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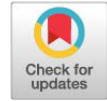

Short Communication

MRSA Clinical Isolates Harboring *mecC* Gene Imply Zoonotic Transmission to Humans and Colonization by Biofilm Formation

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SH and RA collected all samples, performed the experiments and wrote the article. BZ supervised the research work. NA and SK prepared the figures, tables and the initial manuscript.

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ABSTRACT

This study was conducted for the molecular detection of the *mecA, mecC,* and *nuc* gene among MRSA and to investigate biofilm formation among the methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates. A total of 208 different samples were collected and processed for phenotypic and genotypic identification of MRSA. All MRSA isolates were subjected to antibiotics sensitivity, cefoxitin disk diffusion test, and vancomycin minimum inhibitory concentration (MIC) E-test. The MRSA isolates were detected for the presence of *mecA, mecC,* and *nuc* genes. Congo red agar (CRA) method was used to assess the ability of isolates to form biofilms. The results of the study showed that the prevalence of MRSA was 48%. The MRSA isolates were highly resistant (100%) to penicillin, β lactamase inhibitors, cephalosporins, and macrolides. All the MRSA isolates were susceptible to vancomycin antibiotic drugs. Cefoxitin (30 µg) disk diffusion test showed 100% sensitivity and specificity for the identification of MRSA phenotypically. A total of 100 MRSA clinical isolates were positive for the *mecA* and *nuc* gene. Only 3 MRSA isolates were positive for *the mecC* gene. Congo red agar method showed that 20 (20%) isolates formed moderate biofilm while 80 (80%) isolates were non-biofilm forming. Multi drugs resistant and *mecC* gene-positive MRSA isolates are rapidly emerging in Pakistan. Therefore, the *mecC* gene should be detected along with the *mecA* gene for the identification of MRSA clinical isolates. It also requires early identification of biofilm formation and necessary interventions for its effective treatment and control.

Staphylococcus aureus generally colonizes humans and animals. An estimated 25-50% of the general population are nasal carrier and 10-20% are persistent carriers of *S. aureus* (Parveen *et al*., 2020). Over the last few years, methicillin-resistant *Staphylococcus aureus* (MRSA) has increased significantly in community and hospital-related settings. Asia has the highest number of MRSA infections worldwide (Chen and Huang, 2014). MRSA strains are potentially lethal that mediate virulence by adhesions, production of enzymes, immune modulators, and toxins (Watkins et al., 2012). Antibiotics resistance in MRSA isolates is a global concern. It typically takes place as a spontaneous genetic mutation or may acquire a genetic material such as transposon, integron, plasmid, or gene cassette. MRSA isolates are broadly resistant to multiple drugs; aminoglycosides, fluoroquinolones, chloramphenicol, trimethoprim/sulfamethaxazole, macrolides, and β lactamases due to the presence of *mecA* gene on these isolates (Taj *et al*., 2010). Molecular epidemiological studies have revealed that community acquired CA-MRSA and hospital-acquired HA-MRSA are phenotypically and genotypically distinct (Watkins *et al*., 2012). MRSA isolates carry *the mecA* gene which encodes for methicillin-resistance binding proteins (PBP2a) (Harkins *et al*., 2017). *The mecA* gene mediates methicillin resistance that is transmitted by the staphylococcal cassette chromosome *mec* (SCCmec) with a size of 21-67 kbp (Nezhad *et al*., 2017). MRSA isolates that colonize and infect various animals are an important cause of zoonotic

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infections in humans (Tariq *et al.*, 2020). Such MRSA strains harbor novel homolog *mecC* of *mecA*.

S. aureus produces an extracellular thermostable nuclease, encoded by *nuc* gene, which is one of the most distinguishing and successful characteristics that might be used for distinguishing *S. aureus* from other *Staphylococcus* spp. This suggests that *nuc* gene is a specific marker gene and PCR is a useful method for identifying this gene in *S. aureus* (Sahebnasagh *et al.*, 2014). *nuc* gene is strongly associated with the production of enterotoxin and it can be considered as an indicator of infection with enterotoxin producer *S. aureus* (Karimzadeh and Ghassab, 2020). In 2011, a new methicillin resistance determinant, the *mecC* gene, was identified in *S. aureus* isolates recovered from humans and dairy cattle. The *mecC* determinant was able to produce low-level resistance to β-lactam antibiotics such as cefoxitin and oxacillin.

Staphylococci are associated with biofilm-mediated infections. Biofilms are linked to numerous human diseases such as chronic lungs, skin lesions, and ear infections. Biofilms tend to colonize medical devices such as catheters and implants. According to the National Institute of Health (NIH), more than 80% of all microbial infections develop biofilms. These types of infections are difficult to diagnose and treat as they are the leading cause of increased hospitalization, accelerated healthcare expenses, and expanded mortality and morbidity (Rabin *et al.*, 2015). Within a biofilm, bacteria communicate with each other by the production of chemotactic particles or pheromones, a phenomenon called quorum sensing (Hassan *et al.*, 2011; Javed *et al.*, 2020). These biofilm-forming bacteria can be up to thousand fold more resistant to antibiotic treatment than planktonic bacteria (Gebreyohannes *et al.*, 2019). Due to the complex pathogenicity and therapeutic importance, the present study is conducted for the molecular detection of *mecA, mecC*, and *nuc* genes and investigation of biofilm formation among MRSA clinical isolates.

