IDENTIFICATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* USING TOUCHDOWN PCR AND PHENOTYPIC METHODS FROM PATIENTS AND HOSPITALS ENVIRONMENTS IN DIFFERENT IRAQI CITIES

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ABSTRACT

The study was aimed to evaluate the prevalence of MRSA in some Iraqi hospitals and determine the most powerful methods for identification of MRSA, in order to achieve the, 278 samples were collected from different hospitals in Iraq in various intervals, 204 out of 287 were identified as *Staphylococcus aureus* by conventional cultural methods and microscopic characteristics and 177 isolates are identified as MRSA by using HiCrome MeReSa Agar Base medium, but 154 of 177 (87%) isolates are methicillin resistance in sensitivity test. MRSA isolates were highly resistant to β-lactam antibiotics and considered multidrug resistant (MDR) in percent of (94.9%). Touchdown PCR used to identify the isolates, 97.05% were identified as *Staphylococcus aureus,* while 80.88% as MRSA.

Key words: penicillin, antibiotics susceptibility, molecular analysis, MDR
***Part of Ph.D. dissertation of the 1st author.**

مجلة العلوم الزراعية العراقية -2021 :52 (6):1356-1364 زيد وقندلا

تشخيص المكورات العنقودية الذهبية المقاومة للميثيسيلين باستخدام تفاعل سلسلة البلمرة الهابطة والطرق المظهرية من عينات معزولة من مرضى وبيئات المستشفيات في العراق

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المستخلص

هدفت هذه الدراسة الى تقييم انتشار بكتريا المكورات العنقودية الذهبية المقاومة للميثيسيلين في بعض المستشفيات العراقية وتحديد الطرق الفعالة لتشخيصها، ولتحقيق هذا الهدف تم جمع ٢٧٨ عينة من مستشفيات مختلفة في العراق وفي فترات زمنية مختلفة، وقد شخصت ٢٠٤ عينة من اصل ٢٧٨ عينة على انها بكتريا المكورات العنقودية الذهبية و ١٧٧ عزلة منها كانت مقاومة للميثيسيلين باستخدام وسط اغار الكروموجينك، لكن ظهرت ١٥٤ من اصل ١٧٧ عزلة مقاومة للميثيسيلين باختبار الحساسية. هذه البكتريا من نوع مقاومة الادوية المتعددة وبنسبة (٩٤،٩%) وكانت مقاومة لمضادات البيتا لاكتام بنسبة عالية. وقد أظهرت نتائج التشخيص باستخدام تقنية سلسلة تفاعل البلمرة الهابطة ان ٩٧,٠٥% من العينات تابعة الى بكتيريا المكورات العنقودية الذهبية بينما كانت ٨٠٪ مقاومة للميثيسيلين.

الكلمات الافتتاحية: البنسلين، اختبار الحساسية، التحليل الجزيئي مقاومة ادوية متعددة.
***جزء مستل من أطروحة الدكتوراه للباحث الأول**

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INTRODUCTION

Human skin and mucosal microbiota contain *Staphylococcus aureus* which is a common opportunistic pathogen found in human body that causes significant infections (35). The percentage (20–30)% of the general population contain *Staphylococcus aureus* in the nasal mucosa (2). *Staphylococcus aureus* pass into bloodstream, heart, joints, device-related infection and bones during the disruptions of mucosal and cutaneous barriers (35, 21, 28, 2, 3). Although strains of *S. aureus* have been emerged and developed resistance to some antibiotics, some strains still sensitive to more commonly known antibiotics. Methicillin resistant *Staphylococcus aureus* (MRSA) are resistant to antibiotic methicillin and others related antibiotics (28, 14). MRSA is an opportunistic pathogen that produce different kinds of infections that vary from mild to invasive, life-threatening infections (17). United Kingdom is the first place where MRSA reported in, during 1960's after the usage of methicillin in the healthcare system (19), then have been reported in different regions (10, 23, 16). Penicillin-binding protein 2a (PBP 2a) that is coded by gene known as *mecA* and confer methicillin-resistance in MRSA that results in resistance to beta-lactam antibiotics such as Oxacillin, Cloxacillin and Methicillin (16, 20). Staphylococcal cassette chromosome *mec* (*SCCmec*) is a mobile genomic island that contains *mecA* gene which is inserted in the chromosome of MRS (10). There are 13 *SCCmec* types have been reported and designated types I-XIII according to differences in their size and structural organization (20). Increase carriage of MRSA may be caused by prolonged exposure to the hospital environment (2). Among health care workers, work-related factors for MRSA carriage include work experience and area of services, employment in places have high prevalence of MRSA patient and always contact with them, high workload poor hygiene (3). Physicians, clinical microbiologists, and public health officials within the same country and across other regions can have benefits from knowing MRSA antimicrobial resistance. The input is also useful for decisions regarding specific therapy of pathogen, formulary of hospital, and target-oriented infection domination policies (11, 27, 7, 5, 12). In addition, MRSA surveillance researches accomplished in community settings are important to best known the molecular and clinical epidemiology of emerging MRSA isolates (33), so, the main objective of current study is to specify the antibiotic susceptibility profiles and MRSA spreads in Iraqi hospital and its link with risk factors in healthcare workers at hospitals, as well as determine the most powerful methods for identification of methicillin resistance *S. aureus.*

