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COMPARATIVE EFFICACY OF *AZADIRACHTA INDICA* ORGANIC EXTRACTS AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* CLINICAL ISOLATES

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ABSTRACT

The current study targeted the isolation and molecular identification of methicillin-resistant *Staphylococcus aureus* (MRSA) from various clinical samples (n=100). The samples were processed for bacterial isolation based on cultural, morphological and biochemical profiles followed by amplification of *nuc* gene encoding for thermostable nuclease of *S. aureus*. The isolates were screened phenotypically for methicillin resistance using cefoxitin or oxacillin discs and the genomic DNA of each isolate was screened for *mec*-A gene. Minimum inhibitory concentration (MIC) of vancomycin was also determined for each MRSA isolate. Organic extracts of *Azadirachta indica* leaves were prepared using ethanol, methanol and chloroform solvents followed by determination of *in vitro* activity of each extract against the MRSA isolates. A total of 47 isolates were identified as Staphylococci based on cultural, morphological and biochemical profiles, whereas 41 isolates were confirmed as *S. aureus* based on *nuc* gene. Phenotypically 13/41 isolates showed resistance to cefoxitin or oxacillin discs and 13/13 isolates were found positive for *mec*-A gene, hence termed as MRSA. All the isolates were sensitive to vancomycin and showed MIC (0.5-2µg/ml). The mean zone of inhibition was measured as 15.38±1.39 mm and 15.92±1.71 mm for ethanolic and methanolic extracts at 40% concentration, respectively. Whereas the highest zone was measured as 18.38±1.71 mm for chloroform extract at 60% concentration. Altogether, the results indicated that MRSA isolates pose a significant public health threat. However, organic extracts showed significant activity against MRSA isolates along with vancomycin as potential antimicrobial drug.

Keywords: *Azadirachta indica*, Methicillin-resistant *Staphylococcus aureus* (MRSA), Minimum inhibitory concentration (MIC), Thermostable nuclease, Vancomycin

INTRODUCTION

Staphylococcus aureus is gram positive cocci that is responsible for multiple infections in the hospital setting including wound infection, pneumonia, septicemia and skin/ soft tissue infections among children and adults, across the world (Tan et al. 2019; Naeem et al. 2021). *S. aureus* is considered as commensal as well as pathogenic bacteria which are leading cause of wound infections along with *Pseudomonas aeruginosa* and other bacteria (Akinduti et al. 2022). Previously, it was described that the staphylococci isolated from skin or infections were treated with different routine antimicrobial agents including beta lactam drugs (Anwar et al. 2018). One of the previous studies showed that ivermectin has anti-staphylococcus activity against cefoxitin resistant and cefoxitin susceptible isolates at the concentrations of 6.25 and 12.5 µg/ ml (Ashraf et al. 2018).

Currently, there are several studies that described the emergence of antimicrobial resistance among clinical isolates in different regions of the world (Idrees et al. 2023; Tsuji et al. 2024; Asadpour and Ghazanfari 2019). Previously, beta lactam drugs including cefoxitin, ceftriaxone or cefoxitin were considered as first choice for the treatment of staphylococci infections. Later, there were multiple studies which described the occurrence of methicillin-resistant *S. aureus* (MRSA) or methicillin-resistant *S. epidermidis* (MRSE) among community or hospital acquired infections (Tan et al. 2019; Anwar et al. 2018; Ashraf et al. 2018). The mechanism of methicillin resistance is mediated by *mec*-A gene that encodes penicillin-binding protein (PBP-2a) and ultimately the beta lactam antibiotics could not exert their activity against *mec*-A harboring staphylococci (Idrees et al. 2023; Naeem et al. 2021). One of the studies described the effectiveness of linezolid and determined the minimum inhibitory concentration of vancomycin against MRSA clinical isolates (Anwar et al. 2018). Linezolid and vancomycin were effective *in vitro* against MRSA clinical isolates.

