Carriage of Enterotoxin Genes by Methicillin-Resistant Staphylococcus aureus and Anti-Staphylococcal Activity of Local Citrus Fruits

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ABSTRACT

Background: Staphylococcus aureus (S. aureus) is one of the leading causes of skin infections and food poisoning worldwide. Citrus fruits enriched with phytochemicals can exhibit anti-staphylococcal activity.
Objective: The study aimed to investigate in vitro susceptibility of citrus fruits against S. aureus harboured enterotoxin genes among food handlers, personnel, and the environment.
Methods: S. aureus was isolated from food handlers, personnel, and the environment. Methicillin-Resistant Staphylococcus aureus (MRSA) screening was performed using cefoxitin antibiotic discs. The antimicrobial activity of citrus fruits, including Citrus limon (Lemon), Citrus pseudolimon (galgal), Citrus tangerine (fruiter), Citrus sinensis (malta), and Citrus paradisi (grapefruit), was analyzed by performing well-diffusion assay and broth microdilution method. Genes for toxins production, sea and seg were detected using a Polymerase Chain Reaction (PCR).
Results: Out of 200 swab samples, 50 (25%) S. aureus were isolated using the biochemical battery. Antibiotic susceptibility testing (AST) and cefoxitin disc revealed 12 (24%) MRSA strains. Most strains were sensitive to Citrus limon, followed by Citrus pseudolimon, Citrus tangerine, Citrus sinensis, and Citrus paradisi. PCR results detected carriage of the sea gene in 4 (8%) isolates, and 20 (40%) isolates harboured the seg gene.
Conclusion: The prevalence of S. aureus, mainly MRSA, was high in the environment. These resistant strains' carriage of sea and sea enterotoxin genes increases their virulence and leads to untreatable infections. The antibacterial activities of citrus fruits should be explored using adavance methods.

Key Words: Staphylococcus aureus, MRSA, Enterotoxin, Citrus fruits, Anti-staphylococcal activity.

Original Research Article

Introduction:

Citrus fruits are enriched sources of nutrients and bioactive elements such as essential oils, phenolics, alkaloids and vitamins [1]. Phytochemical constituents of citrus fruits have antioxidant, anti-inflammatory and antimicrobial properties that protect against many diseases [2]. Pakistan is blessed with an ideal climate for growing a wide range of vegetables and delicious fruits [3]. However, very few studies have discussed the antimicrobial activities of citrus fruits in detail regarding their principal bioactive constituents and antimicrobial interactions.

S. aureus is one of the significant causes of skin infections and leads to high rates of death in the world [4,5]. S. aureus produces staphylococcal enterotoxins (SEs), which are pyrogenic toxins of the superantigen family. SEs and SE-like toxins (SEls) are the most notable virulence factors associated with food poisoning [6]. Staphylococcal food poisoning (SFP) results in nausea, diarrhea, emesis, and prostration [7]. SEA is a potent gastrointestinal toxin; a minimal amount of 100ng is enough to cause toxicity [8]. The situation has worsened due to antibiotic resistance which makes treatments difficult.

MRSA causes many infections, including skin and soft tissue infections and invasive infections like pneumonia, meningitis, and lung abscess. Several S. aureus strains have developed resistance against both β-lactam and non-β-lactam antibiotics. Infections due to these strains confer a significant public health threat in the healthcare system [9]. S. aureus acquires multiple genes by mobile genetic elements; the mecA gene responsible for methicillin resistance and SEA and SEG for enterotoxin secretions [10]. Previous literature revealed that the genetic elements that encode virulence factors may be the culprit of antibiotic resistance in S. aureus strains. In this scenario, when the world faces the challenge of antimicrobial resistance, plant-based antimicrobials can be promising therapeutic options with no side effects, unlike other synthetic drugs. Thus, this study is aimed to investigate the antimicrobial effects of citrus fruits in MRSA isolated from environment and genetic analysis of their enterotoxin genes.

