Phenotypic and Genotypic (*mec*A gene) Characterization of Borderline Oxacillin Resistant *Staphylococcus aureus* (BORSA) Isolated in Al-Hilla City

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Abstract

Out of 130 clinical samples, a total of 37 (28.4%) *Staphylococcus aureus* isolates were recovered, from skin samples, ear, blood and throat, and both urine and vagina as (75.6), (8.1%), (5.4%), and (10.8%), respectively. Results showed that 21 (56.7%) isolates were identified as β-lactam resistant. The susceptibility to 25 antibiotics were tested using disc diffusion test resulting in 80% of 21 bacterial isolates were resistant to 22 antibiotics but they were sensitive to teicoplanin, netilmicin and chloramphenicol. For detection of Borderline oxacillin resistance *S.aureus* (BORSA), all of 21 isolates were tested by using oxacillin disc (1mcg). Results showed that all 21 isolates were resistant to oxacillin and identified as MRSA as they were resistant to both oxacillin and cefoxitin. Two–fold agar dilution susceptibility method to oxacillin and vancomycin were performed for 21 isolates. All 21 isolates showed resistance to oxacillin, and 16 (%76) were sensitive to vancomycin while 5 (%24) decreased susceptibility to vancomycin (VISA). All 21 isolates had *mec*A gene and identified as MRSA and no BORSA detected in this study which lack *mec*A gene.

الخلاصة

تم عزل ٣٧ (٢٨,٤%) من المكورات العنقودية الذهبية من ١٣٠ عينة سريرية، كانت ماخوذة من الجلد، الاذن، من الدم والبلعوم، و من الادرار والمهبل وبنسب ٧٥,٦%، ٨,١%، ٥,١% من الدم والبلعوم، و ١٠,٨% من الادرار والمهبل على التوالي. بينت النتائج ان ٢١عزلة (٥٦,٧%) كانت مقاومة للبيتالاكتام. تم اختبار استجابة ٢١ عزلة من المكورات العنقودية لـ ٢٥ مضاد حيوي بواسطة فحص انتشار الاقراص فتبين انها مقاومة بنسبة ٨٠% لـ ٢٢ مضاد حيوي لكنها كانت حساسة للتيكوبلانين ، نيتليمايسين و كلورامفينيكول . للكشف عن المكورات العنقودية المتوسطة المقاومة للاوكساسيلين (BORSA) ، تم اختبار ٢١ عزلة من المكورات العنقودية باستخدام قرص الاوكساسيلين. وأظهرت النتائج أن ٢١ عزلة بكتيرية كانت مقاومة للأوكساسيلين. واعتبرت مكورات عنقودية مقاومة للمثيسيلين كونها مقاومة للأوكساسيلين والسيفوكسيتين. تم اجراء اختبار التركيز المثبط الادنى على الوسط الصلب للاوكساسيلين والفانكومايسين على الـ ٢١ عزلة حيث وجد ان هذه العزلات كانت مقاومة للاوكساسيلين وان ١٦ (٧٦%) كانت حساسة للفانكومايسين بينما ٥ (٢٤%) متوسطة المقاومة للفانكومايسين (VISA). اظهرت النتائج ان ٢١ عزلة كانت تمتلك مورثة *mec*A لذلك تم اعتبارها مقاومة للمثيسيلين (MRSA) ولم يتم الكشف عن عزلات BORSA في هذه الدراسة والتي تفتقر الى وجود هذا الجين.

Introduction

Borderline oxacillin resistance *Staphylococcus aureus* (BORSA) hyperproduce extracellular β-lactamase; however, in contrast to ORSA strains, BORSA strains tend to have a normal repertoire of PBPs, with expression of PBP 2a conspicuously absent. BORSA resistance thought to be at least in part related to β-lactamase hyperproduction [1]. However, [2] has shown that, although quantitatively normal, the major PBP components of certain BORSA (particularly PBPs 1 and 2) may manifest relatively low affinities for methicillin. BORSA initially described non-heteroresistant strains of *S.aureus* with oxacillin MIC ≤2 mg/L, which produce β-lactamases and are rendered fully susceptible to PRP by β-lactamase inhibitors [3,4,5]. Subsequent BORSA strains described have had higher oxacillin MICs (4-8 mg/L) [4]. The proportion of BORSA among clinical isolates of *S.aureus* varies (1.4%-12.5%) but is usually 5%. A BORSA infection outbreak among dermatology patients with severe skin diseases has also been reported [6]. For BORSA-associated infections, β-lactam antimicrobial drugs, including high-dose penicillinase-resistant penicillins (PRPs) (e.g., cloxacillin) or β-lactam/β-lactamase--inhibitor combinations (e.g., ampicillin / sulbactam) are regarded as treatments of choice [7,3,4]. Cystic fibrosis (CF) patients with *mec*A positive MRSA most commonly acquire their infection through person to person transmission whereas BORSA is likely preferentially selected out from endogenous MSSA in CF patients due to persistent antibiotic pressure [8]. The aim of this study was to determine the prevalence of BORSA isolates in Hilla City and patterns of resistance to antibiotics that its owned.

