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RESEARCH ARTICLE

The Prevalence, Molecular Characterization and Antimicrobial Susceptibility of *S. aureus* Isolated from Impetigo Cases in Duhok, Iraq

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Abstract:

Background:

Staphylococcus aureus is one of the most important opportunistic pathogens. Impetigo is the common contagious bacterial infection of the skin caused by *S. aureus*.

Method:

Samples were taken from 204 patients with impetigo disease. *S. aureus* isolates were tested for their antimicrobial susceptibility. Genomic DNA of *S. aureus* was used to transform *E. coli* HB101 strain and expression capability of *S. aureus* plasmids in transformed *E. coli* was investigated. 68.62% (140/204) of the specimens were nonbullous impetigo and 31.38% (64/204) were bullous impetigo. *S. aureus* strains were isolated from 41.66% (85/204) of impetigo cases (82.35% from nonbullous and 17.65% from the bullous impetigo). There was an inverse relationship between the incidence of *S. aureus* isolated and age.

Result:

Three biotypes of *S. aureus* were identified based on their fermentation of different sugars. All isolates were resistant to penicillin and most isolates were resistant to ampicillin (95.3%), amoxicillin, (94.11%) and cephalexin (90.95%). Most isolates were sensitive against vancomycin and rifampicin (98.83%). 5.88% (5/85) of *S. aureus* isolates were identified as MRSA. A maximum of 5 markers from *S. aureus* isolates were capable to be expressed in transformed *E. coli* HB101 strains. The incidence of impetigo caused by *S. aureus* is comparable with reports from elsewhere. *S. aureus* isolates showed multidrug resistance against antibiotics.

Conclusion:

Plasmids of *S. aureus* are capable to show its expression in *E. coli* HB101. Molecular study is needed to investigate the role of plasmids in different patterns of multi drug resistance.

Key words: Impetigo, *S. aureus*, antibiotic resistance, plasmid transfer, Duhok, Iraq.

INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community acquired infections [1]. The severity of these infections varied from local simple wounds to severe systemic diseases. This pathogen causes a range of diseases, including skin abscesses, wound infections, osteomyelitis, endocarditis, pneumonia, bacteremia and toxemic syndromes [2]. Impetigo is a common contagious bacterial infection of the skin caused by *S. aureus*. This infection is mostly seen in children, especially in tropical countries [3]. Impetigo spreads from person to person through

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direct contact with the discharge from the sores [4]. Two types of impetigo are diagnosed, nonbullous impetigo which represents a host response to the infection, and it is characterized by reddened sores with honey-yellow crusting on them, and bullous impetigo which is caused by staphylococcal toxin and no host response is required to manifest clinical illness, and it is characterized by bullae large thin-walled blisters that contain clear or cloudy yellow fluid [4].

S. aureus is one of the most successful pathogens to acquire antibiotic-resistance mechanisms [5]. Methicillin-resistant *S. aureus* (MRSA) is an important pathogen causing poisoning and toxin mediated infections. The resistance in MRSA is related to a chromosomal *mecA* gene that specifies the production of an abnormal penicillin–binding protein called PBP2a, resulting in resistance not only to methicillin but to all β–lactam [6]. Most plasmids have a narrow host range but some can replicate in a wider host range [7]. The genetic exchange of plasmids containing antibiotic resistant genes among organisms is believed to play a crucial role in the evolution of antibiotic resistant bacteria. Staphylococcal β-lactamase is carried on plasmids and can be inducible or non-inducible with antibiotic contact [8]. Plasmid mediated drug resistance in *S. aureus* to chloramphenicol, gentamycin, tobramycin and kanamycin has been documented [9]. The aim of this study was to determine the occurrence of impetigo cases and the occurrence of multidrug resistant *S. aureus* in these cases in Duhok city. Additionally, this work aimed to examine the expression ability of *S. aureus* plasmids in *E. coli* HB101 by transformation.

MATERIALS AND METHOD

Sample Collection

A total of 204 specimens were collected from patients with impetigo disease at Azadi General Hospital in Duhok city from November 2008 to April 2009. Samples were collected either by using sterile disposable cotton swabs moistened in brain heart infusion (BHI) broth or by sterile forceps from infected skin. The study was conducted with the approval of ethics committee of the University of Duhok, School of Science.

Identification and Characterization

The samples were cultured in BHI broth and incubated overnight at 37˚C. Samples were subcultured on blood agar and mannitol salt agar (MSA) and incubated at 37˚C for overnight. The isolates were identified as *S. aureus* based on morphology, Gram stain, catalase test, coagulase test, DNase test and mannitol salt agar fermentation. Suspected *S. aureus* colonies were further identified using API® Staph Kit **(**Biomerieux, France).

