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Antibacterial Activity of Fruit Juices on Methicillin Resistance *Staphylococcus aureus*

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ABSTRACT: *The drug-resistance behavior of Methicillin-resistant Staphylococcus aureus (MRSA) has made it difficult to treat. This study aimed to discover some fruit juice's anti-MRSA potential and the presence of enterotoxin genesin MRSA. MRSA strains were confirmed phenotypically by the disc diffusion method. The well-diffusion method and Minimum inhibitory concentration (MIC) were used to determine the antibacterial effects of fruit juices. Congo red test and ring tests were performed to analyze the biofilm-forming ability. PCR detected staphylococcal enterotoxin genes Sea and Seg. A total of 90 strains, of which 26 non-clinical and 64 clinical samples were processed. Ciprofloxacin (CIP) was highly resistant to MRSA in both groups. In clinical isolates, Citrus reticulate has shown the maximum antibacterial activity against 67.1 % MRSA, whereas, Punicagranatum was most effective for 46.1% strains isolated from non-clinical sources. Punicagranatum and Citrus reticulate were inhibiting the growth of MRSA at the concentration of >64 µl/ml in both groups. Both Congo red and ring tests determined the biofilm-forming ability in 57.60 % and 65.30 % of non-clinical strains, respectively. Enterotoxin-producing gene Sea was detected in 8% of MRSA in the non-clinical group and only 2 % in the clinical group. Seg and the co-existence of both genes were found in the same ratio in both groups. This study showed that fruit juices, especially pomelo, orange, and pomegranate, have high antibacterial activity. Enterotoxin genes play a role in the spread of infections caused by MRSA.*

Keyword: Staphylococcus, MRSA, antibiotic resistance, medicinal plants, enterotoxins

INTRODUCTION

Methicillin resistance *Staphylococcus aureus* (MRSA) has become one of the most widespread pathogens in healthcare-associated settings. Due to the extensive usage of antibiotics, it has become a community-acquired deadly pathogen (Dadashi et al., 2018). Throughout the world, MRSA threatens livestock (Sharma et al., 2019). The primary causes behind the spread of pathogenic MRSA are the hospital staff and infected patients (Chukwunoso et al., 2018). *S. aureus* has caused severe outbreaks (Bennett and Monday, 2003; Fanoy et al., 2009; Bennett et al., 2013). Penicillin was used to cure infections caused by Staphylococcus species (Siddiqui and Koirala, 2020). But in no time, they become resistant to penicillin (Vestergaard et al., 2019). The horizontal gene transfer by staphylococcal cassette chromosome mecC caused resistance to methicillin. The mobile genetic element consists of genes like *mecA*or *mecC,* translated into proteins and pathogenicity (Lee et al., 2018). Alteration of the structure of penicillin-binding sites means no β lactam antibiotic can bind with them (Fetsch and Johler, 2018; Veslasco et al., 2018; Alsharif et al., 2021). As a result, food borne MRSA shows resistance against penicillin and cefoxitin. At the same time, hospital-acquired pathogenic MRSA has become multidrug-resistant (Velasco et al., 2018; Algammal et al., 2020). People with weak immune systems are highly vulnerable to hospital-acquired MRSA infections (HA-MRSA) (Chukwunonso et al., 2018). HA-MRSA infections are common in patients with extended hospital stays after surgery (Barcudi et al., 2020). These strains carry SCC *mec* types I, II, or II. They resist antimicrobial agents like macrolides, aminoglycosides, fluoroquinolones, lincosamides, etc. (Klein et al., 2019). Community-acquired MRSA (CA-MRSA) is transmitted mainly from a person who was recently discharged from hospital or community members. From the nasal cavity to the skin, CA-MRSA is present everywhere. During food preparation, the food handlers shed *S. aureus* into food and transfer it to other individuals. So sneezing and improper food handling are causes of CA-MRSA (Fooladvand et al., 2019). Different virulence factors determine the infection-producing capacity of MRSA; they include toxins, adhesins, Panton-Valentine Leukocidins (PVL), hemolysins, etc. (Karmakar et al., 2016). These virulent factors are involved in causing tissue damage, beating the host's immune response, and eventually

