The impact of *Staphylococcus aureus* genomic variation on clinical phenotype of children with acute hematogenous osteomyelitis

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Abstract

**Background:** Children with acute hematogenous osteomyelitis (AHO) have a broad spectrum of illness ranging from mild to severe. The purpose of this study is to evaluate the impact of genomic variation of *Staphylococcus aureus* on clinical phenotype of affected children and determine which virulence genes correlate with severity of illness.

**Methods:** De novo whole genome sequencing was conducted for a strain of Community Acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA), using PacBio Hierarchical Genome Assembly Process (HGAP) from 6 Single Molecule Real Time (SMRT) Cells, as a reference for DNA library assembly of 71 *Staphylococcus aureus* isolates from children with AHO. Virulence gene annotation was based on exhaustive literature review and genomic data in NCBI for *Staphylococcus aureus*. Clinical phenotype was assessed using a validated...
severity score. Kruskal-Wallis rank sum test determined association between clinical severity and virulence gene presence using False Discovery Rate (FDR), significance <0.01.

Results: PacBio produced an assembled genome of 2,898,306 bp and 2054 Open Reading Frames (ORFs). Annotation confirmed 201 virulence genes. Statistical analysis of gene presence by clinical severity found 40 genes significantly associated with severity of illness (FDR ≤0.009). MRSA isolates encoded a significantly greater number of virulence genes than did MSSA (p < 0.0001). Phylogenetic analysis by maximum likelihood (PAML) demonstrated the relatedness of genomic distance to clinical phenotype.

Conclusions: The *Staphylococcus aureus* genome contains virulence genes which are significantly associated with severity of illness in children with osteomyelitis. This study introduces a novel reference strain and detailed annotation of *Staphylococcus aureus* virulence genes. While this study does not address bacterial gene expression, a platform is created for future transcriptome investigations to elucidate the complex mechanisms involved in childhood osteomyelitis.

Keywords: Microbiology, Pediatrics, Pathology, Infectious disease, Genetics, Immunology

1. Introduction

Community acquired *Staphylococcus aureus* is the leading cause of acute hematogenous osteomyelitis (AHO) among children worldwide [1, 2, 3]. There is substantial variation in clinical presentation and response to treatment of affected children ranging in spectrum from mild to severe [3, 4, 5, 6, 7]. Some children require only a short course of antibiotics and brief hospitalization without the need for surgery, whereas others require intensive care, undergo multiple surgical procedures, experience prolonged hospitalization, and receive long-term antibiotic therapy [7, 8]. Differences in bacterial virulence behavior may contribute to this variation in clinical presentation among these otherwise healthy, immunologically competent children [9, 10, 11, 12].

Previous studies have attempted to elucidate the pathogenetic mechanisms of *Staphylococcus aureus* virulence based on a variety of candidate genes, in isolation or combination, which may play a role in disease pathogenesis [13, 14, 15, 16, 17, 18, 19]. The advent of next generation sequencing allows for a greater discriminatory power to aid in this investigation, however, there are current limitations posed by the lack of a suitable reference strain for DNA library assembly. The majority of clinical isolates for which whole genome sequencing data is publicly available in the GenBank database of the National Center for Biotechnology Information (NCBI)
have been isolated from skin and soft tissue infections or from adults [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31]. Of the two reference strains which are commonly used for USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) research, TCH1516 and FPR3757, neither are ideal to study AHO [32, 33]. TCH1516 was obtained from an adolescent described as having “severe sepsis syndrome” without osteomyelitis [32]. FPR3757 was isolated from the wrist abscess of a 36-year-old HIV-positive white male with a history of injection drug use from San Francisco, California [33]. In a recent study of *Staphylococcus aureus* isolates obtained from children with AHO within a single community, phylogenetic analysis utilizing TCH1516 and FPR3757 as reference strains demonstrated substantial genetic distance of the all study isolates from either reference strain [9].

