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Original Article

Abundance and diversity of methicillin-resistant bacteria from bathroom surfaces at workplaces using CHROMagar media, 16S, and dnaJ gene sequence typing

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Abstract: University campus communities consist of dynamic and diverse human populations originated from different regions of the country or the world. Their national/global movement to and from campus may contribute to the spread and buildup of methicillin-resistant (MR) bacteria, including MR *Staphylococci* (MRS) on high-touch surfaces, sinks, and toilets. However, studies on MR bacteria contamination of surfaces, sinks, and toilets are scarce in workplaces outside of healthcare settings. Hence, little is known whether university communities contaminate campus bathrooms by MR bacteria. This study evaluated the abundance, identity, and phylogenetics of MR bacteria grown on CHROMagar MRSA media from bathrooms at workplaces. We collected 21 sink and 21 toilet swab samples from 10 buildings on campus and cultured them on CHROMagar MRSA media, extracted DNA from MR bacteria colonies, sequenced PCR products of 16S and dnaJ primers, determined the sequence identities by BLAST search, and constructed a phylogenetic tree. Of 42 samples, 57.1% (24/42) harbored MR bacteria. MR bacteria were more prevalent on the sink (61.9%) than in the toilet (52.2%) and in male bathrooms (54.2%) than in female bathrooms (41.7%). The colony count on the bathroom surfaces of 42 samples varied in that 42.9% (18/42), 33.3, 14.3, and 9.5% of samples harbored 0, 100, and > 1000 MR bacteria colonies, respectively. Of MR bacteria sequenced, BLAST search and phylogenetic analysis showed that *Staphylococcus* accounted for 60% of the MR bacteria and the rest were non-*Staphylococci*. Of *Staphylococcus* carrying MR (n = 15), 53.3% were *S. hemolyticus* followed by *S. lugdunensis* (26.7%), *S. epidermidis* (8%), and a newly discovered *S. borealis* in 2020 (4%). Of non-*Staphylococci* MR bacteria, 20% accounted for *Sphingomonas koreensis*. Campus bathrooms serve as a reservoir for diverse bacteria carrying MR, which pose a direct risk of infection and a potential source of horizontal gene transfer. To reduce the health risk posed by MR bacteria in high traffic areas such as bathrooms additional environmental monitoring and improved decontamination practices are needed.

Keywords: Bathroom hygiene, methicillin-resistance, molecular epidemiology and ecology, public health, workplaces

Introduction

Travel drives the spread of antimicrobial-resistant (AMR) bacteria (or AMR genes) [1-3]. Universities have dynamic populations of diverse students traveling back and forth to different areas of the world [4-6]. The movements of workers and students to and from workplaces and campuses have contributed to the spread of pathogens such as COVID-19 [7]. People (travelers) can also carry and spread AMR bacteria such as methicillin-resistant *Staphylococci* (MRS) via the skin [8-11], respiratory tract [12, 13], digestive tract [14, 15], and urinary tract [16-18]. Patients traveling to hospitals can carry AMR bacteria and contaminate high-touch surfaces [19], bathroom sinks [20], and toilets [21, 22].

Reports on MR bacteria are rising from diverse ecologies and many surfaces such as sinks at hospitals [23], but less is known about MR bacteria in workplaces outside of healthcare settings concerning contamination of surfaces,

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sinks, and toilets on-campus by university communities. Toilet hygiene and decontamination of bathroom surfaces can control the buildup and spread of AMR bacteria [24], but many workplaces like college campuses do not conduct regular monitoring and surveillance of bathrooms to validate the efficacy of decontamination practices. Therefore, this study evaluated the abundance, identity, and phylogenetics of MR bacteria grown on CHROMagar MRSA media from bathrooms at a college campus by 16S and dnaJ gene sequence typing.

