strong historic focus on hospital samples, there is growing interest into the epidemiological consequences of community carriage, emphasizing the HA/CA divide [8,9,10].

Abbreviations: ACC, ambulatory care center; CA, community acquired; HA, hospital acquired; BF, Bayes Factor; MLST, multi locus sequence type; PFGE, pulse field gel electrophoresis; ST, sequence type.

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Table 1
Demographic information of participants (a) and positive donors (b) by race. Asterisks denote positive numbers that were significantly different than expected ($p \leq 0.05$).

a)

Racial categories	Females	Males	Total	Percentage
White	49	25	74	52.48
Asian Pacific Islanders	8	3	11	7.80
Black or African American	22	8	30	21.28
American Indian or Alaskan Native	0	0	0	0.00
More than one race	6	1	7	4.96
Unknown	16	3	19	13.48
Total of all subjects	101	40	141	100.00

b)

Racial categories	Females	Males	Total	Percentage
White	13	6	19	65.5
Asian Pacific Islanders	4*	0*	4	13.7
Black or African American	1*	2	3	10.3
American Indian or Alaskan Native	0	0	0	0.00
More than one race	0*	0	0	0
Unknown	0*	3*	3	10.3
Total of all subjects	18	11	29	100.00

Systematic investigations into the genetics of hospital and community isolates of *S. aureus* have failed to recover evidence to differentiate these categories [11]. Though phylogenetic investigations suggest little divergence across healthcare environments, protein expression does vary across [12,13]. Furthermore, the loci coding for certain proteins are entirely present or absent in the genomes of strains associated with one environment or the other [7]. In order to address this disconnect, further genetic investigations of *S. aureus* are necessary. Increased taxon sampling, especially from varied origins, is beneficial in refining the results of investigations of relationships between sampled organisms and may serve to resolve conflicting conclusions [14]. As the current nomenclature of *S. aureus* relationships is centered on the locality of strain isolation, additional sampling schemes encompassing novel localities may serve to clarify evolutionary relationships.

Classically, samples of *S. aureus* representing the 'clinical' group have been drawn from hospital settings [15]. Medical clinics, also known as ambulatory care centers (ACCs), are facilities that share similarities between both traditional hospital settings as well as nonclinical, community environments [16]. Little research has been conducted into pathogen assemblage within ACCs. This is especially relevant in the case of *S. aureus*, given importance of origin of isolation for categorization of isolates. Epidemiological studies into *S. aureus* within ACCs have been performed, though no phylogenetic information incorporating these centers is available [17]. Ambulatory care clinics may act as interfaces between hospitals and communities, as it has been previously demonstrated that medical attendants (such as those operating in ACCs) can bridge *S. aureus* outbreaks between distinct environments [18]. Studies that include *S. aureus* isolates from ACCs will explore previously uninvestigated environment, as well as aid in resolving confusion between HA and CA nomenclature and *S. aureus* genetics overall.

Staphylococcus aureus population structure and systematics has an extensive history. Pulse field gel electrophoresis (PFGE) results in the highest discriminatory power between *S. aureus* strains, but cannot be reliably reproduced between laboratories and across studies [19,4]. Conversely, Multi Locus Sequence Typing (MLST) produces standardized results, but a reliance on slowly evolving housekeeping genes limits discriminatory power at local spatial scales [20–24]. Attempts to find a standardized method with a high discriminatory power have incorporated virulence factors such as Staphylococcal protein A (spa) and clumping factors (clf) typing [24,25]. While *spa* typing and MLST have been used in the thorough investigation of *S. aureus* population structure within the community and hospitals [24,21], no investigation has yet sought to leverage these tools in exploring novel environments such as ACCs.

Here, we have performed an evolutionary analysis of seven MLST gene fragments and *spa*, incorporating samples taken from a representative example of previously uninvestigated ACC, with the aim of better understanding how population structure of *S. aureus* varies across environments. Isolates were taken from employees of the University of Central Florida's Health Center, a representative ACC. This sample site was additionally attractive as it was correlated to a previous community cohort, allowing us to eliminate the effects of geographic distance, which can influence the resulting phylogeny [25,10]. We confirm that neither HA or CA strains demonstrate monophyly, while additionally showing ambulatory care centers contain an admixture of samples associated with both other sources.

Material and methods

Ethics statement

Samples were collected from willing donors, under the guidance of University of Central Florida's Institutional Review Board (IRB) approved procedures. Written, informed consent was acquired from all donors prior to sampling. All investigators involved in sample collection were properly instructed and granted Collaborative Institutional Training Initiative (CITI) certification.

