Biofilm-forming, Antimicrobial Resistance and Whole Genome Sequencing (WGS) Profiles of Hospital- and Community-acquired Methicillin-susceptible and -resistant *Staphylococcus aureus* Isolates

Saeed Khan[1], K. Sung[1], M. Park[1], M. Nawaz[1], G. Dereje[1], K. S. Phillips[2], S. Park[2], P. S. Vagnone[3], S. Agnihothram[4], I. Sulaiman[5], and S. Foley[1]
[1]Division of Microbiology, National Center for Toxicological Research, US FDA, Jefferson, AR, [2]Center for Radiological Devices and Health, US FDA, Silver Spring, MD,
[3]Minnesota Dept. of Health, MN, [4]Center for Biologics Evaluation and Research, US FDA, Silver Spring, MD, [5]Southeast Food and Feed Laboratory, US FDA, Atlanta, GA.

FDA U.S. FOOD & DRUG ADMINISTRATION
NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH

ABSTRACT

Staphylococcus aureus is primarily known to cause infections of the skin and soft tissues. It can also cause necrotizing pneumonia, sepsis, osteomyelitis, and urinary tract infections. *S. aureus* has gained this notoriety due to the acquisition of toxin and virulence genes and biofilm-forming ability. Hospital-associated methicillin-resistant *S. aureus* (HA-MRSA) accounts for most invasive infections, higher morbidity and mortality rates and requires longer hospital stays than hospital-associated methicillin-susceptible *S. aureus* (HA-MSSA) and community-associated MRSA (CA-MRSA). In this study, we present the antimicrobial resistance (AR) profiles, biofilm formation capability, and draft whole genome sequences of 9 HA-MSSA, 27 HA-MRSA and 8 CA-MRSA isolates. *S. aureus* AR profiles were determined by disk-diffusion assays. Biofilm formation was studied using crystal violet assay. WGS assays were performed on a MiSeq platform using 2 x 300-bp paired-end runs. Multilocus sequence typing (MLST), *spa* and SCC*mec* typing were conducted with MLST v2.0, *spa*Typer v1.0, and SCC*mec*Finder v1.2, respectively. HA-MSSA and CA-MRSA isolates exhibited resistance to ampicillin, erythromycin, and penicillin. HA-MRSA isolates were resistant to ampicillin, erythromycin, levofloxacin, oxacillin, and penicillin. Three of the 9 HA-MSSA, 3 of the 8 CA-MRSA, and 20 of the 27 HA-MRSA isolates exhibited high biofilm-forming activity. Most of the isolates used in the study harbored 59-85 virulence and 39-49 AR genes. Virulence genes also included genes involved in biofilm formation. HA-MSSA showed diverse MLST (ST5, ST8, ST15, ST59, ST96, ST398, ST1390) and *spa* (t008, t242, t338, t437, t571, t2802) types. HA-MRSA exhibited ST5, ST8, ST105, ST231, and ST2253, and *spa* t002, t008, t1154, t045, and t586 types. Most HA-MRSA harbored large SCC*mec* type II elements but also contained smaller SCC*mec* type IV. CA-MRSA harbored SCC*mec* types II and IV. Among the 27 HA-MRSA isolates, 18 of the 27 were SCC*mec* type II and 9 were SCC*mec* type IV. CA-MRSA possessed ST8, ST59, ST72, ST231, and ST2725, and *spa* t008, t024, t190, t693, t1784, and t2365 types. Three CA-MRSA strains exhibited an ST8-SCC*mec*-IVa pattern, which represented a major clone in North America. Moreover, one of the MSSA isolates had an MLST type ST398, which is frequently found in livestock and patients with septicemia and bloodstream infections. All the isolates used in the study possessed multiple AR and virulence markers, and biofilm production ability, and exhibited strong potential for causing disease.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) are major sources of hospital-acquired infections and are of concern due to their involvement in high incidences of morbidity and mortality worldwide. Resistance to methicillin is conferred by *mecA* gene that was first discovered in 1961 among nosocomial isolates of *S. aureus* and since then it has been independently transferred multiple times into *S. aureus* chromosome. Healthcare-acquired MRSA (HA-MRSA) strains pose serious challenges to treatment because they also exhibit resistance to multiple other antibiotics including macrolides, fluoroquinolones, and some of the last resort antibiotics such as vancomycin and linezolid. These organisms cause a variety of infections such as boils, furuncles, styes, impetigo and other superficial infections in humans. They are also known to cause serious infections such as pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, mastitis and meningitis in immunocompromised individuals. Among life-threatening diseases, MRSA have been implicated in unusually invasive pathogenic diseases like necrotizing fasciitis, severe septicemia, and necrotizing pneumonia. A serious concern about the treatment of MRSA infections is their ability to form biofilms in medical equipment and devices. An enhanced biofilm formation in a clinical strain of MRSA has been shown in an endotracheal tube (ETT) that is routinely used in 40%–50% of patients admitted to intensive care units. Their attachment to the ETT surface affects gene expression that helps the bacteria to form bioflms (10). Several studies have shown a close link between *S. aureus* ETT bioflms and ventilator-associated pneumonia (VAP). The ability of HA-MRSA to form biofilms on medical devices can result in invasive infection, sepsis, morbidity and mortality, as such they remain a serious public health concern that needs to be tackled by understanding the physiology of biofilm formation and development of remedial measures. Another form of MRSA known as community-acquired MRSA or CA-MRSA is also prevalent in humans which is widely believed to spread from person to person contact. People infected with MRSA or those working in the hospital settings may spread MRSA in the community.