rising in number (Tang et al., 2019). Staphylococcal enterotoxins are involved in causing stomach infections and enhancing resistance to different chemicals (Tarisse et al., 2021). These food poisoning agents are divided into 22 subtypes, from (SE) A-E, G-I, K-T, and Y (Etter et al., 2020). Among all the serotypes SEA, SEE, and SEG are dominant. SEA and SEG are responsible for causing 95% of cases of food poisoning (Guidi et al., 2018). The primary source of toxins is improperly cooked and stored meat sources. Sea and seg work independently and with other toxins, making conditions even worse (Manyi-Loh et al., 2023). In 2017, the World Health Organization (WHO) issued a list of organisms based on their resistance to the available antibiotics. MRSA is one of the twelve pathogens that seriously impact human health (Asokan et al., 2019). Many phenotypic and molecular studies are conducted in Pakistan to check the prevalence of MRSA. The prevalence rate of HA-MRSA infections in Pakistan is 51% lower than in European countries and the United States of America (Rafay et al., 2020).Scientists are investigating medicinal plants to find new therapeutic options for MRSA's drug resistance. Some plants like lime, pomelo, orange, and tangerine have antibacterial properties, helping to cure several infections and diseases. Rhizomes of ginger, leaves of oak, and pomegranate are used as sources of antibacterial agents against MRSA (Okwu et al., 2019). Citrus plant's flavonoids, limonoids, and phenolic compounds are also good antibacterial agents. Some non-citrus fruits like pomegranate and apple also show bactericidal properties. It is reported that apples have antimicrobial activity against *E. coli, P. aeruginosa, S. serovar, S. aureus,* and *B. cereus* (Chen et al., 2018). Extensive research has been carried out on HA MRSA, but less attention has been paid to CA-MRSA, such as food handlers. Very little has been known about MRSA's carriage frequency, biofilm-forming capacity, and antimicrobial resistance profiles isolated from community settings. The current study emphasized the above-mentioned gaps and the antibacterial activity of citrus and non-citrus fruit juices. The study aimed to determine the prevalence of MRSA isolates in health and community settings. Moreover, to characterize their antimicrobial resistance profiles by disc diffusion or agar dilution techniques. We determined the presence of *Sea* and *Seg*genes. The biofilm-forming abilities of isolates were also determined. The study was done to assess the

antimicrobial activity of citrus and non-citrus fruits against MRSA isolated from three different settings (environment, food handlers, and non-food handlers).

MATERIALS AND METHODS

This cross-sectional study was conducted at the Institute of Microbiology and Molecular Genetics, University of Punjab, Lahore, from September 15, 2020, to March 31, 2021.

Sample collection

A total of 160 non-clinical samples were collected from the food handlers of different cafes, hotels, markets, non-food handlers, and the environment(basin, tabs, and locks). All the samples were proceeded according to bacteriology protocols. Out of which,26 MRSA were used for further analysis. To compare the characteristics of non-clinical samples with clinical ones, 64 isolated clinical strains of MRSA were obtained from Citi Lab and Research Centre, Lahore. The clinical strains were isolated from wounds, skin, pus, ear swabs, urine, CSF, and tracheal swabs. The current study was done on 90 isolated MRSA from clinical and non-clinical settings.

Antimicrobial susceptibility testing (AST)

AST was performed on Muller Hinton agar plates according to the Clinical Laboratory Standard Institute (CLSI) guidelines. Eleven antibiotics, Tetracycline (TE), Ciprofloxacin (CIP), Cefoxitin (FOX), Cephalexin (CN), Fusidic Acid (FA), Triglycine (TGC), Co-trimoxazole (SXT), Linezolid (LZN), Clindamycin (DA), Erythromycin (E), and Chloramphenicol(C), were applied to see the resistance of MRSA against those antibiotics. Results were noted by measuring the zone of inhibition to see whether the strains were susceptible or resistant to applied antibiotics according to CLSI 2019 guidelines.

