ORIGINAL ARTICLE

Correlation between Methicillin Resistance Gene Presence, Biofilm Production and Antibiotics Pattern of *Staphylococcus aureus* Wound Isolates and the Activity of Possible River Water Bacteriophage Against Them

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ABSTRACT

Background: *Wound infections with Staphylococcus aureus strains, a potential health care problem, especially after methicillin-resistant Staphylococcus aureus (MRSA) occurrence, which poses a significant challenge for healthcare workers.* ***Objective:*** *is to identify the relationship between the existence of the gene mecA within isolated bacterial cells and the phenomenon of multiple antibiotic resistance profiles of these strains. The study also explored the potential therapeutic activity of river water bacteriophage.* ***Methodology:*** *Among other bacterial species isolated from wound infections, 34 strains of S. aureus were isolated. After the screening for mecA gene using the Polymerase Chain Reaction Technique (PCR), the antibiotic susceptibility profiles of S. aureus isolates were determined by the disk diffusion method to identify the relationship between the two parameters. Furthermore, the possibility of Bacteriophage existence against isolated S. aureus was investigated.* ***Results****: A strong correlation between the existence of the gene mecA and the phenomenon of multiple antibiotic resistance profiles, particularly beta-lactams as well as commonly prescribed antimicrobial agents was present. Moreover, the results of the bacteriophage spots showed the possible presence of bacteriophage against S. aureus wound isolates.* ***Conclusion:*** *There was significant relationship between bacterial possessing of gene mecA, their biofilm production ability and their multidrug resistant phenomenon. River water bacteriophage could be used to treat pathogen bacteria included in this study.*

INTRODUCTION

Staphylococcus aureus wound infections represent an important healthcare challenge, particularly after the discovery of antibiotic resistance strains[1]. *S. aureus* is a pathogen known for its ability to produce several infectious diseases, from superficial skin infections to systemic severe conditions like sepsis and pneumonia[2]. The bacterial capability to produce severe infections belongs to its possession of many virulence factors, and its ability to easily acquire resistance to more than one antibiotic. Recently, Multidrug resistance *S. aureus* has become a serious health problem worldwide. It is considered a significant pathogenic bacteria causing both nosocomial and community-acquired infections[3].

Among the various strains, methicillin-resistant *S. aureus* (MRSA) was considered a critical health threat due to its clinical behavior to beta-lactam antibiotics[4]. This resistance is primarily conferred by the mecA gene, the gene that responsible of bacterial ability to decrease the effectiveness of antibiotics that arsenal with beta rings within their chemical structures, by altering penicillin-binding protein (PBP2a)[5,6]. The rise of antibiotic-resistant strains like MRSA complicates infections, like wound infections, treatment and increases the risk of prolonged hospital stays, higher medical costs, and greater morbidity and mortality rates[7,8].

The key factor behind the severity of MRSA is biofilm production, which enables the pathogen to evade human body immunity mechanisms, and moreover, increase the bacterial resistance to antibiotic drugs. The performance of biofilm could be enhanced by low doses of antibiotic drugs, acidity, temperature, and the level of oxygen in the environment[9]. By biofilm formation, the bacteria become embedded in matrices of bacteria polysaccharides, which give bacteria a protection arsenal, as the outer layers protect the inner

layers of the bacterial population inside biofilm matrices. Furthermore, the production of the biofilm participates in the infection severity, as it improves the thrive of bacterial virulence inside the biofilm matrices[10]. Many serious infections was attributed to the ability of MRSA production of biofilm structures, form skin infections (SSTIs) to the deeper systemic infections including endocarditis and osteomyelitis[11].

Bacterial infections with MRSA strain are considered a main problem in hospitals and healthcare places, where aggressive antibiotic resistance strains have been identified[12-15]. The rising of antibiotic resistance prevalence has prompted an urgent search for alternative therapies that can effectively combat resistant bacteria[16,17]. One such alternative is bacteriophage therapy, which uses viruses that specifically infect and lyse bacterial cells[16]. Bacteriophages are abundant microorganisms that cannot live and reproduce without attaching bacterial cells of their specific species, their environments could be any as bacterial hosts are existing[18]. River water is the bacteriophage environment and its isolated phage have shown a potential effect on bacterial pathogens, including antibiotic-resistant strains[19]. Previous studies have demonstrated the bacteriophages' killing efficiency to various drug-resistant bacteria, highlighting their potential role as an adjunct or alternative to traditional antibiotics[16]. Natural water sources bacteriophage have gotten the attention of microbiology scientists recently. Bacteriophage capability to lyse antibiotic-resistant *S. aureus* strains presents a promising avenue for novel therapeutic strategies in the face of increasing antimicrobial resistance[19].