In this study, we have used the NextGen sequencing techniques, proteomic profiles, and adhesion assays to explore the diversity of MRSA isolates of human clinical origin, some of which have caused fatal infections, to understand the factors responsible for biofilm formation, persistence of infection, virulence and pathogenicity markers, which might lead to the development of strategies to manage and control *S. aureus* infections and understand their evolution.

MATERIALS & METHODS

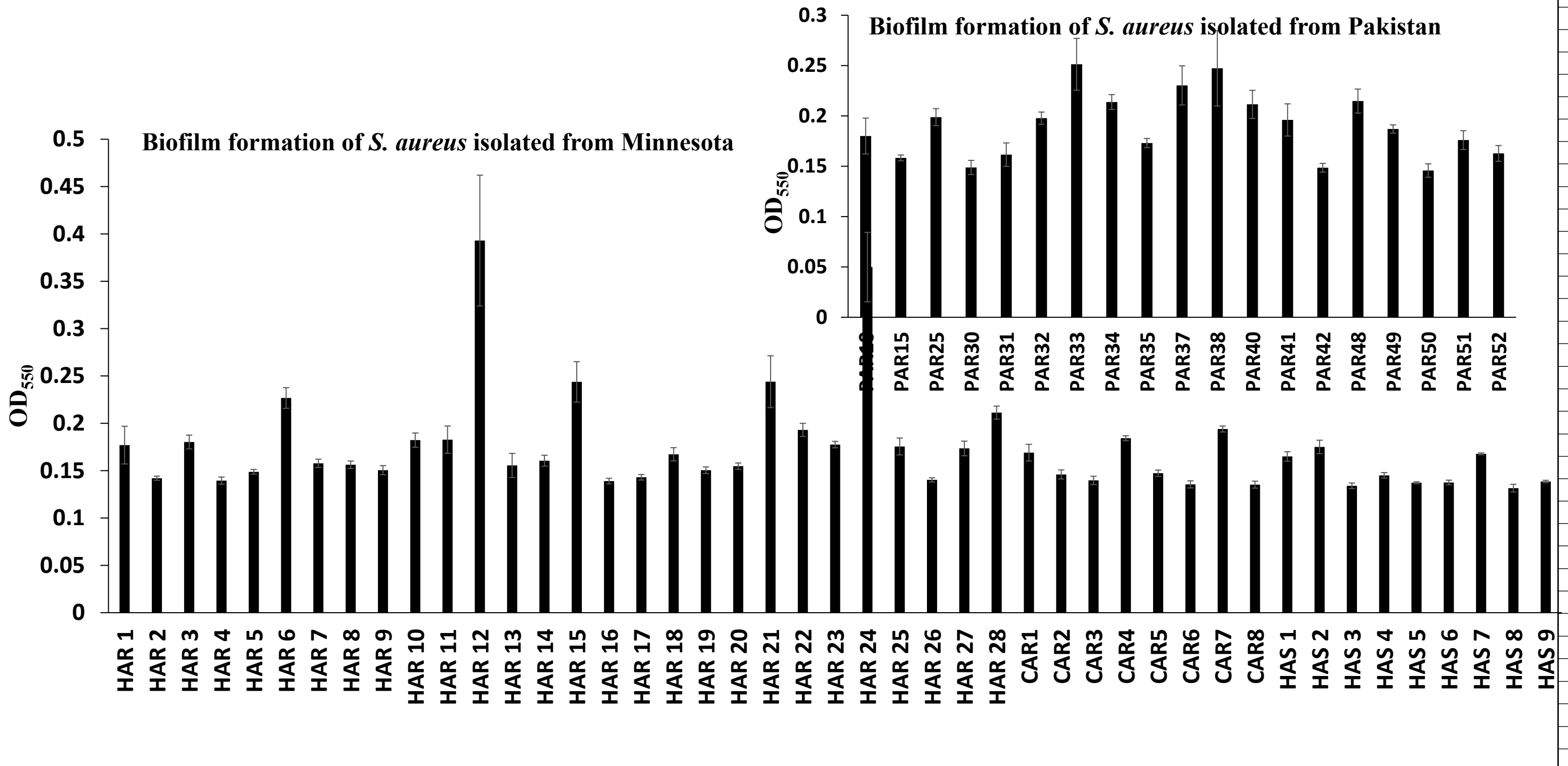
Bacterial Strains: Nineteen nasal and perirectal swabs of patients were obtained from a tertiary health care facility in Karachi, Pakistan. These were subjected to VITEK testing at the FDA's National Center for Toxicological Research (NCTR). The identification was done by API Staph kit as per manufacturer recommended procedure (VITEK 2 System Version 05.01 bioMerieux). Forty-Six *S. aureus* isolates obtained from Minnesota Department of Health and comprising of 29 HA-MRSA, 8 CA-MRSA and 9 HA-MSSA, were also used in the study.

