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In vitro efficacy of *Azadirachta indica* leaf extract against methicillin resistant Staphylococci isolated from skin infection

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Abstract: In this cross-sectional study, the isolation and identification of Methicillin Resistant *Staphylococcus aureus* (MRSA) and Methicillin Resistant *S. epidermidis* (MRSE) was described from skin infections (n=100). Initial isolation was done by conventional procedures followed by amplification/ sequence analysis of 16S rRNA. Methicillin resistance was determined using cefoxitin discs and resistant isolates were screened for *mec*-A gene followed by Minimum Inhibitory Concentrations (MIC) determination of vancomycin. In second phase, we investigated extract of *Azadirachta indica* leaves using Fourier Transformed Infrared Spectroscopy (FTIR-Spectroscopy) and investigated *in vitro* activity. Initially, total of 28 *Staphylococci* were identified. 16S rRNA gene sequence confirmed *S. aureus* (22), *S. epidermidis* (3) and *S. saprophyticus* (3) isolates. Cefoxitin discs showed (7/22) MRSA, (3/3) (MRSE) and none of the methicillin resistant *S. saprophyticus*. MRSA and MRSE isolates showed presence of *mec*-A gene. However, all isolates were sensitive to vancomycin MIC (0.5-2µg/mL) and sensitive to Linezolid. FTIR- Spectroscopy of *A. indica* indicated the presence of *azadirachtin* and *nimbolinin*. The mean zone of inhibition was measured 14.23±1.37 and 13.66±0.70 against MRSA and MRSE isolates, respectively. Altogether, MRSA and MRSE is significant public health concern. However, vancomycin and linezolid were found effective and extract of *A. indica* showed *in vitro* effects.

Keywords: Methicillin resistant *Staphylococcus* spp., MIC, vancomycin, *Azadirachta indica.*

INTRODUCTION

Staphylococcus aureus and other non-aureus *Staphylococcus* are the causative agents of multiple infections including pneumonia, septicemia, skin/soft tissue infections and toxic shock syndrome (Cuny *et al*., 2015). *Staphylococci* are the commensal as well as opportunist pathogens and are the leading cause of bacteremia and are described among almost 30% of the human population (Tong *et al*., 2015). The *staphylococci* infections are treated with different antibiotics i.e. Aminoglycosides, chloramphenicol, clindamycin, fluoroquinolones, macrolides and other β-Lactam antibiotics (Anwar *et al*., 2018). The emergence of resistant strains could not be neglected in hospital and community-acquired infections throughout the world. For example, Methicillin Resistant *S. aureus* (MRSA), non-aureus Methicillin Resistant *Staphylococcus* (NA-MRS) including METHICILLIN RESISTANT *S. epidermidis* (MRSE) and Vancomycin Intermediate/ Resistant *S. aureus* (VISA/ VRSA) are serious public health concerns (Mohamed *et al*., 2016; Anwar *et al*., 2018). Methicillin resistance is based on resistance to β-Lactam antibiotics including methicillin, cefoxitin and cephalosporins, whereas the *S. aureus* isolates which are susceptible to these antimicrobials are termed as methicillin susceptible *S. aureus* (Gurusamy *et al*., 2013). The molecular mechanism involves the presence of *mec*-A gene which encodes for a penicillin-binding protein (PBP-2a) that has a lower affinity for β-Lactam antibiotics as described in detail previously (García- Álvarez *et al*., 2011). The use of vancomycin or linezolid is an excellent substitute for the treatment of infections caused by resistant *Staphylococcus* (Wootton *et al*., 2001; Corey *et al*., 2014). However, the *Staphylococci* strains also showed resistance to vancomycin and resulted in the emergence of VISA/ VRSA strains that is mediated by *van*-A or *van*-B genes of the *Staphylococci* (Tiwari *et al*., 2009). Resistance to vancomycin is also critical as *van*-A or *van*-B genes are plasmid-encoded and are taken up from *Enterococcus* spp. Therefore, molecular identification of *van*-A or *van*-B has little significance in the detection of VISA/VRSA strains. However, the determination of Minimum Inhibitory Concentration (MIC) resulted in accurate identification of VISA/VRSA strains (Anwar *et al*., 2018). The treatment of the MRSA, NA-MRS, MRSE, VISA or VRSA strains requires an accurate diagnosis along with the selection of appropriate antimicrobial agents based on molecular or phenotypic resistance patterns as described (Corey *et al*., 2014; Anwar *et al*., 2018). At present, a lot of research is required to understand the molecular mechanism of resistance, emergence, spread and alternative treatment remedies for *Staphylococci* isolates.