Materials and Methods

Bacterial isolates:

Thirty seven *S.aureus* isolates were obtained from clinical samples in Al-Hilla/Iraq during the period from October 2012 to 25 of January 2013. Clinical samples were collected from the main three hospitals in Al-Hilla city (Al-Hilla teaching hospital, Babylon maternity and pediatrics teaching hospital and Marjan hospital), in addition to some private clinic. Clinical isolates were as follows: skin swabs (19), wound (5), burn (4), ear (3), blood (1), throat (1), urine (2), and vagina (2). These bacterial isolates were identified as *S.aureus* based on their morphology, Gram-staining, catalase properties. Vitek 2 system and coagulase test were performed to identify *S.aureus* isolates.

Primary detection of BORSA by oxacillin disc:

Use oxacillin (1mg) disc for detection BORSA in the present study.

Antimicrobial Susceptibility Test:

1- Screening for β-lactam Resistance:

Screening for β-lactam resistance (ampicillin and amoxicillin) were determined according to [9]. All the 37 *S.aureus* isolates were subjected to β-lactam resistance screening test as a phenotypic selection test. Results were determined according to presence or absence of colony patch.

2- Disc diffusion test (DD test):

The antimicrobial susceptibility patterns of isolates to different antimicrobial agents was determined and interpreted according to [5]. Disk diffusion test was used against 20 antibiotics, the following antimicrobial agents were obtained (from Oxoid, U.K) as standard reference disks as known potency for laboratory use: pencillin (10mcg), oxacillin (1μg), cloxacillin (1mcg), cefoxitin (30mcg), ampicillin (l0mg), amoxicillin-clavulanate (20/10mg), ampicillin-sulbactam (10/10mcg), cefepime (30mcg), cefotaxime (30mcg), cefotetan (30 mcg), ceftazidime (30mcg), ceftriaxone (30mcg), cephalothin (30mcg), imipenem (10mg), meropenem (10mg), teicoplanin (30mcg), netlimicin (30mcg), tobramycin (10mcg), azithromycin (15mcg), and tetracycline (30mcg).

3- Determination of minimum inhibitory concentration (MIC):

The MIC test for each oxacillin and vancomycin was determinated according to [10].

the MIC was recorded as the lowest concentration of the antimicrobial agent that completely inhibit growth or that concentration (µg/ml) at which no more than two colonies were detected. The MIC values were compared with the breakpoints recommended by [5].

DNA extraction:

Chromosomal DNA was extracted according to the genomic DNA purification kits supplemented by manufacturer company (Geneaid, UK).

Detection *mec*A gene in the present study:

*Mec*A gene were detected by PCR. Each 25µl of PCR reaction mixture contained 2.5µl of upstream primer, 2.5µl of downstream primer, 2.5µl of nuclease free water, 5µl of DNA extraction, and 12.5µl of master mix. Thermal cycling conditions are showen in the table1. The primer of *mec*A used generated 533bp fragments. The amplification products were separated in 1% agarose gel containing ethidium bromide. DNA ladder 100bp (Bioneer, Korea) consist of 13 linear double-stranded DNA fragment with size 100-2000bp used for compare. After electrophoresis, the gel was photographed under UV light.

