Detection of Tetracycline *tet(k)* Gene in Clinical *Staphylococcus aureus* Isolates

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Abstract. This paper concerned with the identification of *S. aureus* and detection of *tet(k)* gene using PCR technique. A total of fifty samples were collected from different clinical sources: 20 from burns, (10) surgical wounds, (10) dental carries, and (10) urine samples from patients who were admitted to West Erbil Emergency and Rizgary teaching Hospitals during 1 October to 17 December 2020. Isolates were identified using conventional method and confirmed by VITEK2 compact system. Twenty out of Fifty isolates were identified as *Staphylococcus aureus*. Disk diffusion method was done for antibiotic resistance, all *S. aureus* isolates were showed resistance to antibiotic in different percentage: 100% for AMC, AP, AX, and PG, 90% for ME, 65% for CAZ, 65% for TM, 60% for T, 60% for E, 50% for CRO, 45% for CTX, and S, 45% for C%15, for KF and 10% for GM while all isolates were sensitive to vancomycin. PCR technique results of partial fragments of *tet(K)* gene using 360bp primer were shown that 7 (35%) of isolates were *tetk* positive, while 13 (65%) of the isolates were *tetk* negative.

1. Introduction

*Staphylococcus aureus* is a major cause of nosocomial infections and remain a versatile and dangerous pathogen in human. The frequency of both communities acquired and hospital acquired staphylococcal infections have increased steadily [1]. *S. aureus* is coagulase-positive, often hemolysis blood and produce a variety of extracellular enzymes and toxins [2]. In humans, infection with *S. aureus* may cause suppuration, abscess formation, a variety of pyogenic infections and even fatal septicemia. It can also cause food intoxication due to elaboration of heat-stable enterotoxin [3]. Antimicrobial resistance is an increasing threat afflicting hospitals worldwide [5]. Antimicrobial drug resistance in hospitals is driven by failures of hospital hygiene, selective pressures created by overuse of antibiotics, and mobile genetic elements that can encode bacterial resistance mechanisms [6]. Methicillin -resistant *S. aureus* (MRSA) isolates were once confined largely to hospitals, other health care environments, and patients frequenting these facilities [7]. A large number of tetracycline resistance genes have been identified. The main mechanisms conferring resistance to tetracycline to bacteria are active efflux proteins, ribosomal protection proteins and enzymatic inactivation. PCR-based molecular methods are often preferred for determination of antibiotic resistance genes. Therefore, the availability of sensitive and specific methods for the accurate detection of antibiotic resistance in these multi-drug resistant pathogens has become an important tool in clinical diagnosis and permits timely implementation of effective antimicrobial therapy, preventive control strategies, screening of the patient contacts and staff, and appropriate disinfection measures which in turn reduce the costs [8]. Therefore, this paper concerned with isolation and identification of *S. aureus* from different clinical specimen and study both antibiotic resistance pattern and detection of *tet k* in *S. aureus* isolates using PCR technique.

2. Materials and Methods

2.1. Samples collection

Samples were collected from 50 patients who were admitted to West Erbil Emergency, Emergency, and Rizgary teaching Hospitals during the period from 1 October 2020 to 17 December 2020. The age of patients ranged from one to 45 years. Samples were taken from different sites: burns, surgical wounds, dental carries and urine. The samples were obtained by rubbing the inflamed or discharged wound, burn,
or decayed teeth by a sterile disposable swabs with normal saline to keep samples fresh while transporting it to the laboratory for further processing. Urine samples were collected by taking a loop full from the urine sample and streak it directly on the culture media.

2.2. Identification of the isolates
Identification of these isolates was carried out using microscopically, morphological, biochemical tests and VITEK2 compact system [9]; [10]; [11].

2.3. Antimicrobial susceptibility test (Disk diffusion method)
This test was performed according to Schwalbe et al., [12] Antibiotic impregnated discs with required concentration were dispensed on the surface of Mueller-Hinton agar medium that has been spread with a pure bacterial suspension of $10^5$ CFU/ml. After incubation, inhibition zones were measured and translated into predetermined categories as susceptible, intermediate, or resistant.

2.4. Genomic DNA extraction
A Presto™ Mini gDNA Bacteria Kit was used for genomic DNA extraction from S. aureus isolates. A loop full of bacteria were incubated over night in a tube containing LB broth. The kit’s instructions was followed carefully to obtain a good DNA extracts. The purity of extracted DNA was between 1.8-2.0.