Materials and methods

Sample collection

To explore the abundance and diversity of MR microbes in bathroom sinks and toilets on a college campus, we purposively chose to collect samples from high-traffic bathrooms (both men's and women's) on a university campus in New York during the semester in buildings where classes are held. We collected a total of 42 bathrooms i.e. high-traffic bathrooms, from 10 buildings resulting in 21 sink swabs (n = 10 for male, 10 for female, and 1 for both genders) and 21 toilet swabs (n = 10 for male, 10 for female, and 1 for both genders). During sampling, the swabs were rubbed several times against the surface of the sink drain or the inside of the toilet bowl, shipped in ice-cold, and spread plated on a selective CHROMagar MRSA media (DRG International, Springfield, NJ, USA) within fifteen minutes of collection. CHROMagar MRSA media was chosen because it has sensitivity and specificity values close to 100% [25]. The institutional research board (IRB) ethical committee decided that the project does not need IRB review since sink and toilet swab samples were collected from public bathrooms and the project does not meet the definition of human subject research as defined by 46.102 (https://www.hhs.gov/ohrp/regulations-and-policy/regulations/45-cfr-46/revised-common-rule-regulatory-text/index.html#46.102).

DNA extraction

We extracted the DNA of MR bacteria colonies grown on CHROMagar MRSA by vortexing vigorously in 300 µl TENT buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 5% [v/v] Triton X100, pH 8.0) followed by boiling for 10 min, chilling on ice for 10 min, and centrifuging at 10,000 rpm for 10 min by Eppendorf Centrifuge 5418R (Eppendorf, Enfield, Connecticut). Finally, we preserved the supernatant containing DNA at -20°C [26, 27].

16S and dnaJ gene amplification by PCR

Each 25 µL PCR reaction mixture consisted of genomic DNA (~50-100 ng), 12.5 µL of 2× Thermo Scientific™ Phusion Flash High-Fidelity PCR Master Mix (Fisher Scientific, USA), 1 µL forward primer (10 uM), 1 µL reverse primer (10 uM), and the remainder was DNase/RNase free ultrapure water. All primers were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, Iowa). We used T100 Thermal Cycler PCR machine (Bio-Rad, Hercules, California) for performing all PCR reactions.

16S primers: These 16S RNA gene targeting primer pairs amplify all bacteria phylum universally. They were 27-F (TPU1)-5'-AGAGTTTGATCCTGGCTCAG-3' and U1492R-5'-GGTTACCTTGTTACGACTT-3'. The PCR condition was 95°C for 3 min initially followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and finally 72°C for 7 min [28, 29].

dnaJ primers: The SA-(F)-5-GCCAAAAGAGACTATTATGA-3' and SA-(R) degenerate primer - 5-ATTGYTTACCYGTTTGTGTACC-3 were used as they amplify *Staphylococci* species. The DNA PCR amplification was performed in two steps. The first step had initial denaturation at 94°C for 3 min followed by 5 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s. The second step had 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 60 s, and 72°C for 3 min [30, 31].

The 1400-1500 bp PCR of the 16S gene [28, 29] and 920 bp PCR product of the dnaJ gene [30, 31] were resolved by gel electrophoresis using 0.8% and 1% gels, respectively, at 100 v for 90 min. We used GelRed dye (Millipore Sigma, Burlington, Massachusetts) for staining the gels due to its safety and sensitivity [32, 33] and visualized using ChemiDoc Imaging Systems (Bio-Rad, Hercules, California) under UV light.