Antimicrobial Susceptibility: Antibiotic-coated discs containing ampicillin (10 µg/ml), cefazolin (30 µg/ml), ciprofloxacin (5 µg/ml), erythromycin (15 µg/ml), gentamicin (30 µg/ml), kanamycin (30 µg/ml), oxacillin (1 µg/ml), penicillin (10 U), vancomycin (30 µg/ml), lincomycin (2 µg/ml), streptomycin (10 µg/ml), rifampin (5 µg/ml), and tetracycline (30 µg/ml) were acquired from Remel Fisher Scientific, USA. Susceptibility of the various *Staphylococcus* isolates to different classes of antibiotics were determined using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) at 37ºC. Interpretation of the data was performed according to CLSI guidelines.

Biofilm Formation: Two microliters of overnight cultures of Staphylococcal isolates in Trypticase Soy Broth (TSB) were diluted in 200 µL TSB containing 0.25% glucose in individual wells of polystyrene, flat-bottomed 96-well plates. The plates were incubated at 37°C for 24 h. Next, the planktonic cells were removed, and the wells were washed three times with autoclaved distilled water. Subsequently, 125 µl crystal violet solution (0.1% wt/vol) was added to all wells. After 15 min, the excess crystal violet was rinsed off by placing the plates under water three times. Finally, after drying the plates, bound crystal violet was released by adding 125 µL of 30% acetic acid in water. Absorbance was measured spectrophotometrically at 550 nm for quantification. Standard deviation was calculated and data with error bars was plotted. Good biofilm-forming stains, *S. epidermidis* RP62A and *S. aureus* TCH1516 and poor biofilm-forming stains, *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923, were added to each plate as reference standard, respectively. Experiments were run in triplicate and higher A_{550} values were recorded as a measure of biofilm formation.

Comparative Analysis of Genomic Data from Planktonic and Biofilm Cells: To understand the genomic differences among strong and poor biofilm producing *S. aureus* isolates from hospital and community setting, it is important to decipher their genomic make up. This was achieved by using the following methods.
The genomic DNA of MRSA isolates was extracted using a Master Pure Gram-positive DNA purification kit (Epicentre Biotechnologies). A TruSeq DNA library preparation kit (Illumina) and TruSeq paired-end cluster kit were used for the DNA preparation and cluster generation for sequencing on a HiSeq system, respectively. An Illumina HiSeq 2500 system was used for sequencing. *De novo* assembly of high-quality paired-end reads (100 bp in length) was conducted using the CLC genomics workbench 6.5.1 (CLC Bio), and further genome annotation was performed using an annotation method GeneMarkS in the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

