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Title: **Comparative Analysis of Methicillin-resistant Staphylococcus aureus through Whole Genome Sequencing (WGS) in the Largest Tertiary Care Hospital in Peshawar, Pakistan**

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Research Article

Comparative Analysis of Methicillin-resistant *Staphylococcus aureus* through Whole Genome Sequencing (WGS) in the Largest Tertiary Care Hospital in Peshawar, Pakistan

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Abstract

Methicillin-resistant Staphylococcus aureus (MRSA) is a globally disseminated antibiotic-resistant pathogen that causes mild to severe infections. The population structure of MRSA is highly clonal. Individual genetic clones harbor specific plasmids and determinants for antibiotic resistance and virulence. Therefore, whole genome sequencing (WGS) of five MRSA isolates of the predominant genetic lineage was performed to gain insight into the most prevalent endemic strains. Sequencing libraries were arranged in accordance with the Nextera XT DNA Library Prep Guide (Illumina). WGS was performed on Illumina NextSeq 500 platform with 2×151bp using a NextSeq Mid-Output Kit. The sequenced genome was characterized for spa type, MLST type, SCCmec type, plasmid, antibiotic resistance gene, and virulence gene. This characterization was performed by publicly available bioinformatics tools, available on the website of the Center for Genomic Epidemiology. All the isolates were characterized as ST8/t064-SCCmecIVa (2B) clones of MRSA. A total number of 13 resistance genes, 11 virulence genes, and 6 types of plasmids were identified in the sequenced isolates of MRSA. Furthermore, the sequenced isolates showed a similar pattern of distribution for antibiotic resistance genes and virulence genes. WGS revealed that the isolates were genetically closely

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related and showed a similar pattern of distribution for different genetic markers. Therefore, these strains could be an accidental case cluster or cluster of an outbreak.

Keywords: methicillin-resistant *S. aureus*, molecular typing, resistance genes, virulence genes, Whole Genome Sequencing (WGS)

Introduction

Staphylococcus (S.) aureus is commonly found living on the skin, nostrils, and the pharynx of healthy adults. It is a cause of many diseases, whose severity ranges from common community-associated skin infections to fatal bacteremia [1]. Recently, Methicillin-resistant *S. aureus* (MRSA) strains have been responsible for about 25%–50% of all clinical infections caused by *S. aureus* in many countries [2]. The population structure of MRSA is highly clonal. The information regarding the dissemination pattern of the different clones of MRSA has been gathered largely through molecular typing methods. These methods encompass pulsed-field gel electrophoresis (PFGE), *S. aureus* protein A (*spa*) typing, and multilocus sequence typing (MLST) [3]. However, these methods cannot distinguish the strains of the same lineage [4]. Therefore, whole genome sequencing (WGS) is a preferred method, with superior power of discrimination, for the evolutionary studies of a specific lineage, its transmission and nosocomial outbreak [5]. Furthermore, WGS can be used for a better understanding of MRSA pathogenesis by delineating the genes responsible for resistance and virulence [6].

MRSA has formed diverse clones since its discovery. Although the most commonly reported clones throughout the world fit into one of the five main clonal complexes (CCs) encompassing CC5, CC8, CC22, CC30, and CC45. CC5 and CC8 are the most prevailing CCs among the major CCs throughout the world. CC45 is more common in USA and Europe, whereas CC30 is mostly distributed in the USA and the U K. In Asian countries, CC8, CC5, and CC22 are the most commonly distributed CCs. Latin America is predominantly populated with CC5, CC8, and CC30, whereas CC8, CC5, and CC30 are the prevalent lineages in Africa. The occurrence of CC8 has been only sporadically reported from Australia and Africa [7].

In developing countries such as Pakistan, there is very limited data available about the molecular epidemiology of MRSA. Usually, there is no infection control program and monitoring system in place for the typing of

infectious pathogens including MRSA. Hence, the current study was conducted to perform the WGS of the MRSA isolates, selected from the most prevalent strains of MRSA (MRSA t064/IV unpublished data) in the largest tertiary care hospital in the province. It provides helpful and in-depth knowledge of the most frequent endemic strains of MRSA in this hospital.