Juice extract preparation

Citrus fruits, *Citrus maxima (pomelo)*, *Citrus reticulate* (orange), *Citrus aurantifolia* (lime), and non-citrus fruits, *Malus domestica* (apple), *Punicagranatum* (pomegranate) were chosen and collected. Fruits with no cuts or bruises were selected for sampling. Juices were extracted by grinding them into a pestle, and motor and Coarse filtration was done. All the process is carried on under sterilized conditions.

Agar well diffusion assay

Muller Hinton plates were used for agar diffusion assay. A 100 µl of 24-hourculturein broth equal to 0.5 McFarland's standard solution was spread over the plates with a cotton swab to make an even lawn. The Pasteur pipette was used to make wells an equal distance. In the respective well, 100 µl of each fruit was loaded, and plates were incubated for 24 hours at 37°C. Clear zones against the selected strain were recorded for MIC to show antibacterial activity.

Minimum inhibitory concentration (MIC)

MIC was performed to measure the minimum amount of juice inhibiting bacterial growth. Bacteria were grown in TSB, and density was adjusted with the McFarland Standard. Juices of different concentrations were prepared. For MIC, a 96-wellmicrotiter plate was used. In this, 1st column was used as blank. In the second column, only autoclaved broth was added to check sterility. From the 4th to 12th column, 100µl broth was added. Then 100 µl juices were added to 1st well. To make dilutions of 2 µl/ml, 8 µl/ml, 32 µl/ml, 128 µl/ml, and 512 µl/ml concentrations, from 1st well of the column, transfer 100µl into 2nd well and so on. 20µl of test strains were added. In 2nd last row, bacterial cultures were used as the positive control. Then 20µl of bacterial strain was added to each well. Plates were incubated at 37°C for 24 hours. The next day, the reading was taken at 600nm by an ELISA plate reader.

Biofilm formation tests

Two phenotypic tests were performed to indicate whether the strains were biofilm producers.

1. Congo Red Agar test

Strains were streaked on Congo red agar plates and incubated for 24 hours at 37°C. After 24 hours, black colonies were selected as biofilm producers, and pink were indicated as non-biofilm formers.

2. Ring test

A ring test was performed to check the ability of bacteria for biofilm production. Strains were inoculated in Tryptic soy broth and incubated for 24 hours at 37°C. The next day, without mixing, the content of the tube, which was not attached to the tube, was transferred to the falcon tubes. Falcons were centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was treated with 100 µl of crystal violet for 5 minutes. The solution was centrifuged again, and the pellet was treated with 200 µl of 0.85% NaCl

three times. The liquid solution was discarded. Glacial acetic acid was added to remove the leftover strain and assimilation of the pellet. At this stage, a clear ring was seen in the biofilm former strain's falcon. Optical density was taken at 523 nm by spectrophotometer. Then, the tube in which cells were attached tightly was tested. Normal saline was added to those tubes and centrifuged. All the other steps were the same, except we added 100 µl of crystal violet. Again, optical density was noted (Crabbé et al, 2019).

Polymerase chain reaction (PCR)

For the PCR reaction of *Sea* and *Seg* genes, 2µl of DNA extracted by heat lysis method (Kim et al., 2020) was added into the PCR tube, and 13µl of master mix containing $MgCl_2$, dNTPs, PCR buffer, forward and reverse primers, and Taq polymerase. The primer details and PCR conditions are described in Table 1. Gel electrophoresis was done for genomic DNA and PCR products. 2% gel was used for PCR products.

Table 1: Primer sequence for *Sea* and *Seg*genes with annealing temperature

Sr. No.	Genes		Nucleotide sequence	Product size	Annealing temp.	Ref.
1.	*Sea*	Forward	5'-GCAGGGAACAGCTTTAGGC-3'	512	47°C	(Løvseth et al., 2004)
		Reverse	5'GTTCTGTAGAAGTATGAAACACG-3'			
2.	*Seg*	Forward	5'-CGTCTCCACCTGTTGAAGG-3'	328	47°C	(Løvseth et al., 2004)
		reverse	5'-CCAAGTGATTGTCTATTGTCG-3'			

RESULTS

Antibiotic Susceptibility Testing (AST)

Out of 90 MRSA strains, CIP was highly resistant in n=10 (38.4%) non-clinical and n=63 (98.4%) clinical isolates. MRSA from the non-clinical group showed maximum resistance for TE n=8 (30.7%), FOXn=7 (26.9), and CN n=5 (19.2%). While in the clinical group, the maximum resistance was towards SXT and FOX n=57 (89.0%), FA n=56(87.5%) as given in Table. 2.