Biofilm Assay



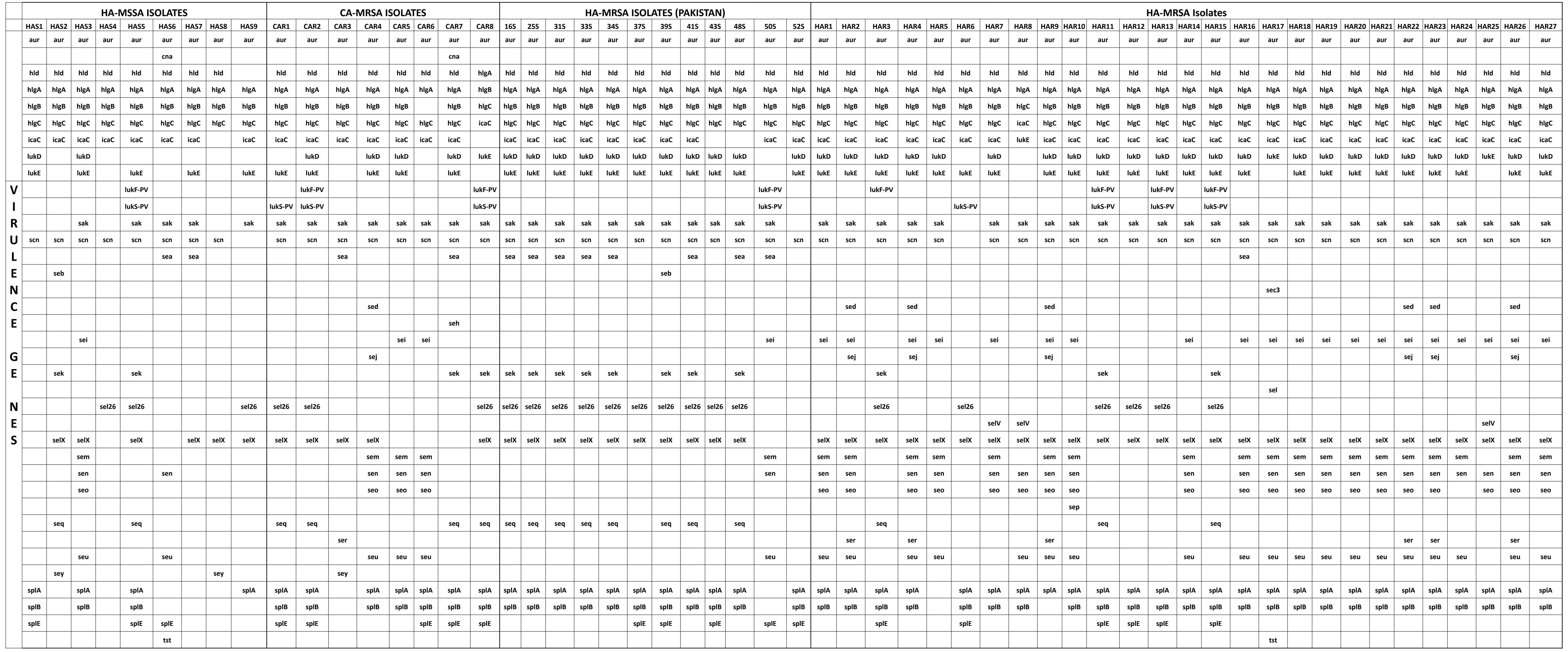
WGS Analyses of MSSA and MRSA Isolates

Isolate	MLST (ST)	spa type	SCCmec type	GenBank Acc. #
HAS1	ST15	NA	NA	JAHNGX000000000
HAS2	ST59	t437	NA	JAHNGY000000000
HAS3	ST5	t242	NA	JAHNGZ000000000
HAS4	ST398	t571	NA	JAHNHA000000000
HAS5	ST8	t008	NA	JAHNHB000000000
HAS6	ST1390	t338	NA	JAHNHC000000000
HAS7	ST96	t2802	NA	JAHNHD000000000
HAS8	ST59	NA	NA	JAHNHE000000000
HAS9	ST8	t008	NA	JAHNHF000000000
HAR1	ST231	t002	II(2A)	JAHNFW000000000
HAR2	ST5	t002	II(2A)	JAHNFX000000000
HAR3	ST8	t008	IVa(2B)	JAHNFY000000000
HAR4	ST105	t045	II(2A)	JAHNFZ000000000
HAR5	ST105	t586	II(2A)	JAHNGA000000000
HAR6	ST8	NA	IVa(2B)	JAHNGB000000000
HAR7	ST231	NA	II(2A)	JAHNGC000000000
HAR8	NA	t002	II(2A)	JAHNGD000000000
HAR9	ST231	t002	II(2A)	JAHNGE000000000
HAR10	NA	t1154	II(2A)	JAHNGF000000000
HAR11	ST8	NA	IVa(2B)	JAHNGG000000000
HAR12	ST2253	t008	IVg(2B)	JAHNGH000000000
HAR13	ST8	t008	IVa(2B)	JAHNGI000000000
HAR14	ST105	t002	II(2A)	JAHNGJ000000000
HAR15	ST8	t008	IVa(2B)	JAHNGK000000000
HAR16	ST5	t002	IVa(2B)	JAHNGL000000000
HAR17	ST5	t002	IVc(2B)	JAHNGM000000000
HAR18	ST105	NA	II(2A)	JAHNGN000000000