Methodology

The current cross-sectional study was conducted in the Department of Medical Lab Technology, University of Haripur, Haripur, Pakistan. A total of five non-repetitive MRSA isolates were anonymously collected in the year 2019 from pus specimens in the Lady Reading Hospital (LRH), Peshawar, Pakistan. LRH is the largest tertiary care public sector hospital located in the center of Peshawar, the capital city of Khyber Pakhtunkhwa (KP) Province, Pakistan. It has 30 departments and 1751 beds. All the *S. aureus* isolates were collected from the routine work of the microbiology laboratory of the hospital. The isolates were identified as *S. aureus* by mannitol fermentation and tube coagulase test. While Methicillin-resistance was determined by E-test against Cefoxitin, according to the Clinical and Laboratory Standards Institute [8].

Whole Genome Sequencing (WGS)

MRSA isolates were incubated overnight on an individual blood agar plate (SSI Diagnostica). A 1µl loop of culture was incubated for 30 minutes at 37°C in 180µl enzymatic lysis buffer containing 10 units/ml lysostaphin (Sigma-Aldrich) and 20mg/ml lysozyme (Sigma-Aldrich). Afterwards, DNA was extracted and quantified using the DNeasy Blood & Tissue Kit (Qiagen) and the Qubit 3.0 Fluorometer (Invitrogen), respectively.

Sequencing library preparation was conducted in accordance with the Nextera XT DNA Library Prep Guide (Illumina). The libraries were sequenced on a NextSeq 500 platform (Illumina) with 2×151bp using a NextSeq Mid-Output Kit.

WGS data were analyzed by using publicly accessible online bioinformatics tools available on the website of the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). Antibiotic resistance genes, both acquired and chromosomal mutations, were determined by the application of ResFinder 3.1 [7]. Furthermore, virulence genes were identified by using VirulenceFinder 2.0 [9], plasmids were

detected through PlasmidFinder 2.0 [10], SCC*mec* typing was performed by SCC*mec*Finder 1.2 [11], and MLST was assigned by MLST 2.0 server.

Results

All the five isolates were able to ferment mannitol and were positive for the tube coagulase test. Moreover, the minimal inhibitory concentration of the tested MRSA isolates against Cefoxitin was higher than 8µg/ml, as measured by the E-test. All the isolates of MRSA were classified as ST8/t064-SCCmecIVa (2B) genetic clones.

A total of 13 resistance genes were detected through ResFinder in the sequenced data. Wherein, 12 genes of antibiotic resistance were found in all the 5 sequenced isolates of MRSA. While, *aadD* gene, which is responsible for aminoglycoside resistance, was found only in one isolate. Out of the 13 resistance genes, 4 genes encompassing *gyrA*, *grlA* for ciprofloxacin resistance, *ileS* for mupirocin resistance, and *fusA* for fusidic acid resistance were present on the chromosome. Whilst, the rest of the 9 genes were acquired, as presented in Table 1.

Eleven potential virulence determinants were recognized by VirulenceFinder, in which 4 genes were responsible for exoenzyme, 2 for host immune evasion components, and 5 for encode exotoxin. Exoenzymes include aureolysin and serine protease, both help in tissue destruction and are present in the core genome. Hence, they are usually recognized in the *S. aureus* and MRSA strains. Host immune evasion components include staphylokinase and staphylococcal complement inhibitors, responsible for clot dissolution and complement inhibition, respectively. These toxins encompass the γ-hemolysin component responsible for hemolysis and leukocidin. It also plays a vital role in white blood cell destruction. Eventually, 11 potential virulence genes were detected in each genome of the sequenced isolates and tabulated in Table 1.

PlasmidFinder detected six plasmids including pDLK1 of the rep10 family, pKH12 of the rep21 family, MSSA476 of the rep7 family, pWBG753 of the rep20 family, pWBG749 of the rep24 family, and pLW043 of the rep15 family in the five sequenced genomes of MRSA. Wherein, each plasmid belonged to an individual rep family. Three of the six plasmids encompassing pDLK1, pKH12, and MSSA476 were identified in all of the five genomes.