2.5. Detection of tet(k) gene in S. aureus clinical isolates
The standard PCR assay was performed using the DNA amplification instrument Master cycler gradient (Eppendorf, Germany) to detect tet(k) gene. The tet(k) - specific primer pairs used for amplification of 360 base pair (bp) fragment are: Forward, 5'-GTGCACAATAGTTATAGT-3' and Reverse, 5'-GTAGTGACAATAACCTGCTA-3' (Bühlmann et al., 2008). A volume of 20µl deionized distilled water (ddH2O), 1.3 µl Reverse primer, 1.3µl Forward primer and 2.5 µl of extracted DNA (template) was added to the ready to use PCR reagent tube (Bioneer, South Korea) which contains the following (for the 20µl reaction): 1U Top DNA polymerase, 250 µM of each: dNTP (dATP, dCTP, dGTP, dTTP),10 mMTris-HCl (pH 9.0),30 mM KCl, 1.5 mM MgCl2, Stabilizer and tracking dye. The thermal cycling protocol for PCR was comprised as described by [13]: 1. Initial denaturation at 95°C for 3 minute. 2. Thirty three cycles of: Denaturation at 95°C for 30sec, annealing at 54°C for 30 seconds, elongation at 72°C for 30sec and final extension at 72°C for 4 minutes.

2.6. Detection of PCR products
About 5µl of the amplified products were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide under UV trans illuminator and photographed. The amplicon (PCR product) generated from S. aureus gene sequences by this PCR method was a DNA fragment of 360 bp length. Therefore, a positive PCR test should yield a 360 bp DNA fragment which appeared as an intense band on an ethidium bromide stained agarose gel. The molecular size of the band was verified by comparing its migration to that of a DNA marker (100bp DNA ladder) run on the same gel. A negative PCR product did not produce any visible band on the gel [13].

3. Results and Discussion
3.1. Isolation and Identification of S. aureus
twenty isolates were identified among 50 different clinical specimens including: 20 burn swab, 10 wound swab, 10 urine and 10 from dental carries. The identification of the isolates were carried out using conventional method based on cultural characteristics, cell morphology, Gram stain reaction biochemical properties and VITEK2 compact system. All isolates were able to grow on mannitol salt agar (selective media for Staphylococcus) (figure 1). S. aureus has the ability to change the color of the media from Pink-Orange to yellow, because it can ferment the mannitol which present in the medium that leads to change in the color [14]. circular, smooth, yellow to golden colonies raised on blood agar with various degrees of hemolysis (mostly beta hemolysis). Prepared smears of S. aureus isolates appeared as purple single, diplo, and grape like Gram positive cocci under light microscope. All isolates were positive for catalase, coagulase and DNase. All isolates were identified as S. aureus by VITEK2 compact system with over 85% probability percentage.
3.2. Antibiotic resistance pattern of *S. aureus* isolates

Antibiotic Sensitivity test by disk diffusion method for 50 isolates of *S. aureus* was done against 20 commonly used antibiotics (AMC, AP, AX, C, CAZ, CIP, CRO, CTX, DA, E, GM, KF, L, ME, PG, RA, S, T, TM, VA). The resistance percentage of *S. aureus* isolates varied for different antibiotics used in this study as shown in table (1). The results revealed that the resistance was 100% for AMC, AP, AX, and PG and 90% for ME. Resistance percentage for other antibiotics were 65% for CAZ, 65% for TM, 60% for T, 60% for E, 50% for CRO, 45% for CTX, and S, 45% for L, 40% for RA, 40% for DA, 35% for CIP, 35% for C, 15% for KF, and 10% for GM while all isolates were sensitive for vancomycin. Our results are in agreement with that of [15] that found that *S. aureus* isolates from patients with urinary tract infections were highly resistant to ampicillin and amoxicillin. The results of [16] showed that (77%) of isolates were oxacillin-resistant *Staphylococcus aureus* and exhibited multiple resistances to other tested antibiotics which is close to our results. [17] reported that the resistance patterns of *S. aureus* were: for levofloxacin (20%), for norfloxacin (16%), for ofloxacin (18%), for ciprofloxacin (16%), for levofloxacin (14%) and for nalidixic acid (50%), while the results of [18] revealed that (8.10%) of *S. aureus* isolates were amikacin resistant, (100%) of isolates were amoxicillin resistant (86.48%) of isolates were ampicillin resistant, (54.05%) were resistant to (cephotaxim, erythromycin, tetracycline), (21.62%) of isolates were methicillin resistant (MRSA), and (10.81%) were vancomycin resistant. [19] found that *S. aureus* isolates from Africa were: 54% resistant for trimethoprim, 21% for sulfamethoxazole and 19% for trimethoprim/sulfamethoxazole, while [20] reported that a total of 94 cases from 2010 to 2012 were diagnosed to have *S. aureus* infection using conventional bacteriologic methods. From these cases, 38 (40.6%) were identified as MRSA and 37 (39.4%) were inducible clindamycin resistant. In England, surveillance of surgical site infections has been running since 1997. During the 5 year period between January 2003 and December 2007, at least one causative microorganism was reported for 77% of surgical site infections. The most common organism was *S. aureus* (accounting for 38% of surgical site infections), of which 64% were MRSA. However, between October 2008 and September 2009, the proportion of *S. aureus* isolates (accounting for 31% of surgical site infections) that were methicillin resistant decreased to 32%. This decrease in surgical site infections due to MRSA in England appears to mirror the decline in MRSA bacteremia [21]. The antimicrobial agents are losing their efficacy because of the spread of resistant organisms due to indiscriminate use of antibiotics, lack of awareness, patient noncompliance and unhygienic condition. It is the need of the time that antibiotic policies should be formulated and implemented to resist and overcome this emerging problem. Every effort should be made to prevent spread of resistant organisms. There are multiple factors, which contribute to the global spread of resistance. Decreasing unnecessary antibiotic use, with narrow spectrum agents, improving compliance...
with therapy, decrease in use of antibiotic in animal and agriculture, and improving infection control all have a role in confronting this problem [22].

