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Original article

Random amplified polymorphic DNA technique (RAPD) for typing of *Staphylococcus aureus* causing infection in intensive care units of Tanta University Hospitals

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ABSTRACT

Background: Healthcare-associated infections appear in a patient receiving medical attention at a hospital or other healthcare facility and develops infections that weren't present when they were admitted. One of the most important organisms causing infections acquired in hospitals is *Staphylococcus aureus (S. aureus)*. For many bacterial species, Random Amplified Polymorphic DNA (RAPD) is a low-cost but effective typing technique. **Objectives:** To determine the genotypic polymorphism, the level of genetic relatedness, and the antimicrobial sensitivity and resistance among various *S. aureus* isolates. **Methods:** The following tests were performed on 50 samples that were obtained using strict aseptic precautions: sample culture, isolate identification using colony morphology, Gram stained film, biochemical responses, and antibiotic susceptibility testing. After RAPD-PCR, Syngene gene tool software was used to analyse the fingerprint pattern. **Results:** The most prevalent isolated organism was *S. aureus.* as it represented (70%) of isolated pathogens. Vancomycin and linezolid were effective against *S. aureus* isolates. The isolates of *S. aureus* were more common in nasal swab (42.9%) followed by wound (28.5%) then urine &endotracheal aspirate (14.3%). Patterns of RAPD-PCR of *S.aureus* isolates generated with primer GEN1-50-01 (5'GTGCAATGAG-3') resulting in several polymorphic bands and a dendrogram was created. **Conclusions:** *S. aureus* is an important cause of HCAI, Health care workers represent an important source of HCAI, RAPD –PCR technique was an easy and rapid one to perform with a good discriminatory power in typing (fingerprinting) of *Staphylococcus aureus*.

Introduction

Patients receiving medical care in a hospital or other healthcare facility may get healthcare-associated infection (HCAI) that was not present at the time of admission. These infections include occupational infections among health care workers. They can even develop after patients have been discharged from the hospital [1]. These infections are also intimately tied to invasive medical equipment used in modern healthcare, such as ventilators and catheters [2].

According *to Markwart et al. (2020*) [3], there are seven HCAs per 100 hospitalised patients in rich nations and ten in developing countries who can contract an infection related to healthcare. Patients in intensive care units (ICUs), burn units, people receiving organ transplants, and newborns are populations that are at high risk. The percentage

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of infected patients in the ICU is typically as high as 51%, according to the Extended Prevalence of Infection in Intensive Care (EPIC II) research [4].

The setting in which treatment is provided, the patient's vulnerability and health, and staff and healthcare professionals' lack of awareness of such widespread infections are all risk factors for HCAs [5].

One of the most major pathogens causing hospital-acquired infections is *Staphylococcus aureus*, a facultative anaerobic gram-positive bacteria. In the nares and skin of healthy humans, *S. aureus* is frequently present (30–60%) of them [6]. It contributes to a variety of infections, including urinary, respiratory, and surgical site infections [7]. In order to analyze variation among *S. aureus* isolates, numerous phenotypic and genotypic techniques have been employed. Random Amplified Polymorphism PCR is one of them [8,9].

For many bacterial species, Random Amplified Polymorphic DNA (RAPD) is a strong yet reasonably priced typing technique. PCR amplification of random genomic DNA segments using a single (universal) primer with any nucleotide sequence produces DNA fragments known as RAPD markers. If two isolates have the same number of bands in their restriction patterns and the corresponding bands have the same apparent size, they are said to be genetically indistinguishable. Closely related strains or clones were identified by profiles that showed (3 band variances). Similarity of *S. aureus* isolates of 65% or more indicated the likelihood of a common ancestor (potentially related isolates) [10].

Aim of the Work:

To determine the genotypic polymorphism, the level of genetic relatedness, and the antimicrobial sensitivity and resistance among various *S. aureus* isolates.

Methodology

Study design and testing:

A cross sectional study, which involved both patients and staff in intensive care units at Tanta University Hospitals, was conducted in the department of medical microbiology and immunology at the faculty of medicine , Tanta University.

All research participants provided their written consent before beginning the study.

