

# Molecular markers for the detection of pathogenic and food poisoning potential of methicillin resistant *Staphylococcus aureus* isolated from wounds of hospitalized patients

Rana Adnan Ali<sup>1</sup>, Mahtab Ahmad Khan<sup>1,2\*</sup>, Aftab Ahmad Anjum<sup>3</sup>, Mian Muhammad Khubaib Sattar<sup>4</sup>, Arslan Sarwar<sup>5</sup>, Tehreem Ali<sup>5</sup>, Madeeha Tariq<sup>3</sup> and Aqeela Iqbal<sup>6</sup>

<sup>1</sup>Department of Pharmacology, Faculty of Pharmacy, University of Lahore, Lahore, Pakistan

<sup>2</sup>Faculty of Pharmacy, University of Central Punjab, Lahore, Pakistan

<sup>3</sup>Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan

<sup>4</sup>Department of Microbiology, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

<sup>5</sup>Faculty of Life Sciences, University of Central Punjab, Lahore, Pakistan

<sup>6</sup>The Children's Hospital and The Institute of Child Health, Lahore, Pakistan

**Abstract:** Pathogenic strains of *Staphylococcus aureus* are mostly resistant to methicillin and they can cause severe infections. The current study was planned to assess the food poisoning potential of pathogenic, methicillin resistant *Staphylococcus aureus* by molecular detection of enterotoxin A (*Eta*) gene. A total of 100 septic wound samples from patients admitted in surgical ward (n=50) and burn unit (n=50) of Mayo Hospital Lahore were collected aseptically. These samples were processed primarily for bacterial growth on nutrient agar and purified on mannitol salt agar where twenty (20) samples showed pin-point colonies with yellow discoloration of media. Moreover, isolates were further characterized on the basis of microscopic appearance and biochemical assays where fourteen (14) isolates were declared *Staphylococcus*. DNA of these isolates were subjected to 16S rRNA gene amplification and sequences of *S. aureus* were submitted to NCBI GenBank viz., MW344063.1, MW341438.1, MW344064.1, MW344065.1, MW341439.1, MW341440.1, MW345971.1, MW345972.1, MW345973.1, MW716458.1. All the isolates (n=10) demonstrated molecular confirmation of pathogenicity and methicillin resistance by amplification of *Coa* and *mecA* gene. Out of these ten isolates, three amplified enterotoxin A (*Eta*) gene were confirmed. It is concluded that enterotoxin A of *S. aureus* which causes food poisoning is present in pathogenic, methicillin resistant *S. aureus* isolated from various wounds infections.

**Keywords:** *Staphylococcus aureus*, methicillin resistant, septic wounds, food poisoning, *Coa*, *mecA*, *Eta*.

## INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an unrestricted health threat since 1961 and has extended across the world as an important source of hospital acquired infections (Mbogori *et al.*, 2013). MRSA shares a reasonable stake of nosocomial burden in hospitals. *Staphylococcus aureus* is gram positive cocci and non-spore forming facultative anaerobes. The biochemical profile of *S. aureus* is catalase-positive, coagulase - positive, oxidase -negative and can tolerate high salt concentration and resistant to heat treatment (Oliveira *et al.*, 2018). Being natural inhabitant of skin microbiota, it usually causes infections especially in immune-compromised individuals along with septicemia, pyaemia, localized skin and other body infections. This heterogeneous microorganism has been declared as causative agent of diseases ranging from life threatening endocarditis, toxic shock syndrome to minor skin infections (Adalbert *et al.*, 2021). *Staphylococcus aureus* commonly causes skin and soft tissue infections such as sepsis, pneumonia, endocarditis, osteomyelitis, and

wound infections (Frana *et al.*, 2013). This usually occurs in people predisposed through injury or illness. Those already colonized are at high risk of becoming infected with *S. aureus* because of certain kind of infections including skin abscesses, post-operative wound infections, septicemia and pneumonia. This ubiquitous bacterium is a significant pathogen due combination patterns of toxic-mediated virulence, antibiotic resistance and invasiveness (Kadariya *et al.*, 2014).