HAR19	ST105	t002	II(2A)	JAHNGO000000000
HAR20	ST5	t002	IVc(2B)	JAHNGP000000000
HAR21	ST5	t003	II(2A)	JAHNGQ000000000
HAR22	ST496	NA	II(2A)	JAHNGR000000000
HAR23	NA	t458	II(2A)	JAHNGS000000000
HAR24	ST5	t003	II(2A)	JAHNGT000000000
HAR25	ST105	t002	II(2A)	JAHNGU000000000
HAR26	ST231	t586	II(2A)	JAHNGV000000000
HAR27	ST105	t002	II(2A)	JAHNGW000000000
CAR1	ST8	t008	IVa(2B)	JAHNFO000000000
CAR2	ST8	t190	IVa(2B)	JAHNFP000000000
CAR3	ST59	t2365	IVg(2B)	JAHNFQ000000000
CAR4	ST231	NA	II(2A)	JAHNFR000000000
CAR5	ST72	t693	IV(2B)	JAHNFS000000000
CAR6	ST72	NA	IVa(2B)	JAHNFT000000000
CAR7	ST2725	t1784	IVa(2B)	JAHNFU000000000
CAR8	ST8	t024	IVa(2B)	JAHNFV000000000
16S	ST239	t632	III(3A)	JAHNFC000000000
25S	ST8	t064	IV(2B)	JAHNFD000000000
31S	ST239	t030	III(3A)	JAHNFE000000000
33S	ST239	t030	III(3A)	JAHNFF000000000
34S	ST239	t030	III(3A)	JAHNFG000000000
37S	ST8	t064	IVa(2B)	JAHNFH000000000
39S	ST8	t064	IVa(2B)	JAHNFI000000000
43S	ST30	t148	III(3A)	JAHNFJ000000000
41S	ST239	t030	III(3A)	JAHNFK000000000
48S	ST239	t030	III(3A)	JAHNFL000000000
50S	ST503	t138	IVg(2B)	JAHNFM000000000
52S	ST15	t1509	IVa(2B)	JAHNFN000000000