Traditionally, herbs and medicinal plants are widely used

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throughout the world. Among these *Azadirachta indica* (Neem) which is a native plant in Pakistan, India and other Southeast Asian countries (Anwar *et al*., 2018) has been used for hundreds of years. Further, different commercial products (soaps and hand wash etc.) are available which are supplemented with several contents from *A. indica* plant. The extracts of the leaves were found effective in different skin infections (Anwar *et al*., 2018) and has potential effect against induced colitis in rat model (Ruslie and Darmadi, 2020). This effect is contributed to different active ingredients present in *A. indica* extracts (Bolade *et al*., 2018). Therefore, the following objectives were targeted (a) to find out the prevalence of *Staphylococci* infections among patients attending Nishtar Medical University & Hospital, Multan and District Head Quarters Hospital, Faisalabad, (b) molecular confirmation of the isolates, (c) molecular basis of the resistance, (d) antimicrobial resistance profiles and (e) *in vitro* activity of *A. indica* leaves extracts against resistance isolates.

MATERIALS AND METHODS

Study area and processing of samples

This cross-sectional study was approved (*Study No. 019259 and IRB No. 259 vide Ref. No. GCUF/ERC/1759, Dated 10-10-2017*). The sterile cotton swabs, soaked in normal saline were collected from the skin infections from the patients attending or admitted in the Nishtar Medical University & Hospital, Multan (n=50) and District Head Quarters Hospital, Faisalabad (n=50). All the collected samples were shifted to the Lab under controlled temperature conditions and streaked on blood, nutrient and mannitol salt agar (Oxoid-UK) followed by incubation at 37°C for 24-48 h. The bacterial growth was initially identified microscopically followed by coagulase and catalase tests as described (Cappuccino and Sherman 2014; Ilyas *et al*., 2016; Anwar *et al*., 2018).

Molecular identification of samples

Initially identified *Staphylococcus* isolates were subjected to DNA extraction using the GeneJET Genomic DNA Purification Kit (Thermo Scientific-UK). Briefly, a single colony of *S. aureus* isolate was inoculated into the nutrient broth and incubated overnight at 37°C followed by centrifugation at 5000g for 10 min. The bacterial pellet was re-suspended into the lysis buffer and bacterial lysis was targeted by adding lysis solution and proteinase-K enzyme. Finally, after the washing, the DNA was collected from the spin columns using sterile Eppendorf tubes. The quantity of DNA was measured using Colibri Microvolume Spectrophotometer (Titertek-Berthold, Germany). PCR was performed using template DNA and Master mix (Thermo Scientific-UK) using Thermal cycler (BIO RAD, T100™ Thermal Cycler, California) and targeting 16S rRNA gene using forward primer (27F- 5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer (1492R- 5′-TACGGYTACCTTGTTACGACTT-3′) (Dupont *et al*., 2016). Following the electrophoresis (1.5% agarose with 1X Tris-EDTA buffer) the amplified products were dispatched to Macrogen, Korea for sequencing. The sequences were analyzed and compared with the existing GenBank database (http://blast.ncbi.nlm.nih.gov/). The phylogenetic position of the isolates was determined using Molecular Evolutionary Genetic Analysis software (Mega-X) for phylogenetic analysis as described by Kumar *et al*., 2018.

Phenotypic and molecular antibiotic susceptibility profiling

The isolates which were confirmed on molecular levels were subjected to phenotypic identification of methicillin resistance using cefoxitin discs (Oxoid, UK) along with antimicrobial susceptibility profiles (against linezolid, tigecycline, ciprofloxacin, tobramycin and amoxicillin/clavulanate) using Mueller Hinton agar (Oxoid-UK) plates as described (Anwar *et al*., 2018). The isolates were also subjected to determine the MIC of vancomycin using E-strips (Oxide M.I.C.EVALUATOR™, UK) as described (Anwar *et al*., 2018). Briefly, the E-strips were applied with the lowest concentration onto the plate and incubated at 37°C for 16-18h and the results were observed as vancomycin sensitive/intermediate/resistant *S. aureus* (VSSA/VISA/VRSA). The phenotypically identified cefoxitin resistant isolates were also subjected to the molecular identification of *mec*-A gene. For this purpose, DNA was amplified according to the conditions described above targeting *mec*-A gene using forward primer (5′- CCAGATTACAACTTCACCAGG-3′) and reverse primer (5′-CCACTTCATATCTTGTAACG-3′) as described by Alharthi *et al*., 2016.