Materials and Methods

Collection and Identification of Staphylococcus aureus

This cross-sectional study was conducted at the Institute of Microbiology and Molecular Genetics, University of Punjab, Lahore, Pakistan. Two hundred samples were collected from June 2019 to December 2019; using sterile cotton swabs moistened in saline water. Sampling sites included the hands and nares of food handlers 80, students 100 and the environment 20. Samples were cultured on Mannitol Salt Agar (MSA) media, and routine bacteriological analysis was performed to identify S. aureus.

Antibiotic Susceptibility Testing

The antibiotic susceptibility testing was performed according to the Kirby-Bauer Disc Diffusion method. Eight antibiotics were used; Penicillin (P 10U), Amoxicillin (AMC 10µg), Tetracycline (TE 30 µg), Cefoxitin (FOX 10 µg), Gentamycin (CN 10 µg) and Ceftriaxone (CRO 30 µg). All the zone diameters were interpreted per the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2018).

Phytochemical Analysis of Fruit

Juices were sterilized by using 0.45µm sterile syringe filters. The phytochemical constituents of the raw juices were determined by treating them with different reagents. Alkaloids, carbohydrates, coumarins, flavonoids, steroids, and glycosides were tested [11-13]. To test alkaloids, 1 ml extract juice was treated with Dragendroff's reagent and 1% HCL. The appearance of a red precipitate confirmed its presence. Fehling A and B were treated with juice in 1:1 proportion and heated in a flame, establishing the carbohydrates' presence by the appearance of a brick-red precipitate. 10% sodium hydroxide (NaOH) and 1 ml chloroform were treated with 1 ml juice extract. The appearance of the yellow colour showed the presence of coumarins. Production of yellow colour after adding a few drops of ferric chloride solution 1ml juice extract indicated the presence of flavonoids. To confirm the glycosides presence, 1 ml of the extract was mixed with 0.5 ml chloroform and 0.5 ml conc. and heated. A red-brown colour developed in the presence of glycosides. Mixing 1 ml extract with 0.5 ml chloroform and heated. The red colour indicated the presence of steroids.

Well Diffusion Assay

Screening of citrus juice for antibacterial activity against S. aureus isolates was performed by agar well diffusion assay. Using sterile swabs, culture broth diluted to match 0.5 McFarland standards was streaked onto Muller Hinton Agar (MHA). Wells were made by using a sterile cork borer, and 20 µl of each juice was diffused into separate wells. The plates were incubated at 37^{o} C. After 24 hrs, zones of inhibitions were measured for each of the juice in millimetre (mm).

Minimum Inhibitory Concentration

The broth micro-dilution method evaluated the Minimum Inhibitory Concentration (MIC) of all the S. aureus isolates. A 96-well microtiter plate was used, and all the samples were treated in triplicates. The nutrient broth was taken as sterility control, raw juice as a negative control, while a bacterial inoculum was considered as a positive control. Five different concentrations (60 µl/ml, 70 µl/ml, 80 µl/ml, 90 µl/ml and 100 µl/ml) of the juices were added for each bacterial isolate. The micro-titer plates were incubated at 37 °C for 24 hrs. Interpretation of MIC was done by naked eyes and measuring optical density (OD).

Genetic analysis of resistance and Enterotoxin causing elements

DNA extraction was done through the Cetyltrimethylammonium bromide (CTAB) method [14]. Molecular detection of methicillin resistance was carried out by mecA gene detection [15]. The mecA gene was amplified using the following thermocycler conditions: pre-denaturation at 94 °C for 5 minutes, 35 cycles each of denaturation at 94 °C, annealing at 52 °C and extension at 72 °C for 30 seconds each, followed by a single cycle of post-extension at 72 °C for 10 minutes. Enterotoxin genes, including sea and seg, were also detected [16]. The reaction mixture contains 0.5 µl of each primer, 10 µl of Master Mix (Taq, dNTPs, and Buffer included), and 7 µl RNase free water. The total volume was made up to 20 µl. A known control for each gene was used in all reactions. The annealing temperature for the sea and seg multiplex PCR was optimized at 47 °C. Amplified products were visualized in 2% agarose gel on a UV illuminator. PCR product of 552 bp for sea gene and 331 bp for seg gene were confirmed.