Sequencing 16S and dnaJ gene PCR products of MR bacteria

The 16S and dnaJ PCR products (10 µL for each sample) were sequenced with (5 µL of 5

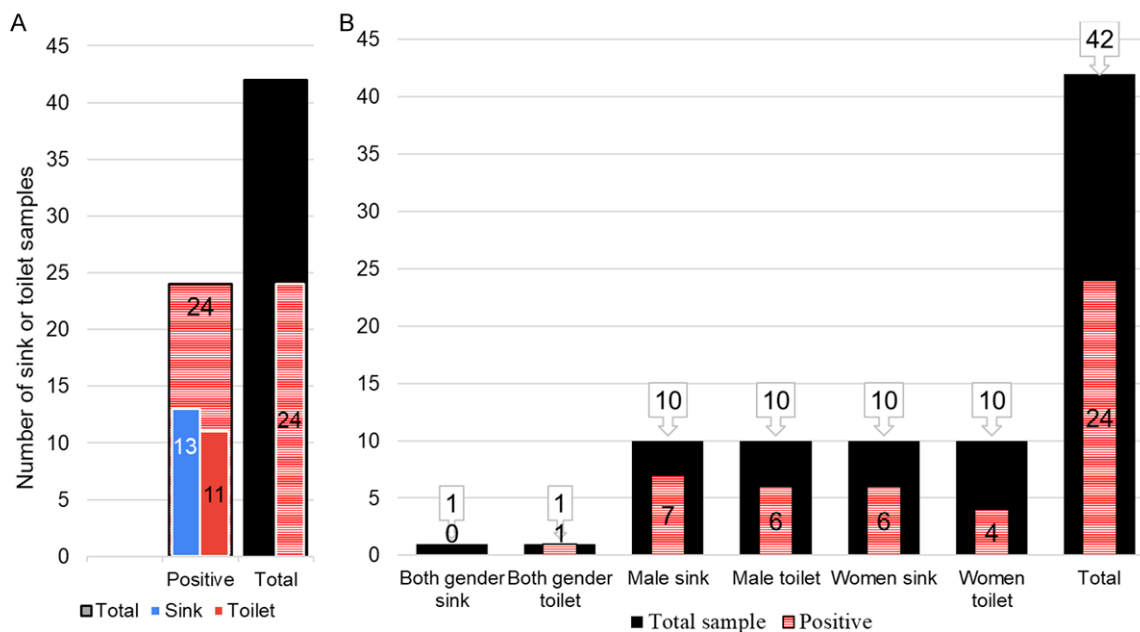

Figure 1. Distribution of MR bacteria on the surfaces of sink and toilet (A) and bathroom surfaces of males vs. females (B). Of 42 total samples tested, 57.1% (n = 24) harbored MR bacteria.

µM for each sample each primer) by Eurofins Genomics (Louisville, KY, USA).

Bioinformatics and phylogenetic tree of 16S and dnaJ gene sequences

Each DNA sequence chromatogram file (".ab1") was visualized, trimmed, and edited using UGENE software based on chromatogram quality [34]. BLASTn at NCBI was used to search and identify the taxonomy (species) of our test DNA sequence. After BLAST, sequences of several candidate species that match each of our test DNA sequences were imported to MEGA-X software [35]. Multiple sequence alignments were generated with MUSCLE in MEGA-X software for each of our DNA sequences [36, 37]. Phylogenetic trees were generated using the neighbor-joining to identify the species of our MR bacteria isolates [38]. We constructed an additional phylogenetic tree with MUSCLE in MEGA-X using the imported sequences from NCBI that scored the highest similarity (identity matching) with our MR bacteria isolates. Phylogentic trees were visualized and edited for clarity in Evolview software [39].

Data analysis

Data nalaysis was conducted using SPSS software version 29.0.1.0 to evaluate and compare the presence and absence of MRS or non-*Staphylococci* methicillin-resistant (MR) bacteria in the collected samples using a Fisher's exact test. The abundance of MR bacteria (CFU/ml) in the different sample sources was compared by the non-parametric Mann-Whitney Test and Wilcoxon Rank Sum Test. Differences in proportions of positive samples for MR bacteria as well as differences in counts of MR bacteria (CFU/ml) per sample were considered statistically significant with p-value ≤ 5% and 95% confidence interval.