Antibiotic Sensitivity Test

All *S. aureus* isolates were tested against 13 different antimicrobial drugs (Bioanalyse, Turkey) as listed in Table **1**. Screening for antibiotic resistance was performed using disk diffusion assay according to Bauer *et al*. [10]. Bacterial suspensions were prepared in 1.0 ml of sterile saline solution. 0.5 ml of suspensions (optical density of 0.3 at 600nm) was spread on Mueller-Hinton agar (Oxoid Limited, Hampshire, England) and then incubated at 37˚C for 24 hours. The inhibition zones were measured according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [11].

Table 1. A list of the thirteen tested antimicrobials, their classes, and the concentrations used for susceptibility testing.

Class		Antimicrobial	Symbol	Disk potency/ µg
β-lactams	Penicillins	Penicillin	PG	10
		Amoxicillin	Ax	30
		Methicillin	Met	5
		Ampicillin	AMP	30
	Cephalosporins	Cephalexin	CL	30
		Cephotaxime	CTX	30
Fluoroquinolones		Ciprofloxacin	CIP	5
Macrolides		Erythromycin	E	15
Fusidanes		Fusidic Acid	FA	10
Lincosamides		Lincomycin	L	2
Rifamycin		Rifampicin	RA	5
Sulfonamides		Trimethoprim	TMP	5
Glycopeptides		Vancomycin	VA	30

Plasmid Extraction and Gel Electrophoresis

Plasmid DNA of *S. aureus* was isolated according to Lema *et al*. [12]. Briefly, *S. aureus* cells were harvested from an overnight 10 ml culture and the pellet was suspended in 1ml TE buffer pH 7.4 (10mM of Tris-base and 1mM of EDTA). Suspended cells were distributed into 1ml tubes and centrifuged at 6000 rpm for 5 minutes. The pellet was suspended in 200µl acetone and incubated on ice for 5 minutes then centrifuged at 6000 rpm for 5 minutes. The pellet was resuspended in 100µl of TE buffer (contain lysozme 2mg/ml) and incubated at 37˚C for 1 h. 300µl of TESN (lysis) buffer with pH 7.4 (10mM of Tris-base, 1mM of EDTA, 1% of SDS and 6 M of NaI) as added and incubated at 65˚C for 5 minutes, then 300µl isopropanol was added and incubated for 5 minutes at room temperature. The mixture was centrifuged at 6000 rpm for 2 minutes and the pellet was resuspended in 500 µl of isopropanol 40% and centrifuged at 10000 rpm for 10 minutes. The pellet was resuspended in 1ml of ethanol 70% and centrifuged at 10000 rpm for 10 minutes. Finally, the pellet was suspended in 100µl of TE buffer and stored at -20˚C. The DNA was analyzed by 1.5% agarose gel electrophoresis (80 V in 1× TAE buffer).

Transformation

DNA transformation was achieved according to Sambrook *et al.* [13]. The preparation of the competent cells and DNA uptake were achieved as follows:

Preparation of Competent Cells

Single colony of *Escherichia coli* HB101 strain was inoculated into 100ml nutrient broth and incubated at 37˚C for 3h and then was incubated on ice for 10 minutes. Cells were harvested by centrifuge at 4000 rpm for 10 minutes at 4˚C. The pellet was suspended in 10 ml of 0.1M calcium chloride. The mixture was centrifuged at 4000 rpm for 10 minutes at 4˚C. The pellet was suspended in 2 ml of 0.1M calcium chloride and kept on ice.

DNA Uptake

10µl of the genomic DNA of *S. aureus* was mixed with 100µl of the competent cells of *E. coli* and incubated on ice for 30 min. Tubes were exposed to heat shock at 45˚C for 90 seconds, then to ice for 1-2 minutes. 800µl of nutrient broth was added and incubated at 37˚C for 45 minutes. Transformed *E. coli* colonies were screened on nutrient agar containing 100mg/ml penicillin. Transformed cells were tested for their acquisition of resistance antibiotics. The susceptibility profile of transformants was compared with that of the *S. aureus* isolate and *E. coli* HB101 strain used in transformation.

RESULTS

Isolation and Characterization of *S. aureus*

A total of 204 specimens were collected from patients clinically diagnosed with impetigo at Azadi general hospital in Duhok city. Out of 204 cases, 140 (68.62%) specimens were nonbullous impetigo and 64 (31.38%) were bullous impetigo. Prevalence rate of *S. aureus* was 41.66% (85/204). The incidence of *S. aureus* isolated from nonbullous impetigo included 70 isolates (82.35%) and 15 isolates (17.65%) were from the bullous impetigo specimens. Twenty isolates were selected randomly and confirmed as *S. aureus* by API Staph System test. Based on their fermentation to different sugars, these twenty isolates were classified into three biotypes (Table **2**).

Table 2. The *S. aureus* biotypes according to their fermentation to different sugars.