Table 2: Antibiotic Susceptibility Testing of MRSA isolated from Non-clinical and clinical samples

Antibiotics	MRSA in Non-clinical N=26n (%)	MRSA in clinical N=64n (%)
DA	0	3 (4. 6)
E	2 (7.6)	13 (20.3)
CN	5 (19.2)	52 (81.2)
SXT	2 (7.6)	57 (89.0)
TGC	1 (3.8)	9 (14.0)
LZD	1 (3.8)	4 (6.2)
TE	8 (30.7)	20 (31.2)
FA	3 (11.5)	56 (87.5)
C	1 (3.8)	9 (14.0)
CIP	10 (38.4)	63 (98.4)
FOX	7 (26.9)	57 (89.0)

Well diffusion Assay

in the non-clinical group, n=23 (88%) showed resistance toward *Citrus reticulate*, and n=24 (92.3%) resisted *Malus domestica*. However, in the clinical group, the maximum MRSA n=39 (61%) showed resistance to *Citrus aurantifolia*, and n=41 (64%) resisted *Malus domestica*. In the non-clinical group, maximum sensitivity was demonstrated for *Citrus maxima* n=5 (19%) and *Punicagranatum* n=14 (46%). Most MRSA was sensitive to *Citrus maxima* n=39 (61%), *Citrus reticulate* n=43 (67%), and *Punicagranatum* n=31(48%) in the clinical group as given in Table 3.

Table 3: Antibacterial activity of fruit juices by well diffusion assay

Citrus fruits	Non-clinical N=26n (%)		Clinical N=64n (%)	
	Resistant	**Sensitive**	**Resistant**	**Sensitive**
Citrus maxima (Pomelo)	21 (80.7)	5 (19.2)	25 (39.0)	39 (60.9)
Citrus *aurantifolia* (Lime)	22 (84.6)	4 (15.3)	39 (60.9)	25 (39.0)
Citrus reticulate (Orange)	23 (88.4)	3 (11.5)	21 (32.8)	43 (67.2)
Non-citrus fruits				

Punicagranatum (Pomegranate)	14 (53.8)	12 (46.1)	33 (56.2)	31 (48.4)
Malus domestica (Apple)	24 (92.3)	2 (7.6)	41 (64.0)	23 (35.9)

Minimum Inhibitory Concentration

MIC was performed to check the minimum inhibitory concentrations of fruit juices to kill MRSA. In the non-clinical group, *Citrus maxima* inhibited the n=18 (69.2%) strains at >64µg/ml concentration. And n=4 (15.3%) was only sensitive to non-citrus fruit *Punicagranatum.* While in the clinical group, *Citrus maxima* were inhibiting n=5 (7.8%) of strains at the 32µg/ml concentration. And n=4 (6.2%) strains were sensitive to *Citrus reticulate* at >64µg/ml concentration as in Table 4.

Table 4: Antibacterial activity of fruit juices at two different concentrations

Fruits	**32 µl/ml**	**≥64 µl/ml**
Clinical group		
Citrus maxima	5	3
Citrus reticulate	0	4
Citrus aurantifolia	0	2
Punicagranatum	0	3
Malus domestica	2	1
Non-clinical group		
Citrus maxima	1	18
Citrus reticulate	2	12
Citrus aurantifolia	1	0
Punicagranatum	3	4
Malus domestica	2	1

Biofilm production Tests

Non-clinical groups have a higher ratio of biofilm formers than clinical groups. From the non-clinical group, 57.60% (n=15) of isolates have shown moderate biofilm-forming ability in the Congo Red test. While among the clinical group, the percentage was a little low. Only 51.10% (n=32) of isolates were biofilm formers.

