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Identification and Characterization of *Staphylococcus aureus* 16s rRNA Gene Isolate from Environmental and Clinical Sources

Manar Ahmed Ehmud[1], Athar Saleh Mteran[1], Mustafa H. Al-Furaiji[1], Ghaidaa Sabry Hassoon[1] **10.18805/ajdfr.DRF-506**

ABSTRACT

Background: One of the most well-known and widespread bacterial pathogens, *Staphylococcus aureus*, causes hundreds of thousands to millions of more harmful, invasive infections each year in addition to an unusual number of uncomplicated skin infections.

Methods: A total number of 168 clinical and environmental samples of *Staphylococcus aureus* were characterized by morphological, microscopical and biochemical testing and VITEK® 2 Compact. All identified clinical isolates were 100% resistant to Penicillin and also highly resistant to Erythromycin 97.5% and higher sensitivity to Chloramphenicol 95%. Environmental isolates were 100% resistant to penicillin and 65% to erythromycin; the ciprofloxacin had a higher sensitivity percentage of 100%. Environmental isolates showed 80% multidrug resistance, while clinical isolates showed 100%.

Result: All the identified isolates were subjected to detection of nuc and 16S rRNA genes using PCR and all isolates were found to harbor nuc and 16S rRNA genes. By using the nucleotide sequence of the 16S rRNA gene region, two new local isolates were obtained and registered in GenBank. Blast Tool in the Geneious software was used to draw Distance Tree. A novel local strain of *S. aureus* isolated from clinical sources (MIM2022) was genetically closely associated with a novel local strain of *S. aureus* isolated from environmental sources (MIM2023); based on genetic distance, clinical isolate (MIM2022) was genetically homologous with environmental isolate (MIM2023), indicating that environmental and clinical isolates likely belong to the same strain. Studies show that isolates of *S. aureus* can move between different niches.

Key words: 16S rRNA and Nuc gene, Multidrug resistance, PCR, *Staphylococcus aureus*.

INTRODUCTION

One of the most well-known and widespread bacterial pathogens, *Staphylococcus aureus,* causes hundreds of thousands to millions of more harmful, invasive infections each year, in addition to an unusual number of uncomplicated skin infections (Mohamed *et al*., 2019). Rasigade *et al*., 2014). The global spread of infectious bacteria that are resistant to many first-line medications is highly concerning and might have negative effects on people's health, the economy and society (Al-Hamdani *et al*., 2024; Ventola, 2015). As a result, the World Health Organization (WHO) has designated *Staphylococcus aureus* as a high-priority antibiotic-resistant pathogen for the purpose of prioritizing the research and development of new antibiotics. *S. aureus* has an annual downfall of 20-50 cases/100,000 individuals and a mortality rate of 10-30% (Kumari *et al*., 2024); (Nirwan *et al*., 2022). This is more fatal than AIDS, TB, and viral hepatitis combined (Klevens, 2007; van Hal *et al*., 2012). The anterior nares and vestibules contain the most enormous reservoir (Sakr *et al*., 2018). Nasal colonization was previously assumed to be the cause of bacteremia in 30% of people (Tong *et al*., 2015). In numerous *in vitro* and *in vivo* infection models, it has been demonstrated how important these characteristics of virulence are during infection (Cheung *et al*., 2021; Lindsay, 2019). The synthesis of multiple distinct virulence factors that accomplish the same objective can be a waste of scarce resources and detrimental to survival since pathogenic *S. aureus* is typically prevalent in environments with restricted natural resources (Tam and Torres, 2019). However, should one of the virulence variables become inefficient, this redundancy can guarantee the bacteria's survival. Alternately, the bacteria may have gained certain virulence characteristics that appear to be redundant during the history of their evolution and can more effectively adjust to different diseases or colonization areas. The wastewater that is discharged to wastewater treatment plants (WWTPs), particularly those from hospitals and areas where intensive livestock farming is conducted, can include these bacteria (Al-Jadir *et al*., 2022; Al-Musawy *et al*., 2021). Due to their exposure at work, there is a significant risk that WWTP employees will contract *S. aureus* and transmit antibiotic

[1]Water, Environment and Renewable Energy Center, Scientific Research Commission, Ministry of Higher Education and Scientific Research, Baghdad, Iraq.