No. of isolate (%)	GLU	FRU	MNE	MAL	LAC	TRE	MAN	XLT	MEL	NIT	PAL	VP	RAF	XYL	SAC	MDG	NAG	ADH	URE
11 (55)	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	+
6 (30)	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	+	-	+	-
3 (15)	+	+	+	+	+	+	+	-	-	+	-	-	+	-	+	+	+	-	-

GLU: Glucose, FRU: Fructose, MNE: Mannose, MAL: Maltose, LAC: Lactose, RE: Trehalose, MAN: Mannitol, XLT: Xylitol, MEL: Melibiose, NIT: Nitrate reduction, VP: Acetoin Production, RAF: Raffinose, XYL: Xylose, SAC: Sucrose, URE: Urease, MDG: α -methyl-D- glucoside, NAG: N-acetyl-glucosamine, ADH: Arginine dihydrolase.

The percentage of *S. aureus* prevalence in age group from few months to 5 years was 50.66% and increased to 55.55% in the age group of 6-10 years. Then the percentages were continually decreased to 14.3% in the age group of more than 30 years (Table **3**).

Table 3. The incidence of *S. aureus* isolates from impetigo cases in different age groups.

Age group years	No. of impetigo cases	No. of *S. aureus* isolates (%)
0-5	75	38 (50.66)
6-10	54	30 (55.55)
11-20	43	12 (27.9)
21-30	25	4 (16)
≥ 31	7	1 (14.3)

Antibiotic Susceptibility Test

All 85 isolates of *S. aureus* were tested for their susceptibility against thirteen different antibiotics. Results indicated that all isolates were resistant to penicillin G, and most isolates were resistant to ampicillin (95.3%), amoxicillin (94.11%) and cephalexin (90.95%). On the other hand, most isolates were sensitive against vancomycin and rifampicin (98.83%) (Table **4**).

Table 4. Antimicrobial resistance pattern of *S. aureus* isolates.

Antibiotics	No. of resistant isolates (%)
Penicillin	85 (100)
Ampicillin	81 (95.3)
Amoxicillin	80 (94.11)
Cephalexin	77 (90.58)
Lincomycin	57 (67.05)
Cefotaxime	41 (48.23)
Erythromycin	10 (11.76)
Ciprofloxacin	5 (5.88)
Methicillin	5 (5.88)
Trimethoprim	3 (3.52)
Fusidic Acid	2 (2.35)
Vancomycin	1 (1.17)
Rifampicin	1 (1.17)

Out of 85 isolates, 5(5.88%) were found to be methicillin resistant *S. aureus* (MRSA).

The Plasmid Profile of *S. aureus* Isolates

In our study, 10 different isolates showed multi drug resistance (MDR) against at least three different antibiotics. These isolates were selected and the plasmid profile was investigated. All tested isolates contained two small plasmids represented by two bands (Fig. **1**).

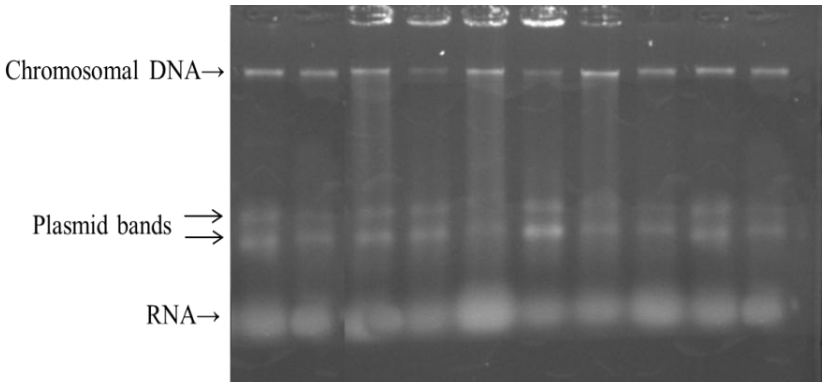

Fig. (1). Agarose gel electrophoresis (1.5% agarose) of purified DNA from different MDR *S. aureus* isolates.

Transformation

In order to investigate the expression ability (antibiotic resistance) of the genetic marker in *E. coli*, genomic DNA from MDR *S. aureus* isolates was used to transform *E. coli* HB101 rifampcin resistant competent cells. All ten antibiotic resistant markers were checked for their transformation to *E. coli* HB101. Transformants *E. coli* were screened on nutrient agar supplemented with different antibiotics. Results showed that only a maximum of 5 markers (penicillin, ampicillin, amoxicillin, cephalexin and lincomycin) were capable to transfer transformation from *S. aureus* isolates which have different patterns of MDR (Table **5**).

Table 5. Transforming of *S. aureus* genetic markers to *E. coli* HB101.