Results

Of 42 samples, 57.1% (n = 24) harbored MR bacteria. MR bacteria were more prevalent on the sink swabs (61.9% = 13/21) than on the toilet swabs (52.2% = 11/21) (**Figure 1A**). 61.9% of male bathrooms and 47.6% of female bathrooms harbored MR bacteria. Of 24 MR-positive samples, male bathrooms (i.e., sink and toilet combined) had higher MR bacteria (54.2% = 13/24) than female bathrooms (41.7% = 10/24). We isolated 1, 7, 6, 6, and 4 positive samples for MR bacteria from the toilet of both genders bathroom, the sinks of male bathrooms, the toilets of male bathrooms, the sink of female bathrooms, and the toilet of female bathrooms, respectively (**Figure 1B**). Overall, the proportions of MR bacteria in male

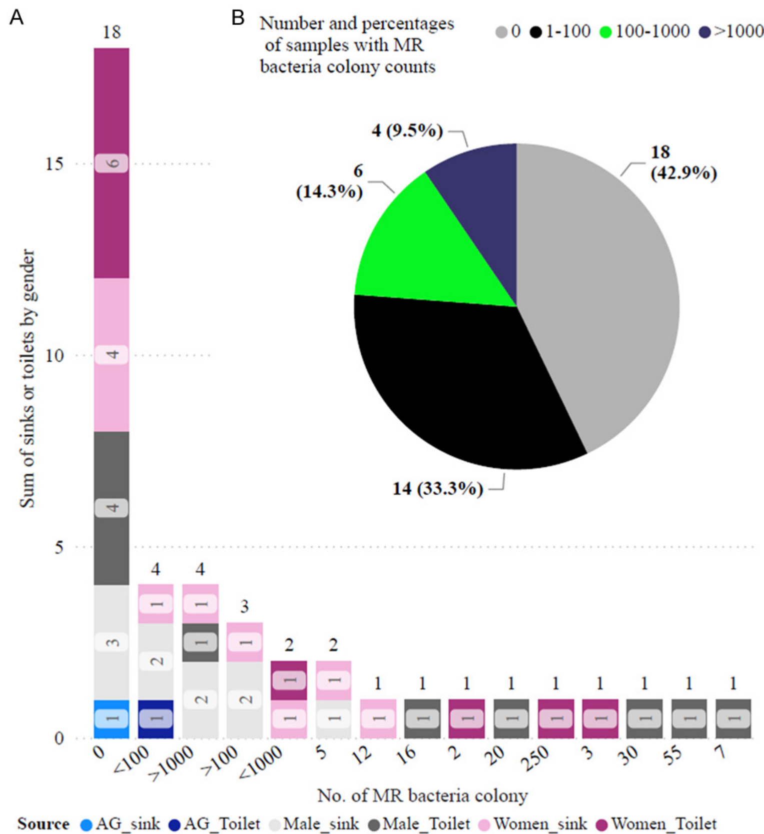

Figure 2. Counts of MR bacterial colonies grown on CHROMagar MRSA. A. MR bacterial colony counts on the x-axis and the number of MR-positive sink and toilet samples on the y-axis. AG = All gender bathroom. B. Number and percentage of samples with zero, 1-100, 100-1000, and > 1000 MR bacterial CFU counts.

or female bathrooms ($X^2 = 0.921$; $P > 0.05$) and in sink or toilet ($X^2 = 0.389$; $P > 0.05$) were not significantly different, indicating that the prevalences of MR bacteria are similar in the sinks and toilets of male and female.

Counts of MR bacteria colonies grown on CHROMagar MRSA media

The Mann-Whitney (mean rank) and Wilcoxon W (Sum of ranks) tests for MR bacteria colony counts were not significantly ($P > 0.05$) different in male bathrooms (mean rank = 22.25; sum of ranks = 445) vs. female bathrooms (mean rank = 18.75; sum of ranks = 375). Similarly, the colony counts of MR bacteria were not significantly ($P > 0.05$) different between sink (mean rank = 23.71; sum of ranks = 498) vs. toilet (mean rank = 19.29; sum of ranks = 405). Of the 4 bathrooms with > 1000 CFU MR bacteria, 2 of them were from the male sink, 1 from the male toilet, and 1 from the female sink (**Figure 2A**).