*S. aureus* produces toxins that may cause such diverse manifestations as septic shock, gastroenteritis, toxic shock syndrome and scalded skin syndrome. A wide range of *S. aureus* virulence factors includes enzymes i.e. coagulase, protease. Lipases, nucleases and catalases that help it to evasion from immune system and many exotoxins such as four hemolysis (alpha, beta, gamma and delta), enterotoxins and toxic shock syndrome toxin (TSST) (Adalbert *et al.*, 2021). Several strains of *S. aureus* produce enterotoxin A, B, C, D and E which can lead to food poisoning and also resistant to methicillin and  $\beta$ -lactams antibiotics because of *mecA* gene that encodes penicillin binding protein PBP2a (Batista *et al.*, 2013). Coagulase gene (*coa*) protein is a clotting factor secreted

\*Corresponding author: e-mail: raomahtab@yahoo.com

by *S. aureus* that acts as a virulence factor as well phenotypic determinant (Effendi *et al.*, 2019). Multiplex PCR assay can be used for the detection of *mecA*, *coa* and *Staphylococcus* enterotoxin genes (Rodriguez-Lopez *et al.*, 2020)

*Staphylococcus* food borne disease (SFD) is one of the most prevalent food borne disease from food contamination with preformed enterotoxins from *S. aureus* (Kadariya *et al.*, 2014). In several food products, including retail raw meat, presence of *S. aureus* suggest that customers are at potential risk of developing colonization of *S. aureus* and eventual infection (Wu *et al.*, 2018). The presence of pathogens in food products poses potential hazards to consumers and causes significant economic loss. Nausea, vomiting with or without diarrhea and stomach cramps are signs of SFD. Preventive measures include proper handling and processing of food, cold chain maintenance, adequate equipment for cleaning and disinfection, cross contamination prevention in home and kitchen and contamination prevention from farm to fork (Ucar *et al.*, 2016).

## MATERIALS AND METHODS

### Collection of Samples

Patients having postoperative surgical wound with pus discharge, diabetic foot wound and burn wound were included for the study. Only those patients were selected which had the history of taking broad spectrum antibiotics and hospitalized for more than 5 five days with special concern of the nosocomial infection caused by MRSA. Those patients who were having wounds with no drainage or were newly admitted and operated and did not want to participate in the study were excluded from the study. Samples from septic wounds of patients (n=100) admitted in Surgical ward and Burn unit (n=50, each) of Mayo Hospital, Lahore were screened for presence of pathogenic enterotoxin producing methicillin resistant *Staphylococcus aureus* (MRSA) after taking written consent from patients. The samples were collected from the post-operative surgical wounds with pus discharge along with diabetic foot wounds and burn wounds which had the history of taking broad spectrum antibiotics and hospitalized for more than 5 five days with special concern of the nosocomial infection caused by MRSA (Biswas *et al.*, 2010). Sterile swabs were used for sample collection by rubbing on the wound after removing of the pus, if any, and transported to Quality Operational Laboratory, Institute of Microbiology, University of Veterinary and Animal Sciences, Lahore in transport medium at 4°C.

### Isolation, Identification and Molecular Confirmation

Initially all samples were cultured on nutrient agar to confirm the presence of bacterial growth. The colonies