The Tanta University Faculty of Medicine's ethics and research committee granted their approval for this work. Code of approval for the protocol: N: 3409-9-20.

All patients were subjected to:

1: History taking including : age , sex ,underlying disease ,onset, course and duration of illness ,antibiotic course of treatment.

2: Clinical examination: including general examination with special emphases for signs of infection such as fever >38, chills, rigors, erythema, swelling and tenderness in SSI.

Inclusion criteria:

Medical personnel and patients hospitalized to Tanta University Hospital's intensive care units (ICUs) displaying symptoms of hospital acquired illnesses (infections occur on or after the third day of admission).

Exclusion criteria:

Patients from outpatient clinics. Cases showing manifestations of infections before the third day of admission.

Microbiological study:

Sampling: Under strict aseptic conditions, 50 samples were collected. The samples were labelled and delivered as quickly as possible to the microbiology and immunology department laboratory. The samples comprised wound swabs, nasal swabs, endotracheal aspirates, urine samples, and others. The 50 samples then underwent the following:

Culture of samples on nutrient agar, blood agar and MSA (Oxoid, UK) then incubation at 37 for 24 hours [11]. Isolates were identified by using; Colony morphology , Gram stained film. Biochemical reactions (catalase test - coagulase test) [12,13] . The isolates were examined for antibiotic sensitivity using the Kirby Bauer-disk diffusion method over Mueller Hinton agar [14].

RAPD-PCR analysis [15] :

DNA extraction (Spin column based nucleic acid purification). From overnight growth by DNA extraction kit according to manufacturer recommendations (QIAGEN, Germany). Amplification of DNA fragments with RAPD

primer. According to manufacturer recommendations (TIB MOLBIOL, Germany).

Ten randomly designed oligonucleotide primer with 50% G+C content, the GEN1-50-01 (5'GTGCAATGAG-3') was selected for the RAPD analysis as they provide reproducible and discriminatory banding patterns. PCR reactions for the RAPD assays were performed in 25 µL volumes containing 20 ng of genomic DNA, 2.5 µL of 10x PCR buffer, 0.5 µL of 10 mM dNTPs, 1.5 µL of 25 mM MgCl2, 1 unit of Taq polymerase (Promega Co, USA) and 5 pmol of primer. The Amplifications were carried out in a thermal cycler (BIO-RAD, USA). The cycling parameters were 4 min at 94°C for pre-denaturation, 45 cycles each of 1 min at 94°C for denaturation, 1 min at 36°C for annealing, 2 min at 72°C for extension and a final extension at 72°C for 8 min. Amplified products were resolved by electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5 µg mL-1) and photographed under UV transilluminator (UVitec, UK) A100 bp DNA ladder was used as a DNA fragment size marker in all gels.

Fingerprint pattern analysis:

The patterns RAPD-PCR bands generated were analyzed using Syngene gene tool software version 4.3.14.0.(Cambridge ,UK) which involves main 8 steps: Pre-Processing Stage , Automatic and Semi-Automatic Detection of Lanes, Automatic and Semi-Automatic Detection of Bands, Ladder Size Detection , Calculating the Molecular Weights of the Bands of Unknown Size, Band Matching Algorithm, Clustering of the Bands .We were able to identify the size, quantity, and configuration (pattern) of gel bands thanks to this software. Using the underweighted pair group method with arithmetic averages (UPGMA) and dice similarity co-efficient, RAPD-PCR profiles were arranged into a dendrogram (phylogenetic tree).

Results:

Table 1. Distribution of studied subjects according to epidemiological factor (n=50).

Epidemiological factor	No	%
Age		
20-30 years	10	20.0%
40-50 years	25	50.0%
60-70 years	15	30.0%
Sex		
Male	30	60.0%
Female	20	40.0%
Studied cases		
Patients	40	80.0%
Health care workers	10	20.0%
Previous antibiotic intake		
Positive	30	60.0%
Negative	20	40.0%
Underlying chronic disease		
Present	30	60.0%
Absent	20	40.0%

50% of studied subjects (n=25) aged between 40-50 years old. Previous antibiotic intake was positive among 60% of studied cases. 60% of studied cases were suffering from chronic underlying disease (n=30).