Bacterial isolates

One hundred and forty one healthy employees of the University of Central Florida's Health Center underwent pre-screening for bacterial isolates. Donors' volunteered demographic information is provided in Table 1. Isolates were collected via donor insertion of a cotton swab into both nostrils and circulation for approximately 5–10 s. Of the screened donors, 29 (20.5%) resulted in positive identification of *S. aureus*. Positive samples were correlated with demographic information (Table 1). Swabs were immersed in glycerol-Trypticase™ Soy Broth (TSB) solution during transport, followed by plating on Trypticase™ Soy Agar (TSA) containing 5% sheep's blood (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Isolates were incubated at 37 °C for 16 h. Resultant colonies were tested with Staphyloslide™ Latex Test (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) reagent to positively identify cultures as *S. aureus*. Verified *S. aureus* colonies were isolated and inoculated in TSB for an additional 16 h at 37 °C at 250 rpm in preparation for DNA extraction.

DNA extraction and sequence analysis

1.5 mL of each bacterial inoculate was centrifuged at 16,000 g for two minutes. Supernatant was removed, and the remaining pellet was utilized in the extraction protocol. DNA was extracted utilizing GenElute Bacterial Genomic DNA kits (Sigma–Aldrich Co., St. Louis, Missouri), in accordance with manufacturer's instructions. Fragments of seven MLST loci (*arc, aroE, glpF, gmk, pta, tpi, yqiL)* ranging from 402 to 512 base pairs were amplified [21]. Additionally, approximately 500 base pair fragments of *spa* were amplified [24].

Following amplification, PCR products were purified utilizing QiaQuick PCR Purification Kit (QiaGen, Redwood City, Ca). All Sanger Sequencing was performed at University of Arizona's Genetics Core. Forward and reverse reads were visualized with Sequencher 5.1 (Gene Codes Co., Ann Harbor, Michigan). Sequences were organized in MEGA 5.2 and aligned with ClustalW. Sequence Types (STs) were determined for each sample based on alleles identified for each of the seven MLST loci. Alleles were cross-referenced

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Table 2
Partition of gene fragments and codon positions, with associated evolutionary models. Models include Felsenstein 1981 (F81), Hasegawa, Kishino, and Yano 1985 (HKY) and Generalised time-reversible (GTR, Tavare 1986). Variable site distributions are equal unless denoted as invariant (I) or Gamma (G).

Gene fragment	CP1	CP2	CP3
arcC	HKY + I + G	F81 + I	HKY + G
aroE	HKY + I + G	F81 + I	HKY + I + G
glpF	HKY + I + G	F81 + I	HKY + I + G
gmk	HKY + I + G	F81 + I	HKY + I + G
pta	HKY + I + G	F81 + I	HKY + G
tpi	HKY + I + G	F81 + I	HKY + I + G
yqiL	HKY + I + G	F81 + I	HKY + I + G
spa	GTR + I	HKY + I	HKY + I

against the *S. aureus* database curated at MLST.net. Novel alleles – or novel combinations of known alleles – were submitted to the MLST database for curation, whereupon new allele designation and STs were obtained.

Phylogenetic reconstruction

In order to infer the relationships between samples of hospital, community, and ambulatory care center origins, phylogenetic analysis was performed on a concatenated dataset of all eight sequenced loci for all samples. Datasets representing community samples were constructed utilizing 141 samples from a previous study at the University of Central Florida [25]. Gene sequences from strains associated with hospital infection were also included in this analysis. The previously sequenced hospital strains were N315, Mu50, COL, MRSA252, MSSA476, MW2, USA300_FPR3757, NCTC8325, JH1, JH9, Newman, Mu3, USA300_TCH1516, 04-02981, and TW20. The concatenated dataset of all samples was partitioned by gene fragment and codon position, with models of evolution being assigned by Akaike Information Criterion (AIC) within PartitionFinderv1.1.1 [26, Table 2]. Once partitioned, phylogenetic reconstruction of the data was performed using Metropolis-Hastings Coupled Markovnikov Chain Monte Carlo in BEAST v1.8 [27]. Two independent Bayesian Inference runs were carried out using random starting trees. After five million iterations, runs were terminated and visualized utilizing Tacer v1.5 [28]. The most-likely tree was summed utilizing TreeAnnotatorv1.8.2 and visualized with FigTreev1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

In order to test competing hypotheses against the most likely tree, Bayes Factor Topography Testing of a number of alternative tree topographies (Fig. 1) was performed in BEAST. Samples within the phylogenetic reconstruction were forced into monophyly utilizing BEAST's unbreakable priors. Summarized phylogenies were reconstructed for our dataset when all taxa were constrained to be monophyletic by these priors based on their origins (i.e., hospital, clinical, or community). The log marginal likelihood of both the unconstrained, maximum-likelihood tree (B_0) and the constrained hypotheses trees (B_A) were calculated using two methodologies in order to compare results: Bayesian Stepping Stone and Path Sampling. Bayes Factors (BFs) for alternative hypotheses were calculated as $BF = \frac{P(D|M_0)}{P(D|M_A)}$ where $P(D|M_x)$ is the marginal likelihood of model x — essentially a ratio between marginal likelihoods of competing models.