Table 1. Antibiotic Resistance and Virulence Genes, Their Phenotype and Function

Antibiotic Resistance		**Function**	**Virulence Factors**		
Gene (n)	**Phenotype**		**Gene (n)**	**Phenotype**	**Function**
***ant(6)-Ia* (5)**	Aminoglycoside nucleotidyl transferase	Aminoglycoside resistance	*aur* (5)	Aureolysin: member of the thermolysin family of zinc-dependent metalloproteases	Cleave the plasma proteinase inhibitors, α_1-antichymotrypsin and α_1-proteinase inhibitor and to activate prothrombin in human plasma
***aph(3')III* (5)**	Aminoglycoside-30-O-phosphoryltransferase				
aac(6')aph (2'')	Aminoglycoside-60-N-acetyltransferase/200-O-phosphoryltransferase				
***aadD* (1)**	Adenyltransferase				
***mecA* (5)**	Penicillin-binding protein D (PBPD)	β-lactam resistance	*splA* (5), *splB* (5), *splE* (5)	Serine proteas: classic catalytic triad of trypsin-like serine proteases	Proteolytic and protease activity
***blaZ* (5)**	Penicillinase				
***erm(C)* (5)**	Erythromycin ribosomal methylase	Macrolide resistance	*Sak* (5)	Staphylokinase: N-acetylmuramyl-L-alanine amidase	Forming a plasminogen activating complex and initiates the fibrinolytic cascade
***tet(M)* (5)**	Ribosomal associated protection protein	Tetracycline resistance	*Scn* (5)	Staphylococcal complement inhibitor (SCIN) protein	Inhibiting the complement cascade
***dfrG* (5)**	Trimethoprim-resistant dihydrofolate reductase	Trimethoprim resistance	*hlgA* (5) *hlgB* (5)	γ-hemolysin chain precursor	Host cell lysis
***gyrA* (5)**	DNA gyrase	Ciprofloxacin resistance	*hlgC* (5)	γ -hemolysin component	Host cell lysis
***grlA* (5)**	DNA topoisomerase IV				
***ileS* (5)**	Additional isoleucyl-tRNA-synthetases	Mupirocin resistance	*lukD* (5)	leukocidin D component	Inducing dermonecrosis and inflammation
***fusA* (5)**	EF-G	Fusidic acid resistance	*lukE* (5)	leukocidin E component	Inducing dermonecrosis and inflammation

Moreover, PlasmidFinder revealed that the genome of one isolate contained 5 plasmids encompassing pDLK1, pKH12, MSSA476, pWBG753, and pWBG749. While, the genome of another strain harbored four plasmids including pDLK1, pKH12, MSSA476, and pLW043. The rest of the three genomes were found to be the carriers of only 3 plasmids including pDLK1, pKH12, and MSSA476.

MLST server and *spa* typer assigned Sequence type (ST) 8 and *spa* type t064 to all the isolates, respectively. All the isolates harbored SCC*mec* type Iva (2B), revealed by SCC*mec*Finder.

Discussion

The current study subjected to testing and analysis the five isolates of MRSA, which were selected from the most prevalent lineage of MRSA in the largest tertiary care hospital of the province. These isolates were subjected to WGS for delineating the genes responsible for antibiotic resistance and virulence. Moreover, plasmids were also identified and SCC*mec* element was harbored by the sequenced isolates of MRSA. Finally, their genetic lineage was determined by the MLST server. There is hardly any data available about the WGS of MRSA from Pakistan. This study certainly could not address the issue of insufficient data regarding infectious bacterial pathogens including MRSA. However, it may contribute to an in-depth understanding of the circulating clone of MRSA in the largest tertiary care hospital of Peshawar.

In LRH, antibiotic resistance was detected using the disc diffusion method. Consequently, there is no understanding of the origin of resistance and virulence genes in terms of whether they are acquired or chromosomal. Subsequently, the concerned genes and mechanisms remained obscured. Hence, the current study provides an insight into the origin and mechanism of the gene harbored by the endemic MRSA strains for resistance and virulence.