Table 2. Distribution of studied subjects according to the isolated pathogen (n=50).

Isolated pathogen	No	%
S. aureus	35	70.0%
Coagulase negative Staphylococci	7	14.0%
Pseudomonas aeruginosa	5	10.0%
Acinetobacter	3	6.0%

S. aureus was the most common isolated organism in the studied cases it represented (70%, n=35).

Table 3. Antimicrobial susceptibility of *S. aureus* isolates (n=35).

Drug	Disc content microgram	Susceptible (N)	%
Cefoxitin	30	25	71.0%
Ceftaroline	30	25	71.0%
Vancomycin	- (Minimal inhibitory concentration (MIC) Breakpoints µg/mL)	35	100.0%
Gentamycin	10	20	57.0%
Azithromycin	15	25	71.0%
Clarithromycin	15	25	71.0%
Erythromycin	15	25	71.0%
Tetracycline	30	20	57.0%
Doxycycline	30	25	71.0%
Ciprofloxacin	5	25	71.0%
Norfloxacin	10	10	29.0%
Ofloxacin	5	25	71.0%
Nitrofurantoin	300	10	29.0%
Linezolid	30	35	100.0%

All *S. aureus* isolates were susceptible to linezolid and vancomycin. Nitrofurantoin and norfloxacin were effective against every urine isolate (n = 5).. The prevalence of *MRSA* was (28.5%, n=10).

N.B 1. Testing with oxacillin on a disc is unreliable for *S. aureus*.

2. *Staphylococcus spp.* may become resistant to quinolones when treated for an extended period of time; hence, isolates that were previously susceptible may change within 3–4 days of starting treatment, necessitating further testing.

3.Classification according to clinical and laboratory standard institute Vancomycin resistance *S. aureus* (*VRSA*) is currently characterised as a MIC 16mcg/mL, vancomycin intermediate *S. aureus* (*VISA*), and vancomycin susceptibility (MIC 2mcg/mL).

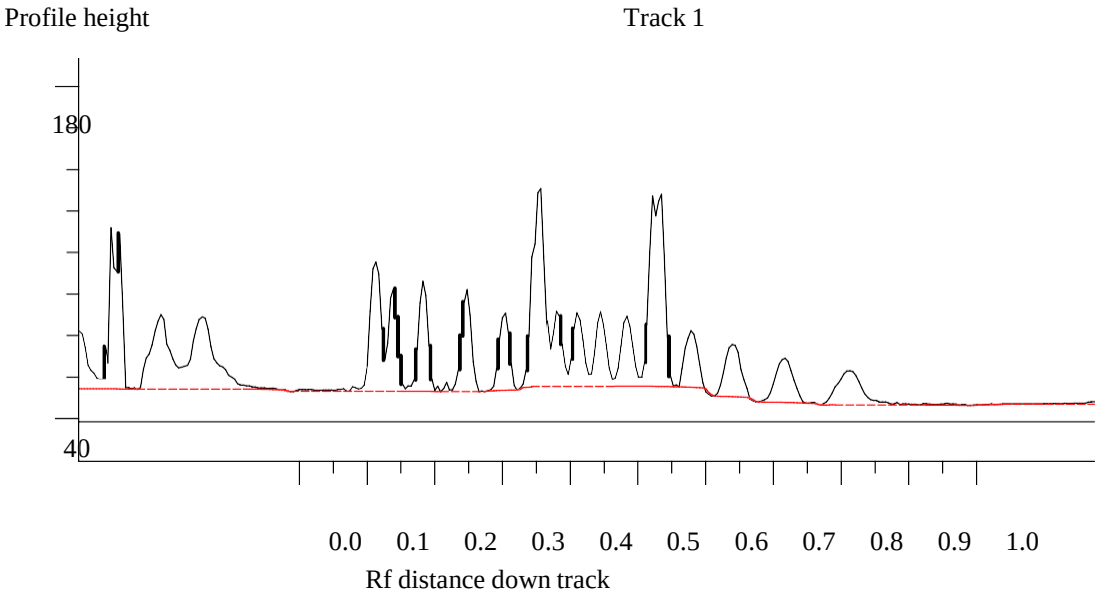
.4. To make the vancomycin suspension, 500 mg of vancomycin powder were dissolved in 10 ml of sterile distilled water (50 mg/ml), and a further 1:10 dilution was carried out twice (0.5 mg/ml).