Corresponding Author: Mustafa H. Al-Furaiji, Water, Environment and Renewable Energy Center, Scientific Research Commission, Ministry of High Education and Scientific Research, Baghdad, Iraq. Email: alfuraiji79@gmail.com
ORCID: https://orcid.org/0000-0002-7373-9370

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resistance in the environment, which poses a serious threat to public health (Ferguson *et al*., 2016; Kozajda and Jeżak, 2020). The strongly conserved 16s rRNA gene is the most widely used gene marker for genus and species identification, in addition to its taxonomic significance in bacteria and archaea (Callahan *et al*., 2017; Kim and Chun, 2014). The information found in the variable regions of 16S rRNA differs between species, which makes the detection particular (Dias *et al*., 2020).

In order to distinguish between *Staphylococcus aureus* isolates, this study will use 16S rRNA gene sequencing.

MATERIALS AND METHODS

One hundred sixty-eight samples were obtained from various sewage treatment facilities in Baghdad utilizing sterile techniques, including submersion of gloved hands in 250 mL screw-top, sterilized wide-mouth vials. All samples were processed within 24 hours of collection or immediately following arrival at the laboratory.

Clinical samples from Centric Kid's Technical Hospital The collection of specimens was accomplished by rotating a sterile swab in (Nasal, hands) and then placed in the transport media and collected in an ice box until transported to the laboratory between November (2021) and January (2022) (Thapaliya *et al*., 2017) and then Identification of *S. aureus* by VITEK 2 Compact system GP. All experiments were conducted at the laboratories of the Ministry of Science and Technology in Baghdad-Iraq.

The susceptibility to antibiotics was examined in all verified *S. aureus*. The Kirby-Bauer method was applied in accordance with the Clinical and Laboratory Standards Institute's recommendations, involving standard disk diffusion on Mueller-Hinton agar incubated at 37°C for 24 hours (James *et al*., 2024), Cefoxitin, Erythromycin, Amikacin, Azithromycin, Chloramphenicol, Ciprofloxacin, Clindamycin, Penicillin, Gentamicin, Linezolid, Nitrofurantoin, Rifampin, Tetracycline, Tigecycline, Tobramycin and Trimethoprim/Sulphathiazole were among the 16 antimicrobial disks used to test the susceptibility of *S. aureus* isolates the National Committee Clinical for Laboratory Standards standard recommendations for recording experiment findings were followed.

Positive samples of Staphylococcus species based on morphological and biochemical assays were subsequently handled for molecular analysis using a PCR-based test; genomic DNA was obtained using the Wizard Genomic DNA construction (Promega, Madison, WI). Following the extraction of genomic DNA from bacterial colonies, the PCR method-which is far more sensitive than the most widely used microbial culture and stainingtechniques-is used for recognizing the nuc gene and 16S rRNA gene techniques-is used for recognizing the nuc gene and 16S rRNA gene.

Then, PCR was performed utilizing universal 16SrRNA oligonucleotide primers: R- 5-AGA CCC GGG AAC GTA TTC AC -3′ and F-5- GTG CCA GCA GCC GCG GTA A -3′ and also primer R- 5 -CGT AAA TGC ACT TGC TTC AAG -3′ and F- 5′ -TCA GCA AAT GCA TCA CAA ACA G -3 for nuc gene (Haque *et al*., 2017). In the finished volume of 25 μl, the reaction combination comprises 1 μl of bacterial DNA, 9.5 μl of nuclease-free and 1 μl of each primer. The PCR master mix had this combination added to it in line with the production handbook (GoTaq, Promega, USA). Use a thermal cycler (Lab Net, USA) to do the following procedure: A thermocycler was used to complete 30 cycles, with each cycle consisting of three steps: denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min.

Conventional electrophoresis was used to assess amplified products and a Gel documentation image analysis system was used to identify bands (Bio-Rad, USA). The universal primer-amped products had sizes of 1000 bp, which suggests that the bands were made of the 16S rRNA gene.

Utilizing an automated DNA sequencer ABI3730XL, the PCR product was transferred to Macrogen Corporation-Korea for Sanger sequencing of the 16S rRNA gene. After receiving the findings *via* email, we analyzed them using specialist tools.

RESULTS AND DISCUSSION

First, traditional techniques were used to identify clinical isolates. All isolated strains of Gram-positive cocci were sub-cultivated on nutrient agar (HiMedia Laboratories Pvt. Ltd) and biochemical tests were then investigated overnight at 37°C before being promptly cultured on both Manitol salt agar and blood agar plates by direct streaking procedures (Gajdács and Urbán, 2019).