Preparation and processing of A. indica extract

In the last phase, fresh leaves of *A. indica* were collected, identified and washed with sterilized water followed by dehydration of the leaves at room temperature. The dry leaves (100 g) were ground gently using pestle and mortal and soaked in 200 mL of chloroform for 48 h and kept at room temperature followed by filtration using Whatman filter paper (Sigma-Aldrich, UK) (Siddiqui *et al*., 2003; Anwar *et al*., 2018; Bolade *et al*., 2018). The filtrate was subjected to Fourier Transformed Infrared Spectroscopy (FTIR-Spectroscopy) for the determination of the active ingredients of the extract. Briefly, the extract was frozen at -80^{o} C followed by lyophilization and finally the infrared absorption spectrum was recorded on FTIR spectrophotometer (Alpha, Bruker, California, USA) in the region of 4000 to 400 cm^{-1}(Bolade *et al*., 2018). This extract was used against MRSA and MRSE isolates using agar well diffusion assay (each isolate was processed in triplicates). The zone of inhibition (>12 mm) was considered as positive efficacy of the extract against MRSA and MRSE isolates (Dahiya *et al*., 2012; Anwar *et al*., 2018).

Table 1: Distribution and characteristics of bacterial isolates

S No.	Characteristics	District Head Quarters Hospital, Faisalabad	Nishtar Medical University & Hospital, Multan
1	Total Number of samples	50	50
2	Initial growth on different media	47	48
3	Colony characteristics (blood agar)	circular & convex	circular & convex
4	Colony characteristics (nutrient agar)	circular & convex	circular & convex
5	Colony characteristics (mannitol salt agar)	circular & convex	circular & convex
6	Gram's stain & morphology	positive & cocci	positive & cocci
7	Coagulase positive	10	11
8	Coagulase-negative	3	4
9	Catalase positive	10	12
10	Catalase negative	3	3
11	α-Hemolysis	4	3
12	β-Hemolysis	9	12
13	*Staphylococcus*	13	15
14	*S. aureus* (16S rRNA)	10	12
15	*S. epidermidis* (16S rRNA)	3	-
16	*S. saprophyticus* (16S rRNA)	-	3
17	Methicillin-resistant *S. aureus* (MRSA)	4	3
18	Methicillin-resistant *S. epidermidis* (MRSE)	3	-
19	*mec*-A gene containing MRSA	4	3
20	*mec*-A gene containing MRSE	3	-

Table 2: Susceptibility profiles of methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE) clinical isolates

S No.	Isolates	Antimicrobials							Neem extract Zone of inhibition(mm)
		FOX	VAN	LZD	TGC	CIP	TOB	AMC	Mean±SD*
1	MRSA-01/DHQ/FSD	R	1µg/mL	S	R	R	R	R	14.23±1.37
2	MRSA-02/DHQ/FSD	R	0.5µg/ml	S	S	S	R	S	
3	MRSA-03/DHQ/FSD	R	1µg/ml	S	R	R	S	R	
4	MRSA-04/DHQ/FSD	R	0.5µg/ml	S	R	R	R	R	
5	MRSA-01/NMU/MTN	R	1µg/ml	S	R	S	S	S	
6	MRSA-01/NMU/MTN	R	1µg/ml	S	R	S	S	R	
7	MRSA-01/NMU/MTN	R	2µg/ml	S	R	R	S	R	
8	MRSE-01/DHQ/FSD	R	0.5µg/ml	S	S	S	S	S	13.66±0.70
9	MRSE-01/DHQ/FSD	R	0.5µg/ml	S	R	S	S	S	
10	MRSE-01/DHQ/FSD	R	1µg/ml	S	S	S	S	S	

*mean zone of inhibition (>12 mm) was considered a positive effect. FOX=Cefoxitin (≥22mm zone of inhibition=Sensitive), VAN=Vancomycin Minimum inhibitory concentration using E-strips (≤2MIC=Sensitive), LZD=Linezolid, TGC=Tigecycline, CIP= Ciprofloxacin, TOB= Tobramycin, AMC= Amoxicillin/Clavulanate, S=Sensitive, R=Resistant

STATISTICAL ANALYSIS

The obtained data was tabulated in Microsoft Excel spread sheet using Microsoft 365 software and Mean±SD was calculated (Anwar *et al*., 2018).