The purpose of this study is to evaluate the impact of genomic variation of *Staphylococcus aureus* on clinical phenotype of affected children and determine which virulence genes correlate with severity of illness. A secondary purpose is to carefully annotate candidate virulence genes within the *Staphylococcus aureus* genome following de novo assembly of the whole genome of a novel isolate of MRSA which caused severe AHO in a child in the Southwestern United States.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center Dallas and Children’s Medical Center of Dallas (IRB# STU 032013-022).

2.2. Clinical phenotype of the child from whom the reference strain was isolated

UTSW55 was isolated from an otherwise healthy 12 year old female with osteomyelitis of the right proximal femur, septic arthritis of the right hip, and adjacent pyomyositis. She had sustained a recent minor fall which was followed by fever and pain in the hip region. She was hospitalized in septic shock with disseminated infection (bacteremia, deep venous thrombosis of the right iliac vein, septic pulmonary emboli, pneumonia and a urinary tract infection). She had eight consecutive days of positive blood cultures. Cultures were positive for clindamycin-sensitive MRSA. She was treated with five days of inotropic support, two weeks of mechanical ventilation and five surgical procedures. Her initial hospitalization was 51 days, 19 of which were intensive care. A total hip replacement was ultimately performed due to avascular necrosis, chondrolysis, and a pathologic femoral neck fracture. Severity of illness scored (Table 1) as 10 out of 10 (average 2.9, standard deviation...
Health Information Management (HIM) scoring methods determined a Medicare Severity Diagnosis Related Group (MS DRG) score of 5.4 (average 1.7, range 0.7\textendash}5.5, standard deviation 0.9); All Patient Refined Diagnosis Related Groups Case Mix Index (APR-DRG CMI) of 5.8 (average 1.7, range 0.6\textendash}8.3, standard deviation 1.4); Severity of Illness (SOI) score of 4.0 (average 2.3, range 1.0\textendash}4.0, standard deviation 0.7); and Risk of Mortality (ROM) score of 4.0 (average 1.3, range 1.0\textendash}4.0, standard deviation 0.8).

2.3. Study population

Previously healthy children birth to 18 years who were admitted and confirmed to have AHO due to methicillin-sensitive or methicillin-resistant *Staphylococcus aureus* (MSSA or MRSA, respectively) between January 1, 2009 and March 1, 2014 were retrospectively studied. AHO was defined as infection diagnosed within 2 weeks of the onset of symptoms. Diagnosis was confirmed by magnetic resonance imaging (MRI), positive cultures, and histopathology of bone obtained from the site of infection. Bacterial isolates from infected bone were obtained under sterile conditions in the operating room, plated on sheep blood agar, and incubated at 37°C. Bacterial isolates were then catalogued and stored at –80 °C in the microbiology lab. Excluded from the study were children with underlying medical conditions associated with immunocompromise, children with AHO caused by any other bacterial organism, culture-negative cases, and children with history of surgery or penetrating inoculation as the cause of infection.

### Table 1. Modified severity of illness scoring system for AHO [5, 6].

| Scoring parameter                          | Criteria                  | Points | Total |
|--------------------------------------------|---------------------------|--------|-------|
| Initial CRP (mg/dL)                        | \(>15\)                   | 2      | 0\textendash}2 |
|                                            | 10 to 15                  | 1      |       |
|                                            | \(<10\)                   | 0      |       |
| CRP hospital day 4\textendash}5 (mg/dL)   | \(>10\)                   | 2      | 0\textendash}2 |
|                                            | 5 to 10                   | 1      |       |
|                                            | \(<5\)                    | 0      |       |
| CRP hospital day 2\textendash}3 (mg/dL)   | \(>10\)                   | 2      | 0\textendash}2 |
|                                            | 5 to 10                   | 1      |       |
|                                            | \(<5\)                    | 0      |       |
| Band percentage of WBC                     | \(\geq1.5\%)              | 1      | 0\textendash}1 |
|                                            | \(<1.5\%)                 | 0      |       |
| Febrile days on antibiotic                | \(\geq2\)                 | 1      | 0\textendash}1 |
|                                            | \(<2\)                    | 0      |       |
| ICU admission                              | Yes                        | 1      | 0\textendash}1 |
|                                            | No                         | 0      |       |
| Disseminated disease\(^a\)                | Yes                        | 1      | 0\textendash}1 |
|                                            | No                         | 0      |       |
| Total severity of illness score            |                           |        | 0\textendash}10 |