Table 1 primer sequence and thermal cycling conditions

Primer	Primer Sequence (5'-3')	Product size (bp)	PCR condition	Reference
*mec*A1	AAA ATC GAT GGT AAA GGT TGG	533	94°C 5min 1x	[26]
*mec*A2	AGT TCT GCA GTA CCG GAT TTG		94°C 30sec 55.5°C 30sec 40x 72°C 1min	
			72°C 5min 1x	

Results and Discussion

Isolation and Identification of *Staphylococcus aureus* isolates:

A total of 130 clinical samples were collected during this study, 37 (28.4%) *Staphylococcus aureus* isolates were recovered during this study, the higher percentages were from the skin 51% while the lower percentages were from the blood and throat samples (Table 2). The majority of *S.aureus* isolates was detected in the skin and soft tissue (SSTs), wound, and burn infections as 51.3%, 13.5% and 10.8%, respectively. This is due to the fact that *S.aureus* is the almost-universal cause of furuncles, carbuncles, and skin abscesses and worldwide is the most commonly identified agent responsible for skin and soft tissue infections. *S.aureus* skin and soft tissue infections frequently begin as minor boils or abscesses and may progress to severe infections involving muscle or bone and may disseminate to the lungs or heart valves (i.e., endocarditis) [11]. The present result was approximately similar to a result study conducted in 2004 in emergency departments in 11 US cities found that MRSA was isolated from 59% of patients with skin and soft tissue infections [12]. Also, in the present study the existence of *S.aureus* isolates in urine and vagina was 5.4% for each one. on the other hand *S.aureus* from urine samples is often secondary to staphylococcal bacteremia arising elsewhere (e.g., endocarditis) [13]. Also use urinary tract instrumentation and the presence of an indwelling catheter increase the risk of *S.aureus* carriage in the urinary tract [14]. [15] who found that *S.aureus* in urinary tract infection was 82% had undergone recent urinary catheterization. The study of [16] was confirmative to the result of the present study where, they found that *S.aureus* in genitourinary tracts was 7.1%. Based on a study of [17] comparing superantigen profiles of *S.aureus* vaginal colonizing isolates from 1980 and 1981 to 2003–2005, the increased incidence of TSS was most likely due to the increase in the prevalence of vaginal *S.aureus* (from 12 to 23%) or non-menstrual TSS associated cases, instead of an increasing proportion of TSST isolates in vaginal colonization strains**.** In the present study the existence of *S.aureus* isolates in throat and blood were 2.7% for each one. This low percentage may be due to few numbers of samples collected. [18] they were found that %38 of persistent carriers in throat while only 5% were preferential anterior naris. In the present study the percentages of occurrence of *S.aureus* in ear infection was 8.1%. Also, the present result was in accordance with [19] who found low number of otitis media caused by *S.aureus*. However the study of [20] showed that 49% of patients were infected with otitis media.

Table 2 Numbers and percentages of *S.aureus* isolates recovered from different sources of infections

Source of samples	No. of samples	No. of *S.aureus* isolates	Percentage %
Skin swabs*	37	19	51.3
Wound swabs	25	5	13.5
Burn swabs	20	4	10.8
Ear swabs	29	3	8.1
Blood swabs	4	1	2.7
Throat swabs	6	1	2.7
Urine swabs	7	2	5.4
Vagina swabs	2	2	5.4
Total number	130	37	100

* skin swabs represented folliculitis, boils, and abscesses

Primary screening of β-lactam resistant isolates:

All the 37 *S.aureus* isolates were subjected to β-lactam resistance screening test as a phenotypic selection test. Such two β-lactam antibiotics (ampicillin and amoxicillin) were selected because they are the most commonly used antibiotics in the therapy of bacterial infections, compared to other β-lactam antibiotics. A part from their therapeutic usage, these antibiotics can provide a comprehensive primary screeing of β-lactam resistant isolates, because the isolates that is resistant to carbenicillin and cephalosporin, is already resistant to ampicillin and amoxicillin [21]. The results showed that 21 isolates (56.7%) of *S.aureus* were resistant to both antibiotics. All these isolates were able to grow normally in presence of ampicillin and amoxicillin that refer to most of the *S.aureus* isolates (about 90% of the isolates) coming from several infectious sources (nosocomial infections and other anatomical sites) were resistant to penicillin due to producing β-lactamases, and the existence *mec*A gene in chromosomal DNA of *S.aureus* codifies a protein (PPB2a) that confers resistance to synthetical penicillin [22].

Antibiotic susceptibility by disc diffusion method (DD method):

In this study, 25 antibiotics performed to all 21 *S.aureus* isolates for testing their susceptility and to identify the most effective one against *S.aureus*, because indiscriminate using of multiple broad spectrum antibiotic may associated with increased risk of MRSA infection [23]. The results revealed that all 21 *S.aureus* isolates showed high resistance (100%) to pencillin and ampicillin as shown in (Figure1).