RESULTS

Out of 100 swab samples, 95 were found positive for bacterial growth. A total of 28 isolates were identified as *Staphylococci* based on microscopic examination and coagulase and catalase test. Molecular data of 16S rRNA gene confirmed *S. aureus* (22), *S. epidermidis* (3) and *S. saprophyticus* (3) isolates. Detailed characteristics of the isolates are described in table 1. Molecular identification of all *Staphylococcus* isolates indicated the presence of 16S rRNA gene and resulted in 100% base pair similarity with available NCBI database. Further, all the phenotypically identified MRSA and MRSE were positive for *mec*-A gene as shown in fig. 1. One of the MRSA sequences was submitted to GenBank vide GenBank Accession No. MN453615.1 (https://www.ncbi.nlm.nih.gov/nuccore/MN453615.1). All the *S. aureus* strains were sensitive to vancomycin by using the E-Strips method and

the measured MIC was ranged 0.5-2µg/mL for each MRSA and MRSE (table 2). The phytochemical analysis of *A. indica* aqueous extract as determined by FTIR-Spectroscopy indicated the presence of bands at 3255cm^{-1} assigned to O–H stretching, the peak at 2,971cm^{-1} for -CH_3 stretching, 1,547cm^{-1} for C=C group stretching and 1300 cm^{-1} for stretching of -CH_3 group. These bands are indicative of the presence of *azadirachtin* and *nimbolinin* (Bolade *et al* 2018), potent tetranortriterpenoid as shown in fig. 2. The antimicrobial activity of the extract against MRSA and MRSE isolates is shown in table 2. Briefly, all MRSA and MRSE isolated were observed as sensitive against *A. indica* extract and showed zone of inhibition (>12 mm).

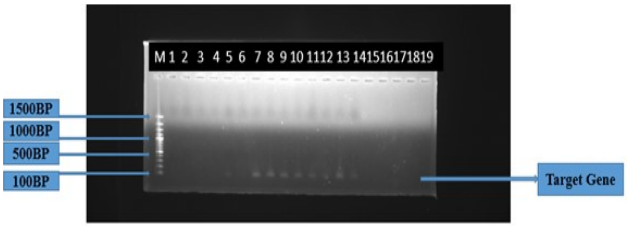

Fig. 1: Molecular identification of *mec*-A gene, M=100 bp marker, Lane 1=Negative Control, Lane 2-6=methicillin-susceptible *S. aureus* (MSSA), Lane 7-13=methicillin-resistant *S. aureus*, Lane 14=methicillin-resistant *S. epidermidis* (MRSE)

DISCUSSION

Antibiotic resistance among *S. aureus* isolates has emerged as public health concern throughout the world (Monecke *et al*., 2011; Tong *et al*., 2015). The resistance mechanism has expanded to several classes of antibiotics including methicillin, hence termed as MRSA which is mediated by chromosomal encoded gene *mec*-A (Alharthi *et al*., 2016). A similar mechanism of resistance is also observed in other species of *Staphylococcus* i.e. MRSE as described recently (Nobrega *et al*., 2018). Further, the resistance has also been reported against vancomycin which is mediated by plasmid-encoded genes *Van*-A or *Van*-B, hence termed as vancomycin-intermediate/ resistant *S. aureus* (VISA/VRSA) as described by Mohamed *et al*., (2016). However, the incidence of VRSA is not as high as MRSA (Anwar *et al*., 2018).

At present, significant work has been reported in Pakistan in different regions regarding the resistance to different antibiotics. However, in this study *Staphylococcus* isolates were analyzed based on 16S rRNA gene followed by the screening of methicillin-resistant isolates by amplification of *mec*-A gene. Further, all MRSA and MRSE isolates were screened for resistance to vancomycin by determining the MIC against each isolate. For this purpose, a total of 100 swab samples were collected from Nishtar Medical University & Hospital, Multan (n=50) and District Head Quarters Hospital, Faisalabad (n=50). A total of 95 samples showed bacterial growth on nutrient agar medium. Further, based on cultural, microscopic and biochemical tests, 28 isolates were identified as *Staphylococcus*. Molecular identification and characterization also confirmed *S. aureus* (22), *S. epidermidis* (3) and *S. saprophyticus* (3) strains. Collectively, a high prevalence was observed, as one of the previous studies also described the increased emergence of *S. aureus* from Faisalabad (Anwar *et al*., 2018). Another study described that 16S r RNA nucleotide sequence analysis with the existing database of *S. aureus* is a confirmatory characterization tool for different bacterial isolates (Woo *et al*., 2001).