Population structure: MLST data

Sequence Types were grouped based on clusters sharing six of seven MLST alleles in common with at least one other member of the cluster to account for bacterial recombination. Clustering was performed in eBURSTv3 [29], which provides for a query set of clinical samples to be tested against the reference set of community and hospital samples. Bootstrap support for clusters were acquired after 1000 resampled iterations.

In order to visualize population structure at MLST loci without masking recombination at a single locus, PopART (http://popart.otago.ac.nz) was utilized to produce a minimum spanning network of all mutational steps.

Results

Multilocus sequence typing demonstrates similarity between isolates of varying origins

MLST analysis of the 29 samples isolated from our sampling scheme within the UCF Health center identified 10 different sequence types, one of which was new. Of these 10 sequence types, 5 were previously recovered in a sampling of the adjacent community. Of the remaining 5 samples, 4 were previously identified as hospital-associated strains (105, 109, 20, 1) and 1 was novel. Overall, carriage rate within the ACC cohort was 20.6%. $\chi 2$ tests of demographic information indicated that carriage was not evenly distributed across groups (Table 1). Notably, carriage was 10% for non-Hispanic black donors. This difference was predominately driven by a low carriage rate of 4.5% by non-Hispanic black females. Non-Hispanic black males had a carriage rate of 25%. Asian/Pacific Islanders had a significantly higher than average carriage rate at 36%. This result was driven by females within this group (50%). No tested male Asian/Pacific Islander was positive for *S. aureus* carriage. Neither age of donor nor length of employment were significant predictors of carriage, though length of employment did approach significance ($p = 0.07$, Fig. 2), the result of an inverse correlation between extensive employment within the health center and likelihood of carriage (Fig. 2).

Of these 10 sequence types, the most prevalent was ST30, accounting for 9 of 29 isolates (31%). Sequence types 5 and 45 were the second and third most commonly identified sequence types following ST30, with 6 (21%) and 4 (14%) instances respectively. These results are roughly comparable with the prevalence of prominent sequence types in the prior UCF sampling [25].

Phylogenetic reconstruction of MLST data shows lack of differentiation between taxa

Bayesian analyses of MLST loci were conducted in order to establish any signal of divergence existing between isolates of *S. aureus* originating from hospital, community, and ambulatory clinics. The genetic similarities of our samples of clinic, hospital, or community origin evidenced itself in the most likely phylogenetic tree (Fig. 3). In the unconstrained tree, no monophyly between samples of ACC, hospital, or community origin were recovered; clades containing combinations of all three origins were recovered with strong nodal support. Investigation into the demographic information supplied by donors did not identify a causal relationship to explain these patterns.

In order to compare the credibility of this result with competing hypotheses, Bayesian Hypothesis Testing was performed. Calculations for BFs were adopted from Raftery [30]. Interpretation of BF were informed by Posada and Buckley [31], stating that BF exceeding 150 are considered very strong indication of a less likely tree, with those falling between 12 and 150 were strong, and those between 3 and 12 were positively indicative. Bayes Factors below this threshold were considered insignificant. Based on this interpretation of BF, all trees representing competing hypotheses were significantly worse at representing the data than the most likely, unconstrained tree (Table 3).