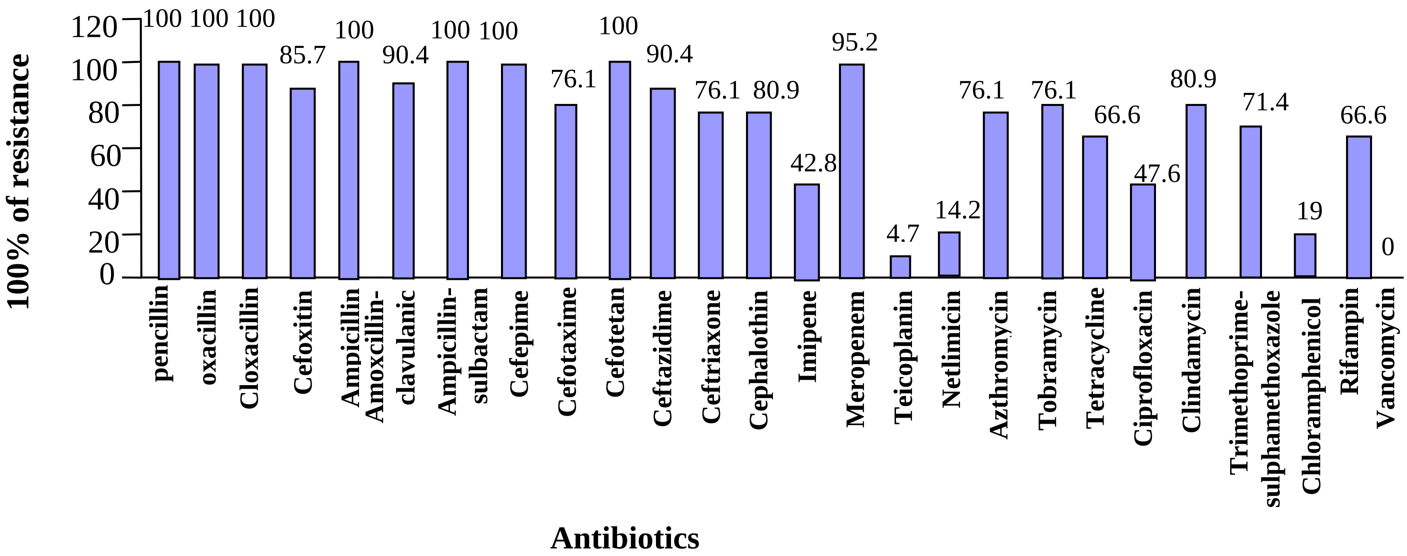

Figure 1 Resistance of 21 *S.aureus* isolates to different antibiotics by DDT

These results were conformative with [24] who found that (100%) of *S.aureus* isolates were resistant to ampicillin. Resistance to amoxicillin-clavulanic acid was (90.4%) in this study. [25,26] were found that BORSA was sensitive to the amoxicillin-clavulanic acid disk. In the present study, the highly resistance to ampicillin-sulbactam (100%). The mechanism of resistance to β-lactam antibiotic is mostly due to either production of β-lactamases that hydrolyze β-lactam ring, or lack of penicillin receptors on cell wall and / or alteration in their permeability to β-lactam antibiotics preventing the uptaking of them [27]. Results also showed that the resistance rate to both of oxacillin and cloxacillin resistance were 100% (Figure1). Results of Cephalosporins; cephalothin (1st generation), cefotetan, cefoxitin (2nd generation), cefotaxime, ceftriaxone, ceftazedim, (3rd generation), and cefepime (4th generation) showed that percentages of *S.aureus* isolates resistant was substantial to these antibiotics: 80.9%, 76.1%, 76.1%, 90.4%, 100%, 100%, 85.7%, respectively. Resistance to cephalosporins mediated by cephalosporinase production that may be producing from this bacteria. Furthermore, β-lactamase that producing by staphylococci may excreted into the surrounding environment by which the hyper-production of β-lactamase will give longer validity and surviving to this bacterium, because the hydrolysis of β-lactams takes place before the drug can bind to PBPs in the cell membrane [28]. In the present study the imipenem and meropenem resistance were 42.8% , 95.2%, respectively (Figure1). [29] detected that meropenem is a well-established carbapenem that is more active than imipenem against gram-negative pathogens and somewhat less active than imipenem against gram-positive pathogens.