This study aims to find out if there is any connection between the existence of the methicillin resistance gene mecA, the bacterial ability of biofilm production, and the antibiotic resistance patterns among *S. aureus* wound isolates. In addition, the possible presence of bacteriophage in river water against isolated pathogens was investigated.

METHODOLOGY

Ethical Approval

This study obtained ethical approval from the Ethical Approval Committee, University of Anbar, Ramadi, Iraq, prior to conducting any research involving human participants. Informed consent was obtained from all patients providing wound samples and/or biopsy samples, ensuring that they were fully aware of the study's purpose, procedures, and potential risks. All data collected were handled confidentially concerning ethical guidelines to protect participant privacy.

Study design

Our study is a cross-sectional aimed at investigating the correlation between the presence of the *mecA* gene in *bacterial isolates* isolated from wound infections and biopsy samples and their antibiotic resistance profiles, as well as the possibilities of river water bacteriophage activity against these strains. The study was conducted in two phases: (1) *in vitro* analysis of bacterial resistance patterns to antibiotics and genetic screening for their resistance to methicillin, and (2) river water bacteriophage testing against this study bacterial isolates.

S. aureus Isolation and Identification

Wound swab samples and biopsy specimens were obtained from patients with wound infections. The patients were residents in Ramadi Teaching Hospital. According to by Macfaddin (2000) recommended method, Wound swab samples and biopsy specimens were collected and transported to the laboratory[20]. They were inoculated onto Mannitol salt agar, blood agar, and MacConkey agar and incubated overnight at 37°C aerobically. *Staphylococcus aureus* strains were detected using the microbiology routine standards protocols, by colonial morphology, gram staining, and detection of bacterial biochemical enzymes, including catalase, slide, and tube coagulase tests. Bacterial biochemical tests were performed with API kits following the manufacturer's instructions (Biomerieux, Montalieu, France). Furthermore, the Viteke 2 Compact system (bioMerieux, Marcy l'Etoeile, France) was utilized to confirm the identification of the bacterial isolates.

Detection of MRSA gene:

To determine if the *bacterial* isolate is methicillin-resistant, Polymerase Chain reaction (PCR) were employed to identify the gene that encoding this important phenomenon (*mecA* gene). Specific primers for the *mecA* gene were used in this assay. DNA extraction was performed using the QIAamp DNA Mini Kit (QIAGEN). The amplification of the gene was carried out with specific primers previously used by de Melo DA, et al.(2020) [21], as detailed in Table 1. The PCR reaction had a volume of 50 µL, consisting of master mix (45 µL), a dNTP mix (0.2 mM of each nucleotide), primers (0.5 µM), Taq DNA polymerase (0.25 U), and 1.5 mM of $MgCl_2$, and the template DNA (5 µL).

The PCR conditions started with 4 minutes hot start at 94°C, as PCR initiation. The next step was the denaturation for 30 cycles at 94°C, which toke 45 seconds. The annealing step spent 45 seconds at 50°C, while extension toke 1 minute at 72°C. The reaction was ended with final extension which was spent 3 minutes at 72°C. The amplicon size was 331 bp, confirming the amplification of the mecA gene.

Table 1: PCR Primers used in this study

Primer	DNA Sequence	Size
mecA -F	AAA ATC GAT GGT AAA GGT TGG C	331 bp
mecA -R	AGT TCT GCA GTA CCG GAT TTG C	

Bacterial Antibiotic susceptibility Test

The *S. aureus* strains were investigated for their sensitivity to 15 different antibiotic agents (Table 2). The bacterial isolates sensitivity to antibiotic agents were investigated using the agar disc diffusion method (Kirby–Bauer method) on sold medium plates[22]. All tested plates were incubated for 24 h at 37°C. The inhibition zone was scaled using metameric ruler. The outcome was classified according to CLSI guidelines[22]. Moreover, the Viteke 2 Compact system (bioMerieux, Marcy l'Etoiele, France) was utilized to confirm the identification of antibiotic susceptibility patterns for each bacterial isolate.