MATERIALS AND METHODS

Collection of samples

The number of collected samples is 278 samples, that's include 139 samples from patients such as pus, sputum, ear infections, burn wounds, wounds, blood and foot ulcers, in addition to 58 from health workers such as epidermis and medical outfits, and 81 from hospitals environments such as infant incubators, blood pressure chuffs, microscopes, door knobs floor, sinks and trash. All samples were collected from local different hospitals in different Iraqi cities (Baghdad, AL-Diwanniyah, Najaf, Nasiriyah, Hillah and Kut) in different intervals (2015, 2016, 2017, 2018, 2019).

Bacterial isolation

Brain heart infusion agar and Mannitol salt agar were used for isolation of *S. aureus.* Depending on the morphological bases the suspected colonies were chosen and isolated for extra diagnostic tests. According to Bergey's manual of systematic bacteriology, the diagnosis of *S. aureus* was achieved (38).

Identification of *Staphylococcus aureus* isolates: Isolates which recorded as positive were assayed by Gram stain, catalase, oxidase as well as coagulase assays. The bacterial isolates which were recorded as mannitol positive, Gram positive, oxidase negative, catalase positive and coagulase positive were determined and identified as *Staphylococcus aureus*, then isolated for extra assays.

Identification of methicillin-resistant *Staphylococcus aureus:* Isolates that were identified as *Staphylococcus aureus* were cultured on HiCrome MeReSa Agar Base medium. The medium is used as a selective medium for MRSA isolation by the by

combining it with cefoxitin supplement (FD259) and MeReSa Selective Supplement (FD229). Positive colonies are detected by it's bluish green color and identified as MRSA and selected for future assays.

Antibiotics susceptibility

The antibiotics susceptibility test was accomplished by using disk diffusion methods as instructed by the Clinical and Laboratory Standards Institute (CLSI) guidelines (37). A disposable wire loop was used, the tops of few bacterial colonies were transmitted to a test tube that contain 5ml of BHIB and incubated at 37C° for (4-6)h until the turbidity appearance. Culture turbidity was estimated at ($1.5\times10^{8)}$ cell/ml by compared it to 0.5 McFarland standard (NO. 0.5) (8). The antibiotics used in this study were shown in Table 1.

Table 1. The antibiotics used in this study

Antimicrobial agent	symbol	Concentration µg/disk
Ampicillin	AMP	25
Amikacin	Ak	10
Azithromycin	AZM	15
Ampicillin\Cloxacillin	APX	30
Bacitracin	B	10
Cefoxitin	FOX	30
Ceftriaxone	CRO	10
Cephalothin	KF	30
Ciprofloxacin	CIP	10
Doxycycline	DO	10
Erythromycin	E	10
Imipenem	IPM	10
Methicillin	MET	10
Norfloxacin	NOR	10
Novobiocin	NV	30
Oxacillin	OX	5
Penicillin-G	P	10
Tobramycin	TOB	10
Vancomycin	VA	30
streptomycin	S	25

DNA extraction

The genomic DNA of the *Staphylococcus aureus* isolates was extracted using Genomic DNA purification kit purchased from (Promega/USA), the purity and concentration were tested, and the gel electrophoresis method was used for testing the integrity of DNA samples.