Results

Bacterial Identification and Antibiotic Susceptibility Testing

A total of 200 samples were collected to determine the anti-staphylococcal activity of citrus fruits against non-clinical isolates of S. aureus. 160 (80%) swab samples were grown as positive cultures on mannitol salt agar media. Out of 160 isolates of Staphylococcus spp, 50 (31.25%) were identified as S. aureus. Among these 50 strains, 19 (38%) were isolated from food handlers, 23 (46%) personnel, and 8 (16%) environments (tabs and door locks). AST results revealed the highest resistance against penicillin 41 (82%), followed by amoxicillin 39 (78%) and tetracycline 23 (46%). Vancomycin, ceftriaxone, cefoxitin, and gentamycin were susceptible to S. aureus (Table 1). Based on cefoxitin susceptibility, 12 (24%) strains were screened as MRSA.

Table I Antibiotic susceptibility against non-clinical Staphylococcus strains

Antibiotics	Concentration	Resistance n	Resistance %	Sensitive (N)	Sensitive (%)
Amoxicillin	30 µg	39	78	11	22
Tetracycline	30 µg	23	46	27	54
Vancomycin	30 µg	0	0	50	100
Cefoxitin	30 µg	12	24	38	76
Gentamycin	30 µg	15	30	35	70
Penicillin	10 U	41	82	9	18
Ceftrioxone	30 µg	6	12	44	88

Phytochemical Screening

Alkaloids were present in all five fruits. High amounts of alkaloids were detected in Citrus tangerina and Citrus sinensis. Carbohydrates were also present in all fruits. Coumarins were present in four juices of citrus fruits except for Citrus pseudolimon . Citrus sinensis and Citrus paradisi contained a high amount of coumarins. Citrus sinensis , Citrus tangerina , and Citrus paradisi had many flavonoids. It was absent in Citrus pseudolimon . Red-brown precipitates of glycosides were only seen in the juices of Citrus pseudolimon and Citrus paradisi . Steroids were slightly detected in Citrus paradisi.

Well Diffusion Assay

Citrus limon 45 (90%) and Citrus pseudolimon 34 (68%) showed the best antibacterial activity among citrus fruits. At

the same time, more than half of S. aureus were resistant against the Citrus paradisi, Citrus tangerina, and Citrus sinensis juices, with percentages of 30 (60%), 28 (56%), and 28 (56%) of the isolates, respectively.

Table II Percentages of resistant/sensitive strains for fruit juices by well diffusion assay

Fruits names	Resistance (n)	Resistance (%)	Sensitive (n)	Sensitive (%)
Citrus pseudolimon	16	32	34	68
Citrus limon	5	10	45	90
Citrus sinensis	28	56	22	44
Citrus tangerina	28	56	22	44
Citrus paradisi	30	60	20	40

Minimum Inhibitory Concentration

Citrus paradisi juice was most sensitive at the lowest concentrations, inhibiting growth at 60 µg/ml of 7 (35.0%) strains. Then, Citrus tangerine juice at 70 µl/ml showed maximum sensitivity against 7 (31.81%) strains. Citrus pseudolimon juice inhibited maximum bacterial growth at 80 µl/ml. MIC of both Citrus sinensis and Citrus limon was highly sensitive at µl/ml 8 (36.36%) and 16 (35.56%) strains, respectively (Table 3).

Table III Minimum inhibitory concentrations for citrus fruit juices

Citrus Fruits (n)	60 µg/ml	70 µg/ml	80 µg/ml	90 µg/ml	100 µg/ml
Citrus pseudolimon (34)	7 (20.5%)	9 (26.47%)	10 (29.41%)	5 (14.70%)	3 (17.64%)
Citrus limon (45)	7 (15.56%)	7 (15.56%)	14 (31.11%)	16 (35.56%)	1 (2.23%)
Citrus sinensis (22)	4 (18.18%)	3 (13.63%)	7 (31.81%)	8 (36.36%)	1 (4.54%)
Citrus tangerina (22)	5 (22.72%)	7 (31.81%)	5 (22.72%)	2 (9.09%)	3 (13.63%)
Citrus paradisi (20)	7 (35.0%)	6 (30.0%)	5 (25.0%)	1 (5.0%)	1 (5.0%)