Biofilm formation test

The MRSA isolates were tested for their biofilm-forming capability using the Microtiter Plate method which was employed to evaluate biofilm formation, as described by Campo-Pérez V, et al.[23]. Briefly, The bacterial isolates were transferred from a fresh overnight growth on solid medium, 5 mL of brain heart infusion (BHI) broth containing 2% sucrose, and incubated at 37ºC for 24 hours, as declared by Mathur, et al[24].

Subsequently, using microtiter plates, 20 µL of bacterial suspension from each isolate, standardized to 0.5 McFarland, was added to 180 µL of BHI broth in each well within the microtiter plate. Plates were incubated overnight at 37ºC. After three time washing with normal saline, each well was covered with 99% methanol (200 µL) and incubated for 15 minute at room temperature.The plate was then air-dried for 30 minutes at room temperature. Following this, 200 µL of 1% crystal violet was added for 15 minutes to stain the biofilm. After discarding the dye and washing it with sterile distilled water, the retained dye was solubilized with 96% ethanol. Microtiter plate reader was used to evaluate biofilm formation at 630 nm, optical density.

Identification of possible river water bacteriophage against MRSA

MRSA isolates were investigated to test their sensitivity to possible river water bacteriophages. A sterile cotton swab was moistened with 0.5 O.D. overnight bacterial broth before preparing a bacterial lawn plate for each *S. aureus isolate.* This test was prepared using a tryptic soy agar medium. Various filtered river water samples, by 0.22 nm microfilter, were spotted on agar plates in droplets of five microliters. The plates were then dried and left 24 h incubation at 37 °C. After incubation time, bacterial lone were investigated for phage effectiveness plaque within the spotted location[16].

RESULTS

Isolation of bacterial pathogens

The *S. aureus* number *among bacterial isolates was 32 strains from 115 patients, which were isolated using either wound swabs or biopsy aspiration.*

Detection of mec A Genes of MRSA using PCR:

Methicillin-resistance was verified by detection of the mecA gene, using PCR. According to PCR results, out of 32 *S. aureus bacterial isolates, 25 (78 %) were confirmed have the* mecA gene (Fig.1).

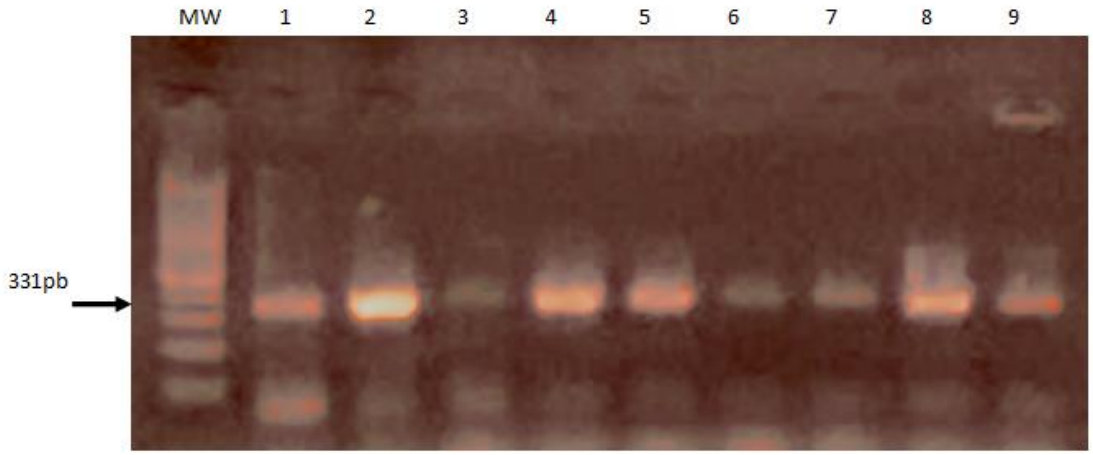

Fig. 1: Identification of mecA gene of *S. aureus isolates* using PCR assay: MW, 100 bp Hyperladder (Bioline), 1-9, mecA gene identification bands (331pb).

Antibiotic susceptibility

The Disk Diffusion test was employed to assess the resistance of antibiotic, and the sensitivity profiles of the 25 MRSA isolates to various antibiotics.

Table 2 illustrates the inhibition zone diameters interpreted based on CLSI Standards (CLSI, 2021) [22]. MRSA isolates had antibiotic resistance the majority of antibacterial drugs as shown in table 2 and fig.2.