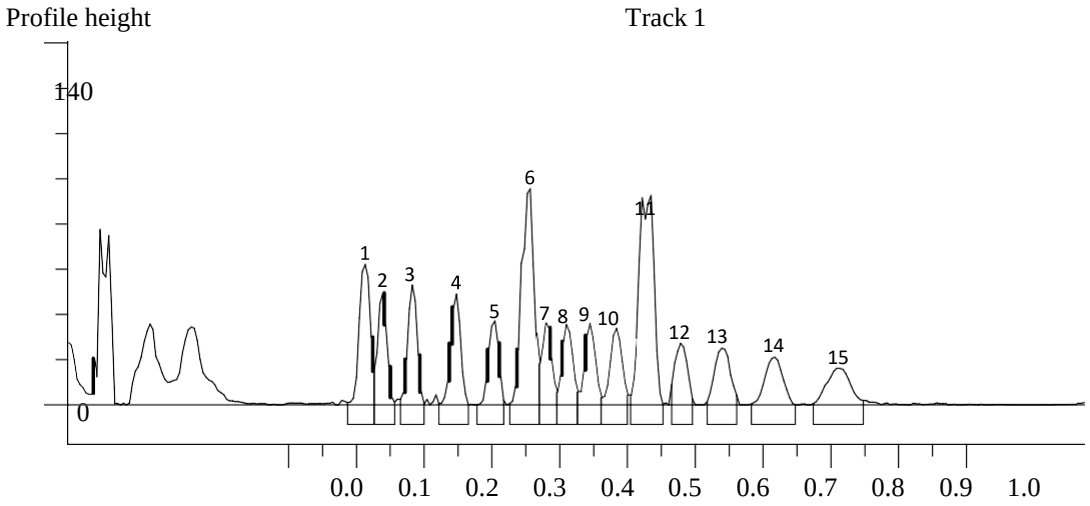
Table 4. Distribution of studied subjects according to the type & sources of specimens (n=35).

Type of specimen	No. of *S. aureus* isolates	%
Nasal swab	15	42.9%
Wound	10	14.3%
Urine	5	28.5%
Endotracheal aspirate	5	14.3%

S. aureus isolates were more common in nasal swab (42.9%, n=15) followed by wound (28.5%, n=10) then urine & endotracheal aspirate (14.3%, n=5).

Figure 1. Tracks of *S.aureus* generated by SynGene soft ware version 4.3.14. Track 1 (standard track)





Track 1				
Number	**Mol. weight**	**Height**	**Raw vol.**	**% Raw vol.**
1	1500.00	62.206	7844.10	8.503
2	1400.00	49.420	5266.76	5.709
3	1300.00	53.075	5712.44	6.193
4	1200.00	49.164	5551.56	6.018
5	1100.00	37.150	4385.41	4.754
6	1000.00	95.543	13310.32	14.429
7	900.00	36.345	3999.76	4.336
8	800.00	35.348	4252.39	4.610
9	700.00	35.764	4344.45	4.710
10	600.00	33.642	4529.67	4.910
11(m)	500.00	82.125	16049.20	17.398
12	400.00	27.305	3836.77	4.159
13	300.00	25.138	4474.71	4.851
14	200.00	21.256	4306.87	4.669
15	100.00	16.279	4382.34	4.751

The Rf value: It is a common abbreviation for relative mobility or retention factor. The Rf is defined as the migration distance of the protein through the gel divided by the migration distance of the dye front. The distance should be measured from the top of the resolving gel to the band of interest, as illustrated on the gel. Use a graphing program, plot the log (MW) as a function of Rf. Generate the equation y = mx + b, and solve for y to determine the MW of the unknown protein. A linear relationship exists between the logarithm of the molecular weight of native nucleic acid, and its Rf.

.Quantity calibration and Raw volume: quantity calibration refers to the initial concentration of the DNA ladder and then you know the amount of DNA in each band. The Raw volume box is read-only: it shows the un calibrated volume calculated from the area of the peak.