One hundred clinical samples, consisting of 45 nasal swabs and 55 hand swabs, were taken from healthcare workers and patients (male and female) of various ages and conditions, including chronic otitis media and wounds.

A 250 mL autoclaved screw-cap jar containing 50 mL of Baird Parker broth was supplemented with water samples and incubated at 37°C for 18 to 24 hours. Following a 24-hour incubation period, 50 ul of broth was placed onto Baird Parker agar (BPA) with egg yolk tellurite enrichment (BD Difco TM, Becton, Dickinson and Company, Sparks, MD). Following 24-48 hours of culture at 37°C, the inoculum was examined for the occurrence of bacterial development (Thapaliya *et al*., 2017). *S. aureus* indicated (black colonies with clear shadows on Baird Parker Aagar). *S. aureus* was confirmed by the catalase examination, the sliding coagulase test and the preservation of *S. aureus* isolates at 80°C.

S. aureus was confirmed using the mannitol fermentation method, colony morphology, Gram stain, Catalase, coagulase and DNase tests. The isolates that were determined to be *S. aureus* were then used in the investigation. Following that, species were determined to use the VITEK 2 (BioMérieux, France) Automation Technology with probable *S. aureus* isolates. 60 *S. aureus*

from clinical and water samples were found using conventional methods and automated system results, (Fig 1).

For clinical isolates 100% (40/40) of isolates were resistant to penicillin, 97% (39/40) to erythromycin, 65% (26/40) to tetracyclin, 45% (16/40) to Cefoxitin and Methprim, 55% (22/40) to Azithromycin, 62.5% (25/40) to tobramycin, 40% (16/40) to Clindamycin, 32.5% (13/40) to Rifampin, 25% (10/40) to Linezolidin, 22.5% (9/40) to Gentamicin, 15% (6/40) to Amikacin, 7.5% (3/40) to Ciprofloxacin and Nitrofuratoin, 5% (2/40) to Chloramphenicol and 2.5% (1/40) to Tigecycline (Fig 3). For environmental isolates, 100% (20/20) of isolates were resistant to penicillin, 65 % (13/20) to erythromycin, 45% (9/20) to Rifampin, Azithromycin and Clidamycin, 40% (8/20) to Cefoxitin, 30% (6/20) to Linezolidin, 20% (4/20) to Ciprofloxacin and Nitrofuratoin, 15% (3/20) to tetracycline and Tobramycin, 10% (2/20) of isolates were resistant to Tigecycline, Chloramphenicol and Gentamicin; all isolates were sensitivity to Ciprofloxacin (Fig 2).

In this study, drug resistance to three or more antimicrobials was defined as multiple drug resistance. Relatively fewer environmental isolates than clinical isolates were present. it was revealed that isolates had 80% resistance to at least three antibiotics and 100% at least resistance to one antibiotic, as illustrated in (Table 1).

The nuc gene was determined by agarose gel electrophoresis and imaged under an ultraviolet (UV) trans-illuminator, as shown in (Fig 4). The amplification indicated a product of 300 bp. We used PCR primers targeted to this gene, as explained by McClure *et al.* (2017). Each isolate was also confirmed at molecular levels.

For this aim, the 16S rRNA gene, one of the S. *aureus*-specific genes, was detected using a single-target PCR assay. Results from gel electrophoresis revealed that every single one of the 60 *S. aureus* isolates contained the 16S rRNA gene (Fig 5). The 16S rRNA gene was utilized to compare with the type strains identified by 16SrRNA gene sequencing at GenBank using Geneious software for the purpose of identification. The identified isolates' nitrogen bases matched those of the closest isolates novel local isolates were obtained from clinical and environmental samples and two isolates were registered in GenBank. Distance Tree Using the Blast Tool in Geneious Software (Fig 6).

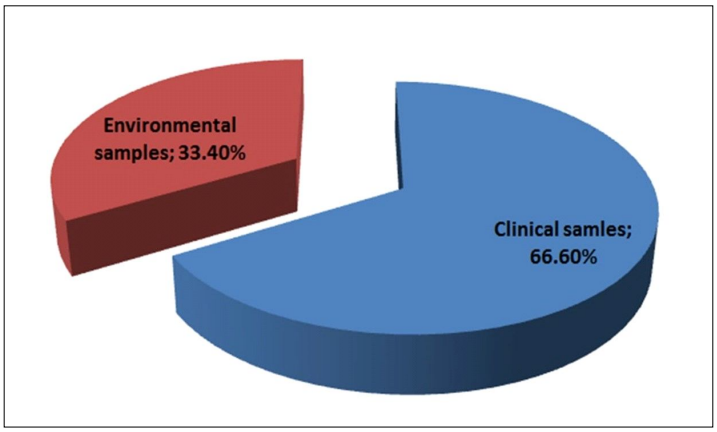


Fig 1: Clinical and environmental isolates percentage from sample.