\(^a\)DVT, septic pulmonary embolism, pneumonia, endocarditis, multi-focal infection.
2.4. Clinical data

Clinical and laboratory data were collected to calculate severity of illness for each child using the previously validated method based on CRP values (mg/dL) on admission, at 48 hours, and at 96 hours; febrile days on antibiotics; band percentage of the white blood cell count (WBC); ICU admission; and evidence of disseminated disease (multi-focal infection, deep venous thrombosis, septic pulmonary embolism, pneumonia, or endocarditis) (see Table 1) [5, 6]. Tables 2a, 2b and 3 provide relevant details of the clinical disease of the affected children, aggregated into groups according to severity of illness and antibiotic resistance of the causative organism.

2.5. Specimen processing and PacBio of UTSW55

A frozen specimen of UTSW55 was thawed and inoculated on blood-agar. Isolated colonies were grown overnight in Brain Heart Infusion (BHI) medium at 37 °C in nephlo flasks. Genomic DNA was extracted with DNeasy blood and tissue kits (Qiagen, Germantown, MD) with lysostaphin replacing lysozyme. Quality of the genomic DNA was assessed on 1% agarose gels and measured with the use of Picogreen. A total of 4.4 mg DNA was sent to the sequencing service at the University of Washington (https://pacbio.gs.washington.edu/). De novo whole genome sequencing and assembly was conducted for this isolate using the PacBio Hierarchical Genome Assembly Process (HGAP) from 6 Single Molecule Real Time Sequencing (SMRT) Cells.

For all other isolates, frozen specimens were thawed and inoculated on blood-agar at 37° for 24 hours. Isolated colonies were transferred to enriched Mueller Hinton Broth Media overnight. DNeasy Blood and tissue kits (Qiagen) were used for DNA extraction, with lysostaphin replacing lysozyme.

2.6. Next generation sequencing of clinical isolates

A range of 2–5 ug of isolated genomic DNA, quantified by Picogreen, was submitted to the UT Southwestern Genomics Core (see http://genomics.swmed.edu/) from each study isolate for sequence analysis. Sequencing libraries were produced using TruSeq adaptors (Illumina, Inc., San Diego, CA) with inserts of 300–500 bp in length. DNA quality was analyzed with a Bioanalyzer (Agilent technologies). Sequencing was performed on an Illumina HiSeq 2000 using a paired-end protocol in which 100 bp sequences were obtained from each end of the sequencing library fragments. An average of 6.9 million reads was obtained for the study samples, for an average of 243-fold coverage of the Staphylococcus aureus genome. Sequencing protocols and basic information concerning the sequence generation are available from the UT Southwestern Genomics Core website.
Table 2a. Comparisons of clinical isolates (Grouped by severity of illness and antibiotic resistance) according to clinical parameters of the affected children.