Sea and Seg Enterotoxin Genes

PCR results expressed the presence of enterotoxin genes in 50 S. aureus, including MRSA and MSSA (Table 4). Among 12 MRSA, 2 (16.67%) strains with sea and 5 (41.60%) with seg genes were detected. However, MSSA strains had a high frequency of seg gene 15 (39.70%). The coexistence of sea/seg genes was detected in only one strain (Table 4 and Figure 1).

	Sea	Seg	Sea/Seg +	Sea/Seg -
MRSA (n=12)	2 (16.66%)	5 (41.60%)	1 (8.33%)	4 (33.33%)
MSSA (n=38)	2 (5.26%)	15 (39.70%)	0	21 (55.26%)

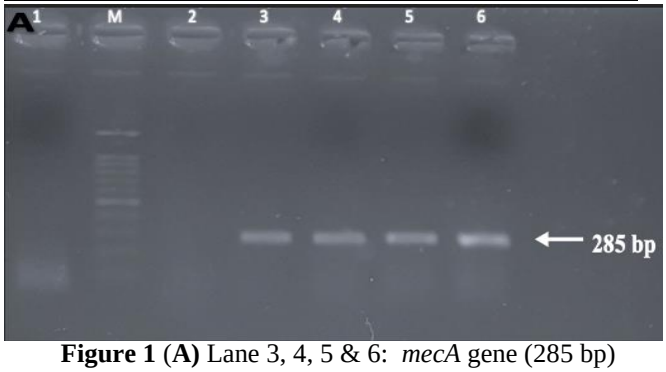

Figure 1 (A) Lane 3, 4, 5 & 6: *mecA* gene (285 bp)

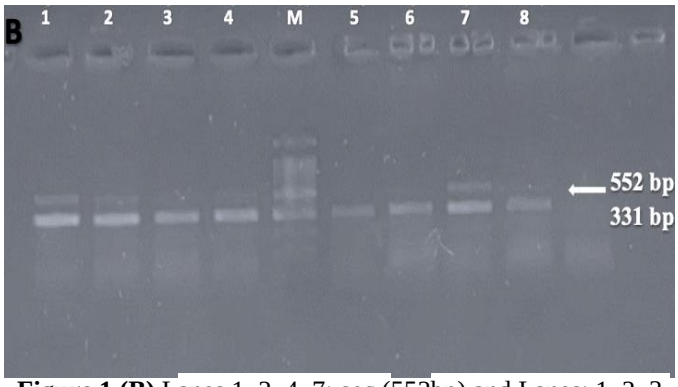

Figure 1 (B) Lanes 1, 2, 4, 7: *sea* (552bp) and Lanes: 1, 2, 3, 4, 5, 6, 7, 8 *seg* (331 bp)

Discussion

Citrus fruits are a well-appreciated therapeutic option due to their anti-inflammatory, antioxidant and antimicrobial properties. In contrast, the antibacterial effects of these fruits against pathogens have not been extensively studied [17]. S. aureus, as an opportunistic pathogen, can cause severe invasive infections, including bacteraemia, pneumonia, osteomyelitis and septic arthritis [18]. This study investigated the anti-staphylococcal activity of different citrus fruits and the carriage of enterotoxin genes by S. aureus strains isolated from non-clinical samples.

Out of 200 swab samples, 25% of cultures were identified as S. aureus in this study. These percentages vary in different studies. Sexton et al., isolated 70% S. aureus strains from patients and their isolation rooms. He also observed that the strains isolated from patients and their environment were closely related. This indicates environmental strains' contribution to infection spread [19].