Figure 2. Dendrogram generated from RAPD-PCR analysis of the *S. aureus* isolates using primer GEN-50-01(5'GTGCAATGAG-3') showing similarity level between isolated tested strains tracks in relation to each other and the standard track.

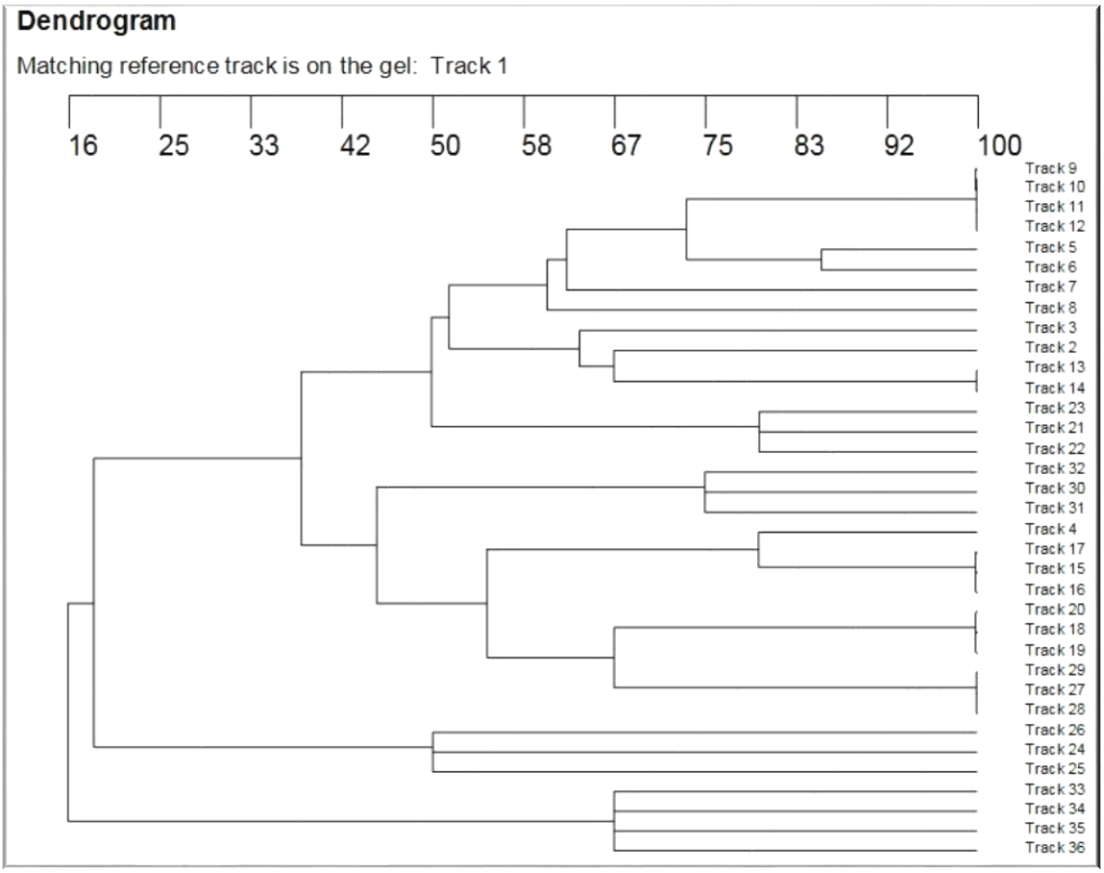


Table 5: Clustering of *S. aureus* strains that located in 12 clusters (8 clusters with 100% similarity & 4 clusters with 75% similarity level).

Clusters	Tracks	Similarity level	Common band
1	9,10,11,12	75%*	500 bp
2	5,6		
3	7,8		
4	13,14		
5	2,3	100%*	
6	30,31,32		
7	15,16,17		
8	18,19,20		
9	21,22,23		
10	24,25,26		
11	27,28,29		
12	33,34,35,36		

* Detected by UPGMA (un weighted pair group of arthmetic mean) &Dice similarity co-efficient.

*Track 4 was un related strain to that clustering.