Table 1. Resistance percentage of S. aureus to antibiotics.

| Antimicrobial agent                  | Antibiotics | % of resistance | No. of resistant isolates |
|--------------------------------------|-------------|----------------|--------------------------|
| Amoxicillin                          | AMC         | 100            | 20                       |
| Amoxicillin+ clavulanic acid         | AP          | 100            | 20                       |
| Ampicillin                           | AX          | 100            | 20                       |
| Cefotaxime                           | C           | 35             | 7                        |
| Ceftazidime                          | CAZ         | 65             | 13                       |
| Ceftriaxone                          | CIP         | 35             | 7                        |
| Cephalothin                          | CRO         | 50             | 10                       |
| Chloramphenicol                      | CTX         | 45             | 9                        |
| Ciprofloxacin                        | DA          | 40             | 8                        |
| Clindamycin                          | E           | 60             | 12                       |
| Erythromycin                         | GM          | 10             | 2                        |
| Gentamicin                           | KF          | 15             | 3                        |
| Lincomycin                           | L           | 45             | 9                        |
| Methicillin                          | ME          | 90             | 18                       |
| Penicillin G                         | PG          | 100            | 20                       |
| Rifampin                             | RA          | 40             | 8                        |
| Streptomycin                         | S           | 45             | 9                        |
| Tetracycllin                          | T           | 60             | 12                       |
| Trimethoprim                         | TM          | 65             | 13                       |
| Vancomycin                           | VA          | 0              | 0                        |

3.3. Detection of tet(k) in S.aureus isolates

All isolates were analyzed by PCR to detect the presence of tet(k) gene using forward and reverse primers described by [13]. The results showed that 7 isolates (35%) were harboring the tet(k) gene (360 bp), while 13 isolate (65%) were lacking the tet(k) gene (figure 1). These results shows contrast with the results of disc diffusion method for tetracycline, 65% of isolates were resistant to tetracycline while only 35% of it were harboring tet(k) gene. This may be due to that the resistance may conferred by other class of tetracycline gene like tet(K), tet(M), tet(O) and tet(L) that not detected in our study. Our results agree with that obtained by [13] Duran et al., (2012) who reported 36% of MRSA were positive for tet(k) gene. In contrast to our results [23] Adwan et al., (2014) reported that 76% isolate of methicillin resistant S. aureus were tet(k) positive. [24] found that only 58 (44.61%) from total 130 S. aureus isolates showed tet(k) gene positive.

A large number of tetracycline resistance genes have been identified. There are 38 acquired tetracycline resistance genes that are known and all use one of three strategies to render the bacteria resistant. These include (1) efflux proteins, (2) ribosomal protection proteins and (3) enzymatic inactivation of tetracycline. The majority of these genes (60%) code for energy-dependent efflux pumps, and different bacterial genera tend to have the same efflux or ribosomal protection genes [25]. This indicates that tetracycline resistance genes can be transferred among the bacterial population. In fact, resistance to tetracycline in most bacteria is due to the acquisition of new genes; these genes tend to be associated with mobile elements such as transposons and plasmids [26].
Figure 2: PCR gene product of tetK gene

Tetacycline resistance genes: tetK, tetM, tetO and tetL are four major genes associated with tetracycline resistance amongst Gram positive bacteria. The tetK and tetL genes code for efflux proteins; these are energy dependent membrane-associated proteins which prevent tetracycline from accumulating within the cell [25]. The other two genes, tetM and tetO, code for ribosomal protection proteins, which reduce the affinity of tetracycline to the ribosome [27].

Our study confirms the usefulness of PCR assay for the detection of antibiotic resistance genes associated with S. aureus infections. The PCR assay offers a rapid, simple, and accurate identification of antibiotic resistance profiles and could be used in clinical diagnosis as well as for the surveillance of the spread of antibiotic resistance determinants in epidemiological studies. Classical methods and molecular approaches especially PCR based techniques were more effective when used together and could provide more accurate and reliable information. Laboratory methods used to detect multidrug resistant bacteria such as MRSA should have high sensitivity and specificity.

4. Conclusion
In this study we can conclude the resistance was 100% for Amoxicillin, Amoxicillin+ clavulanic acid, Ampicillin and PG, 90% for ME while all isolates were sensitive to vancomycin. Results also indicated that 7 (35%) of isolates were tet(k) gene positive.

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