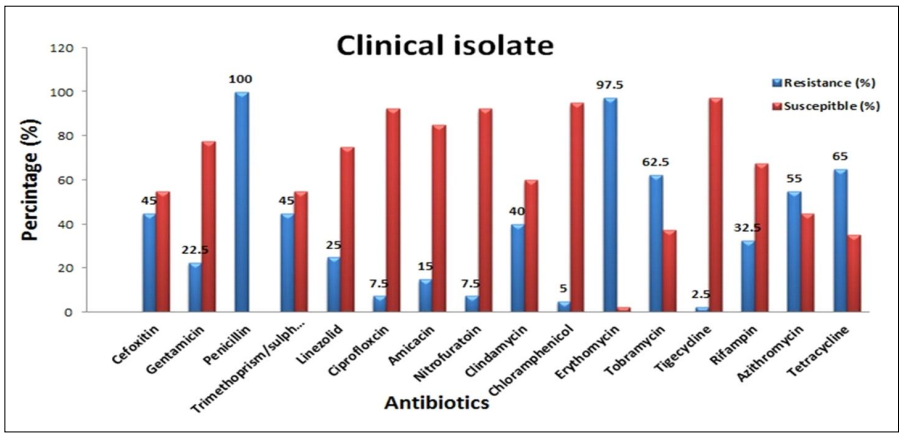


Fig 2: Antibiotics susceptibility test against clinical isolates.

From clinical sources, a new local strain of *S. aureus* was identified. (MIM2022) were genetically homologous with a new local strain of *S. aureus*, which was isolated from environmental sources (MIM2023) with a percentage of homology 100%, where the results show that the sample isolates belonged to a monophyletic group with other *S. aureus* strains, with the closest genetic distance between the two isolates being 0.00. Clinical isolate (MIM2022) has the greatest relative distance to environmental isolate (MIM2023) based on genetic distance, suggesting that clinical isolates of *S. aureus* are likely to be the same strain as environmental isolates.

The aim of the study is to analyze the evolutionary relationships between *Staphylococcus aureus* strains isolated from ecological niches and those from clinical sources. This study attempts to show a possible connection between environmental reservoirs and clinical strains by comparing the genetic features of these isolates, especially the unique local strains MIM2023 (environmental source) and MIM2022 (clinical source). This link may shed light on the spread routes, evolutionary dynamics and the involvement of environmental strains in clinical illnesses.

Clinical and environmental isolates showed high resistance to Penicillin, clindamycin, erythromycin and

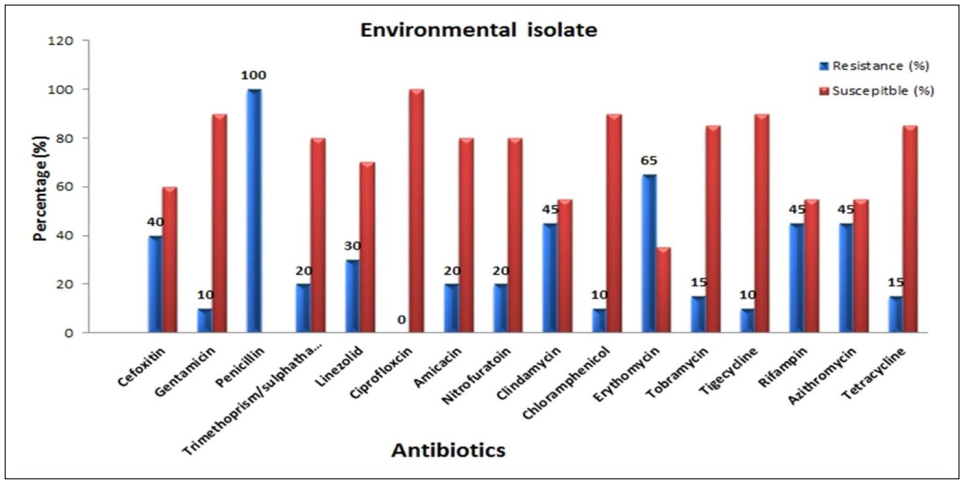


Fig 3: Antibiotics susceptibility test against environmental isolates.