from nutrient agar were purified on mannitol salt agar (Oxoid, UK) by four way streaking method along with discoloration of media (fig. 1) Microscopic characteristics were confirmed by gram's staining. The isolates were characterized as *Staphylococcus aureus* on the basis of biochemical assays including hemolysis on blood agar, catalase, coagulase, oxidase, methyl red, vogesproskauer and citrate utilization by following the Bergey's manual of systemic bacteriology (Vos *et al.*, 2011; Sharma *et al.*, 2011). Deoxyribose Nucleic Acid (DNA) of the biochemically confirmed isolates was extracted by following the manufacturer's recommendation of DNA extraction kit (GeneAll® Exgene™). The extracted DNA was visually confirmed by agarose gel electrophoresis containing 0.8% concentration of agarose in the gel. The isolates were further confirmed by polymerase chain reaction (PCR) amplification of 16S rRNA gene by using primer sequences (Forward: 8F1P: 5'-AGTTTGATCCTGGCTCAG-3'; Reverse: XB4: 5'-GTGTGTACAAGGCCCGGGAAC-3') as described by Asghar and colleagues (Asghar *et al.*, 2016). Reaction mixture was prepared by mixing 12.5µL of 2X PCR master mix, 1 µL of each forward and reverse primers along with 2 µL of template DNA and 8.5 µL of DNase, RNase free water to make the final volume of 25 µL. For amplification, the mixture was run in thermocycler at 94°C for 10 minutes, 35 cycles were at 94°C/1 minute, 55°C/1 minute, 72°C/2 minutes and final extension at 72°C for 10 minutes. The amplified product was visually confirmed by agarose gel electrophoresis containing 1.5% agarose in the gel. Moreover, the amplicons were subjected to nucleotide sequences by sanger di-deoxy method and FASTA files were received. These sequences were submitted to NCBI GenBank with the following accession number; MW344063.1, MW341438.1, MW344064.1, MW344065.1, MW341439.1, MW341440.1, MW345971.1, MW345972.1, MW345973.1, MW716458.1. Phylogenetic analysis of these isolates was carried out using MEGA X. The present molecular based study was carried out by using Thermocycler (Kyratex, Australia, Model: SC300G-R2).

### Molecular confirmation of pathogenicity by coagulase (coa) gene

The isolates confirmed as *Staphylococcus aureus* by 16S rRNA gene amplification were subjected to polymerase chain reaction (PCR) amplification of (*coa*) gene for confirmation of pathogenic strain of *Staphylococcus aureus* by using (Forward: Coag2: 5'-CGAGACCAAGATTCAACAAG-3'; Reverse: Coag3: 5'-AAAGAAAAACCACTCACATCA-3') primer sequences (Aqib *et al.*, 2017). PCR reaction mixture was prepared for a final volume of 25 µL and amplified in thermocycler initially at 94°C for 10 minutes followed by 35 cycles of 94°C/45 Seconds, 54°C/1 minute and 72°C/2 minutes along with final extension at 72°C for 10 minutes. The amplicons were visually confirmed by

ethidium bromide in agarose gel electrophoresis containing 1.5% agarose concentration.

#### **Molecular confirmation of methicillin resistance by *mecA* Gene**

Molecular confirmed pathogenic *Staphylococcus aureus* were subjected to amplification of *mecA* gene for detection of methicillin resistance genes by using (Forward: GMECAR-1: 5'-ACTGCTATCCACCCTCAAAC-3'; Reverse: GMECAR-2: 5'-CTGGTGAAGTTGTAATCTGG-3') primer sequences (Vatansever *et al.*, 2016). PCR reaction mixture was prepared for a final volume of 25  $\mu$ L by adding 2  $\mu$ L of template DNA in 12.5  $\mu$ L 2X master mixture along with 1  $\mu$ L of each forward and reverse primer and approximately 8.5  $\mu$ L of DNase and RNase free water. The mixture was amplified in thermocycler at same conditions as used above for *Coa* gene and visualized by ethidium bromide in 1.5% agarose gel electrophoresis.

#### **Molecular confirmation of enterotoxin A (*Eta*) gene**

Detection of food poisoning causing isolates among pathogenic isolates was carried out using amplification of Enterotoxin A (*Eta*) gene using (Forward: GETAR-1: 5'-GCAGGTGTTGATTTAGCATT-3'; Reverse: GETAR-2: 5'-AGATGTCCCTATTTTTGCTG-3') primer sequences. Source of all primers was ABI Lab, Lahore (Rasheed and Hussein, 2020). Total volume of 25  $\mu$ L PCR reaction mixture was used for each isolate of MRSA. Reaction mixture was made by mixing 12.5  $\mu$ L 2X master mixture, 8.5  $\mu$ L LPOR water, 2  $\mu$ L DNA sample, 1  $\mu$ L each forward and reverse primer. PCR reaction conditions were used as initial denaturation at 94°C for 05 minutes followed by 35 cycles of 94°C for 2 minutes, annealing at 54°C for 2 minutes and 72°C/1 minutes along with final extension at 72°C for 07 minutes. The amplicons were visually confirmed by ethidium bromide in agarose gel electrophoresis containing 1.5% agarose concentration.