Overall, 42.9, 33.3, 14.3, and 9.5% of the 42 samples harbored zero, 1-100, 100-1000, and ≥ 1000 MR bacterial colony counts (**Figure 2B**).

Sequence analysis of MR isolates by BLAST and phylogenetics

We selected 25 MR representative isolates, amplified them by PCR using 16S and dnaJ primers, sequenced them, and searched similar sequences using BLASTn. *Staphylococcus* genera accounted for 60% (15/25) of the MR bacteria and the rest 40% were non-*Staphylococci* bacteria. Of non-*Staphylococci, Sphingomonas koreensis* accounted for 20% (5/25). Of MR *Staphylococcus* genera, *S. hemolyticus* was 53.3% (8/15) followed by *S. lugdunensis* (26.7% = 4/15), *S. epidermidis* (8%), and *S. borealis* (4%). In this study, 52% (13/25) and 16% (4/25) of the sequenced MRS isolates had 100% and 99.8% match with a specific pathogen sequence deposited database at the NCBI, respectively (**Figure 3**).

We summarized the final DNA fragment lengths (bp) of the sequenced products of our isolates that we used for searching in the NCBI list of the closest relative taxa in NCBI to each of our isolates, and percentage (%) of DNA sequence similarity between our isolates and their closest relative taxa at the NCBI (**Figure 4**). For example, one isolate (*S. borealis*) matched *S. borealis* strain 51-48, which was previously a member of *S. haemolyticus* strain and became a new species in 2020. Sequencing using 16S provided us with a high-quality product with a fragment length ranging from 119-986 bp (on raw sequence data). This fragment is a perfect match to the 150-1071 chromosomal region of *S. borealis* strain 51-48. Similarly, 5, 2, and 1