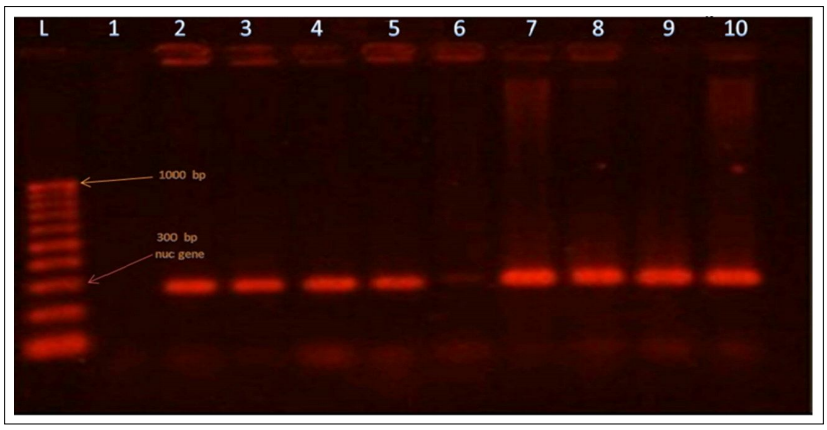
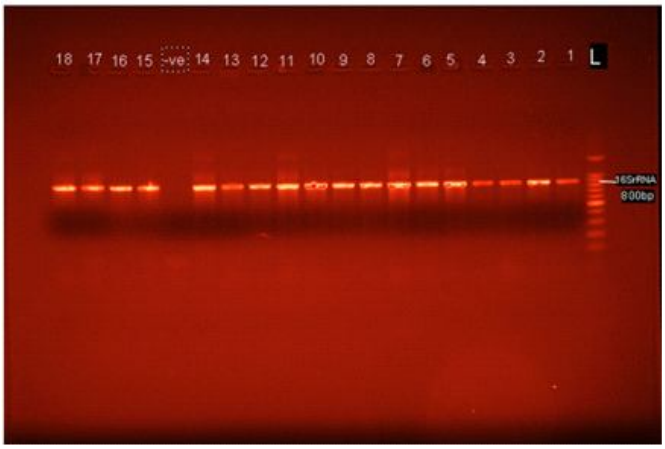


Fig 4: The virulence factor-encoding gene (nuc) of *S. aureus* can be detected using agarose gel photos in samples that have an amplicon size of 300 bp or less and are (nuc) gene positive.

Table 1: Multidrug resistance patterns of *S. aureus* isolated from clinical and environmental samples.

Resistance	Clinical isolates (n=40)		Environmental isolates (n=20)		Total (n=60)	
	No.	(%)	No.	(%)	No.	(%)
Fully sensitive	0	0	0	0	0	0
At least one antibiotic	40	100	20	100	60	100
At least three antibiotic (MDR)	40	100	16	80	56	93.3

azithromycin, and approximately all isolates were multi-drug resistant; these results agreed with the study of Akya *et al*. (2020). Additionally, numerous studies have demonstrated that antibiotic-resistance genes are frequently found in wastewater treatment facilities (Bergeron *et al*., 2016); (Everage *et al*., 2014); (Naquin *et al*., 2015). One of the main elements contributing to the spread of antimicrobial resistance in the environment is horizontal gene transfer (Kristiansson *et al*., 2011). Horizontal gene transfer is regarded as a non-random approach to genome innovation because it heavily depends on internal and external variables (D'Costa *et al*., 2011). According to Kateete *et al*. (2010) and Gumaa *et al*. (2021) the identification of *S. aureus* using PCR amplification of the nuc gene and 16S rRNA is considered a gold standard method. The novel local strains (MIM2022) and (MIM2023) support a previously published study from Sudan that discovered a genetic similarity between various *S. aureus* strains around the world and isolated strains (Ali *et al*., 2019). 16S rRNA analysis is regarded as an effective discrimination method for differentiating unrelated isolates (Al-Obaidi *et al*., 2018). A host is anticipated to switch from



L: Ladder (100 bp); (-ve) lane: Negative control; 1 to 18 lanes: 16S rRNA gene positive clinical and environmental isolate.