#### **Ethical approval**

All the experimental work has been approved by the Institutional Review Board (IRB) of King Edward Medical University, Lahore under the reference number 901/RC/KEMU dated 23/11/2020. Written consent was taken from patients before taking the sample.

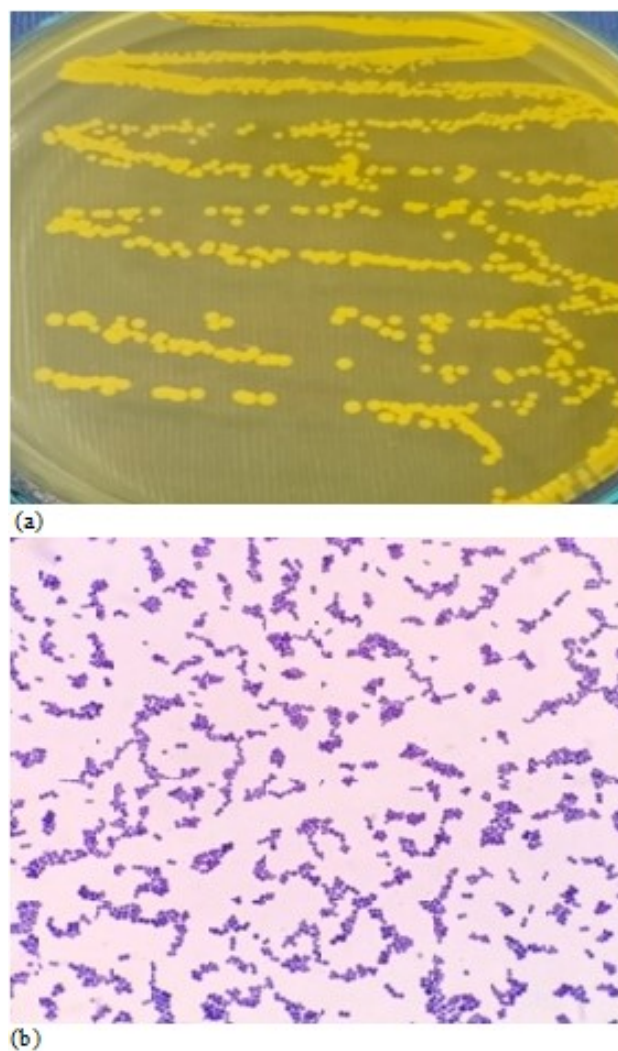
#### **STATISTICAL ANALYSIS**

The data of biochemical and molecular confirmation of *Staphylococcus aureus* were added and statistically analyzed by two-tailed Z-test in the Statistical package for social sciences (SPSS) version 21.0 software for comparison of treatment groups and results were computed as frequency (n) and percentages (%). A two-tailed  $p < 0.05$  was considered as statistically significant.