PCR amplification

A thermal cycler (BioRad, USA) were used to amplify PCR reactions. The mixtures of reaction was set up as follows: (1X) of GoTaq®Green Master Mix (Promega/USA), that composed of MgCl2, deoxynucleotides (dNTP), Taq DNA polymerase, reaction buffer, and green and yellow dyes that used to observe progress during electrophoresis. Various concentration of every used primer (10 pmol), (50-80)ng of DNA template then sterile deionized D.W was added to obtain a final volume. The PCR products were separated on agarose gels (2% agarose, 1µ of ethidium bromide (10 mg/ml), 1X TAE) and analyzed on OWL Electrophoresis System (Thermo, USA).

Touchdown PCR for *nuc* gene detection

The *Staphylococcus aureus* isolates confirmed by Touchdown-PCR using specific primer for *nuc* gene which was designed according to Brakstad et al (9). The Amplicom size and primer sequence were listed in Table 2. The program was adopted in PCR analysis of primer for *nuc gene* as shown in Table 3.

Touchdown PCR for *mecA* gene detection

Detection of Methicillin resistance was done by Touchdown-PCR with specific primer for *mecA* gene of *Staphylococcus aureus* isolates. This technique was used to confirm the detection of Methicillin resistant *S. aureus* (MRSA) isolates by using specific primers which was designed according to Zhang et al (40). The primer sequence and its amplicon size were listed in Table 2. The program was adopted in PCR analysis of primer for *mecA* gene as shown in Table 4

Table 2. Primers sequence and amplicon size used in this study

primer	Sequence 5'→3'	Amplicon size	References
nuc-F	GCGATTGATGGTGATACGGTT	276bp	9
nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
mecA-F	GTGAAGATATACCAAGTGATT	147bp	40
mecA-R	ATGCGCTATAGATTGAAAGGAT		

F: Forward **R: Reverse**

Table 3. The program of *nuc* primers amplification used in Touchdown PCR analysis

Steps	Temperature	Time	Cycle No.
Initial denaturation	95C°	2min.	1
Denaturation	95C°	30sec.	20
Annealing	62C° (- 0.5 at each cycle)	45sec.	
Extension	72C°	45sec.	
Denaturation	95C°,	30sec.	10
Annealing	52C°	45sec.	
Extension	72C°	45sec.	
Final extension	72C°	3min.	1
Hold	4C°, ~	~	1

Table 4. The program of *mecA* primers amplification used in Touchdown PCR analysis

Steps	Temperature	Time	Cycle No.
Initial denaturation	95C°, 2min.	2min.	1
Denaturation	95C°	30sec.	20
Annealing	50C° (- 0.5 at each cycle)	45sec.	
Extension	72C°	45sec.	
Denaturation	95C°	30sec.	10
Annealing	40C°	45sec.	
Extension	72C°	45sec.	
Final extension	72C°	3min.	1
Hold	4C°	~	1

RESULTS AND DISCUSSION

Identification of *Staphylococcus aureus*

Out of a total of 278 patients and common surfaces samples 204 (73.38 %) samples were found positive for *Staphylococcus aureus* by conventional culture method and microscopic characteristics. Morphologically, 204 isolates showed smooth, translucent, creamy, yellow pigmented colonies on mannitol salt agar and fermented mannitol. Microscopically examination showed that the bacterial cells positive for gram stain reaction and appeared as grape like clusters, nonsporeforming and non-motile. Several biochemical tests were performed, and the results demonstrated that 204 isolates gave positive results for catalase, coagulase, and negative for oxidase.

Identification of methicillin resistant *Staphylococcus aureus*

Isolation of methicillin resistant *staphylococcus aureus* was achieved by culturing the isolates on HiCrome MeReSa Agar Base which provided with Cefoxitin supplement (FD259) and MeReSa Selective Supplement (FD229). The use of these medium supplements aid to inhibit all MSSA isolates and allowed the growth all MRSA isolates, 177 of 204 isolates are developed bluish green color and identified as MRSA as shown in Figure 1. Using of chromogenic agar promotes the isolation and detection of MRSA from primary isolation plates during 24h after enrichment, without needing for extra biochemical tests (22). This method is cost effective, save time and supply powerful outcomes that are mimic PCR method results (39). The authors also mentioned that a concentration of 4 mg of cefoxitin/liter promote the inhibition of all MSSA isolates and the growth of all MRSA isolates (13). Alzaidi, (6) identified MRSA by using chromogenic agar and noticed that, among the 192 *S. aureus* isolates, 126 (65.6%) were MRSA of which 100 (66.6%) and 26 (61.9%) from the patients and environment, respectively, Nasser et al (26) showed that 77.9% were identified as MRSA from all *S. aureus* isolates in Indian hospitals.