| Variable                          | Summary statistics |
|-----------------------------------|--------------------|
|                                  | 1 (MSSA 0−5)       | 2 (MSSA 6−10)     | 3 (MRSA 0−5)       | 4 (MRSA 6−10)       |
| N                                 | Mean ± std, med (range) | N Mean ± std, med (range) | N Mean ± std, med (range) | N Mean ± std, med (range) |
| LOS (days)                        | 20  5.72 ± 3.22, 4.85 (2, 16.54) | 7  14.16 ± 7.18, 10.08 (8, 27) | 13  6.34 ± 2.03, 7 (2.43, 8.64) | 30  22.63 ± 11.14, 21.03 (7.75, 51) |
| TLOS                              | 20  5.87 ± 3.11, 5 (2.69, 16.54) | 7  14.3 ± 7.35, 10.08 (8, 27) | 13  6.34 ± 2.03, 7 (2.43, 8.64) | 30  24.39 ± 14.22, 21.03 (7.75, 51) |
| ICU LOS                           | 20  0.15 ± 0.67, 0 (0, 3) | 7  1.14 ± 1.57, 0 (0, 4) | 13  0 ± 0, 0 (0, 0) | 30  5.8 ± 7.12, 1.5 (0, 22) |
| Days of persistent bacteremia     | 20  0.65 ± 0.99, 0 (0, 4) | 7  3.14 ± 1.86, 4 (1, 6) | 13  1.08 ± 1.04, 1 (0, 3) | 30  3.83 ± 3.24, 3 (0, 14) |
| CRP initial (mg/dL)               | 20  6.42 ± 5.48, 4.58 (0.68, 21.9) | 7  25.87 ± 8.08, 24.2 (14.5, 38.2) | 13  8.13 ± 5.53, 7 (0.1, 16.2) | 30  25.38 ± 10.17, 23 (9.8, 55.6) |
| CRP 48 hours (mg/dL)              | 20  5 ± 2.84, 4.74 (0.88, 13) | 7  22.63 ± 7.32, 21.9 (10.5, 33) | 13  5.82 ± 3.26, 5.1 (0.1, 10.2) | 30  23.63 ± 10.76, 20.55 (11.8, 67.7) |
| CRP 96 hours (mg/dL)              | 20  2.05 ± 1.69, 2 (0, 7.2) | 7  16.49 ± 8.28, 16 (6.3, 32.9) | 13  2.91 ± 2.14, 2.6 (0, 7) | 30  19.63 ± 9.39, 19.9 (2.5, 39.7) |
| Band % of white blood cell count  | 20  1.42 ± 2.83, 0 (0, 12) | 7  14.06 ± 7.56, 12.2 (2.5, 27) | 13  2.64 ± 5.17, 0 (0, 17.4) | 30  14.38 ± 15.73, 10.6 (0, 64) |
| Febrile days on Antibiotic        | 20  1.0 ± 1.34, 0.5 (0, 5) | 7  2.29 ± 0.76, 2 (1, 3) | 13  0.54 ± 0.88, 0 (0, 3) | 30  7.93 ± 7.64, 6 (0, 41) |
| Number of surgeries               | 20  0.85 ± 0.59, 1 (0, 2) | 7  1.43 ± 0.79, 2 (0, 2) | 13  1 ± 0.71, 1 (0, 2) | 30  2.37 ± 1.43, 2 (0, 6) |
| Days until CRP <2.0 mg/dL         | 20  5.25 ± 3.08, 5 (0, 11) | 7  14.0 ± 5, 14 (7, 20) | 13  6.31 ± 3.15, 6 (0, 14) | 30  24.93 ± 24.99, 17.5 (8, 141) |
| Antibiotic duration (days)        | 20  38.35 ± 13.76, 34.5 (20, 74) | 7  63.29 ± 40.65, 48 (28, 138) | 13  53 ± 33.11, 42 (28, 151) | 30  73.03 ± 52.73, 48.5 (21, 265) |
| SIS                               | 20  1.45 ± 1.47, 1 (0, 5) | 7  8.0 ± 1.15, 8 (6, 9) | 13  1.77 ± 1.48, 2 (0, 4) | 30  8.33 ± 1.49, 8 (4, 10) |

One-way ANOVA was used to compare 4 groups, followed by post-hoc Tukey (which accounts for multiple comparisons). Likewise, a nonparametric Kruskal Wallis test was used to compare 4 groups.

LOS (length of stay); TLOS (total length of stay including readmission days); ICU LOS (length of intensive care stay); CRP (C-Reactive Protein); SIS (severity of illness score).
2.7. Genome assembly and gene prediction

Genome assembly for the isolates was performed using the A5-miseq microbial genomic assembly pipeline specifically designed for Illumina sequencing data [34]. The assembled isolates had an average contig count of 48 with an N50 length of 607,387. Assembled genomes were then run through the gene prediction tool Glimmer3 (https://ccb.jhu.edu/software/glimmer/) before annotation.