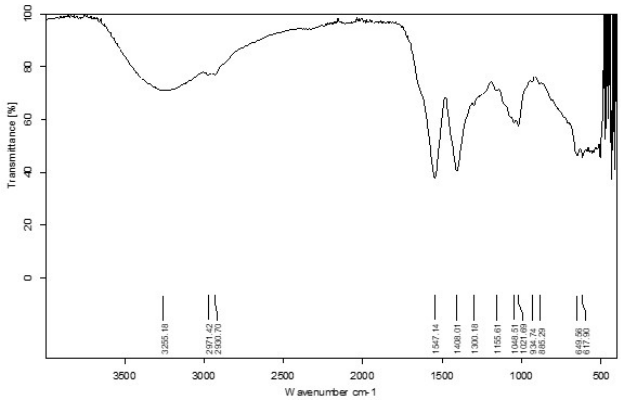

Fig. 2: Full FTIR spectrum of *Azadirachta indica* aqueous extract analyzed in the range of 4000 to 400 cm^{-1} wave number (X-axis) and the function of transmittance (Y-axis)

In the current study, all the *Staphylococcus* isolates were subjected to antimicrobial susceptibility profiles including Cefoxitin, Linezolid, Tigecycline, Ciprofloxacin, Tobramycin and Amoxicillin/Clavulanate along with MIC determination against Vancomycin. We observed that 7/22 *S. aureus* and 3/3 *S. epidermidis* were resistant to cefoxitin, hence, termed as methicillin resistant. However, all the isolates were sensitive to vancomycin with MIC ranged (0.5-2 µg/mL) and linezolid. All the MRSA and MRSE strains were also screened for the presence of *mec*-A gene to investigate the molecular basis of resistance. All the seven MRSA and three MRSE isolates were found positive for *mec*-A gene. Few of the previous studies described that *mec*-A is responsible for resistance against Methicillin (Anwar *et al*., 2018; Nobrega *et al*., 2018); while, Argudin *et al*., (2014) described that sensitive *S. aureus* strains were negative for the presence of *mec*-A gene. These findings were also found similar to one of the previous studies (García- Álvarez *et al*., 2011). Therefore, the presence of *mec*-A gene on chromosomal DNA could be responsible for the expression of Methicillin resistance. Ullah *et al*. (2016) described that MRSA isolates were resistant to penicillin, whereas sensitive to vancomycin. Even in the last decade, a significant prevalence of MRSA isolates was also reported (Dar *et al*., 2006; Tiwari *et al*., 2009). As a matter of concern, the MRSA or MRSE

isolates could be treated as with vancomycin or linezolid (Nobrega *et al*., 2018).

In the second part of the study, phytochemical analysis of an aqueous extract of *A. indica* leaves showed presence of *azadirachtin* and *nimbolinin,* a potent tetranortriterpenoid (Siddiqui *et al*., 2003; Cen-Pacheco *et al*., 2020). Few of the studies also described the phytochemical analysis and identified the similar ingredient using different organic solvents in Pakistan, India, Brazil and other countries (Siddiqui *et al*., 2003; Satdive *et al*., 2011; Quelemes *et al*., 2015; Cen-Pacheco *et al*., 2020). The previous data is based on HPLC or thin-layer chromatography. However, FTIR-spectroscopy was used in the current study for qualitative phytochemical analysis. Previous studies have described Neem extracts for the treatment of diabetic foot and chronic wounds or skin gangrenes (Zafar *et al*., 2008; Wong *et al*., 2013). One of the recent studies described the effects of *A. indica* leaf extracts in induced colitis in rat model (Ruslie and Darmadi, 2020). Some studies have also described the effects of *A. indica* against biofilm formation and resistant bacteria (Dahiya *et al*., 2012; Quelemes *et al*., 2015). However, in the current study, we reported the antibacterial activity of *A. indica* as 14.23±1.37 and 13.66±0.70 against MRSA and MRSE isolates, respectively.