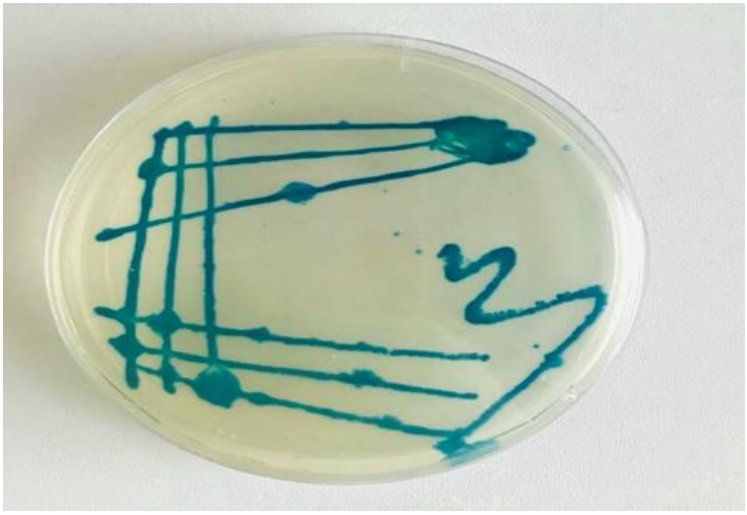

Figure 1. MRSA isolate on HiCrome MeReSa Agar Base medium

MRSA antibiotics susceptibility profiles

The antibiotics susceptibility profile for MRSA was determined by using disc diffusion method, the 177 MRSA isolates which had been identified previously by using HiCrome MeReSa Agar Base were specified as follows: The highest level of resistance was with Penicillin, Cefoxitin, Ampicillin/Cloxacillin and Ampicillin 100% (n= 177/177) followed by Amikacin with 88.70% (n= 157/177), methicillin 87% (n= 154/177), Oxacillin 81.92% (n= 145/177), Azithromycin 60.45% (n= 107/177), Tobramycin 48.58% (n=86/177), Erythromycin 47.45% (n=84/177), Streptomycin 45.19% (n= 80/177), Norfloxacin 37.28% (n= 66/177), Doxycycline 28.24% (n=50/177), Cephalothin 27.11% (n= 48/177), Ceftriaxone 25.42% (n = 45/177) Vancomycin 12.99% (n= 23/177), Bacitracin 11.86% (n= 21/177), Novobiocin 10.73% (n= 19/177) and Ciprofloxacin 5.64% (10/177). All the isolates showed 100% susceptibility towards Imipenem as shown in Figure 2. The highest level of resistance to β-lactam due to *mecA* gene expression and may be to *blaZ* produced of *S. aureus* isolates and their alternative mechanism (34). Aminoglycoside modifying enzymes are the most common mechanism of resistance to aminoglycosides, especially in *S. aureus* (29). Lower resistance ability toward vancomycin; Glycopeptides group can be due to resistance *van* A operon which transformed from *Enterococcus* to *Staphylococcus* isolates that placed in gut (32). The *S. aureus* high susceptibility rate to Imipenem because of its high partiality for penicillin binding protein PBP2 produced by β-lactamase producing bacteria (18). The result also mentioned that (94.9%) of the isolates are multidrug resistant. MDR MRSA is overcome the world, which can be due to excessive use of antimicrobials agent randomly, physical contact with cattle animals and consuming of contaminated animals (36, 37). Abdul-Wahhab, (1), mentioned that 100% of MRSA isolates in Baghdad were MDR.