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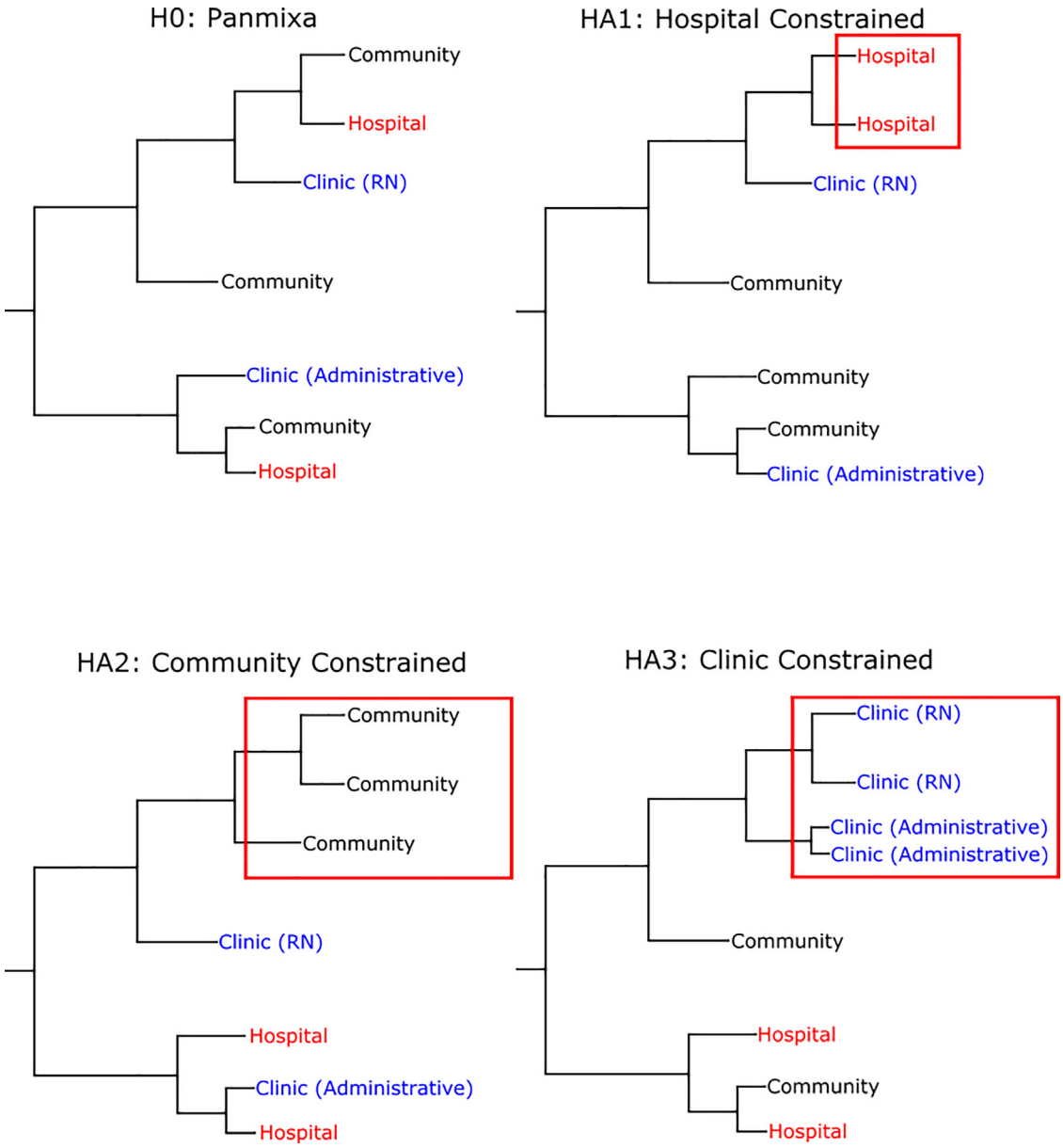

Fig. 1. Schematic representation of all competing hypotheses as calculated utilizing Bayes Factor testing. The null hypothesis of free exchange across environments is represented by H_0. This hypothesis serves as the unconstrained reference tree that constrained alternative hypotheses are tested against. Taxa not contained within boxes are not constrained in respect to each other.

Table 3
Bayesian Factor hypotheses testing of various competing hypotheses. Constrained hypotheses were tested against an unconstrained topology. The unweighted hypothesis was tested and was outperformed by a reconstruction weighted more heavily for MLST loci. Bayesian factors exceeding a value of 150 are considered decisively significant — those exceeding 12 are strongly significant. Higher values indicate less likely hypotheses.

Hypothesis	BF (stepping stone)	BF (path sampling)
Constrained hospital	399	400
Constrained community	862	864
Constrained clinical	852	854
Constrained profession	163	163
Unweighted	128	115

As this investigation was also interested in determining the relative contribution of genetic markers of varied origin (i.e., housekeeping MLST vs variable repeat regions in *spa*) on our reconstruction, additional phylogenies were constructed, weighting the conserved genes more heavily than the hypervariable *spa* locus. Weights were conveyed based on the mean genetic distances between loci. Average genetic distance between all seven MLST loci was 0.6%; between *spa* alleles, comparatively, there was a mean genetic distance of 4.7%. The marginal likelihood of the resulting weighted phylogeny was compared against the unconstrained tree (Table 3). The weighted tree vastly outperformed the unweighted tree in our BF hypothesis testing framework.

MLST eBURST produces clusters containing S. aureus *from all sample categories*

MLST eBURST clustered our samples into 10 distinct groups, with 13 remaining singletons that were not assigned to a cluster (Fig. 4). These singleton STs were most often represented by a single isolate, though ST 20 was not clustered, and contained two individual isolates from our ACC subset. Of our clusters, three (3,