Antibiotic resistant	*S. aureus*	*E. coli* HB101	*E. coli* HB101Transformation
Penicillin resistant	ve+	ve-	ve+
Ampicillin resistant	ve+	ve-	ve+
Amoxicillin resistant	ve+	ve-	ve+
cephalexin resistant	ve+	ve-	ve+
Lincomycin resistant	ve+	ve-	ve+
Rifampcin resistant	ve-	ve+	ve+

ve+: Positive, ve-: Negative

DISCUSSION

Although *S. aureus* is an important pathogen, many healthy people carry it as part of the nose, throat, perineum and skin microbiota [14]. Our results were in agreement with those obtained from other studies which showed that non-bullous impetigo was the most common form of impetigo and that *S. aureus* was the main bacterium that causes non-bullous impetigo [4, 15]. In studies conducted in Kurdistan region, *S. aureus* isolated from different clinical and environmental sources were also characterized into three biotypes [16, 17]. Results revealed that there is an inverse relationship between the percentage of isolated *S. aureus* and patient age. Similar patterns of these results were observed in different studies conducted in Iraq and worldwide [18, 19].

Overuse and misuse of antibiotics have led to increased levels of antibiotics resistance [20 - 22]. Antibiotic susceptibility of isolated *S. aureus* was found to be comparable with other studies conducted locally and worldwide in which *S. aureus* clinical isolates from different hospitals and medical institutions were found to be highly resistant to penicillin G (92% to 100%) and highly sensitive to vancomycin [16, 17, 23]. High resistance to penicillin is due to the ability of *S. aureus* to produce β- lactamase enzymes [24]. It is known that most *S. aureus* clinical isolates are β-lactamase producers [23]. *S. aureus* is also highly sensitive against rifampcin because this antibiotic specifically inhibits bacterial RNA polymerase [25].

MRSA is an increasing problem worldwide in community and hospital environment [26 - 29]. MRSA colonization rate varied in different countries from about 1% to 35% [22, 26, 27, 30 - 34]. Combination of host, geographical, environmental and bacteria factors could contribute to the nasal carriage of *S. aureus* and MRSA among population. There is evidence suggesting that development of antibiotic resistant human microflora in hospitals is mainly caused by the use of antimicrobial agents by the medical profession [35]. The health care workers usually act as asymptomatic carriers of multiple drug resistance organisms, especially MRSA, and help in its transmission to patients [36].

Studies with *S. aureus* clinical isolates showed that these isolates harbored many plasmids of different sizes. Kloose *et al*. [37] described plasmids with sizes between 2-29kb. Another plasmid profile study was conducted with 88 strains of *S. aureus* isolated causing impetigo cases and showed that most strains harbor different size plasmids between 1.29-25 kb [38].

S. aureus plasmids were found to be expressed in *E. coli* HB101. These results were in agreement with those obtained from different studies carried out worldwide [23, 39, 40]. A study showed that 23 kb plasmid from a selected multidrug resistant strain of *S. aureus* was used to transform a sensitive *E. coli* LE 392 and it was found that *E. coli* LE 392 which was sensitive to ampicillin, amoxycillin and penicillin before transformation became resistant to these drugs [39]. Results revealed that transformants *E. coli* acquired resistance against penicillin, ampicillin, amoxicillin, cephalexin and lincomycin. Resistance to penicillin, ampicillin and amoxicillin is mediated by beta-lactamases, which may also act against first generation cephalosporins, such as cephalexin. Thus, a single genetic marker transferred from *S. aureus* to *E. coli* could be responsible for the acquisition of resistance to these 4 antibiotics. It is revealed that five *S. aureus* plasmids, coding for tetracycline or chloramphenicol resistance, have been transformed, replicated and

expressed into *Bacillus subtilis* [41]. It is believed that the multiple drug resistance of *S. aureus* is due to several drug resistance genes in a single plasmid each with its own different resistance markers [42].

It is worth mentioning that other mobile genetic elements may have been incorporated to *E. coli* cell and even into its chromosome, and more in-depth studies would be needed to identify this event. Furthermore, it would be important to detect the presence of the plasmid in the transformed *E. coli* cell, confirming that the acquired resistance is actually related to the presence of the plasmid. There are many factors that play role in selection of the multi-drug resistant strains such as drug misuse, prescription of antibiotic without culturing, identification, and determination of susceptibility tests of pathogen, and giving of broad–spectrum antibiotics instead of narrow-spectrum antibiotics.

CONCLUSION

This study concluded that the incidence of impetigo caused by *S. aureus* is comparable with reports from elsewhere. Most *S. aureus* isolates showed multidrug resistant phenomena against different antibiotics. Plasmids from these multidrug resistant isolates were capable to transform *E. coli* HB101 strain into multidrug resistant bacteria. Further molecular studies are needed to investigate the role of plasmids in different patterns of MDR.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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