For the Ring Test, the percentages of biofilm formers in the non-clinical group were again higher than in the clinical group. In the non-clinical group, 65.30% (n=17) showed positive results for the Ring test. Only 48.40% (n=31) were positive for the Ring test in the

clinical group. However, 51% (n=33) of strains were non-biofilm former in the clinical group, and 35% (n=9) were non-biofilm formers in the non-clinical (Table. 5).

Table. 5: Frequency of biofilm producers and non-producers by Congo Red and Ring Test

	Non-Clinical N=26		**Clinical N=64**	
	Producer n (%)	**Non-producer n (%)**	**Producer n (%)**	**Non producer n (%)**
Congo Red	15 (57. 6)	11 (42.3)	32 (51.1)	32 (50)
Ring Test	17 (65.3)	9 (34.6)	31 (48.4)	33 (51.5)

Genetic Analysis of *Sea* and *Seg* Genes

Both genes' percentages (independent and combined) were confirmed in all clinical and non-clinical isolates. *Sea* gene was found to be in higher percentage in non-clinical isolates n=2 (8%).However, in clinical isolates, it was present in only n=1(2%) isolate. The *Seg* gene was found to be in nearly the same ratio in both groups, n=13 (50%) in the non-clinical and n=31 (49%) in the clinical group. When the co-existence of *Sea* and *Seg* was checked. It was determined that 20% of strains of both (n=5 non-clinical, n=13 clinical) groups have these enterotoxin genes in co-existence. Percentages are shown in Table. 6.

Table 6: Genetic analysis of Sea and Seg genes

Genes	Non-Clinical N = 26n (%)	Clinical N=64n (%)
Sea	2 (8)	1 (2)
Seg	13 (50)	31 (49)
Co-existence	5 (20)	13 (20)

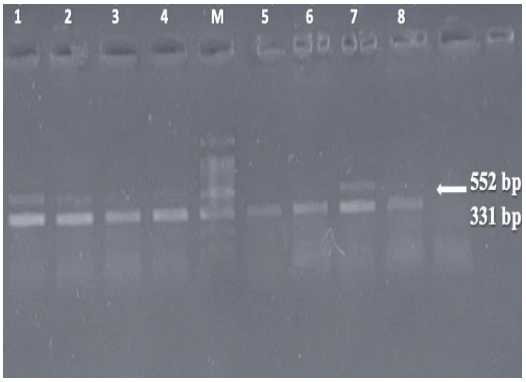

Fig. 1. *sea* (552bp) in lanes 1, 2, 4, 7 and *seg* (331 bp) in lanes 1, 2, 3, 4, 5, 6, 7, 8

DISCUSSION

MRSA is responsible for high antibiotic resistance. In this study, MRSA of both clinical and non-clinical setups, 38.4% and 98.4%,showed maximum resistance towards Ciprofloxacin (CIP), respectively. Antibiotic resistance was recorded as high in the clinical group, as 89% of Strains were resistant to co-trimoxazole (SXT) and Cefoxitin (FOX). However, 95.3% of strains of the clinical group showed sensitivity to clindamycin and, 93.7% were sensitive to Linezolid, 85.9% were susceptible to Triglycine and chloramphenicol. This study's results were different from Manandhar et al., study. He showed in his research that 100% of clinical samples were sensitive to cefoxitin, and 77% were susceptible to ciprofloxacin (Manandhar et al., 2018). It showed that infections caused by MRSA in our region can no longer be treated with the last resort of antibiotics because resistance is relatively high in this region.