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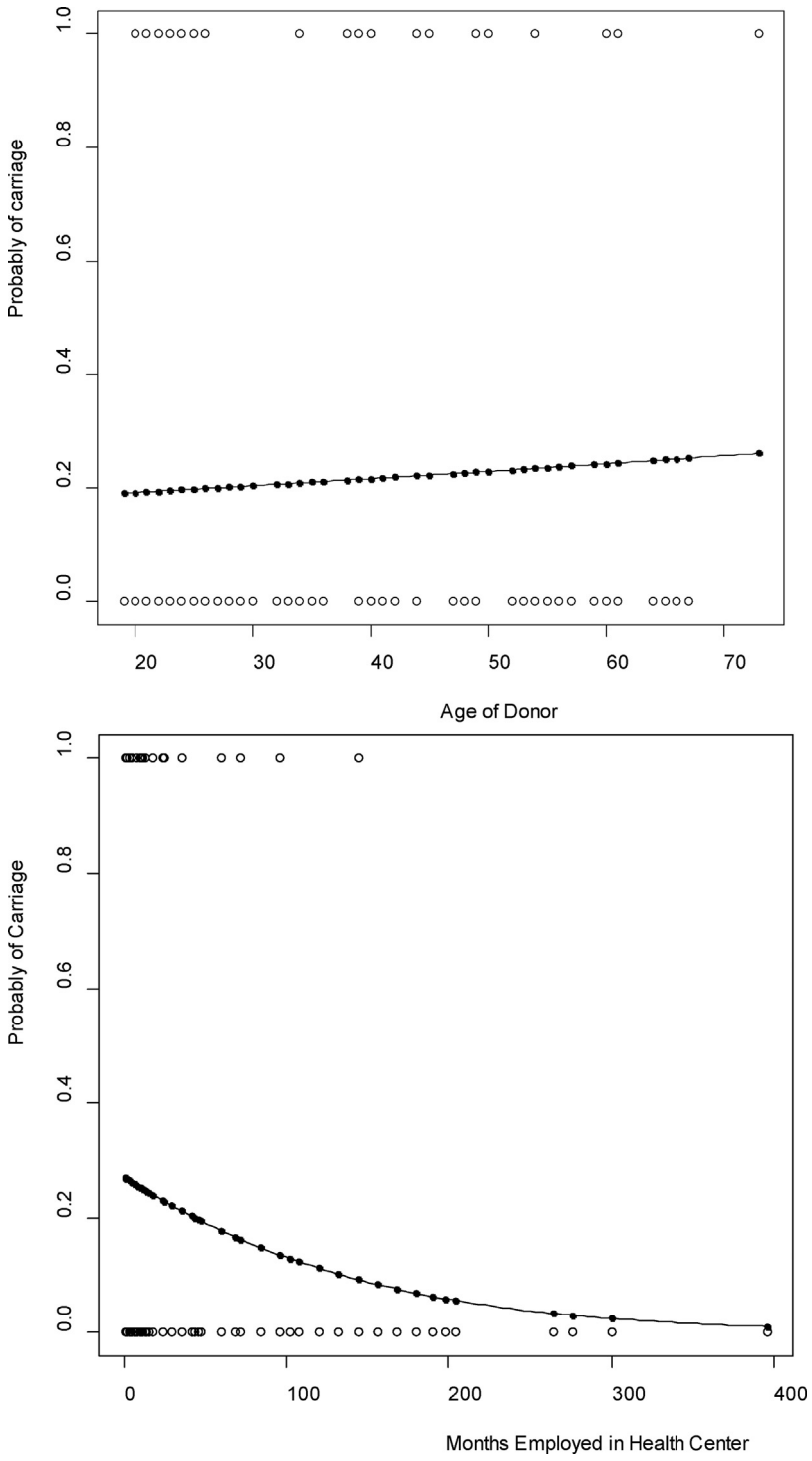

Fig. 2. Logistic regressions of donor characteristics as a predictor for carriage.

7, 9) contained only community samples. All other clusters contained an admixture of all categories of samples. Groups 2 and 8 were founded by clinical STs, with the remaining clusters founded by shared sequence types.

In order to demonstrate inferred haplotypes and interconnect clusters, PopArt was utilized on MLST data in order to build a minimum spanning network (Fig. 5). Unlike the clustering method used in eBURST, a minimum spanning network will visualize relationships between all isolates in the dataset regardless of genetic distance.

Discussion

It was the goal of this study to examine the evolutionary trajectory, relatedness and genetic structure of *S. aureus* in a previously unstudied environment in comparison to previously assessed cohorts. The resulting phylogeny was comprised of numerous polytomies and clades containing isolates from all three environmental origins, suggesting recent common ancestors and low genetic differentiation between putative groups. In all cases, competing hypotheses were found to be conclusively less likely to represent