In the present study, *S.aureus* isolates showed low resistance rate (4.7%) to teicoplanin. Results showed that resistance to netilmicin, tobramycin accounted for 14.2% , 76.1%, respectively (Figure1). [8] found that usage of oral cephalexin and inhaled tobramycin prior to index culture was significantly and independently associated with acquisition of BORSA. All of 21 *S.aureus* isolates 76.1% were found to be resistant to azithromycin. Macrolides inhibit protein synthesis by binding to the 50S ribosomal subunit causing an inhibition of translocation of peptidyl-tRNA and the initial steps of 50S subunit assembly. The spectrum of activity of macrolides include aerobic Gram positive bacteria, including *Staphylococcus* spp. [30]. *S.aureus* isolates results showed 66.6% resistance to tetracycline. [31] explained that 55% of *S.aureus* were resistant to tetracyclines. Disk diffusion test for vancomycin was not performed in this study due to the fact that procedure of disc diffusion cannot differentiate isolates with reduced susceptibility to vancomycin (MIC 8 to 16 µg/ml) from susceptible isolates (MIC $\leq$ 4 µg/ml) even when incubated for 24 hrs.. Additionally, vancomycin resistant *S.aureus* (VRSA) strains (MIC $\geq$ 32µg/ml) may produce only subtle growth around a vancomycin disk according to [5]. In the present study, Out of 21 *S.aureus* isolates, detected by MIC method of vancomycin 16 isolates (76%) were sensitive to vancomycin, while 5 (24%) were reduced susceptible to vancomycin (VISA). And no isolates showed any degree of resistance to vancomycin (Figure1). Vancomycin MICs of the VRSA isolate were consistent with the *Van*A phenotype of *Enterococcus* species, and the presence of the *van*A gene was confirmed by polymerase chain reaction. The DNA sequence of the VRSA *van*A gene was identical to that of a vancomycin-resistant strain of *Enterococcus faecalis* recovered from the same catheter tip. The *van*A gene was later found to be encoded within a transposon located on a plasmid carried by the VRSA isolate [32]. Vancomycin intermediate *S.aureus*, It is also termed GISA (glycopeptide-intermediate *S.aureus*), indicating resistance to all glycopeptide antibiotics. These bacterial strains present a thickening of the cell wall, which is believed to reduce the ability of vancomycin to diffuse into the division septum of the cell required for effective vancomycin treatment [33]. A study of [34] also found high sensitivity (100%) to vancomycin.

Detection of Borderline oxacillin resistant *S.aureus* (BORSA):

Disk diffusion test of BORSA isolates:

Conventional susceptibility test of *S.aureus* have been performed for the detection of resistance to oxacillin and other antibiotics by DDT according to the standard method recommended by [5]. In the present study, the results revealed that all of 21 *S.aureus* isolates did not give inhibition zones range between 11-12 mm of oxacillin resulting in that all of these isolates were resistant to oxacillin (Table 3). [25] isolated BORSA using oxacillin disc (5 mg). Oxacillin is stable under storage condition, and cefoxitin actually is an excellent inducer of the *mec*A gene [35]. According to that, oxacillin and cefoxitin resistant isolates were initially interpreted as MRSA. Therefore, all of the 21 *S.aureus* isolates were subjected for testing with cefoxitin to oxacillin resistance, Results demonstrated that 18 of 21 *S.aureus* were resistant to both of these antibiotics that considered them as MRSA isolates (Table 3). [36] showed that Nineteen strains were classified as borderline according to oxacillin MIC, resistant by oxacillin disk and sensitive to cefoxitin and thirtythree strains were classified as MRSA resistant by oxacillin and cefoxitin disk methods.

MIC of BORSA isolates:

As show in table 3, the MIC value of all 21 *S.aureus* reached to (64 µg/ml) that resulting in all of the isolates were MRSA. According to breakpoints recommended by [5], no BORSA isolates were detected in this study. [37] detected that BORSA was characterized by low levels of resistance 8µg/ml for oxacillin. While, [3,4,38] showed that BORSA can sometimes be confused with CA-MRSA because of similar clinical signs and symptoms and overlapping oxacillin MICs (28 µg/mL and 4-64 µg/mL, respectively).