2.8. Gene presence and annotation

To assess the presence of genes across *Staphylococcus aureus* isolates, reference sequences for genes were retrieved from the NCBI Gene database (https://www.ncbi.nlm.nih.gov/gene) using the taxonomy filter for *Staphylococcus aureus* (ID: 1280). Exhaustive literature review was performed to identify a list of candidate virulence genes. The predicted genes were then aligned to the reference database using the blastp functionality within the DIAMOND program [35]. Due to various levels of sequence similarity between genes, only sequence alignment results with similarity identity scores greater than 0.8 were selected as positive matches. No other filtering criterion was applied to determine presence or absence of gene for a particular isolate. Despite the presence of multiple alternate gene names within the NCBI Gene database, the detailed annotation effort resulted in a unique list of 201 genes, identified within the *Staphylococcus aureus* species, to which predicted protein sequences could be unambiguously assigned.
2.9. Identification of genes associated with severity of illness

The Kruskal-Wallis rank sum test was used to measure the significance of association between the severity of illness scores of affected children and presence or absence of genes within each genome of the associated causative organism [36]. Calculated p-values were further adjusted using False Discovery Rate (FDR) and only genes with adjusted p-values less than 0.01 were considered to be significant [37].

2.10. Phylogenetic tree analysis

Using the single contig of UTSW55 as reference, SNP calling was performed using MUMmer [38]. The haplotypes for each of the study samples were generated with confident homozygous single nucleotide variant (SNV) sites for which the read fractions supporting major alleles were greater than 0.9 for every sample. These haplotypes were used as the nucleotide input for MEGA 6.06 (Molecular Evolutionary Genetics Analysis http://www.megasoftware.net/mega.php) for phylogenetic analysis. Maximum Likelihood (ML) phylogenetic reconstruction was performed with the Jukes-Cantor model using the ML Heuristic Method of Nearest-Neighbor-Interchange (NNI). The initial tree was assembled using maximum parsimony to estimate the evolutionary distances between sequences by computing the proportion of nucleotide differences between each pair of sequences (Fig. 4).

3. Results

3.1. Patient population and reference strain

The clinical severity of illness scores of the study population ranged from 0 to 10 (mean 5.3 ± 3.7). Denovo assembly of UTSW55 using Pacbio HGAP on sequencing reads from 6 SMRT Cells yielded a prefilter read count of 318,329 and mean read length of 5,750. The final assembly of the *Staphylococcus aureus* chromosome

| Variable                  | MSSA 0–5 (n = 20) | MSSA 6–10 (n = 7) | MRSA 0–5 (n = 13) | MRSA 6–10 (n = 30) | *p-value |
|---------------------------|-------------------|-------------------|------------------|-------------------|---------|
| Readmission (rate)        | 1 (5%)            | 1 (14.3%)         | 0 (0%)           | 8 (26.7%)         | 0.060   |
| ICU admission (rate)      | 1 (5%)            | 3 (42.9%)         | 0 (0%)           | 17 (56.7%)        | 0.000   |
| Bacteremia (rate)         | 9 (45%)           | 7 (100%)          | 8 (61.5%)        | 26 (86.7%)        | 0.001   |
| Surgery (rate)            | 15 (75%)          | 6 (85.7%)         | 10 (76.9%)       | 27 (90%)          | 0.071   |
| DVT (rate)                | 0 (0%)            | 0 (0%)            | 0 (0%)           | 29 (96.7%)        | 0.001   |
| SPE or Pneumonia (rate)   | 0 (0%)            | 0 (0%)            | 0 (0%)           | 11 (36.7%)        | 0.011   |

A Fisher’s exact test (*) was conducted to test if there is association among groups and variables of interest. ICU (intensive care unit); DVT (deep vein thrombosis); SPE (septic pulmonary embolism).
had a contig count of 1 with N50 contig length of 2,898,306, plus 4 non-overlapping contiguous sequences with an average length of 35,372 bp, most likely representing plasmids. There were 2,054 assigned ORF’s within the UTSW55 reference genome.

### 3.2. Determination of gene presence and annotation

Extensive review of the literature identified a list of potential virulence genes of *Staphylococcus aureus* (supplementary table S1). These putative virulence genes were then divided into the following categories: adhesins, immune evasion proteins, proteases, regulatory proteins, toxins, and other proteins. This resulted in the identification of 201 unique virulence genes. UTSW55 was found to contain 148 of the 211 putative virulence genes (Table 4), including 22 involved in global virulence regulation and 36 used to evade the immune response. The presence and absence of virulence genes was assessed for each of the study isolates as well as the GenBank strains for which whole genome sequence data is publicly reported (supplementary table S2). Fig. 1 depicts a heat map of the alphabetized list of these *Staphylococcus aureus* virulence genes and their presence or absence among the GenBank and study isolates.