However, few of the staphylococci isolates showed resistance to vancomycin or intermediate susceptibility to vancomycin which were termed as VISA/ VRSA strains. The resistance is mediated by *van*-A or *van*-B genes of the staphylococci (McGuinness, Malachowa, and DeLeo 2017). These genes are plasmid-encoded which could be taken up from *Enterococcus spp*. Hence, it was strongly suggested to determine the MIC for confirmation of VISA/ VRSA strains (Anwar et al. 2018).

Traditionally, herbs and medicinal plants or the extracts of herbs or medicinal were widely used throughout the world. There are several studies that described qualitative or quantitative analysis of herbs or medicinal plant extracts (Akinduti et al. 2022; Altayb et al. 2022; Anwar et al. 2018; Kaseke et al. 2023; Naeem et al. 2021; Sharma et al. 2024; Singaravelu et al. 2019). *Azadirachta indica* (Neem plant) is a traditional native plant in Pakistan, India, southeast Asian countries and some African countries that has been widely used for hundreds of years for its medicinal benefits. There are several studies that described the potential of *A. indica* organic extracts against staphylococci including MRSA or VRSA clinical isolates (Altayb et al. 2022; Anwar et al. 2018; Kaseke et al. 2023; Sharma et al. 2024; Singaravelu et al. 2019). Recently, some of the sentients reported the green synthesis of nano particles using *A. indica* followed by determination of their efficacy against clinical isolates (Gawai et al. 2023; Sharma et al. 2024; Tahir et al. 2023). In the current study, we have described the molecular identification of staphylococci and MRSA isolates from clinical samples in Faisalabad-Pakistan followed by determination of *in vitro* activity of organic extracts of *A. indica* leaves.

MATERIALS AND METHODS

The current study was approved by the Institutional Advanced Study & Research Board and was conducted from November-2022 to June-2023. Sterilized cotton swabs were used to collect the skin wound infection samples (n=100) from the patients attending Allied Hospital, Faisalabad-Pakistan followed by shifting of samples to Lab. The samples were inoculated on bacterial media including Nutrient agar, Blood agar and Mannitol Salt agar (Oxoid-UK) and incubated at 37°C for 24-48 hours. Cultural characteristics were observed for each isolate and bacterial growth was further inoculated on fresh agar plates to obtain purified growth which was initially identified based on Gram's staining followed by coagulase and catalase tests (Naeem et al. 2021). The isolates were processed for DNA extraction GeneJET Genomic DNA Purification Kit (Thermo Scientific-UK) according to the protocol described by the manufacturer. For this purpose, a well isolated bacterial colony was inoculated into nutrient broth followed by overnight incubation. The growth was centrifuged at 5000×g for 5 minutes. The pellet was dissolved using lysis buffer containing lysozyme or proteinase-K enzyme. Finally, the DNA was collected from the spin columns using sterile Eppendorf tubes and stored at -20°C. PCR was performed targeting *nuc* gene encoding for thermostable nuclease enzyme of staphylococci using forward primer (5´-GCGATTGATGGT GATACGGTI-3´) and reverse primer (5´-AGCCAAGCCTTGACGAACTAAAGC-3´) as described by (Brakstad, Aasbakk, and Maeland 1992; Schaumburg et al. 2014). Amplified product was

subjected to gel electrophoresis using 1.2% agarose and stained with ethidium bromide which resulted in a 447bp DNA band. The isolated DNA was also subjected to molecular identification of *mec*-A gene using forward primer (5´-TCCAGATTACAACTTCACCAGG-3´) and reverse primer (5´-CCACTTCATATCTTGTAACG-3´) (Naeem et al. 2021).