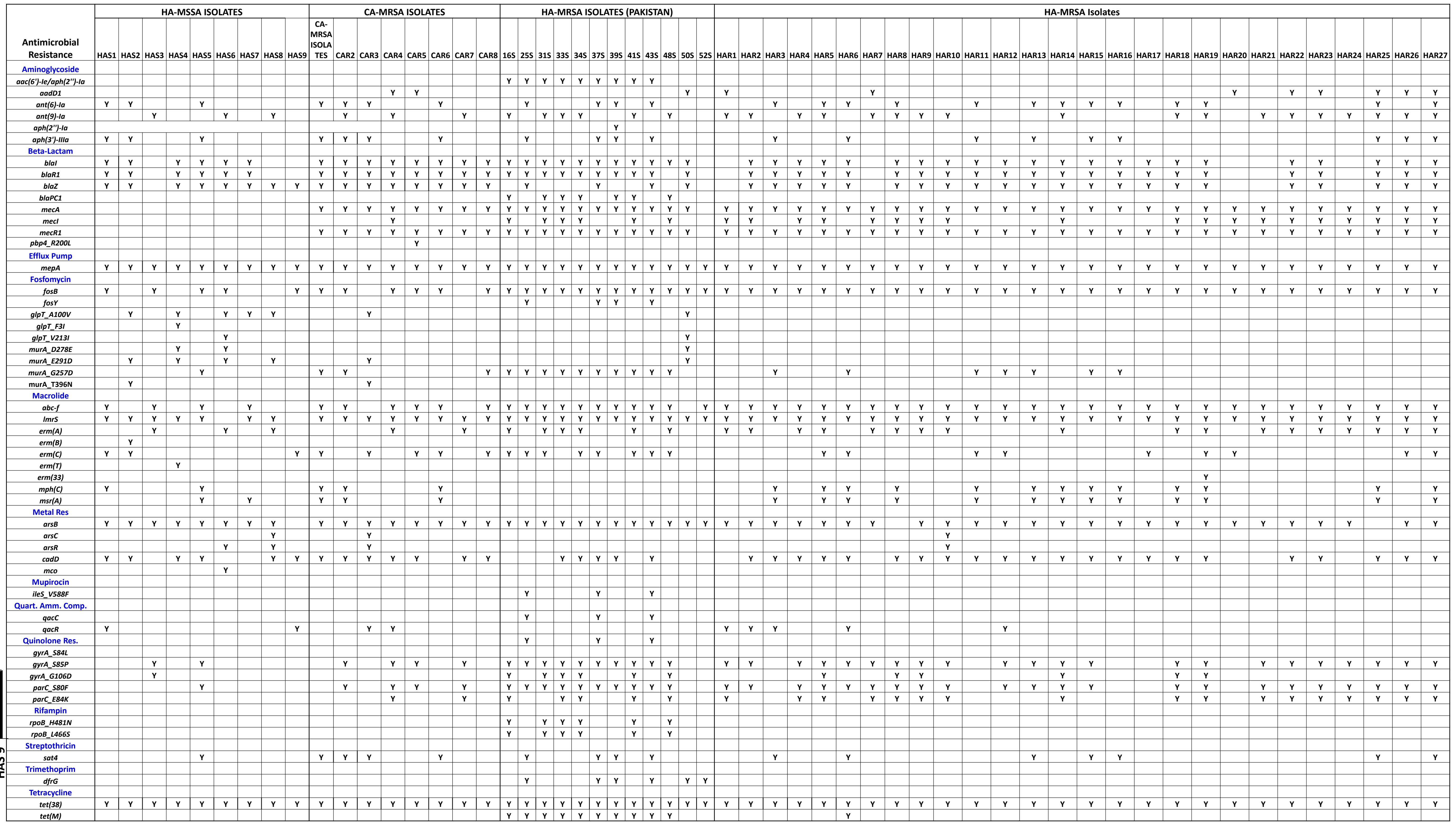
NA- Not available
*Searched by Pathosystems Resource Integration Center (PATRIC)
**Searched by Virulence Factor Database (VFDB)

Virulence Gene Profiles of MSSA and MRSA Isolates

RESULTS



Antimicrobial Resistance Gene Profiles of MSSA and MRSA Isolates



Conclusions

All the isolates were multidrug-resistant and all of them had a tetracycline efflux pump gene tet38 and the multidrug and toxic compound extrusion (MATE) family gene mepA.

Beta lactamases and quinolone resistance genes were present in most isolates but were predominant in MRSA isolates.

ST5 and ST105 were prevalent sequence types in HA-MRSA isolates from the United States. ST8 was common among all the isolates but prevalent among US isolates. Pakistani isolates had ST239 as the most prominent sequence types.

Spa-typing indicated t008 as a common spa types among US isolates. Pakistani isolates predominantly exhibited t030 and to64 Patterns, while the US isolates had t002 and t008 as the predominant spa types.

The most common SCCmec pattern among all the isolates was Iva(2B). However, II(2A) was prevalent among US HA-MRSA isolates and III(3A) being more dominant (7/12) among Pakistani isolates.

All the isolates had multiple virulence genes.

All the isolates had some biofilm-forming ability but some were strong biofilm makers.

Most of the strong biofilm makers had enterotoxin genes, sek, sel26, seq, and delta hemolysin gene hld that regulates the expression of some exoprotein genes.

CONTACT

Dr. Saeed A Khan
Div. of Microbiology
Nat. Ctr. for Tox. Res.
Jefferson, AR 72079
Phone: (870) 543-7197