Monecke et al. [12] reported a DNA microarray based study on MRSA, isolated from patients with bloodstream infection, from the same tertiary care hospital. They reported the prevalence of antibiotic resistance genes including Beta-lactam resistance gene *mecA* and *blaZ*, macrolide and lincosamide resistance gene *msr(A)*, *mpb(C)*, *lnu(A)*, and *erm(C)*, aminoglycoside resistance gene *aphA3*, *aacA-aphD*, and *aadD*, streptomycin resistance gene *sat*, tetracycline resistance gene *tet(K)*, and

trimethoprim resistance gene *dfrA,* respectively. While, the presence of the resistance gene for Beta-lactam, macrolide, aminoglycoside, tetracycline, and trimethoprim was also determined. Also, tet (m) instead of tet (k) for tetracycline resistance, as well as dfrG for trimethoprim resistance rather than dfrA, were identified. The resistance determinants for aminoglycosides were also found to be different in both studies. Furthermore, The genes of resistance for ciprofloxacin, mupirocin, and fusidic acid were identified which were not found in the above mentioned study. Although, the resistance gene for streptomycin was not detected. Moreover, Monecke et al. identified 29 virulence genes containing all the 11 virulence genes detected in the current study. The variation in antibiotic resistance and virulence genes was due to the difference in the genetic lineage of the studied MRSA strains. It was because the MRSA of only one lineage, that is, ST-8 was studied, while the MRSA strains of many lineages were included in the current research.

A recent study in 2019 [13] carried out in Lahore, Pakistan reported the WGS analysis of the MRSA strains with novel MLST type ST5352. The comparable results for antibiotic resistance and virulence genes were also determined in their sequence isolate of MRSA. The study documented the presence of two important virulence genes namely lukF/S-PV and tst1, which affected the severity of the MRSA infection. These two genes are not associated with ST-8 MRSA, therefore, they could not be identified in the current study. Additionally, one plasmid replicon rep20 was identified. It was detected also in one of the sequenced genomes of the currently studied MRSA isolates. While, the presence of SCC*mec* type V was recorded in the previous study, SCC*mec* IVa (2B) was found in all the sequenced genomes in the current study.

A study delineated the antibiotic resistance and virulence genes through the WGS of MRSA ST-8 strain from Tanzania in 2018. It showed a similar distribution pattern of genes responsible for antibiotic resistance and virulence [14]. The similarities among the strains of this study to the current findings suggest the pandemic distribution of ST-8 MRSA.

The five studied MRSA strains were identical in terms of resistance genes, virulence genes, SCC*mec* typing, spa typing, and MLST typing. It indicates the possibility of an accidental case cluster or an outbreak of the studied strain in the hospital and/or region. Therefore, it warrants further studies to overcome this potential health hazard[15].

Plasmids play a vital role in the distribution of resistance and virulence genes in the *S. aureus* population by horizontal gene transfer. Furthermore, specific resistance and virulence genes were associated with a distinctive group of plasmids and those plasmids were then associated with a particular *S. aureus* lineage [16]. The findings of the current study also extend the previous knowledge about the association of resistance and virulence genes with certain plasmid and clonal lineages of MRSA. For instance, pDLK1 and pKH12 carry *ermC* and *blaZ* gene respectively and they were identified in the five sequenced genomes of MRSA. Similarly, other resistance and virulence genes also showed plasmid and lineage specificity, as reported in the literature earlier [17, 18].

As mentioned above, all the five WGS MRSA strains harbored SCCmec type IV elements, which revealed that these strains are community-associated MRSA (CA-MRSA). However, in hospitals and community settings these strains are a major cause of infection [15]. Hence, further studies are required to locate the origin, transmission event, and the outbreak of the circulating strains of MRSA.

The limitation of the current study included relatively small numbers of MRSA strains subjected to WGS.

Conclusion

All the isolates of MRSA were classified as ST8/t064-SCCmecIVa (2B) genetic clone of CA-MRSA. Moreover, comparative analysis showed that the strains were quite closely related and it could be an accidental case cluster or cluster of an outbreak.

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