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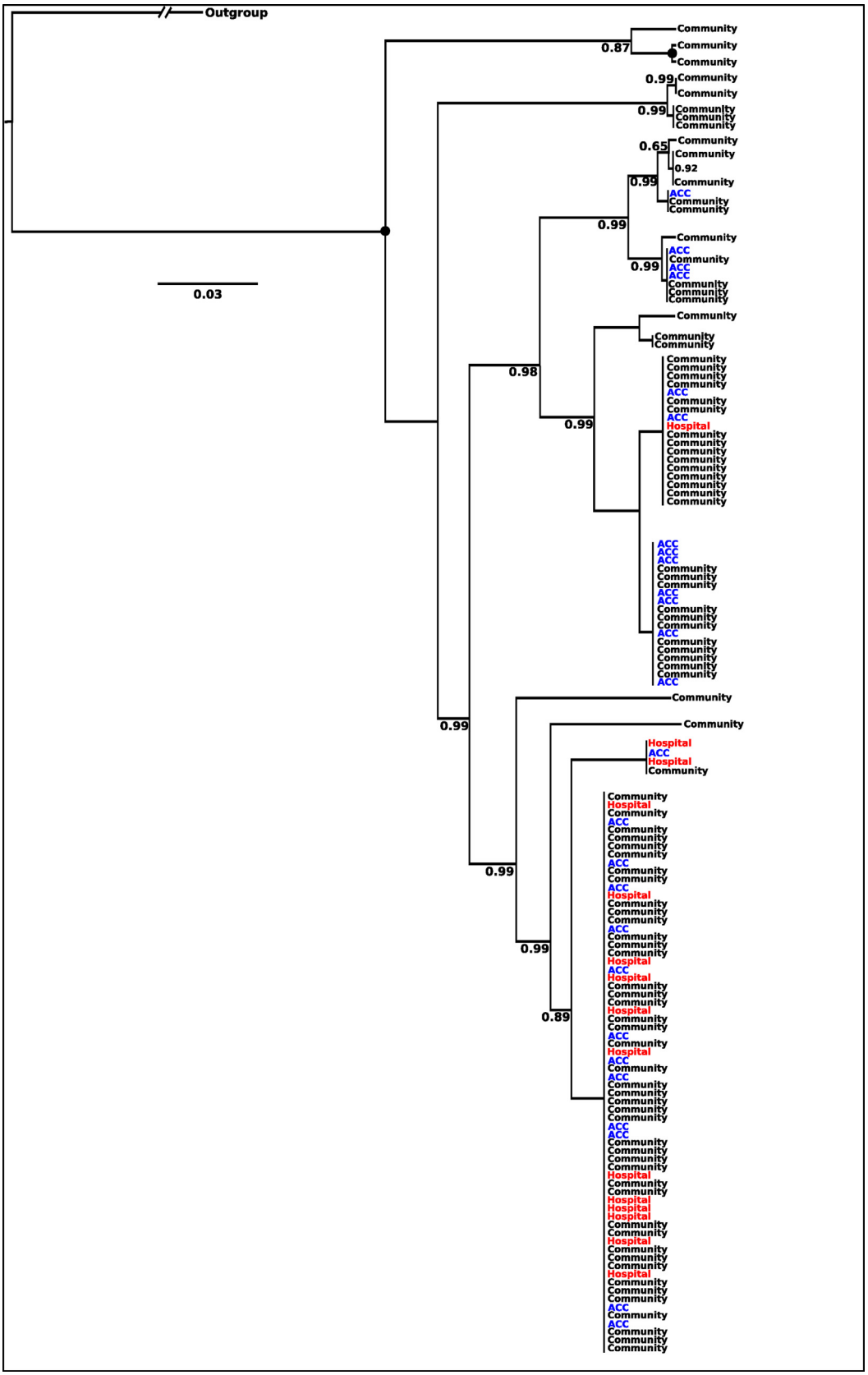

Fig. 3. Most likely unweighted phylogenetic tree. Red taxa labels indicate NCBI hospital samples, blue taxon labels correspond to ACC samples, and black taxon to community samples. Solid circles on nodes indicate posterior probability values of 100.