In this study, amoxicillin and penicillin exhibited maximum resistance against S. aureus. Vancomycin, followed by ceftriaxone, were the most susceptible drugs. In Nigeria, ceftriaxone was also the least resistant antibiotic to S. aureus isolated from students' mobile phones [20]. Out of 50 S. aureus, 24% were identified as MRSA by cefoxitin sensitivity, a reference method for MRSA detection. Cefoxitin resistance results from acquiring the mecA gene by horizontal transfer for modified target proteins and inactivating ß-lactams drugs [21]. In this study, MSSA were higher in number 38 (76%) than MRSA 12 (24%). A systematic review of food contamination in Iran calculated the prevalence of MRSA, ranging from 0% to 32% [22]. In contrast to our results, another study on environmental samples of football team locker rooms found MRSA were more prevalent at 33% than MSSA at 24% [23]. The differences in percentages of MRSA might be due to the sampling surfaces in this study, which were the hands and nasal swabs of food handlers, personnel, and the environment.

Biochemical tests detected phytochemicals like alkaloids, carbohydrates, coumarin, flavonoids, glycosides and steroids in raw juices of citrus fruits. In previous literature, functional groups of these compounds have been detected by advanced techniques like Fourier-Transform Infrared (FTIR) Spectroscopy [24, 25].

Among the citrus fruits, the highest antibacterial activity was observed in the case of Citrus limon juice, inhibiting 90% S. aureus growth. A study reported the antimicrobial properties of Citrus limon and found remarkable antimicrobial properties against S. aureus and Pseudomonas aeruginosa, Escherichia coli, and Candida Albicans [24]. Afroja also supported the

findings by reporting Citrus limon juice was highly sensitive at MIC 12.5 µg/ml concentrations [2].

Over half of the strains were sensitive to Citrus pseudolimon. Citrus sinensis and Citrus tangerina had the same sensitivity values. While most of the strains 60% were resistant to Citrus paradisi juice. On the other hand, Citrus paradisi juices primarily inhibited bacterial growth at its minimum concentrations of 60 µg/ml as compared to other citrus fruits. This showed the solid antimicrobial effects of Citrus paradisi juice. Kim et al. described the antibacterial activity of Citrus paradisi extract by interrupting the glycolysis reactions in bacterial cells. He reported the combination of Citrus paradisi seed extract with melic acid could be an antibacterial agent [26]. However, studies are insufficient to describe the main constituent responsible for the antimicrobial activity of citrus fruits. This needs further investigation to explore the antimicrobial potentials of citrus fruits worldwide, where resistant pathogens and new antibiotics discovery are challenging.

Staphylococcal enterotoxins' (SEs) are one of the significant causes of food poisoning worldwide. Enterotoxins encoded by sea and seg genes can cause severe immunological responses and tissue damage in host. Both toxins show super antigenic and emetic activity. In the present study, the enterotoxigenic genes were detected in 48% of strains more prevalent in MRSA than MSSA (Table 4). Similar results were obtained for toxins genes in 19.12% MRSA isolated from Karachi [27-29]. In Iran, the estimated prevalence of SEs types SEA and SEG in food resources ranged from 13% to 83.3% [22]. This number poses a risk in terms of public health and food safety. Carriage of antibiotics resistance and enterotoxins enhances MRSA's virulence and enables them to cause particular clinical outcomes compared to MSSA strains. Poor hygiene practices cause its dissemination in the environment, consequently, untreatable infections of MRSA. Food handlers with poor personal hygiene are a potential source of infection and serve as a bridge in the journey of enterotoxigenic MRSA in food. The dissemination of these virulence traits further exaggerates the problem. These findings emphases to raise awareness about food-borne diseases and personal hygiene.

Conclusion:

The carriage of both antimicrobial resistance and enterotoxin genes is a growing problem and a threat to a healthy community. Citrus limon juice has the best antibacterial activity against S. aureus among all the tested citrus fruits. Citrus limon could be considered a lead for discovering antimicrobial substances.

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Conflict of Interest

The authors declare that there is no conflict of interest.

MHS: Literature search, data collection, experimental work and first draft

AA: Experimental work, write up, study design, data anaylysis

KN: Write up, data analysis, proof reading

SM: Write up, data analysis, data interpretation

MAR: data analysis, conceptualization of study design

SR: conceptualization of study design, write up, proof reading, supervisor/PI

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