Table 3 Antibiotic resistance 21 *S.aureus* isolates detected by DDT and MIC tests

Isolates designation	Oxacillin1µg (≤ 10 mm) *	Cefoxitin 30 µg (≤ 21 mm) *	Oxacillin MIC (≥ 4 µg/ml) *	Vancomycin MIC (≥ 32µg/ml)*
S2	7	11	64	0.12
S10	No inhibition zone	9	64	0.48
S17	No inhibition zone	10	64	0.96
S19	9	31(s)	64	8
S8	10	14	64	2
S14	No inhibition zone	12	64	0.24
S16	No inhibition zone	10	64	4
S1	8	11	64	4
S5	No inhibition zone	15	64	0.12
S6	9	20	64	4
S13	No inhibition zone	10	64	4
S9	No inhibition zone	No inhibition zone	64	4
W1	No inhibition zone	9	64	8
W3	No inhibition zone	33(s)	64	8
W4	6	12	64	8
W5	10	18	64	8
B1	No inhibition zone	11	64	8
B2	7	22(s)	64	8
B3	No inhibition zone	12	64	8
U2	10	11	64	2
V1	No inhibition zone	10	64	8

* Numbers between brackets refer to the breakpoints recommended by [5]
(s) Letters between brackets refer to the *S.aureus* isolates were sensitive to cefoxitin

Molecular identification and characterization of 21 *S.aureus* isolates:

Detection of *mec*A gene by PCR:

In the light of results mentioned above, all of 21 isolates were selected for genetic study, 18 of these isolates were oxacillin and cefoxitin resistant which occupied the primacy in the resistance of antibiotics and considered as MRSA, other isolates (n =3) uncertain as MRSA or BORSA due to they gave oxacillin resistant and cefoxitin sensitive, thereby selected them for genetic study to detect the gene that considered as marker for MRSA or BORSA. The selected of 21 *S.aureus* isolates were screened for the presence of the *mec*A gene. Monoplex PCR was used in the present study for detection of *mec*A gene. Polymerase chain reaction results detected *mec*A gene (100%) in all of the 21 *S.aureus* isolates (Figure 2a,b,c) resulting in all of these isolates were confirmed as MRSA even the three isolates that were sensitive to cefoxitin. BORSA cannot be detected in this study due to the presence *mec*A gene of oxacillin resistant isolates. In spite of *mec*A gene absent, borderline resistance to oxacillin because of hyper producer to β-lactamase [26]. The *mec*A gene responsible for mediating methicillin resistance in staphylococci [39]. The *mec*A gene carried on the staphylococcal cassette chromosome mec (SCC*mec*). SCC*mec* is inserted into the *S.aureus* chromosome near the origin of replication [40]. Most of antibiotic resistance are transferred by plasmids, while methicillin resistance is chromosomal transferred by transduction [41].

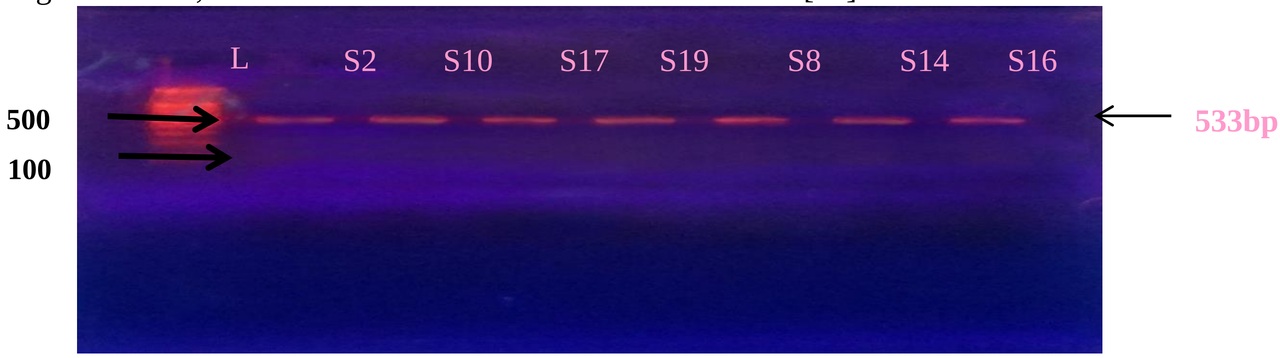

Figure 2a Gel electrophoresis of PCR of *mec*A amplicon product: Lane L: Ladder (٢000-bp), Lanes (S2, S10, S17, S19, S8, S14, S16) *S.aureus* isolates from skin samples.