CONCLUSION

The current study described a higher incidence of *Staphylococci* including MRSA and MRSE from surgical and non-surgical wounds among the patients of tertiary care hospitals in Multan and Faisalabad. The amplification and sequence analysis of 16S rRNA gene offers good molecular identification approach for the identification of different bacterial isolates including *Staphylococcus*. The molecular mechanism of MRSA and MRSE is based on the presence of the chromosomal *mec*-A gene. In the current study, none of the isolates showed resistance to vancomycin as indicated by MIC values. Therefore, it is highly suggested that skin infection should be treated with antimicrobial drugs after antimicrobial susceptibility profiles of the isolated pathogen. The vancomycin should only be suggested in case of incidence of MRSA or MRSE. Further, the use of soap or hand wash containing Neem extract can be used as an alternate home remedy.

REFERENCES

Alharthi AA, Gaber A and Hassan MM (2016). Molecular characterization of *mec*-A and SCCmec genes in pathogenic *Staphylococcus* spp. collected from hospitals in Taif region, KSA. *Biotechnology,* **15**(1): 26-34.

Anwar J, Zahoor MA, Zahoor MK, Siddique AB, Nawaz Z, Rasool MH, Qamar MU, Waseem M, Hussain SZ and Yasmin A (2018). Efficacy of *Azadirachta indica* organic extracts against clinical methicillin resistant *Staphylococcus aureus* isolates. *Pak. J. Pharm. Sci.,* **31**(4-Suppl): 1485-1488.

Argudín MA, Mendoza MC, Martín MC and Rodicio MR (2014). Molecular basis of antimicrobial drug resistance in *Staphylococcus aureus* isolates recovered from young healthy carriers in Spain. *Microb. Pathog.,* **74**: 8-14.

Bolade OP, Akinsiku AA, Adeyemi AO, Williams AB and Benson NJ (2018). Dataset on phytochemical screening, FTIR and GC-MS characterisation of *Azadirachta indica* and *Cymbopogon citratus* as reducing and stabilising agents for nanoparticles synthesis. *Data in Brief.*, **20**: 917-926.

Cappuccino JG and Sherman N (2014). *Microbiology: A Laboratory Manual* (10th edition). Pearson, Boston.

Cen-Pacheco F, Ortiz-Celiseo A, Peniche-Cardena A, Bravo-Ruiz O, Lopez-Fentanes FC, Valerio-Alfaro G and Fernández JJ (2020). Studies on the bioactive flavonoids isolated from *Azadirachta indica*. *Nat. Prod. Res.,* **34**(24): 3483-3491.

Corey GR, Kabler H, Mehra P, Gupta S, Overcash JS, Porwal A, Giordano P, Lucasti C, Perez A, Good S, Jiang H, Moeck G and O'Riordan W (2014). Single-dose oritavancin in the treatment of acute bacterial skin infections. *N. Engl. J. Med.*, **370**(23): 2180-2190.

Cuny C, Abdelbary MH, Kock R, Layer F, Scheidemann W, Werner G and Witte W (2015). Methicillin-resistant *Staphylococcus aureus* from infections in horses in Germany are frequent colonizers of veterinarians but rare among MRSA from infections in humans. *One Health,* **2**: 11-17.

Dahiya P and Purkayastha S (2012). Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. *Indian J. Pharm. Sci.,* **4**(5): 443-450.

Dar JA, Thoker MA, Khan JA, Ali A, Khan MA, Rizwan M, Bhat KH, Dar MJ, Ahmed N and Ahmad S (2006). Molecular epidemiology of clinical and carrier strains of methicillin resistant *Staphylococcus aureus* (MRSA) in the hospital settings of north India. *Ann. Clin. Microbiol. Antimicrob.*, **5**(1): 1-15.

Dupont A, Sommer F, Zhang K, Repnik U, Basic M, Bleich A, Kühnel M, Bäckhed F, Litvak Y, Fulde M, Rosenshine I and Hornef MW (2016). Age-dependent susceptibility to enteropathogenic Escherichia coli (EPEC) infection in mice. *PLoS Pathog.,* **12**: e1005616.