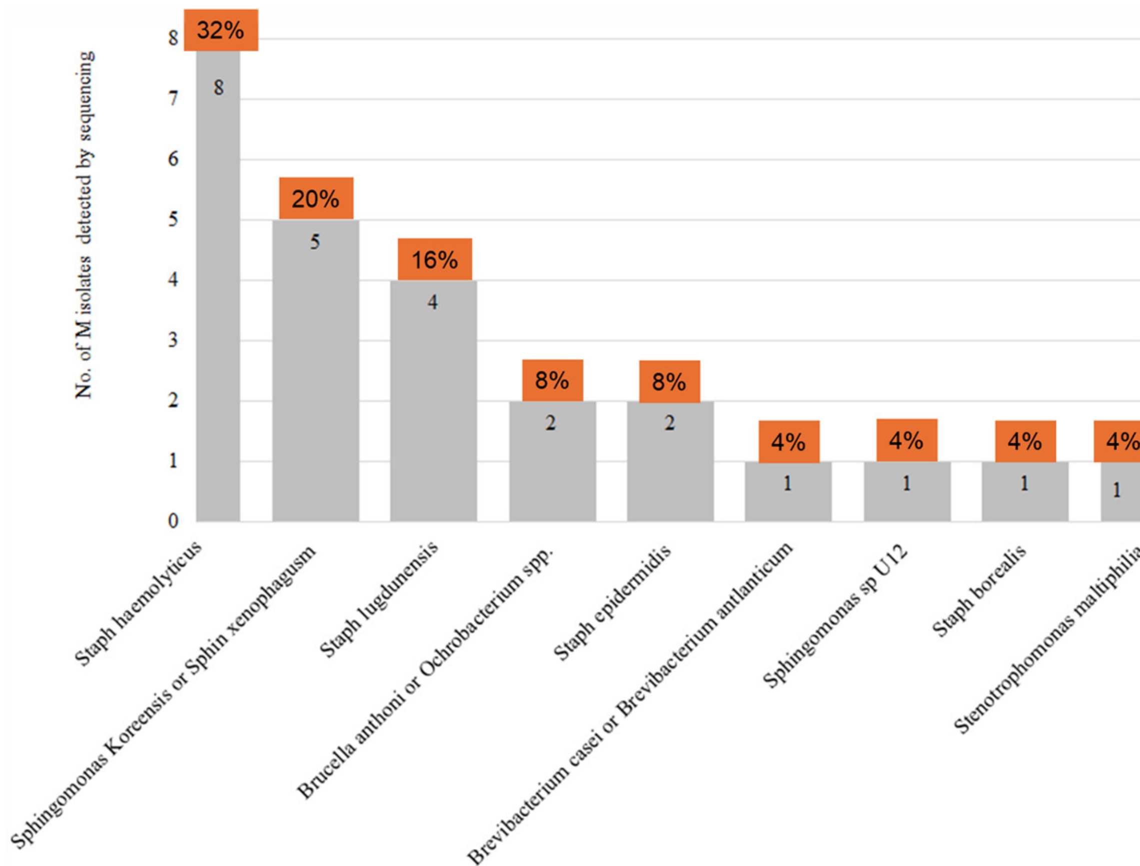

Figure 3. MR bacteria species determination and their prevalences by 16S and dnaJ sequencing. MR bacteria from the bathrooms grown on CHROMagar MRSA media (n = 25 isolates) were sequenced and searched by BLASTn at NCBI to identify bacteria taxa with similar 16S and dnaJ sequences.

of the 8 sequenced MR isolates matching *S. haemolyticus* were relatives of *S. haemolyticus* strain SCAID, *S. haemolyticus* strain Hakim 1980, and *S. haemolyticus* strain J2-28 or *S. haemolyticus* strain OB285, respectively. We also detected four *S. lugdunensis* isolates by dnaJ sequencing (3 isolates) and 16S sequencing (1 isolate) that were 99.9-100% genetic relatives of *Staphylococcus lugdunensis* JICS135.

Discussion

Human skin, respiratory, and digestive tracts carry diverse bacterial species including AMR strains, and spread them through contact, nasal discharges, and feces, resulting in contamination of high-touch surfaces [19], bathroom sinks [20], and toilets [21, 22]. This study aimed to evaluate the abundance and phylogenetics of bacteria grown on CHROMagar MRSA media from bathrooms at workplaces by 16S and dnaJ gene sequence typing.

Of 42 total sinks and bathrooms tested using CHROMagar MRSA, 57.1% harbored MR bacteria. 61.9% of the sinks, 52.2% of the toilet swabs, 61.9% of male bathrooms, and 47.6% of female bathrooms harbored MR bacteria. Although not significantly different in this study, the higher prevalence of MR bacteria in sinks than in toilets may suggest that *Staphylococci* and MRSA are more abundant on the skin (hands) and oro-nasal area than in the intestine and perianal area as previously reported from elsewhere [40]. We observed that MR bacteria prevalence and MR bacteria colony counts were higher in bathrooms of males than females, which agrees with a report that MRSA is higher in males than females from Germany [41] and other parts of the world [42]. We don't know whether males naturally harbor more MR