## **RESULTS**

#### **Isolation and identification**

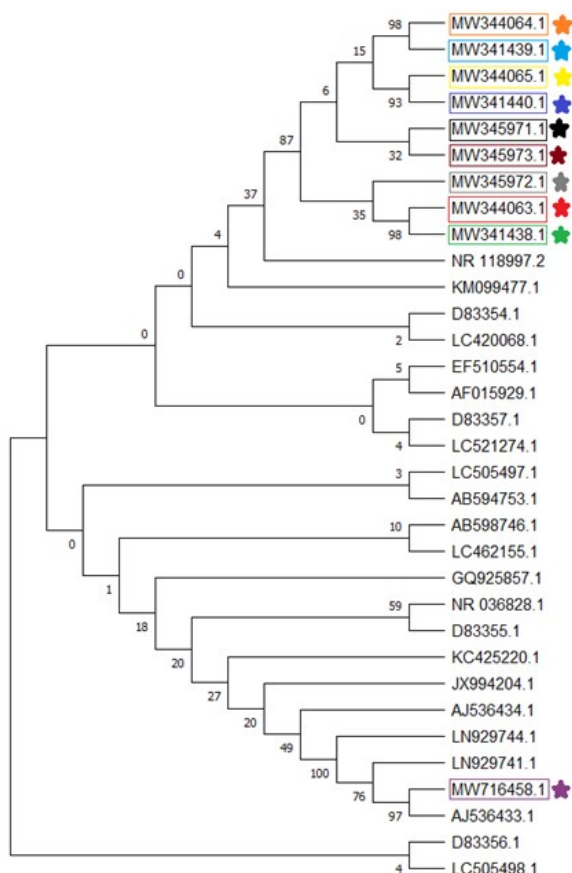
A total of 100 Samples of septic wounds were collected from patients admitted in surgical ward and Burn Units, 50 patients from each group from Mayo Hospital, Lahore. Primarily, all samples were cultured on nutrient agar to check the presence of bacterial growth. The colonies of primary cultures were purified on mannitol salt agar and revealed that out of 100 samples 20 isolates were showing pin point colonies with yellow discoloration of the media. Microscopic appearance of all the isolates was gram positive cocci with cluster arrangement (fig. 1).



**Fig. 1:** Culture characters and microscopic morphology of *Staphylococcus aureus* (a) typical culture growth on mannitol salt agar and (b) microscopic appearance upon Gram staining.

Biochemically confirmed *Staphylococcus* isolates (n=14) were positive for beta hemolysis, catalase, coagulase, methyl red, vogesproskauer while negative for oxidase and citrate utilization test. In present study, overall 14%

(14/100) septic wound samples were confirmed as *Staphylococcus* taken from hospital admitted patients in surgical and burn wards. It has also been observed that 18% (9/50) *Staphylococcus* confirmed samples were from surgical ward and 10% (5/50) from burn ward on the basis of biochemical profile. Furthermore, on the basis of molecular characterization it has been observed that overall 10% (10/100) septic wound samples were confirmed as *Staphylococcus aureus* taken from hospital admitted patients in surgical and burn wards. It has also been observed that 12% (6/50) *Staphylococcus aureus* confirmed samples were from surgical ward and 8% (4/50) from burn ward respectively. These results when subjected to two-tailed Z-test revealed non-significant difference between two groups ( $p > 0.05$ ) (table 1).



**Fig. 2:** Phylogenetic analysis based on 16S rRNA gene sequence of *Staphylococcus aureus*

The genome of biochemically characterized *Staphylococcus aureus* was targeted for 16S rRNA gene amplification and 1500 bps size bands were observed (fig. 3b). Out of 14 biochemically characterized isolates 10 were confirmed as *Staphylococcus aureus* on the basis of 16S rRNA gene sequencing (The procedures of Biochemical Confirmation are same for *Staphylococcus aureus* and *Staphylococcus intermedius* are same and molecular confirmation (16S rRNA gene) is the

confirmation test for *Staphylococcus aureus*). Phylogenetic tree was constructed by using MEGA-X (fig. 2). Phylogenetic analysis was performed through the Maximum Likelihood method, bootstrap consensus tree inferred from 1000 replicates by using 33 16S rRNA sequences from NCBI data base and 10 Pakistani *S. aureus* (colored star) sequences submitted to GenBank. *S. aureus* isolate MW716458.1 was 97% homologous and evolutionary related to AJ536433.1 and 76% evolutionary related to Indian *S. aureus* LN929741.1. *S. aureus* MW344064.1 and MW341439.1 were 98% related to each other and both were 15% evolutionary related to MW344065.1 and MW341440.1. MW345971.1 was 32% homologous to MW345973.1 whereas MW345972.1 was 35% homologous to MW 344063.1 and MW341438.1 while both are 98% evolutionary related to each other.

### Molecular Confirmation of Coagulase (Coa) and Methicillin (mecA) Genes

Coagulase gene was amplified by using specific primers and product was visualized on agarose gel (1.8%) by gel documentation system. All the processed isolates (n=10) demonstrated the band size of 970bps. These isolates were further assessed for the presence of methicillin resistance by using specific primers for amplification of *mecA* gene which was visualized as described above. It was recorded that all isolates (n=10) validated a band size of 163bps (fig. 3c&d).