In the second phase, the bacterial isolates were processed for identification of methicillin resistance using cefoxitin or oxacillin disc (Oxoid-UK) using Mueller Hinton agar (Oxoid-UK) (Anwar et al. 2018). Antimicrobial susceptibility was also determined using linezolid and MIC was also calculated against vancomycin using commercially available E-strips (Oxide M.I.C. EVALUATORTM, UK) (Naeem et al. 2021). In the last phase, organic extracts of *A. indica* (ethanol, methanol and chloroform) were prepared using fresh leaves of *A. indica* (Naeem et al. 2021). Different concentrations of extracts (20, 40 and 60%) were used and the zone of inhibition (>12 mm) was considered as susceptible (Naeem et al. 2021).

RESULTS

Initially, based on cultural, morphological and biochemical profiles, 47/100 staphylococci were identified, whereas *nuc* gene was identified among 41 isolates. Therefore, further tests were performed on these isolates. A total of 13 MRSA isolates were identified based on cefoxitin or oxacillin disc susceptibility, further all these 13 were also found to harbor *mec*-A gene. Out of 100 swab samples, 95 were found positive for bacterial growth. Detailed characteristics of the isolates are described in Table 1. The MRSA isolates were sensitive to vancomycin and linezolid and showed significant susceptibility against ethanol, methanol and chloroform extracts of A. indica. Detailed susceptibility profiles are described in Table 2.

Table 1: Distribution and characteristics of bacterial isolates

Sr. No.	Characteristics	Isolates
1	Number of samples	100
2	Initial growth	71
3	Colony characteristics (nutrient agar)	circular & convex
4	Colony characteristics (mannitol salt agar)	circular & convex
5	Gram's stain & morphology	positive & cocci
6	Coagulase positive	47
6	Coagulase-negative	47
7	Catalase positive	42
8	Catalase negative	5
9	Staphylococci	47
10	*nuc* gene	41
11	Methicillin-resistant *S. aureus* (MRSA)	13
12	*mec*-A gene containing MRSA	13

Table 2: Methicillin Resistant *S. aureus* (MRSA) Susceptibility Profiles against Cefoxitin, Oxacillin, Vancomycin, Linezolid and Organic Extracts of *Azadirachta indica*

Isolates	*FOX*	*OXA*	*VAN*	*LZD*	*Ethanol Extract*			*Methanol Extract*			*Chloroform Extract*		
					60%	*40%*	*20%*	*60%*	*40%*	*20%*	*60%*	*40%*	*20%*
					Zone of inhibition (mm)								
1	R	R	S	S	18	15	15	14	14	12	16	15	17
2	R	R	S	S	15	16	13	16	16	17	21	17	18
3	R	R	S	S	13	15	15	15	15	16	20	18	19
4	R	R	S	S	14	16	14	17	14	15	17	21	18
5	R	R	S	S	18	18	18	19	18	18	17	22	17
6	R	R	S	S	16	17	14	18	16	17	18	18	15
7	R	R	S	S	15	15	16	15	17	16	19	16	17
8	R	R	R	S	14	16	18	17	19	19	17	18	16
9	R	R	S	S	14	15	15	15	16	15	17	19	18
10	R	R	S	S	16	13	16	16	17	13	19	17	21
11	R	R	S	S	13	16	14	14	15	14	17	15	20
12	R	R	S	S	13	13	14	18	17	17	21	19	21
13	R	R	S	S	14	15	17	16	13	14	20	18	17
Mean					*14.85*	*15.38*	*15.31*	*16.15*	*15.92*	*15.62*	*18.38*	*17.92*	*18.00*
SD					*1.71*	*1.39*	*1.60*	*1.57*	*1.71*	*2.02*	*1.71*	*2.06*	*1.71*

FOX=Cefoxitin (≤22mm zone of inhibition=Resistant), OXA=Oxacillin (≤10mm zone of inhibition=Resistant) VAN=Vancomycin (≤2MIC=Sensitive), LZD=Linezolid (≥22mm zone of inhibition=Sensitive), S=Sensitive, R=Resistant