Abbreviation :UPGMA: Unweighted Pair Group Method with Arithmetic Mean: A straightforward clustering technique based on the molecular clock theory, which predicts a constant rate of evolution. After creating the phylogenetic tree (Dendrogram), it requires a distance matrix of the examined taxa, which can be computed using a multiple alignment.

The Dice Coefficient is calculated by multiplying the overlap area by two and dividing the sum of the pixels in both photos.

Discussion

S. aureus, is frequently seen as a colonizer in the healthy people. But it can cause life-threatening infections in high-risk patients as in ICU. Colonization mostly in the nose, but other sites as skin have been identified [16]. The prevalence of *Staphylococcal*-related illnesses and *S. aureus* colonisation are strongly correlated. [17].

Even though *S. aureus* infections can happen to people who are not carriers, they happen far more frequently in people who have *S. aureus* colonisation. *S. aureus*-caused nosocomial pneumonia frequently makes hospitalisation more difficult and can have serious outcomes, especially if it is contracted in the intensive care unit [18,19] During our study as shown in **table (1)** we isolated thirty five *S. aureus* isolates from 50 cases (40 patients, 10 health care workers). Age distribution showed that most isolates were detected in cases aged between 40-50 years old (50%). Previous antibiotic intake was positive among (60%) of studied cases. Also (60%) of studied cases were suffering from chronic underlying disease. These epidemiological factors that detected during our study were supported by other studies [20, 21].

Other studies showed differences in epidemiological factors as following: age >65 years, (37.5%) had used antibiotics in the past, and (75%) had two or more comorbid conditions, such as cancer, diabetes, and cardiovascular disease [22.23].

Health care workers represented an important source of samples during our study (n=10) and they played an important role in transmission of infection among patients.

The distribution of the isolated pathogens during our study as shown in **table (2)** was as the following *S. aureus* was the most common isolated organism in the studied cases it represented (70%) followed by *CoNS* (14%) Other studies show similarity in prevalence of *S. aureus* health care associated infection as it represented the most common cause [24,25].

As regards, *S. aureus* isolates' susceptibility to antibiotics as shown in **table (3)** was as the following: Vancomycin &Linezolid were effective against each and every isolate of *S. aureus*. While sensitivity to (Cefoxitin ,Ceftaroline, Azithromycin, Clarithromycin, Erythromycin, Doxycycline, Ciprofloxacin, Ofloxacin) was about (71%).

All urinary isolates (n=10) were sensitive to nitrofurantoin and norfloxacin. This is in accordance with other studies that showed that many strains of *S. aureus* are already resistant to many antibiotics except vancomycin & linezolid and as a result, the organism has advanced towards becoming an unstoppable murderer [26,27].

The prevalence of *MRSA* in our work was (28.5%,) and were mainly detected in nasal swabs from health care workers this in accordance with another study performed in Iran in which prevalence of *MRSA* was (30%) and was also mainly isolated from hospital staff .As regards, types and sources of samples of *S. aureus* isolated in our study as shown in **table (4)** were as the following *S. aureus* isolates were more common in nasal swab (42.9%) followed by wound samples (28.5%) then urine & endotracheal aspirate (14.3%).. This is supported by *Nikbakht et al* [28].

Another study was performed in Nepal showed the prevalence of *S. aureus* in clinical specimens as following: Tracheal aspirate (0.75%) then urine samples (2.26%) and Wound Swab (23.31%) [29].

In terms of molecular fingerprinting *S. aureus*, RAPD, a PCR approach that uses an arbitrary primer that binds to the nonspecific spots on the DNA strand and amplifies the DNA, was used to carry out the task. After being migrated on an agarose gel, these amplified fragments are examined for differences in the band pattern. This is different from traditional PCR in that it amplifies randomly selected DNA segments that are essentially unknown to the scientist. PCR is frequently used to amplify a known DNA sequence. So, a specific DNA segment is amplified as a result of PCR. SYNGENE software was used to examine the results, and samples were clustered using UPGMA and the dice similarity coefficient. This is consistent with studies that demonstrate RAPD-PCR typing [30,31] .

S. aureus is known to have certain polymorphisms, but in certain situations those that are detected by RAPD-PCR are harder to identify and this may result from point mutations or mobile genetic elements such bacteriophages, plasmids, and transposons [32].