Table2: Antibiotics Susceptibility test of *S. aureus* isolates

Antibiotic Agent	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
Ampicillin	25 (100)	0 (0)	0 (0)
Benzylpenicillin	15 (60)	1 (4)	9 (36)
Oxacillin	23 (92)	0 (0)	2 (8)
Levofloxacin	16 (64)	0 (0)	9 (36)
Moxifloxacin	23 (92)	0 (0)	2 (8)
Erythromycin	23 (92)	0 (0)	2 (8)
Clindamycin	21 (84)	0 (0)	4 (16)
Tetracycline	15 (60)	0 (0)	10 (40)
Fusidic Acid	15 (60)	0 (0)	10 (40)
Teicoplanin	15 (60)	1 (4)	9 (36)
Cefoxitin Screen	9 (36)	0 (0)	16 (64)
Gentamicin	6 (24)	4(16)	15 (60)
Tigecycline	0 (0)	6 (24)	19 (76)
Vancomycin	2 (8)	12 (48)	11 (44)
Rifampicin	14 (56)	1 (0)	11 (44)

P value < 0.01 by ANOVA test

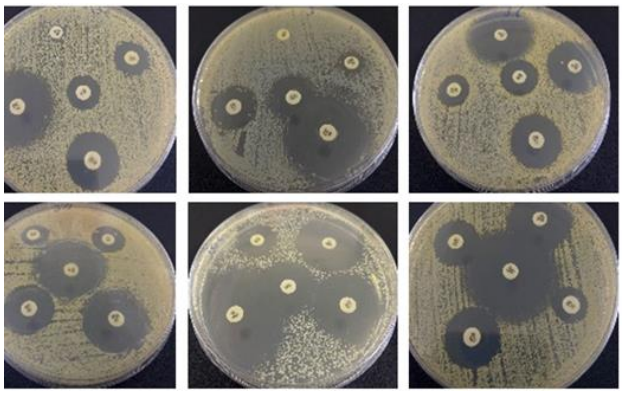

Fig. 2: Disk diffusion test for antimicrobial activity of the *S. aureus* isolates used in this work.

Biofilm production

Microtiter plate (MTP) assay showed majority of MRSA isolates with positive biofilms production (*P* value < 0.01). Eighteen strains (72%) exhibited strong attachment ability, Four strains (16%) showed moderate attachment, and three strains (12%) had weak attachment and no attachment ability at all table 3 and fig. 3.

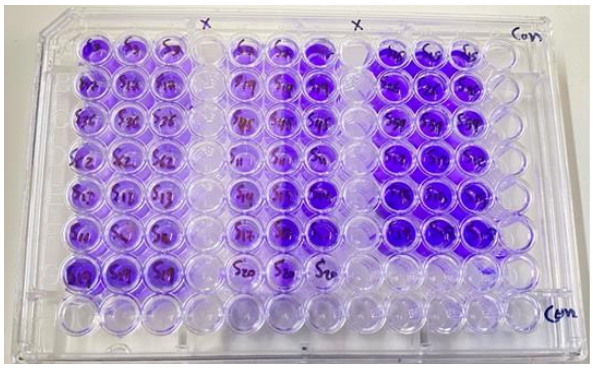

Fig.3: Biofilm test results, using Microtiter Plate (Mtp) assay, of MRSA isolates used in this study.

Table 3: Resistance bacteria, biofilm test, and the sensitivity of bacterial strains to possible water bacteriophage

Sample type	MDR. Bacterial strain No. (%)	positive Biofilm No. (%)	Negative Phage activity No. (%)	Total
Biopsy	15 (60)	13 (52)	3(12)	16(64)
Swab	7 (28)	8 (32)	0 (0)	9 (36)
Total	**22(88)**	**21(84)**	**3(12)**	

P value < 0.01 using ANOVA test

As regard the sensitivity of MRSA isolates to river water bacteriophage. The spot test on sold agar plates outcome showed a range of significant bacterial positive sensitivity to the water bacteriophage (P value <0.01 using T-test) as shown in table 3 and fig. 4.