Materials and methods

A total of 208 different samples including pus, wound swabs, throat swabs, fluids, breasts abscess, ear swabs, urine, and blood were collected. The samples were inoculated onto blood, mannitol salt, and CLED agar. The colonies of *S.aureus* were identified using Gram staining and biochemical tests (catalase, oxidase, DNase, and coagulase). Phenotypic identification of methicillin resistance was achieved by subjecting the isolates to the cefoxitin disk diffusion test with 30 µg disk according to Clinical and Laboratory Standards Institute (CLSI Guidelines, 2017).

After isolation and identification, the isolates were tested for antimicrobial susceptibility by Kirby Bauer disk diffusion method according to the CLSI Guidelines (2017). MRSA isolates were examined for amikacin (30µg), amoxicillin (10µg), ampicillin/sulbactam (10µg), azithromycin (15µg), cefepime (30µg), ciprofloxacin (5µg), clindamycin (2µg), erythromycin (15µg), fusidic acid (10µg), gentamicin (10µg), imipenem (10µg), levofloxacin (5µg), linezolid (30µg), moxifloxacin (5µg), ofloxacin (5µg), tobramycin (10µg) and trime/sulphamethoxazol (25µg). Disks of antibiotics with different concentrations were placed on solid media such as Mueller Hinton agar (MHA) containing the inoculated bacteria. These plates were incubated at 37°C for 48 h.

Vancomycin E-test was performed using a minimum inhibitory concentration (MIC) strip for vancomycin antibiotics susceptibility (VA .016-256 µg/ml) following CLSI Guidelines (2017). The strip was placed on MHA having lawned growth of MRSA incubated at 37°C for 24 h with cefoxitin disk.

The phenotypic production of biofilm in all MRSA isolates was determined by culture in Congo red agar (CRA) plates. The MRSA strains were inoculated on the prepared medium and incubated aerobically for 48 h at 37˚C. The morphology and color of the colonies were noted for biofilm formation.

For molecular identification of MRSA resistance genes DNA was extracted from MRSA positive isolates using the standard method described in the Wizprep ™ GDNA mini kit (Seongnam, Korea). DNA extraction results were confirmed by gel electrophoresis. The three resistance genes of *mecA*, *mecC* and *nuc* genes were amplified using primers sequence of *mecA* F-AAAATCGATGGTAAAGGTTGGC R-AGTTCTGCAGTACCGGATTTGC, *mecC* F-GAAAAAAAGGCTTAGAACGCCTC R-GAAGATCTTTTCCGTTTTCAGC and *nuc* gene F-GCGATTGATGGTGATACGGTTR-AGCCAAGCCTTGACGAACTAAAGC. The product size of these genes was 533bp, 138bp, and 278bp, respectively.

Sequencing was done using commercial sequencing services from Macrogen (Seoul, Korea). Bioinformatics tools (NCBI-nBLAST) were used to determine the similarity index of sequences. MEGA version X software was used to construct the phylogenetic tree by the neighbor-joining method.

Results and discussion

In the present study out of a total of 208 isolates, 100 isolates were identified as MRSA by phenotypic and genotypic identification methods. The prevalence of MRSA was determined as 48% (100/208). In another study conducted by Khan *et al.* (2020) in Islamabad, Pakistan MRSA prevalence was 65% which is higher than our

study. The reason could be differences in the demographic distribution of MRSA in different parts of the country.

The number of MRSA isolated from pus samples was 57%, from wound swab 16 (16%), blood 11 (11%), urine 5 (5/%) throat swab 4 (4%) ear swab 3 (3%), joint fluid 2 (2%) and breast abscess 2 (2%).