### Molecular confirmation of enterotoxin A (Eta) gene

The enterotoxin A (*Eta*) gene was amplified by using specific primers and three out ten *Staphylococcus aureus* samples revealed a band size of 93bps which were visualized by ethidium bromide in agarose gel of 1.8% concentration (fig. 3e).

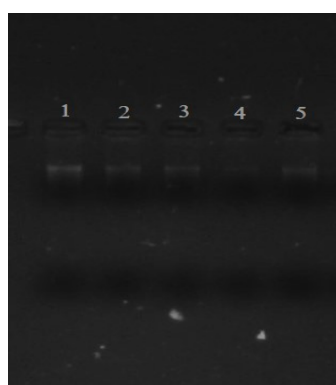
## DISCUSSION

*Staphylococcus aureus* causes multiple types of infection of surgical wounds including incisions, laceration, avulsion, puncture and amputation along with burns of various categories with the isolates resistant to methicillin. The study was undertaken to find the molecular confirmation of pathogenicity along with methicillin resistance and staphylococcal enterotoxin A production potential of the *S. aureus* isolated from different wounds.

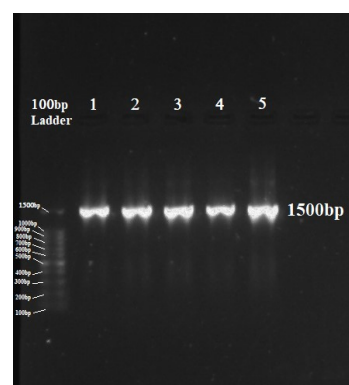
In the present study, 14% and 10% septic wound samples were confirmed for *Staphylococcus* and *Staphylococcus aureus* isolates upon biochemical and molecular profile respectively. Researchers from Nepal have reported that 24.6% samples were cultured positive for *S. aureus* as a predominant bacteria causing wound infection (Shrestha et al., 2018). In this study, biochemically confirmed isolates were subjected to 16S rRNA gene amplification followed by sequencing for molecular confirmation and

**Table 1:** Isolation of *Staphylococcus aureus* from different wound categories

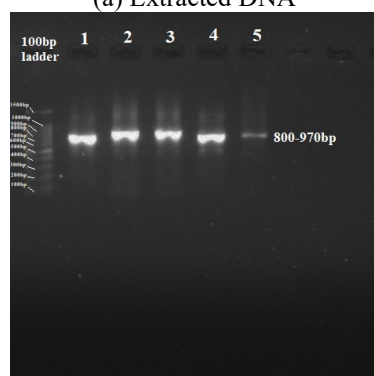
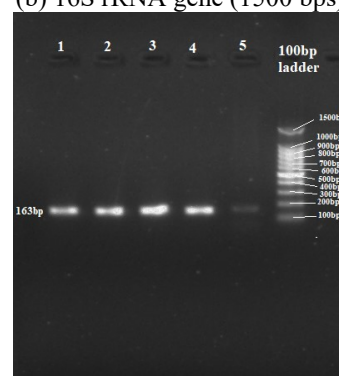
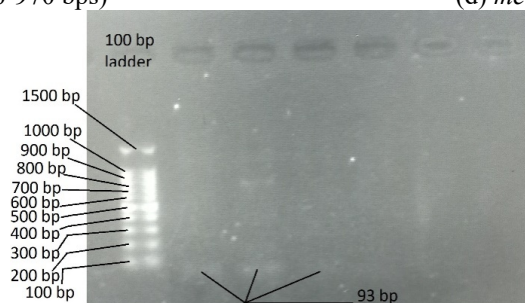
Sr. No.	Ward Name	Wound Category	No. of Samples	Biochemical Confirmation	Molecular Confirmation
					16S rRNA gene
01	Surgical Ward	Incision	10	03	02
02		Laceration	12	02	02
03		Avulsion	05	00	00
04		Puncture	18	03	02
05		Amputation	05	01	00
06	Burn Unit	First Degree	10	02	01
07		Second Degree	22	02	02
08		Third Degree	11	01	01
09		Fourth Degree	07	00	00
Total			100	14	10