Fig 5: Amplification of 16S rRNA gene with previously published primers.

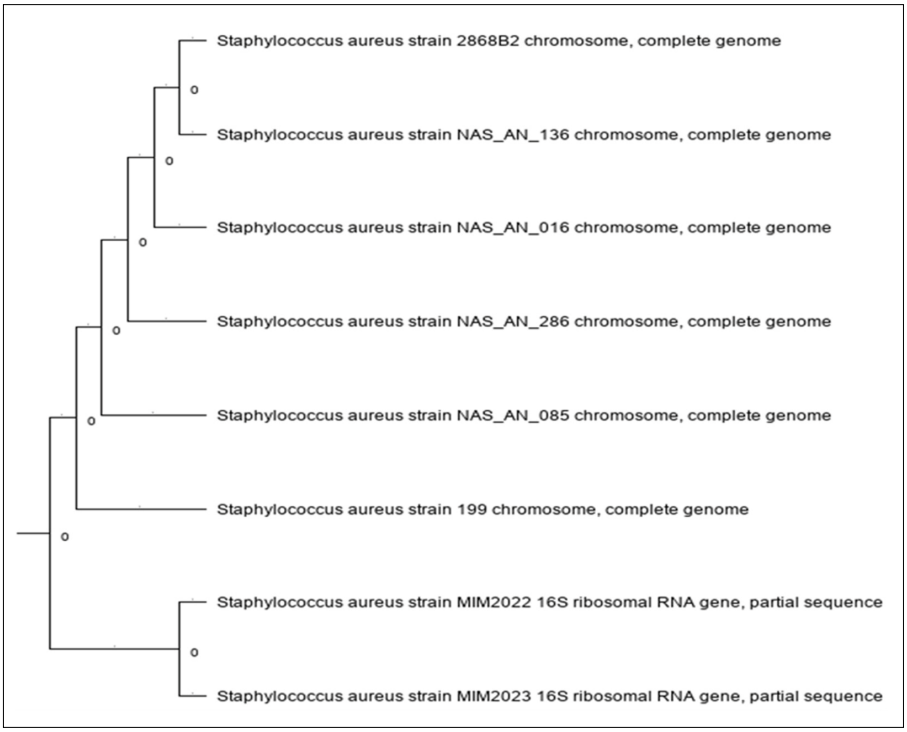


Fig 6: Distance tree using blast tool in the geneious software.

interacting with humans to environmental factors under the strain of adjusting after a protracted engagement with them. The environment and people being in close proximity can encourage host-switching incidents (Haag *et al*., 2019). The risk to public health posed by some pathogens' capacity to spread from one niche to another is significant (Richardson *et al*., 2018). According to the genetic profile, there is a clear genetic link between environmental and clinical *S. aureus* isolates and the direction of *S. aureus* transmission in this study is most likely from patients to the environment. The isolate from clinical sources was genetically closely linked to one *S. aureus* isolate from environmental sources.

Prokaryotes, including *S. aureus*, use the 16S rRNA gene to identify species and build phylogenetic connections because of its extremely conservative sequencing and minimal discrimination against closely related species (Vìtrovsky and Baldrian, 2013). When identified using visual and biochemical characteristics, organisms that resemble *S. aureus* present numerous challenges for species identification. However, the sequencing of the 16S rRNA region is a viable tool for species identification.

The study of 16S rRNA gene sequences to ascertain bacterial phylogeny and taxonomy has been the most popular housekeeping genetic marker for a number of reasons. The function of the 16S rRNA gene has not modified across time, suggesting that random sequence changes are a more precise indicator of time. These factors include (i) its existence in practically all bacteria and (ii) the fact that it frequently exists as a multi-gene family or operons (evolution).

CONCLUSION

All clinical isolates were multidrug resistant. Clinical isolates were highly resistant toward penicillin and Erythromycin and low resistant to Ciprofloxacin, Nitrofurantoin, Chloramphenicol and Tigecycline, while all the environmental isolates were sensitive to Ciprofloxacin. The study got a novel local strain of *S. aureus* that was isolated from clinical sources (MIM2022) and was genetically 100% homologous with a novel local strain of *S. aureus* that was isolated from environmental sources (MIM2023), which demonstrates *S. aureus* isolates' capacity to jump from one niche to another which means poses a significant hazard to public health.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided but do not accept any liability for any content direct or indirect losses resulting from the use of

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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