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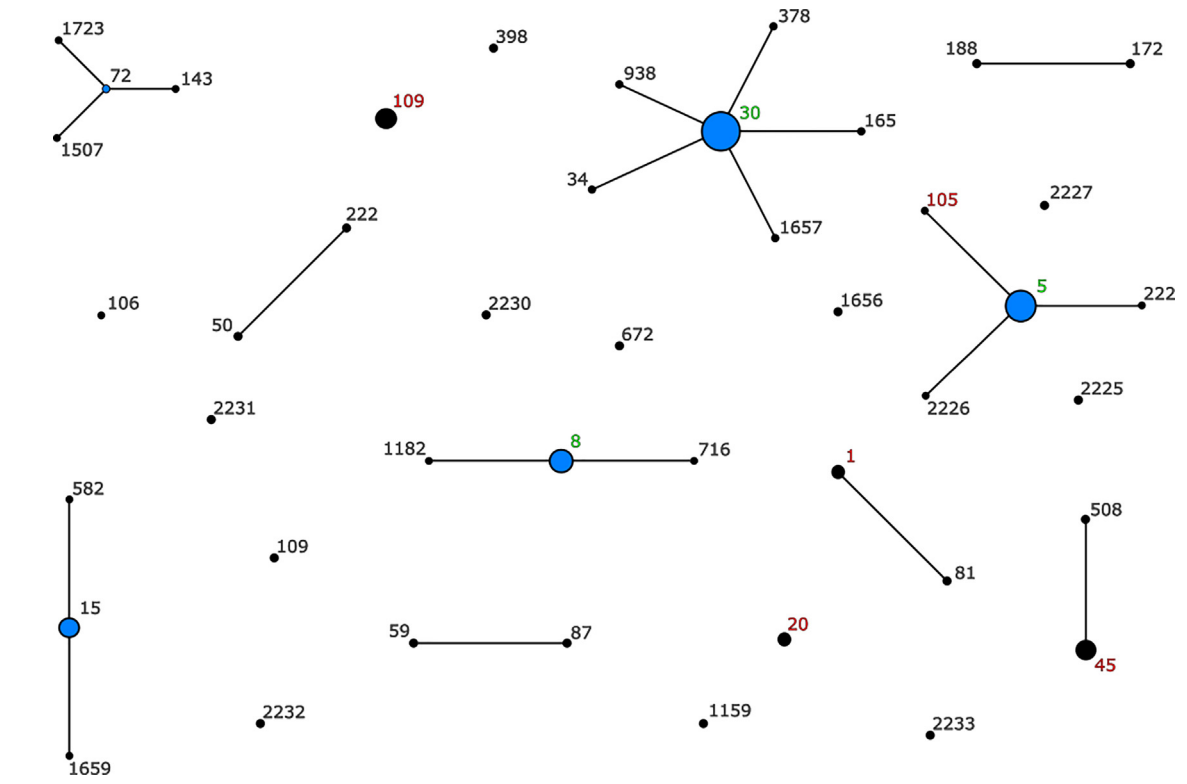

Fig. 4. MLST eBURST of samples. Blue circles represent projected founder strains. Green labels represent strains shared between ACC and community cohorts. Black labels are community only, and red are clinical only. Size of circles are proportional to sampled number of isolates conforming to that strain.

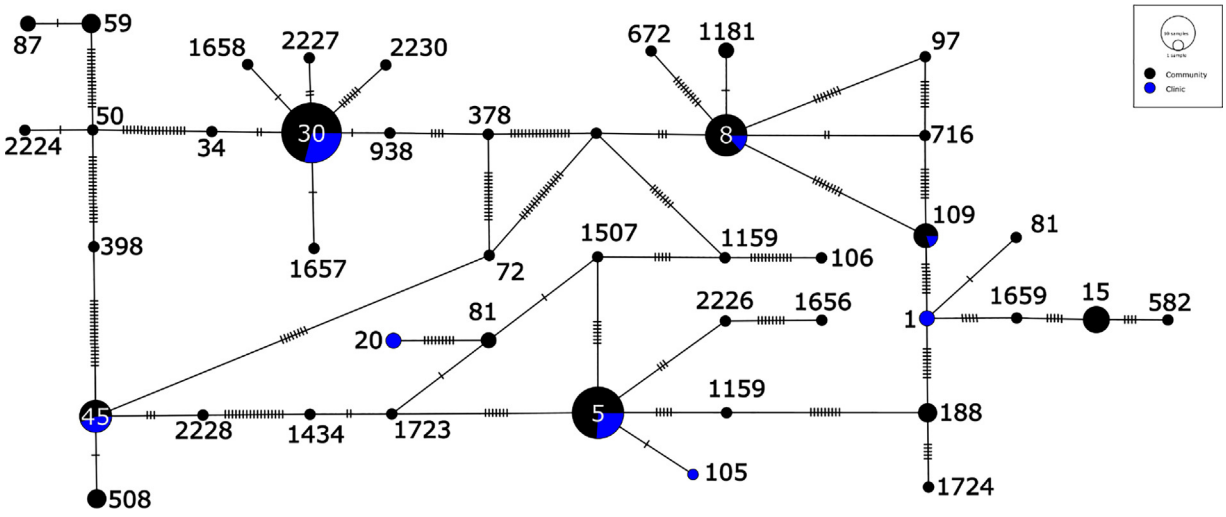

Fig. 5. Minimal Spanning network of MLST data. Dashes represent inferred haplotypes not recovered in this sampling scheme. Numerical labels indicate the STs of isolates. Size of haplotype circle schematics are proportional to number of isolates recovered of that ST. Coloured wedges indicate proportion of ST owed to each sampled origin.

true relationships, including a hypothesis that represents the colloquial community vs. hospital divide.