DISCUSSION

Antimicrobial resistance has emerged as a global concern among community acquired and hospital acquired clinical isolates of staphylococci. (Anwar et al. 2018; Tan et al. 2019; Tsuji et al. 2024; Cardoso Guimarães et al. 2021). The concern is further expanded as emergence of biofilm producing isolates (Akinduti et al. 2022; Cardoso Guimarães et al. 2021). In both cases, the antimicrobial susceptibility patterns showed significant variations and consequently limiting the therapeutic choices for the clinicians. At present, the clinical isolates of staphylococci have demonstrated resistance to different antibiotics including beta lactam drugs (methicillin), termed as methicillin-resistant *S. aureus* (MRSA) (Anwar et al. 2018; Hanif and Hassan 2019; Indrawattana et al. 2013). The molecular mechanism usually involved the occurrence of *mec*-A or *mec*-C plasmid encoded genes (Naeem et al. 2021; Idrees et al. 2023). The isolates which are resistant to methicillin could also show resistance to vancomycin (Asadpour and Ghazanfari 2019; McGuinness, Malachowa, and DeLeo 2017). Vancomycin resistance is mediated by plasmid-encoded genes *Van*-A or *Van*-B (McGuinness, Malachowa, and DeLeo 2017).

Currently, significant research has been reported in Pakistan. However, in the current study staphylococcus clinical isolates were identified based on *nuc* gene and MRSA were identified based on *mec*-A gene followed by *in vitro* evaluation of *A. indica* organic extracts (ethanol, methanol and chloroform) along with determination of vancomycin and linezolid susceptibility profiles.

A total of 100 samples were collected from Allied Hospital, Faisalabad- Pakistan following all ethical guideline and consent of patients or their guardian. Results showed 41/100 clinical staphylococci isolates based on *nuc* gene amplification including 13/41 MRSA isolates, further these 13/13 carried plasmid encoded *mec*-A gene. There have been several studies that reported an increased number of clinical staphylococci among similar settings (Anwar et al. 2018; Ashraf et al. 2018; Hanif and Hassan 2019). The increased occurrence could be attributed towards an unhygienic environment and lack of biosafety or biosecurity measures. In the current study molecular identification was targeted by amplification of *nuc* gene encoding thermostable nuclease among *S. aureus*. The mechanism has been described previously (Brakstad, Aasbakk, and Maeland 1992). The *nuc* gene could also be utilized to characterize highly divergent strains (Schaumburg et al. 2014).

The results showed that 13/41 *S. aureus* isolates were MRSA and carried *mec*-A gene. Previously, it has been demonstrated that MRSA could be identified based on cefoxitin/ oxacillin disc susceptibility or molecular identification of *mec*-A gene (Naeem et al. 2021). However, in the current study we have employed both methods to determine their efficacy. However, it was observed that cefoxitin/ oxacillin and *mec*-A amplification are equally good to determine MRSA strains. All the 13 MRSA isolates showed susceptibility to vancomycin and linezolid. Similar findings were reported previously (Anwar et al. 2018; Naeem et al. 2021). However, there are several reports regarding resistance to vancomycin or linezolid (Akinduti et al. 2022; Hanif and Hassan 2019; McGuinness, Malachowa, and DeLeo 2017). However, in the current study none of the isolates were resistant to vancomycin or linezolid which could be due rationale use of these drugs among hospital settings. All the 13 MRSA isolates showed susceptibility to ethanol, methanol and chloroform extract of *A. indica*. This efficacy is primarily due to the presence of *Azadirachtin* and *Nimbolinin*, potent tetranortriterpenoid compounds which are dissolved in organic extracts (Akinduti et al. 2022; Altayb et al. 2022; Gawai et al. 2023; Singaravelu et al. 2019).

In conclusion, in the current study, we isolated a higher number of *S. aureus* isolates including MRSA from clinical settings in Faisalabad-Pakistan. Cefoxitin/ oxacillin disc susceptibility and *mec*-A gene amplification are promising tools to identify MRSA strains. Further, vancomycin, linezolid and organic extracts showed significant results against MRSA isolates.

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