However, the non-clinical samples showed less resistance to antibiotics than clinical ones. Most of the strains were sensitive to applied antibiotics. In another study in Pakistan, clinically isolated MRSA strains were susceptible to clindamycin, 84.6%, linezolid 96.7%,

Chloramphenicol83.7%, fusidic acid 70.6%, gentamicin 67.7% and tetracycline 56.8%. The resistance was shown by strains against norfloxacin 91.2%, levofloxacin 87.1%, ciprofloxacin 83.9%, azithromycin 78.6%, erythromycin 77.4%, moxifloxacin 69.8% and sulfamethoxazole/trimethoprim 54.9% (Idrees et al., 2023). In this study, the well diffusion and microdilution methods were implicated in investigating the antibacterial activity of fruit juices. The highest antibacterial activity was shown by non-citrus fruit, *Punicagranatum*, 46.1 %, and citrus fruit, *Citrus maxima,*19.2% in non-clinical samples. In clinical samples, 67.2% of MRSA showed sensitivity towards *Citrus reticulate,* and 48.4% were sensitive to *Punicagranatum*. All of this is an indication that extracts of fruit juices can be used for the treatment of infections caused by MRSA. Both *Citrus maxima* and *Punicagranatum* have shown good antibacterial activity. Using natural remedies for medicinal use has no side effects as well.

The biofilm-forming capacity of *S. aureus* imparts its pathogenicity. The two tests performed in this study to see the biofilm-forming ability of MRSA showed that 57. 6% MRSA of the non-clinical group and 51.1% MRSA of the clinical group are biofilm producers in the Congo ring test. And 65.3% and 48.4% MRSA gave positive results for ring tests in non-clinical and clinical groups. It was previously reported in a study in Nepal that 27% of clinical MRSA are moderate biofilm producers. And 11% of non-clinical strains of MRSA also can form biofilms (Manandhar et al., 2018; Mama et al., 2018). However, in this study, we observed that non-clinical strains have more biofilm-forming ability than clinical ones. It indicates that the strains of this study have robust protective mechanisms against antibiotics and host cells. This biofilm production ability of MRSA is responsible for the MDR mechanisms. HA MRSA infections are responsible for CA MRSA infections and the spread of drug resistance in CA MRSA. *S. aureus* is present on the hands, skin, and other body parts. It sheds off while handling food items. There's a need to be careful and maintain a social distance from the patients discharged from hospitals as they are a hub of microorganisms and passive transfer of germs in the community. A study in Iran has also proved that 52.9% of MRSA are strong Biofilm producers.

MRSA is responsible for severe food poisoning as it produces a variety of

toxins. Two important enterotoxins which are considered to be responsible for this are Sea and Seg as the production of toxins is dependent on the presence of genes. When the *Sea* and *Seg* genes were investigated in the clinical and non-clinical samples, it was found that the non-clinical group had a high prevalence of these genes. *Sea* was found in 8% of strains, and *Seg* was present in 51% of strains of the non-clinical group. While in the clinical group, only 2% of strains had the *Sea* gene, and 49% had the *Seg* gene. The percentage of the *Seg* gene was higher in both groups than that of the *Sea* gene. Udo et al reported that in Kuwait, the Seg gene's prevalence was also high, 24%, and *Sea* was present only in 11% of strains (Udo et al., 2009). Yimam et al also found out that in Brazilian MRSA, the *Seg* was present in 29.3% of strains (Yimam et al., 2020). The results of this study's enterotoxin genes were nearly similar to previous studies where it has been shown that the prevalence of enterotoxin *Seg*gene is higher in the MRSA than in the *Sea*. However, in comparing the clinical and non-clinical groups, our results were contrary to the other studies where the prevalence of enterotoxins was higher in the clinical group than in the non-clinical group. Al Jazirah showed that in Sudan, non-clinical samples have 34% of enterotoxin and the clinical group has 40.5% of enterotoxin genes (Ahmed, 2020).

CONCLUSION

In conclusion, enterotoxin genes are disseminated into CA-MRSA and are involved in causing infections difficult to treat. The medicinal plants used in this study have shown successful antibacterial activity results. Hence, they can be sued for combating drug resistance. Moreover, there's a need to initiate regional MRSA surveillance programs to keep track of emerging clones and to reduce the MRSA infection rate.

CONFLICT OF INTEREST

This study is part of the MS thesis of Ms. Hafiza Iqra Abdul Rasheed. All authors declare no conflict of interest.

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