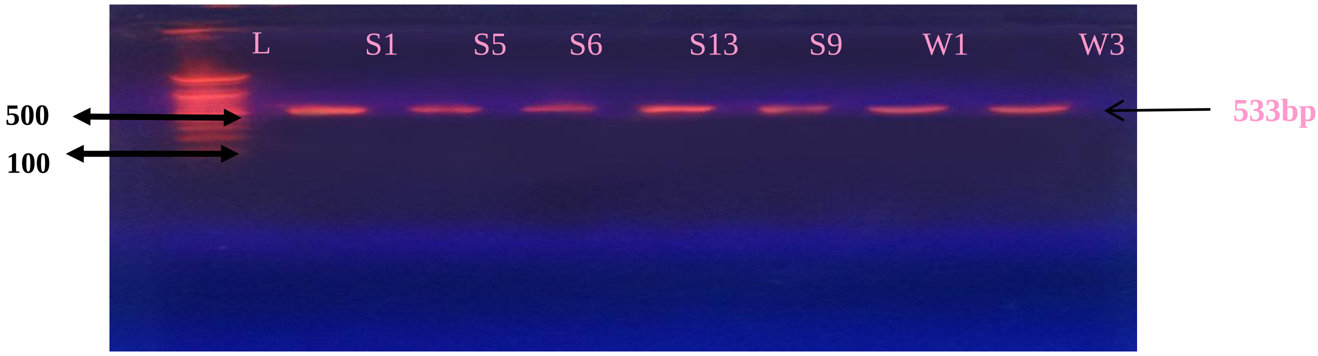

Figure 2b Gel electrophoresis of PCR of *mec*A amplicon product: Lane L: Ladder (2000-bp), Lanes (S1, S5, S6, S13, S9, W1, W3) *S.aureus* isolates from skin and wound samples.

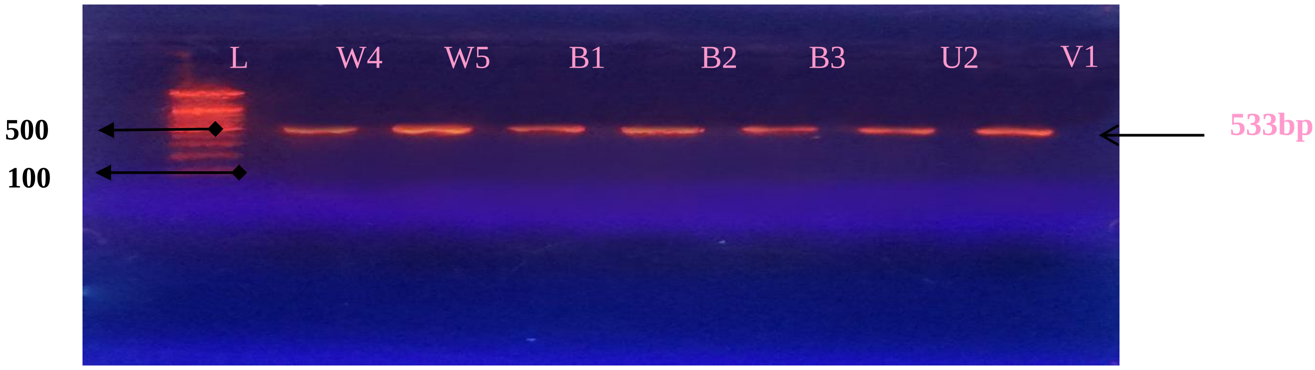

Figure 2c Gel electrophoresis of PCR of *mec*A amplicon product: Lane L: Ladder (2000-bp), Lanes (W4, W5, B1, B2, B3, U2, V1) *S.aureus* isolates from wound, burn, urine and vagina samples.

Conclusions

1. BORSA are not detected in the present study, resulting in all of the oxacillin-resistant *S.aureus* isolates turned into MRSA.
2. MRSA were predominant in skin, wound and burn specimens.
3. MRSA isolates were resistant to 80% of antibiotic mostly against β-lactams while they showed considerable degrees of susceptibility to teicoplanin, netlimicin and chloramphenicol.
4. Some of these MRSA isolates (24%) were detected to be VISA.

Recommendations

1. The use of MIC techniques, and gel-based PCR in the routine work of laboratories, to detect and recognize the genes that are responsible for antibiotic resistance.
2. Antibiotics should not be prescribed (for treatment of *S.aureus* infections) without medical instructions and testing for susceptibility against these antibiotics *in vitro*.

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