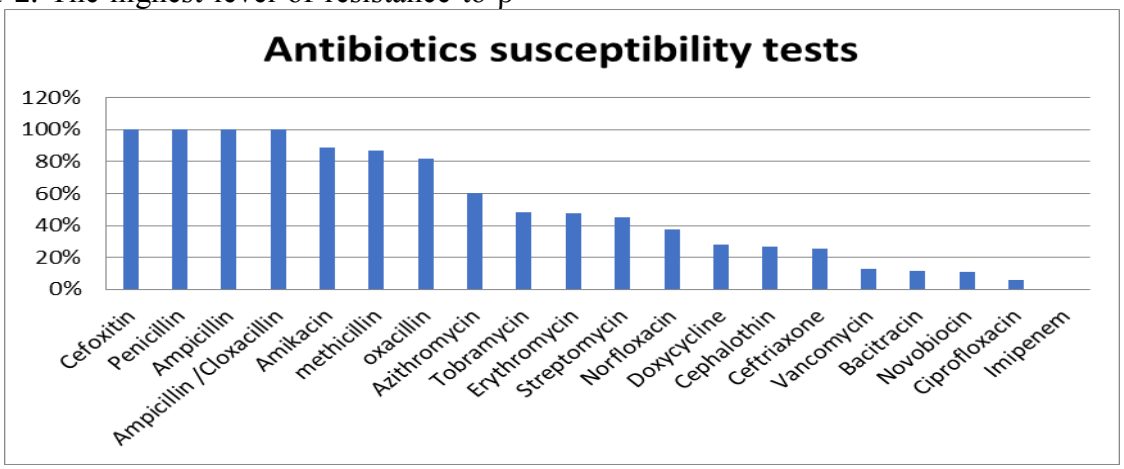

Figure 2. Antibiotics susceptibility test results

Molecular analysis: The *nuc gene* was screened using touchdown PCR technique in order to identify *Staphylococcus aureus* by using specific primers for detection of *S. aureus* isolates and exist of *nuc* gene. The *nuc* gene is baseline in identification and classification of *S. aureus* (4). The MRSA isolates were determined by using touchdown PCR technique by using specific primers for detection *mecA* gene and identification of MRSA isolates. The *mecA* gene responsible for resistance towards β-lactam antibiotics and maybe provide resistance ability toward other classes of antibiotics (24). The results shown that 198 (97.05%) of 204 isolates gave positive result for amplification of *nuc* gene (276bp) and identified as *Staphylococcus aureus* as shown in Figure 3, while 165 of 204 isolates (80.88%) gave positive result for amplification of of *mecA* gene (147bp) and identified as MRSA as shown in Figure 4. The outcomes of this study hinted that, 165 MRSA that identified formerly with classical biochemical tests was amplified with *nuc* and *mecA* genes successfully, what marked that thermo stable nuclease encoding gene is so precise for *S. aureus*, and the protein PBP2A (penicillin-binding protein2A) encoding gene is so precise for MRSA (4). The 165 isolates (80.88%) of 204 isolates were harbored the *mecA* gene, that seems close to local studies of Saleem et al (31) who mentioned that (80.7%) of 140 clinical *S. aureus* isolates were harbored the *mecA* gene, and abdul-wahhab, (1) who reported that (68.96%) among 43 clinical isolates were harbored the *mecA* gene. In other hands, 154/177 MRSA isolates (87%) shown methicillin resistance in susceptibility test and 23 (12.99 %) was methicillin sensitive, this maybe because that occasionally *mecA* gene was expressed invivo but not always invitro, additionally to that, the expression of *mecA* gene is minimum in bacterial cells that are considered as planktonic bacteria, or along these lines, it could be credited to their powerlessness to deliver enough PBP2A as mentioned by Murakami et al (25). According to the results, 177 (100%) isolates where identified as MRSA by using chromogenic agar, and 154 of 177 (87%) were showed methicillin resistance in susceptibility test, while 165 of 177 (93.22%) where harbored the *mecA* gene. The only isolates that contain *mecA* gene consider as MRSA and cannot depended only on phenotypic (susceptibility tests) to determine the MRSA isolates. For the same reason this study depends on molecular diagnosis of *mecA* gene as marker of MRSA isolates because the antimicrobial susceptibility profiles (phenotypic tests) cannot give a certain indicator for determination of MRSA (15). PCR based measures are considered as the highest quality level for the identification of MRSA, because of the heterogeneous resistance by different phenotypic identification strategies showed by numerous clinical samples. Genotypic strategies are more precise in recognizing Methicillin-Resistant *S. aureus* as contrasted with traditional powerlessness strategies (30).