Garcia-Alvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, Curran MD and Parkhill J (2011). Methicillin-resistant *Staphylococcus aureus* with a novel *mec*-A homologue in human and bovine populations in the UK and Denmark: A descriptive study. *Lancet Infect. Dis.*, **11**(8): 595-603.

Gurusamy KS, Koti R, Toon CD, Wilson P and Davidson BR (2013). Antibiotic therapy for the treatment of

methicillin-resistant *Staphylococcus aureus* (MRSA) in non-surgical wounds. *Cochrane Database Sys. Rev.*, **11**: 1-36.

Ilyas S, Qamar MU, Rasool MH, Abdulhaq N and Nawaz Z (2016). Multidrug-resistant pathogens isolated from ready-to-eat salads available at a local market in Pakistan, *Br. Food J.,* **118**(8): 2068-2075.

Kumar S, Glen Stecher, Michael Li, Christina Knyaz and Koichiro Tamura (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.,* **35**: 1547-1549.

Mohamed MF, Abdelkhalek A and Seleem MN (2016). Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular *Staphylococcus aureus*. *Scientific Reports,* **6**(29707): 1-14.

Monecke S, Coombs G, Shore AC, Coleman DC, Akpaka P, Borg M and Kadlec K (2011). A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*. *PloS One*, **6**(4): 1-24.

Nobrega DB, Naushad S, Naqvi SA, Condas LAZ, Saini V, Kastelic JP, Luby C, Buck JD and Barkema1 HW (2018). Prevalence and genetic basis of antimicrobial resistance in non-aureus Staphylococci isolated from Canadian dairy herds. *Front. Microbiol.,* **9**: Article: 256.

Quelemes PV, Perfeito ML, Guimarães MA, Santos RC, Lima DF, Nascimento C, Silva MP, Soares MJ, Ropke, CD, Eaton P, Moraes J and Leite JR (2015). Effect of neem (*Azadirachta indica A. Juss*) leaf extract on resistant *Staphylococcus aureus* biofilm formation and *Schistosoma mansoni* worms. *J. Ethnopharmacol.,* **175**: 287-94.

Ruslie RH and Darmadi D (2020). Administration of neem (Azadirachta indica A. Juss) leaf extract decreases TNF-α and IL-6 expressions in dextran sodium sulfate-induced colitis in rats. *J. Adv. Vet. Anim. Res.*, **7**(4): 744-749.

Satdive RK, Eapen S and Fulzele DP (2011). Bioactive constituents and antimicrobial activity of cell cultures of *Azadirachta indica*. *Int. J. Pharma. Bio Sci.,* **2**(4): 617-628.

Siddiqui SB, Afshan F, Gulzar T and Sultan R (2003). Tetracyclic triterpenoids from the leaves of *Azadirachta indica* and their insecticidal activities. *Chemical and Pharmaceutical Bulletin,* **51**(4): 415-417.

Tiwari HK, Das AK, Sapkota D, Sivrajan K and Pahwa VK (2009). Methicillin resistant *Staphylococcus aureus*: Prevalence and antibiogram in a tertiary care hospital in western Nepal. *J. Infect. Dev. Ctries.*, **3**(09): 681-684.

Tong SYC, Davis JS, Eichenberger E, Holland TL and Fowler, V. G. (2015). *Staphylococcus aureus* Infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.*, **28**(3): 603-661.

Ullah A, Qasim M, Rahman H, Khan J, Haroon M, Muhammad N, Khan A, Muhammad N (2016). High frequency of methicillin-resistant *Staphylococcus aureus in* Peshawar Region of Pakistan. *Springerplus*, **5**(1): 1-6.

Wong S, Manikam R and Muniandy S (2013). Analysis of bacterial diversity in healing and non-healing wounds among Malaysian subjects by phenotypic identification and 16S rDNA sequencing. *Biomed. Res. India,* 24(3), 389-395.

Woo PCY, Leung ASP, Leung KW and Yuen KY (2001). Identification of slide coagulase positive, tube coagulase negative *Staphylococcus aureus* by 16S ribosomal RNA gene sequencing. *Mol. Pathol.*, **54**(4): 244-247.

Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM and MacGowan AP (2001). A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. *J. Antimicrob. Chemother.,* **47**(4): 399-403.

Zafar A, Anwar N and Ejaz H (2008). Bacteriology of infected wounds: A study conducted at children's hospital Lahore. *Biomedica.,* **24**: 71-74.