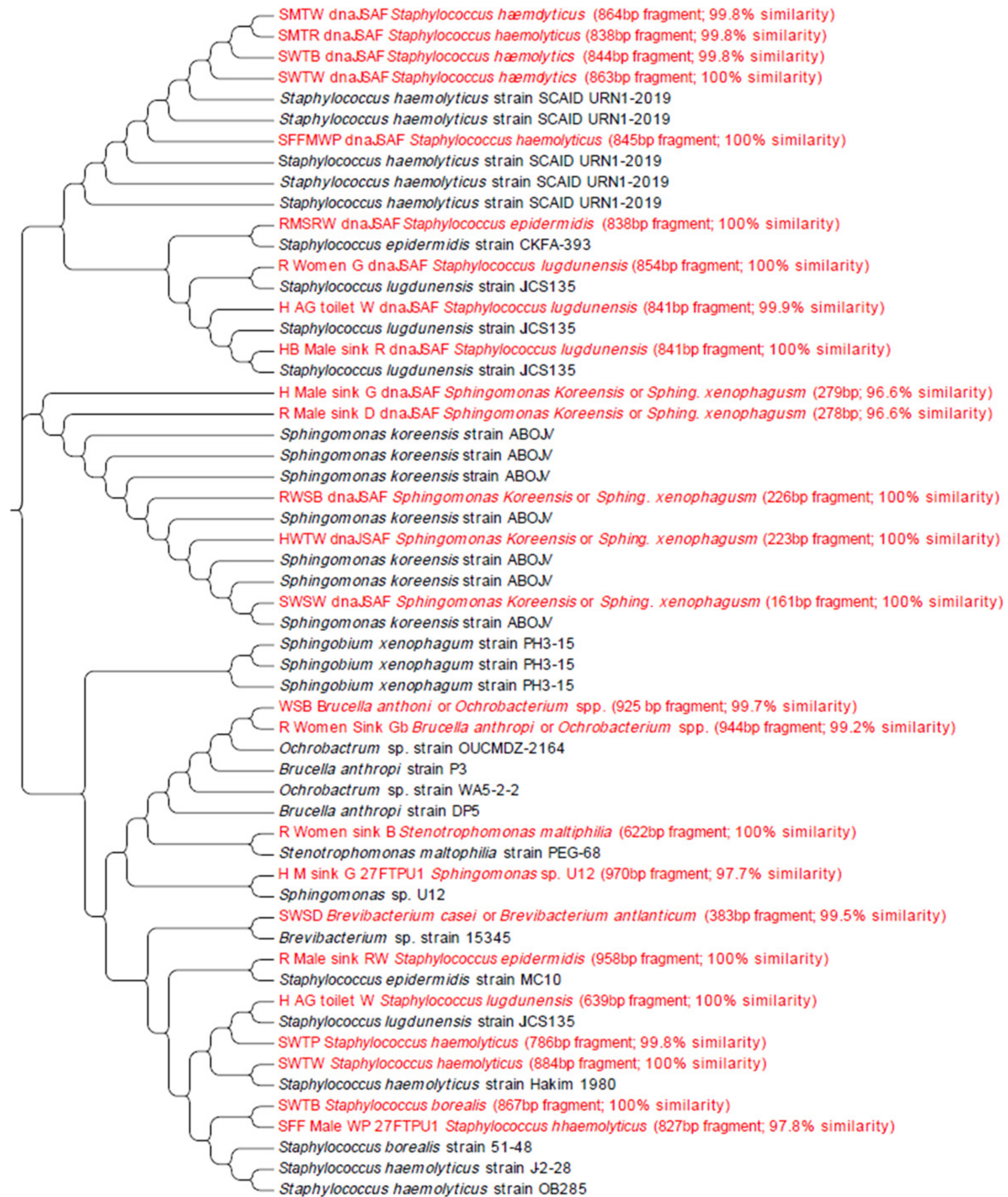

Figure 4. Phylogenetic tree of MR bacteria (n = 25) isolated from male and female bathrooms on campus. The image displays the final quality DNA sequence fragment length (bp) for our isolates and percentage (%) of similarity in DNA sequences of our isolate with their closest relative taxa at NCBI. Those isolates written in red color were our isolates and those written in black color were the closest relative taxa to our isolates from NCBI.

bacteria than females or whether the hygiene of males' bathrooms was poorer than females in terms of cleaning and disinfection.