#### Table 4. Virulence genes of UTSW55 by category.

| Category             | Gene name                                                                 |
|----------------------|---------------------------------------------------------------------------|
| Adhesions            | clfA, clfB, ebpS, fnbA, fnbB, icaA, icaB, icaC, pls, sdrC, sdrE, srtA     |
| Antibiotic resistance| dltA, ileS                                                                |
| Immune evasion       | adsA, aur, capA, capB, capC, capD, capE, capF, capG, capH, capJ, capK, capL, capM, capN, capO, capP, coa, ebbB, hlgA, hlgB, hlgC, lukE, lukF-PV, lukG, lukH, lukS-PV, map, mprF, oatA, sbi, scpA, ssl11nm, ssl6nm, ssl7nm |
| Other                | arlR, arlS, arsB, atl, epiB, epiC, fhuB, fhuD, fmt, geh, graR, graS, htrA, hysA, isaA, isdA, lip, lpl11, lpl2, lpl3, lpl4, lpl7, lpl8, lytR, lysS, nuc, phoU, putP, rot, cebU, sceD, scrA, sigB |
| Protease             | opp3A, opp3C, opp3D, opp3F, sak, splA, splB, splC, splF, sspA, sspB        |
| Regulatory           | agrA, agrB, agrC, arcA, arcB, arcC, arcD, arcR, clpC, clpP, clpX, codY, cshA, essC, saeQ, saeR, saeS, sarS, sarU, sarZ, srrA, srrB |
| Stress resistance    | ahpC, kata, ksgA, plc                                                    |
| Toxin                | ear, eta, hla, hlb, sek, seq, set6, set7, set9, set10, set12, set13, tst   |
3.3. Identification of genes associated with severity of illness

Table 5 lists the 40 genes which were significantly associated with severity of illness of the affected children (p < 0.01). MRSA isolates were found to encode a significantly greater number of virulence genes (p < 0.0001) than did MSSA isolates (Fig. 2A). However, no significant difference in the number of virulence genes was identified when comparison was made between MRSA or MSSA isolates causing mild versus severe illness (Fig. 2B). MRSA isolates demonstrated greater numbers of adhesion, immune evasion, proteases, regulatory, and other protein virulence genes (Fig. 3). There was no difference in the number of toxin genes present between MSSA and MRSA isolates. For each virulence gene class, the genes which were present demonstrated greater variation when comparison was made between MRSA and MSSA than when comparison was made between isolates which had caused mild versus severe illness phenotypes.
### Table 5. Virulence genes significantly associated with severity of illness among 71 children with acute osteomyelitis.

| Gene     | p-value | Adjusted p-value | Gene    | p-value | Adjusted p-value | Gene   | p-value | Adjusted p-value | Gene    | p-value | Adjusted p-value |
|----------|---------|------------------|---------|---------|------------------|--------|---------|------------------|---------|---------|------------------|
| capE     | 0.000   | 0.000            | sarU    | 0.000   | 0.000            | set13  | 0.000   | 0.002            | splB    | 0.001   | 0.004            |
| seg      | 0.000   | 0.000            | sek     | 0.000   | 0.000            | hla    | 0.000   | 0.002            | ccrA    | 0.001   | 0.004            |
| sei      | 0.000   | 0.000            | seq     | 0.000   | 0.000            | lpl1   | 0.000   | 0.002            | cna     | 0.001   | 0.004            |
| sem      | 0.000   | 0.000            | set11   | 0.000   | 0.000            | agrC   | 0.001   | 0.003            | epiC    | 0.001   | 0.004            |
| sen      | 0.000   | 0.000            | car     | 0.000   | 0.000            | lukE   | 0.001   | 0.003            | lpl4    | 0.001   | 0.004            |
| seo      | 0.000   | 0.000            | ssl11nm | 0.000   | 0.000            | hsdM   | 0.001   | 0.004            | set7    | 0.001   | 0.004            |
| set14    | 0.000   | 0.000            | sarH2   | 0.000   | 0.000            | pls    | 0.001   | 0.004            | ssl6nm  | 0.001   | 0.004            |
| set15    | 0.000   | 0.000            | lukF-PV | 0.000   | 0.001            | set12  | 0.001   | 0.004            | cadA    | 0.002   | 0.007            |
| spa      | 0.000   | 0.000            | bbp     | 0.000   | 0.002            | speG   | 0.001   | 0.004            | lpl11   | 0.003   | 0.007            |
| lukS-PV  | 0.000   | 0.000            | fhuD    | 0.000   | 0.002            | splA   | 0.001   | 0.004            | epiB    | 0.003   | 0.009            |
3.4. Phylogenetic analysis of UTSW55