All MRSA isolates were highly resistant to Penicillins (Ampicillin 100%), β lactamase inhibitors (Ampicillin/ Sulbactam 100%) while fourth generation cephalosporin (cefepime) also revealed 100% resistance. Carbapenem (imipenem) showed 100% resistance in all isolates. A comparatively medium level resistance pattern as compared to other antibiotics drugs was observed in aminoglycosides; gentamicin 43 (43%) and tobramycin 55 (55%) but amikacin showed the least resistance of only 1 (1%). It is observed that MRSA isolates were highly resistant to macrolides; azithromycin and erythromycin with 95 (95%) and 92 (92%) resistance respectively. More than 50%MRSA isolates were resistant to quinolones and fluoroquinolones; ciprofloxacin 58 (58%), levofloxacin 61 (61%), ofloxacin 56 (56%), moxifloxacin 62 (62%). Folate pathway inhibitors trimethoprim/ sulphamethoxazole were relatively sensitive and less resistant 16(16%). The resistance pattern of other antibiotics was clindamycin 11 (11%), linezolid (01%), and fusidic acid 13 (13%). Overall, these results revealed amikacin and linezolid as the drug of choice with the highest sensitivity for the treatment of MRSA. The results of our study are comparable to the study conducted by Khan *et al*. (2020) which showed resistance ciprofloxacin 85%, cefoxitin 65%, gentamicin 64%, erythromycin 50% tetracycline 36% sulphamethaxazole/ trimethoprim 26%, clindamycin 26%and rifampicin 20% while high-level susceptibility was observed in linezolid 96%, quinoprstin/dalfoprestin 95% and chloramphenicol 88%. In another study by Kaleem *et al*. (2010) on MRSA antibiotics sensitivity patterns from tertiary care hospitals in Pakistan. In this study, all the MRSA isolates were 100% sensitive to vancomycin, linezolid, and tigecycline. Other antibiotics showed resistance as fusidic acid 35%, tetracycline 36%, doxycycline 59%, macrolides 88%, trimethoprim/ sulphamethoxazole 33%, teicoplanin 6%, chloramphenicol 7%, rifampicin 38%, fluoroquinolones 62% and showed sensitivity 65%, 64%, 41%, 22%, 67%, 94%, 93%, 62% and 38% respectively. Vancomycin E test was performed on all MRSA isolates. The results indicate that all the MRSA isolates were susceptible to vancomycin antibiotic drug that is parallel to previous studies as conducted by Girgis *et al*. (2013) and Kaleem *et al*. (2010). The findings of the present study indicated that vancomycin along with amikacin and linezolid can be the drug of choice to treat MRSA infections (Supplementary Table I).

All the 100 MRSA isolates were sensitive to vancomycin antibiotic drug with a mean minimum inhibitory concentration of 54µg/ml (Supplementary Fig. 1).

20% of the isolates indicated brown colonies (weak biofilm producers), 80% isolates showed red colonies (non-biofilm producers) and none of them depicted black colonies (strong biofilm producers). The results of biofilm formation were different from the previous study which was done by Haddad *et al*. (2018). They reported that 46.5% and 53.5% of isolates were respectively strong and moderately biofilm-formers. Another study reported that 53.8% of MRSA isolates exhibited moderately attached biofilms and 28.5% of isolates were non-biofilm producers (Smith *et al*., 2008). Based on the findings of the present study it was suggested that the susceptibility to methicillin and biofilm formation is not associated with each other (Supplementary Fig. 2).

Molecular analysis revealed that all the 100 MRSA isolates were positive for both *mecA* and *nuc* genes while only 3% (3/100) isolates were positive for the *mecC* gene. All *mecC* genes positive MRSA were also positive for *mecA* gene and detected in pus samples only. The results of our study were parallel to the study conducted by (Pramodhini *et al*., 2011; Skov *et al*., 2013). These studies also conferred 100% sensitivity and specificity of cefoxitin with *mecA* gene. The detection of the *mecA* gene is considered as the gold standard for identification of MRSA by PCR and all the isolates carry the *mecA* gene on the genome as described by Brown *et al*. (2005). The prevalence of *the mecC* gene was 3% as only three MRSA isolates showed positive results for *the mecC* gene by PCR. The results of our investigation are comparable to previous studies on the detection of *mecC* gene-positive MRSA isolates. A similar prevalence rate of *mecC* gene was reported in other regions such as 2% in Austria (Kerschner *et al*., 2015) and 3% in Islamabad Pakistan (Khan *et al*., 2020). The presence of *mecC* gene in MRSA highlights the zoonotic transmission of the organisms due to frequent contact of people with animals. The present study indicates that all the 100 MRSA isolates were positive for *nuc* gene which is parallel to the previous study conducted by Elshimy *et al*. (2018) and Amin *et al*. (2020). They confirmed the presence of *nuc* gene by PCR amplification in all 166 and 50 MRSA isolates, respectively.

NCBI blast analysis showed 96-99% similarities to already submitted sequences in the NCBI data bank. The tree showed an inter-relationship of strain with closely related previously reported *S.aureus* strains.

Conclusion

Multidrug-resistant and *mecC* gene-positive MRSA

isolates are rapidly emerging in Pakistan. Owing to the rapid emergence of *mecC* gene-positive MRSA isolates with zoonotic transmission to humans and its therapeutic and diagnostic importance; the *mecC* gene-positive MRSA clinical isolates can pose serious healthcare problems in the future. It needs immediate attention. Therefore, the *mecC* gene should be detected with the *mecA and nuc* gene for the identification of MRSA clinical isolates. It also requires early identification of biofilm formation and necessary interventions for its effective treatment and control.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210518040546

Statement of conflict of interest

The authors have declared no conflict of interest.

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