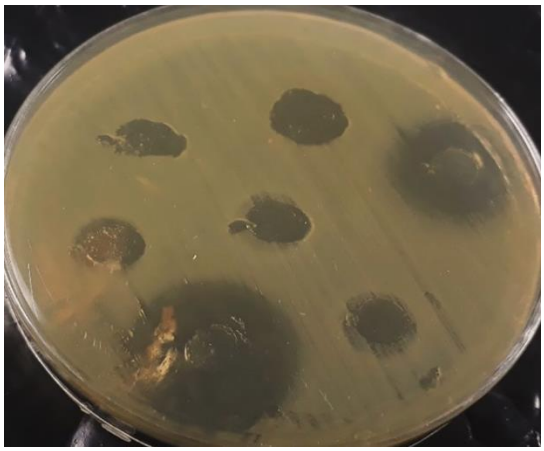

Fig.4: The effect of possible river water bacteriophage on of MRSA isolate bacteria lawn plate.

Moreover, a clear positive relationship connects the ability of bacteria to produce biofilm and their resistance to majority of antibacterial drugs utilized in this research, as shown in table 3.

DISCUSSION

Methicillin-resistant *S. aureus* strains are pathogens of significant health problems and cause both nosocomial and community-acquired serious infections[25]. The reason behind its severity is its possession of important virulence factors that allow the pathogen to create severe infections. Among these virulence factors is bacterial biofilm formation, the key factor behind their severity [9,26].

In accordance with our result, 78.12% of *S. aureus* strains, isolated from wound swabs and /or biopsy samples, were methicillin-resistan strains. This outcome was assured by the detection of the gene mecA within these strains' cells' genomes, using PCR assay. The gene mecA encodes the altered penicillin-binding protein (PBP2a), guiding the reduction of the antibiotics

efficacy[5]. This result is not really surprising sound within the Iraqi population. Both Rasheed & Hussein[26], and Babakir-Mina et al[27], reported that the distribution of MRSA among *S. aureus* strains isolated from Iraqi people was significantly high. MRSA strains are also prevalent in other Middle Eastern communities like Iran and Saudi Aribia[27-29].

Regarding biofilm formation, Most MRSA isolates (84%) were identified as positive biofilm producers. According to previous studies MRSA isolates used biofilm as a key virulence factor for their disease formation, as it protects bacterial cells against antibiotic agents and human body immunity mechanisms[9,11,30-32].

In our work, the results indicate high antibiotic resistance pattern of MRSA isolates to most of antibiotic agents included in this work. *S. aureus* isolates harboring the gene *mecA* were predominantly resistant to a β-lactam antibiotics[33,34]. This resistance pattern is consistent with numerous studies that have demonstrated the importance of the *mecA* gene in the ability of resistance to β-lactam antibiotics by encoding an altered penicillin-binding protein (PBP2a) with reduced affinity for these antibiotics[35]. The significant detection rate emphasizes the role of continuous surveillance in clinical centers and hospitals to track MRSA prevalence and guide treatment decisions[36].

According to our study, the correlation observed between the prevalence of the mecA gene and antibiotic resistance patterns reinforces the critical role of this gene in conferring resistance to antibiotics with beta-lactam ring within their chemical structures[37]. Moreover, our results showed clear positive relationship between the biofilm production capability of the included bacteria and their multidrug resistance, a reason behind their increasing pathogenicity and severity[9]. The antibiotic susceptibility tests revealed a concerning profile of resistance, which complicates treatment options for disease attributed to these strains. The detection of specific resistance patterns in mecA-positive isolates provides essential insights for healthcare professionals, to make informed choices regarding empirical treatment strategies[38].

In regard to bacteriophage as a bactericidal agent, This study provides evidence that bacteriophages are potentially effective at treating wound infections caused by MRSA[16]. Bacteriophage as treatment of infectious diseases caused by bacteria has got great attention from scientists, particularly after the discovery of MDR bacterial pathogens recently[39].

CONCLUSION

Methicillin-resistant strains were prevalent among isolated *S. aureus*. MRSA strains number identified in biopsy samples exceeded MRSA strains identified in wound samples. This result was emphasized using PCR assay detecting *mecA* gene within these strains. Furthermore, Biofilm production was prevalent within isolated MRSA stains. Moreover, MRSA strains were resistant to most antibiotic agents that included within the research. The later Phenomenon had a positive relationship with biofilm formation indicating the role of biofilm in disease formation. Finally, the outcome of this work confirmed the potency of using river water bacteriophage as treatment of infectious diseases caused bacterial pathogens.

Declarations:
Consent for publication: Not applicable
Availability of data and material: Data are available upon request.
Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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