As shown in **table (5), fig. (1&2)** Dendrogram was created from RAPD-PCR analysis using the primer GEN-50-01(5'GTGCAATGAG-3') of the 12 clusters of *S. aureus* strains, 8 of which had 100% similarity and 4 of which had 75% similarity. One strain was unconnected to this clustering, which could be explained by the fact that the patient was an endogenous source (carrier for the infection) and the infection flared again after hospitalisation. Different RAPD-PCR patterns and clustering of *S.aureus* isolates were seen in other studies [33,34]. This might be explained by the use of various primers, the origin of the isolates, or the presence of mutant *S. aureus* strains.

During our study , our research work was conducted using a single primer GEN-50-01 (5'GTGC AATGAG-3'). In other research work ,a greater variety of *S.aureus* strains was found after employing a greater number of primers to analyze their genetic relatedness [35].

We picked RAPD-PCR since it is regarded as a useful method for gathering genomic information. It is an approach that can be used on any genome and is quick, easy, cheap, and simple. No prior understanding of the target sequence is necessary. This supported by [36].

Other studies preferred other methods of typing because of a tendency for a reproducibility problem in the band patterns in RAPD PCR [37,38] . The RAPD reaction settings were well standardised

during our investigation, which reduced the severity of this issue.

A different investigation demonstrated the greater discriminating power and good reproducibility of PFGE. It is referred to as the "gold standard" and is accepted as the proper approach to ascertain strain-specific diversity. In spite of this, we picked RAPD approach since PFGE needs labor-intensive equipment that is uncommon in most molecular biology and microbiology labs and can only run a small number of samples at once [39].

As regards, clustering of samples during our study as we mentioned earlier we had (8) clusters with 100% similarity & (4) clusters with 75% similarity level. We discovered that isolates from a particular ward shared similar RAPD and antibiotic susceptibility patterns. However, several isolates with the same patterns were found in various wards, which may have been caused by the movement of staff and patients.

We also detected isolates with a characteristic RAPD pattern and a distinct pattern of antibiotic susceptibility and vice versa.. This in accordance with another study performed in Egypt [40] this could be due to different history of antibiotic intake among the cases during the study.

Conclusions

S. aureus is a significant contributor to HCAI and can result in potentially fatal consequences, especially in high-risk patients like those in intensive care units. It has a wide range of genetic polymorphism. Health care workers represented an important source of HCAI. RAPD – PCR technique was an easy and rapid one to perform with a good discriminatory power in typing (fingerprinting) of *S. aureus*. It was also a relatively cost – effective technique compared to other methods of genotypic typing of bacteria making it attractive for use in clinical laboratories. This technique made us able to recognize and trace the possible source of infection to establish more effective infection control measures particularly in ICUs.

Recommendations

Frequent screening of health care workers for detection of *S. aureus* is important particularly in cases of recurrent infections in high risk patients as in ICUs. It is recommended to use mupirocin (bactroban) cream to prevent and treat nasal colonization by *S. aureus* particularly among health care workers. As regards RAPD technique , it is better to use more than one primer in the future to overcome the genetic dissimilarity of *S. aureus* particularly when isolated from different sources. It is also recommended to optimize the conditions of RAPD reaction particularly the annealing temperature to decrease the magnitude of reproducibility problem in the band pattern of RAPD PCR.

Ehical statement

The Tanta University Faculty of Medicine's ethics and research committee granted their approval for this work. Protocol approval number N: 3409-9-20.

Procedures used in the research were completely non invasive and did not represent any hazards to participants.

Conflicts of interest

Each author listed in the manuscript had seen and accepted the submission of this version of the manuscript and assumes full responsibility for it. The authors state that they have no financial or non-financial conflicts of interest linked to the work done in the article.

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Authors' contributions

Amira Ezzat (the corresponding author) wrote the main data of the manuscript and prepared the figures and the tables.

Hanan Samir reviewed the manuscript.

Lobna Mohamed reviewed the manuscript.

Ahmed Amin reviewed the manuscript.

Aziza Hassan reviewed the manuscript.

Consent to participate

The research's participants all provided their consent.

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