Based on 16S sequence analysis, 60% of MR was from *Staphylococci* spp. whereas 40% were from non-*Staphylococcus* bacteria. Of *Staphylococci*, the highest prevalence of MR was observed in *S. haemolyticus* followed by in the rank order of *S. lugdunenis*, *S. epidermidis*, and *S. borealis*. *S. haemolyticus* in other parts of the world also harbors greater resistance to

more antibiotics including methicillin than any other coagulase-negative *Staphylococci* such as *S. epidermidis,* and *S. lugdinenis* [8, 43]. MR in most bacteria is due to acquiring mecA gene and the mecA gene sequences of *S. aureus*, *S. haemolyticus*, and *S. epidermidis* are similar by 99.95% [8]. MR *S. lugdunensis* has been reported from several countries [44]. *S. borealis* is a new bacterium that was discovered in 2020, and causes bacteremia and skin problems [45]. The identification of numerous MRS isolates in different bathrooms agrees with a study that reported MR is widespread in coagulase-positive and couagulase-negative *Staphylococci* species [46].

One of the non-*Staphylococcus* MR bacteria isolated by CHROMagr MRSA media from sink and toilet in this study that we subsequently identified by 16S sequencing had a 99.5% sequence similarity with *Brevibacterium casei* or *B. antarticum. B. casei* is a glutin-degrading Gram-positive bacterium in the human intestine [47]. It is a rare opportunistic pathogen in humans [48] that can be treated by vancomycin [49], but some of its strains are vancomycin-resistant [50].

We also identified other MR bacteria isolates from CHROMagar MRSA media that had similarity in their 16S sequences with *Sphingomonas koreensis, Stenotrophomonas maltophilia,* and *Ochrobactrum* ranging from 96.6-100%, 100%, and 99.2-99.7%, respectively. These three species live in the soil and water playing a role in biodegradation and remediation processing, but soil and water can be a source for them to cause opportunistic infections in humans [51-53]. In the USA, an outbreak of *Sphingomonas koreensis* has been reported from the sink and aqueous reservoirs in the hospital plumbing [54], resulting in a waterborne disease of the human central nervous system (meningitis) [55]. *Sphingomonas* spp. are multidrug-resistant including beta-lactams [51]. *Ochrobactrum* species are also a major emerging opportunistic pathogen. *Ochrobactrum* is highly resistant to beta-lactam antimicrobials [56] and cause septicemia, endocarditis, and pneumonia in humans [53, 57, 58]. *Stenotrophomonas maltophilia* is an opportunistic pathogen [52, 59], intrinsically multidrug-resistant [59, 60], including against beta-lactams [59, 61], and its prevalence is also rising in some localities of the USA [62]. These bacteria are Gram-negative, and are, therefore intrinsically resistant to methicillin and vancomycin since their outer membrane and porins do not allow these antimicrobials to penetrate [63].

While 16S and dnaJ sequencing enable strain identification, whole genome sequencing of the isolates grown on CHROMagar MRSA media or metagenomic sequencing of the collected samples would improve the resolution of strain identification. It would be interesting to know how the abundance and distribution of methicillin resistance observed in our study from samples collected from a single campus compares with other college campuses to evaluate the effect of geographic location and student population composition. In this regard, our pilot study has prospects for collaborative research among universities to further study and address the risk of university communities to hazards including MR bacteria at workplaces.

In conclusion, MR bacteria are abundant on the surfaces of 61.9% of the sinks and 52.2% of the toilets on campus indicating people "silently" shed them on campus and pose a risk to the general university communities. MR coagulase-negative *Staphylococcus* species are the major MR bacteria carried by university communities to contaminate sinks and toilets. Conducting monitoring and surveillance of bathrooms in workplace environments such as college campuses is necessary to properly evaluate sanitization and general hygienic practices.

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Disclosure of conflict of interest

None.

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