Phylogenetic Analysis by maximum likelihood (PAML) depicts the relatedness of UTSW55 to the other clinical isolates from children with AHO caused by either MRSA or MSSA within our community. Construction of an unrooted radial dendrogram (Fig. 4) suggests a visible trend of higher severity of illness among predominantly MRSA isolates at one end of the dendrogram in comparison to lower severity of illness scores among predominantly MSSA isolates at the opposite end, with scattered exceptions of severity magnitude and antibiotic resistance throughout the dendrogram (Fig. 4). Using sequences publicly available in GenBank (supplementary table S1), PAML was used to depict the genetic distance of the study isolates to GenBank sequences.

Fig. 2. Number of virulence genes identified among methicillin-sensitive (MSSA) or methicillin-resistant (MRSA) isolates (A). MRSA isolates had a significantly greater number of virulence genes. Number of virulence genes of MRSA or MSSA among isolates from children with severity of illness scores ranging from 0–5 compared with that of children with scores ranging from 6–10 (B). There was no significant difference of the number of virulence genes between severity categories.

Fig. 3. MRSA isolates were found to have greater numbers of adhesion, immune evasion, protease, regulatory, and other virulence genes than were found in MSSA isolates.

Fig. 4. A..
4. Discussion

The pathogenesis of AHO in children is related to the virulence capability of the causative organism, *Staphylococcus aureus* [3, 4, 7, 9, 10, 11, 12]. In order for otherwise healthy children to become infected, the organism must gain access to the circulation, survive in human blood, evade the host defenses, and adhere to deep tissues in a cascade of events supported by hemolysins, leukocidins, toxins, and adhesins [13, 14, 15, 16, 17, 18, 19]. Variation in the clinical spectrum of this disease is not surprising given the array of virulence genes within the organism’s armamentarium which have been discovered to date [13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31]. Following the initial publication of the gene sequence of N315 several investigations were undertaken to explore single gene determinants and genes in combination through polymerase chain reaction (PCR) technology [13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31]. However, discovery has been slow due to the limited discriminatory power of these methods as well as the lack of a relevant reference strain for genomic studies [32, 33]. As new technologies have emerged, it is necessary to reassess the virulence
capability of *Staphylococcus aureus* using next generation tools with improved discriminatory power while utilizing sequence data of a relevant reference strain. Within this context, the addition of complete sequence data of UTSW55 to GenBank (Genbank: NZ_CP013231.1) (Fig. 5) provides necessary foundation for this investigation. Given the evolutionary nature of the *Staphylococcus aureus* genome, the study findings are enhanced by the geographic and chronologic proximity of the reference strain to the community isolates. This study represents the most exhaustive effort to date to accurately annotate the virulence genes of *Staphylococcus aureus*. More importantly, this study has identified 40 virulence genes which are significantly associated with severity of illness among children with AHO [5, 6].