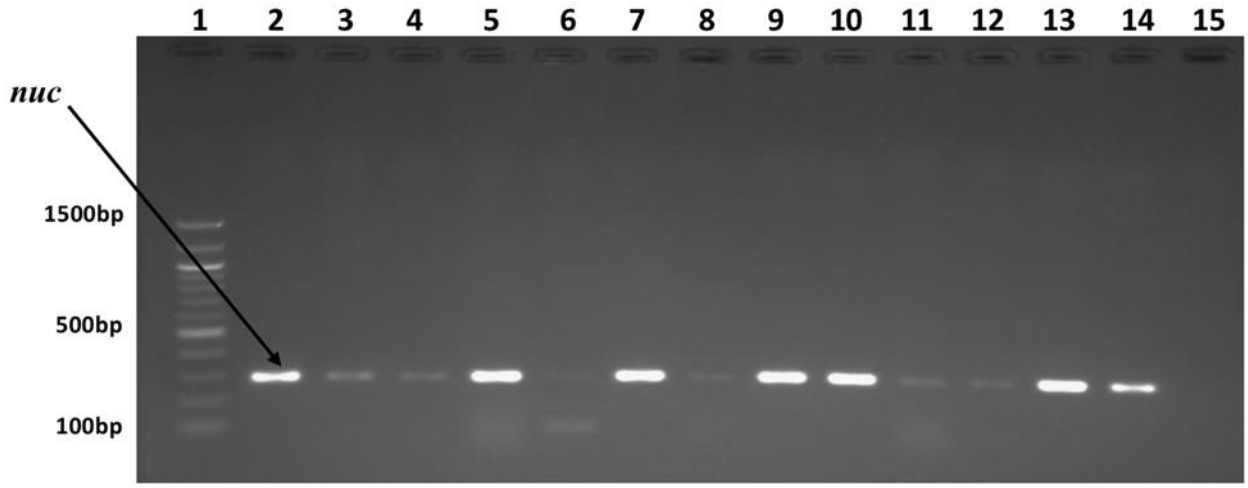

Figure 3. Gel electrophoresis of *nuc* gene (276bp) , lane (1) represents the 100bp DNA ladder, lanes (2-14) represent the MRSA isolates and lane (15) represents negative control, which has been separated on 2% agarose gel (90 V, 1X TBE buffer) for 1hour and has been visualized under U.V. lights post EtBr staining

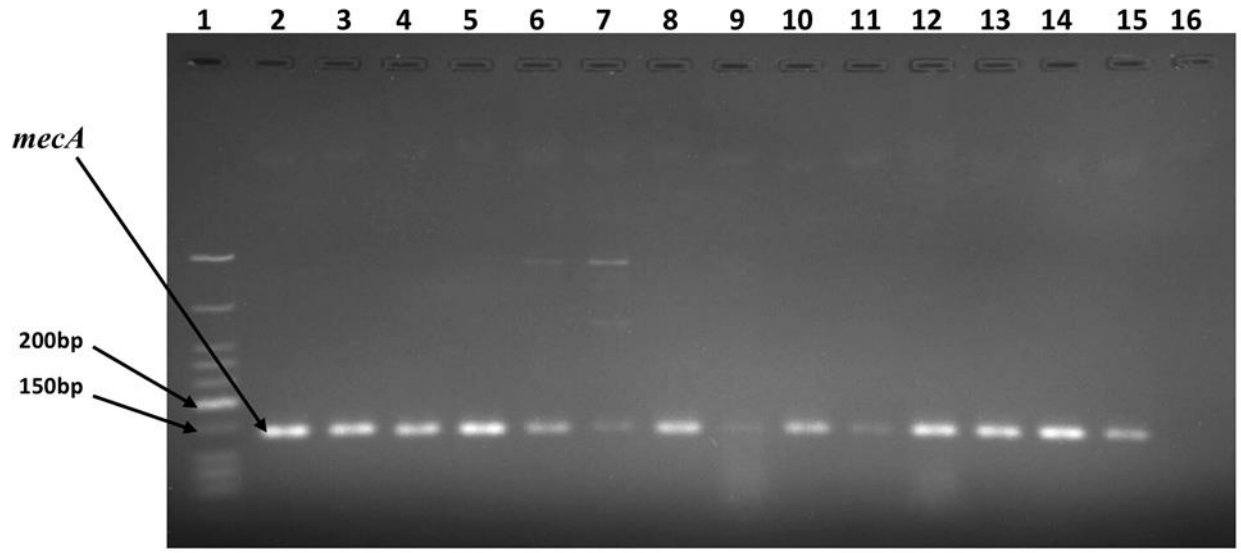

Figure 4. Gel electrophoresis of *mecA* gene (147bp) , lane (1) represents the low molecular weight DNA ladder, lanes (2-15) represent the MRSA isolates and lane (16) represents negative control, which has been separated on 2% agarose gel (90 V, 1X TBE buffer) for 1hour and has been visualized under U.V. lights post EtBr staining

CONCLUSION

According to the results of current study and comparison our results with others, we conclude that the molecular methods consider the precise methods for identification of MRSA, and cannot depends only on phenotypic methods for identification of MRSA.

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