(a) Extracted DNA



(b) 16S rRNA gene (1500 bps)

(c) *Coa* gene (800-970 bps)(d) *mecA* gene (163bps)(e) *Eta* gene (93 bps)

**Fig. 3:** Visualization of DNA bands of *Staphylococcus aureus* on agarose gel (a) Extracted DNA visualized on 0.8% agarose gel (b) Ribosomal 16S RNA amplification resulted in 1500 bps amplicon (c) Coagulase gene amplification resulted in 800-970 bps amplicon (d) Methicillin resistance gene amplification resulted in 163bps amplicon (e) Enterotoxin A gene amplification resulted in 93 bps amplicon; visualized on 1.5% agarose gel.



revealed that 10 isolates were detected as *S. aureus*. Similarly in Baghdad, bacterial isolates from wounds and burns were confirmed as *S. aureus* on the basis of 16S rRNA gene amplification (Al-Alak, 2016). Karmakar and colleagues have confirmed *S. aureus* from the clinical isolates in the hospitalized patients on the basis of 16S rRNA gene amplification (Karmakar *et al.*, 2016). Manaka and coworkers have confirmed the agents responsible for nosocomial infection including *S. aureus* in Gunma, Japan by using universal primers for 16S rRNA gene amplification followed by nucleotide sequencing (Manaka *et al.*, 2017).

In the current study, the isolates confirmed as *S. aureus* after 16S rRNA gene sequencing were further subjected to coagulase (*Coa*) gene amplification for molecular confirmation of pathogenicity and revealed that all the isolates were positive. In a study conducted in India, the coagulase gene was amplified for molecular typing of *S. aureus* isolated from a wide range of samples which also included pus from skin and wounds (Javid *et al.*, 2018). Hamza and co-workers have recorded that 43% strains of *S. aureus* were confirmed as coagulase positive by amplification of *Coa* gene in a public health study conducted in Egypt (Hamza *et al.*, 2015). Similarly, in a teaching hospital at Sudan, coagulase gene was confirmed and its polymorphism was studied from the *S. aureus* isolates obtained from wounds and nasal cavities of the patients (Ibrahim *et al.*, 2019).

The isolates of *S. aureus* were confirmed for the presence of methicillin resistance on molecular basis by amplification of *mecA* gene. The *S. aureus* isolates which contains *mecA* gene are resistant to lactam antibacterial and coded as multi drug resistant. In Giza, Egypt, *S. aureus* isolated from milk samples were subjected to *mecA* gene amplification and 20% of the coagulase positive isolates were containing methicillin resistance gene of band size 147 bps whereas, in our study all the coagulase positive isolates were positive for *mecA* gene with a band size of 163 bps (Hamza *et al.*, 2015). The variation in band size is due to the use of different primer sequences for amplification of *mecA* gene. In a previous study, researchers have revealed that 56.4% isolates were containing *mecA* gene and declared as Methicillin resistant *S. aureus* (MRSA) in a teaching hospital at Sudan (Ibrahim *et al.*, 2019). All the *S. aureus* isolates of sequence type ST88 contains *mecA* gene which were obtained from chronic infected wounds in rural Ghana (Wolters *et al.*, 2020).

All *S. aureus* strains were subjected to amplification of *Eta* gene for assessment of Staphylococcal Enterotoxin A production and food poisoning potential of the isolates and revealed that three isolates (30%) were positive for *Eta* gene. In a study for assessment of major causes of nosocomial infection, 30.2% isolates revealed

staphylococcal enterotoxin A (*sea*) gene amplification (Aggarwal *et al.*, 2019). Similarly, 32.9% isolates of *S. aureus* positive for *Eta* gene which were obtained from clinical specimen in Northern Iran (Mohseni *et al.*, 2018).

## CONCLUSION

It is concluded that *Staphylococcus aureus*, obtained from various categories of wounds and burns showed molecular confirmation of coagulase and methicillin resistant genes. Furthermore, the presence of staphylococcus enterotoxin A (*Eta*) gene among these isolates there indicates its potential to cause food poisoning.

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