As expected, the majority of MRSA isolates within our community are more closely related to USA 300 than to other available reference strains. An unexpected finding was the phylogenetic diversity among the MSSA strains. Other investigators have similarly found CA-MRSA to be relatively homogenous while MSSA isolates from their study population demonstrated greater heterogeneity [32]. The MRSA isolates of this study were found to possess more virulence genes than MSSA isolates. Recent clinical experience in several communities suggest that MRSA causes

Fig. 5. Alignment results of commonly used *Staphylococcus aureus* genomes (TCH1516, COL, MW2, N315, and MRSA252) against the newly assembled UTSW55 reference genome. Annotation locations for 201 genes (Supplemental Table 1) are depicted in the outer circle along with GC content in the inner circle. Alignment identity cutoff of 0.8 was used to determine missing regions in the query genomes compared to the UTSW55 reference.
more severe clinical disease in children [4, 7]. Although this severity is attributed to the antibiotic resistance profile of the bacteria, a more plausible explanation is the increased number of virulence genes among MRSA isolates. The findings of this study support the idea that the antibiotic resistance of the isolate is not the primary driver of severity, but rather a bystander effect.

No single virulence gene has been conclusively identified as responsible for the invasiveness of Staphylococcus aureus. It is suspected that combinations of virulence genes may be involved [11]. Previous investigators have shown that the genes fnbA, cna, sdrE, sej, eta, hlg, and ica are more common among invasive isolates [11]. After an extensive search of the literature and thorough gene annotation, this study reports 201 virulence genes of Staphylococcus aureus (see Supplementary table S1). Among these are 40 which are significantly associated with severity of illness of children with AHO (Table 5). These specific genes will guide future studies of gene regulation (agrC), hemolysins (hla), immune evasion (capE, lukS-PV, lukF-PV, lukE), and the Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) including adhesive surface protein genes of collagen adhesion (cna) and bone sialo-protein binding (bbp). A substantial percentage of children with AHO are bacteremic at initial presentation which suggests that Staphylococcus aureus has acquired mechanisms by which to survive in human blood by liberating free iron through hemolysis (hla) and evading the host neutrophil defense (capE, lukS-PV, lukF-PV, lukE). The organism then must attach to deep structures of collagen and bone (can, bbp) in order to establish the deep infection of bone.

Important limitations of this study include the lack of gene expression data or a mechanistic explanation of relationship of the Staphylococcus aureus genome to the clinical manifestations of infection. It is not possible to equate any of the virulence genes identified as specifically responsible for bone infections or surmise what cascade of events might be responsible for these infections by specific isolates. While the bacterial transcriptome was beyond the scope of this study, promising research using a murine osteomyelitis model demonstrates a differential gene expression of Staphylococcus aureus between in vitro and in vivo conditions [39, 40]. These investigators found 180 genes which were significantly expressed during acute infection with actions involved gluconeogenesis, proteolysis, iron acquisition, evasion of host immune defense and stress response [40].

This study represents the most current and extensive analysis of virulence genes of Staphylococcus aureus to date based on extensive literature review, detailed gene annotation, and phylogenetic analysis. By including all existing GenBank strains for which genomic sequence data is accessible, we have identified a comprehensive list of virulence genes and their penetration across 71 local and 49 remote Staphylococcus aureus strains. Utilizing a framework to objectively categorize severity of illness enhances the clinical relevance of this study specifically to children with...
AHO. The addition of the whole genome of UTSW55 to GenBank (GenBank: NZ_CP013231.1) will facilitate future investigations of the genome, transcriptome and proteotome of *Staphylococcus aureus* isolates from children with AHO. An important question to be addressed in future investigations is whether there is any association between specific virulence factors and long-term clinical outcomes of disease, specifically recurrence of infection, osteonecrosis, growth arrest, or skeletal deformity.

**Declarations**

**Author contribution statement**

Angela Collins, Naureen G. Tareen: Performed the experiments.

Edward K. Wakeland: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Prithvi Raj: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Min S. Kim, Jiwoong Kim: Analyzed and interpreted the data.

Lawson A. B. Copley: Conceived and designed the experiments; Wrote the paper.

**Funding statement**

This work was supported by a grant from the Texas Scottish Rite Hospital for Children (TSRHC), grant # 02-14-1130. The bioinformatics analyses were funded by CPRIT. The funders had no role in study design, data collection, data analysis, interpretation and writing of this paper or in the decision to submit the paper for publication.

**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2018.e00674.

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