Phylogenetic analyses perform best when moderately variable sequences are utilized to build homology statements between pairs of taxa. In instances such as this study, where genetic differentiation is very low, posterior support values tend to suffer. This lack of variation in MLST loci is attributed to their role as housekeeping loci that encode for proteins necessary for *S. aureus* viability, most commonly metabolic enzymes. In our cohort, the average genetic distance between MLST loci was less than a single percentage point (0.6%). This tendency has been noted in previous phylogenetic reconstructions incorporating these loci, including the study that generated our reference community cohorts [10]. The lack of variation in these loci and the resulting potential loss of confidence in analyses in studies at the local scale has been known since the inception of these sites as markers [21]. Efforts have been made to design markers that retain MLST's ability to be exported between labs without sacrificing variability [24,25]. Our inclusion of *spa* in this study is the first phylogenetic reconstruction to utilize this region, and doing so increased the variability across our cohort, as demonstrated by its mean genetic distance of 4.7%. We nonetheless recovered low posterior values at the tips of our tree, resulting in broad polytomies. This variation in our markers also demonstrated itself in the BF we recovered for our weighted tree.

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By informing the reconstruction to weigh autapomorphies at MLST loci more heavily than those in *spa*, we recovered a more likely tree than the unweighted phylogeny alone. This tree mainly increased posterior probabilities at deep nodes; however, and the polytomies at the tips of the tree were still unresolved.

In light of these polytomies, we utilized eBURST algorithms to better associate closely related isolates into clusters. eBURST's model is informed by the fact that bacterial genomes, including *S. aureus,* are subject to recombination events that may result in bacterial isolates differing from their nearest relatives by large portions of a single loci [32]. Contrasting to the large polytomies and low posterior support values of our phylogeny, eBURST clustering resulted in our samples being incorporated in 10 clusters, minus differentiated singletons.

Though eBURST accounts for recombination, it has been shown that *S. aureus* is largely clonal, with variation arising frequently due to point mutation rather than recombination [29]. In order to determine the mutational steps between clusters of isolates, a minimum spanning network utilizing maximum parsimony was performed. Our minimum spanning network largely establishes the same genetic clusters recovered in the eBURST analysis. However, singletons in the network are generally numerous steps away from the nearest clustered group at more than one loci, with a number of equally likely mutational paths likely for their emergence. As it would be unlikely that numerous inferred haplotypes would be missed by both our and previous studies' sampling methods, it is likely that these haplotypes did not directly arise from any of our recovered clusters, and instead represent independent foundation events.

Ultimately, phylogenetic analyses, eBURST clustering, and minimum spanning haplotype networking of our samples all support the conclusion that *S. aureus* nasal isolates taken from ACC employees are not significantly isolated from isolates extracted both from healthy community carriers or hospitalized individuals. This additionally supports the previous findings which assert that nasal carriage community strains were not themselves differentiated from pathogenic hospital isolates [32,33,25] and underscores a lack of differentiation between groupings of *S. aureus* based on locality of sampling.

Our study focused on isolates derived from healthcare employees. The role of healthcare attendants has been routinely investigated for many decades [34]. Comparatively, healthy attendants are more resistant to disease than the sick or immunocompromised patients they care for [34]. Hospital-associated strains of pathogens asymptomatically flourish on the hands of attendants, and have been shown to transfer to new susceptible hosts during care [35]. As predicted by transmission-rate and optimal virulence hypotheses, hospital-associated strains shuttled by attendants demonstrate above average virulence and worsened patient outcomes, emphasizing the need for adequate surveillance [36]. *S. aureus* demonstrated this when a highly virulent outbreak strain bridged a deep-sterilization of an infant ward by sequestering asymptomatically on an attendant before returning to infect neonates post-sterilization [18]. Healthcare assistants operating within ACCs are often recently or simultaneously employed at hospitals, and may potentially bridge *S. aureus* in a similar fashion within ACCs. The fact that we recovered STs identical to HA strains in our clinical cohort implies that these strains have been introduced into our ACC. As patients were not surveyed as part of this study, we cannot definitively conclude that these HA strains were bridged into the community by attendants. However, their presence in our dataset highlights the urgent need for surveillance within ACCs in order to monitor and track HA strains interfacing with community patients in this environment.

Conclusions

It is cleary based on the results of our study that samples of *S. aureus* taken from medical clinics are not significantly differentiated from either hospital or community samples. Therefore, we conclude that medical environments – specifically clinics – may not serve to generate uniquely adapted lineages. Moreover, our study has reiterated recent findings that *S. aureus* may be relatively homogenous genetically across a wide expanse of anthropogenic environments [25]. When describing strain relationships from an evolutionary context in this species, these designations are clearly not sufficient in representing the pattern of relatedness among *S. aureus* isolates. Additionally, recovery of sequence types from within our medical clinic survey demonstrating identical homology to hospital associated strains indicates that ACCs may serve as an under-monitored interface between hospital and community environments.

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Competing interests

None declared.

Ethical approval

The work was approved by the University of Central Florida IRB #BIO-06003582.

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