ISSN: 0067-2904

Phenotypic Detection of Inducible Clindamycin Resistance in MRSA

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Received: 26/11/2024 Accepted: 2/6/2025 Published: 30/6/2026

Abstract

Staphylococcus aureus is a common pathogen associated with community and hospital-acquired infections; it can rapidly develop resistance to new antibiotics. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has severely limited treatment options, necessitating the judicious use of effective antibiotics such as clindamycin. However, routine susceptibility tests often fail to detect inducible clindamycin resistance (ICR), ultimately leading to treatment failures. This cross-sectional study was conducted from September 2023 to June 2024, and it aimed to identify and report the prevalence of ICR phenotypes among MRSA with the d-test. A total of 101 isolates were investigated, of which 85 were *S. aureus* and confirmed using PCR targeting the Sa442 fragment. They were identified as MRSA using the cefoxitin disk diffusion test. Among these, 32.94% showed ICR, of which 21.18% were D-phenotype and 11.76% were D+ phenotype. Constitutive Macrolide-lincosamide-streptogramin B ($cMLS_B$) resistance was observed in 32.94%, including hazy D zone (HD) (24.71%) and resistant (R) (8.24%) phenotypes. Sensitive, non-MLS_B, and macrolide–streptogramin B (MS_B) phenotypes were detected in 27.06%, 3.53%, and 3.53%, respectively. Finally, D-test as part of routine antibiotic susceptibility is important for appropriate clindamycin use, mitigating treatment failures, and reducing the use of broad-spectrum antibiotics in Iraqi hospitals. Therefore, improving patient care in Iraq.

Keywords: MRSA, clindamycin, erythromycin, ICR, MLS_B.

الكشف المظهري عن المقاومة المستحثة للكلندامايسين في المكورات العنقودية الذهبية المقاومة للميثيسيلين

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الخلاصة

المكورات العنقودية الذهبية هي أحد مسببات الأمراض الشائعة المرتبطة بالعدوى المكتسبة في المجتمع والمستشفيات، ولديها القدرة على تطوير مقاومة سريعة للمضادات الحيوية الجديدة. أدى ظهور المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) إلى تقليل الخيارات العلاجية بشكل كبير، مما يتطلب استخدامًا حكيمًا للمضادات الحيوية الفعّالة مثل الكليندامايسين. ومع ذلك، غالبًا ما تفشل اختبارات الحساسية الروتينية في الكشف عن المقاومة المحفزة للكليندامايسين(ICR)، مما يؤدي في النهاية إلى فشل العلاج.

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أُجريت هذه الدراسة المقطعية من سبتمبر 2023 إلى يونيو 2024 بهدف تحديد أنماط المقاومة المحفزة للكليندامايسين والإبلاغ عن انتشارها بين عزلات MRSA باستخدام اختبارD. تم تحليل 101 عزلة، تأكد أن 85 منها MRSA باستخدام السيفوكسيتين و PCRلاستهداف SA442 .من بين هذه العزلات، أظهرت 32.94% مقاومة محفزة للكليندامايسين، حيث أظهرت 21.18% النمط D و11.76% النمط +D. كما تم ملاحظة مقاومة MLS_B الجوهرية ($cMLS_B$) بنسبة 32.94%، بما في ذلك 24.71% بالنمط HD و8.24% بالنمط R. تم اكتشاف أنماط حساسة وغير MLS_B وMS_B بنسبة 27.06% و3.53% و3.53% على التوالي. تؤكد الدراسة أهمية تضمين اختبار D ضمن اختبارات الحساسية الروتينية لضمان الاستخدام المناسب للكليندامايسين، وتقليل فشل العلاج، والحد من استخدام المضادات الحيوية واسعة المدى في المستشفيات العراقية، مما يساهم في تحسين رعاية المرضى في العراق.

1. Introduction

Staphylococcus aureus is a Gram-positive pathogen commonly found on the skin and mucous membranes. It is a major cause of infections in both community and hospital environments. Its ability to rapidly develop antibiotic resistance has led to the emergence of methicillin-resistant *S. aureus* (MRSA), which has become a public health problem globally. It confers resistance towards β-lactams and thus becomes ineffective [1]. The Sa442 DNA fragment is a species-specific DNA fragment that is unique to *S. aureus,* and it is widely used for species identification. It is highly conserved in *S. aureus;* thus, it is a reliable target in PCR detection methods [2]. MRSA infections are often treated with macrolide-lincosamide-streptogramin B (MLSB) antibiotics. Erythromycin was the first macrolide introduced in the 1950s as an alternative option for patients with β-lactam hypersensitivity. Clindamycin is a lincosamide that has long been used to treat staphylococcal infections. Although clindamycin shares a mechanism of action with Erythromycin, they have entirely different structures. It is also used as an alternative option for patients who were allergic to β-lactams. Clindamycin is particularly advantageous due to its availability in oral and intravenous forms, excellent tissue penetration, favorable pharmacokinetics, toxin production inhibition, and cost-effectiveness [3,4]. In staphylococci, resistance to Erythromycin and clindamycin is typically mediated by *erm* genes, which leads to treatment failure due to inducible clindamycin resistance (ICR). While constitutive resistance can be detected by routine susceptibility tests, ICR often goes undetected, resulting in treatment failures. D-test provides a simple and accurate method for ICR detection, which involves merely the placement of erythromycin and clindamycin disks in proximity, as detailed in the methods section. Our study was carried out between September 2023 and June 2024. The motivation for this research stems from George's study, which reported a 5-year-old girl who was subjected to craniosynostosis. The wound site was infected with MRSA and unresponsive to known treatments, and she was given clindamycin therapy to eradicate the infection, although she had left the hospital at that time. After conducting a d-test, they confirmed the incidence of inducible clindamycin resistance [5]. Regular testing for MRSA ICR is crucial to prevent treatment failure and guide the judicious use of clindamycin for multidrug-resistant (MDR) pathogens like MRSA. Our study contributes to improved diagnostic methods and better patient care in settings like Iraqi hospitals.

2. Materials and Methods

The study was approved by the College of Science Ethics Committee (CSEC/1023/0079 on 13/10/2023)

Bacterial Isolates

A total of 101 samples were collected from Baghdad Teaching Hospital, including wounds and burns. They were streaked on mannitol salt agar (Liofilchem, Italy) and incubated for

24h. They were identified as *S. aureus* using standard biochemical tests, including catalase, oxidase, and coagulase, and reidentified by detection of *Sa442*.

Sa442 Detection

The Sa442 DNA fragment is chosen because it is a species-specific for *Staphylococcus aureus*. DNA was extracted using Presto™ mini-DNA kit (Geneaid, Taiwan) following kit's instructions, the primers sequences were: F/5'-CGTAATGAGATTTCAGTAGATAATACAACA-3' R/ 5'-AATCTTTGTCGGT ACACGATATTCTTCACG-3' [2,10]. They were received from Alpha ADN, S.E.N.C. and liquefied with nuclease-free water. The primer was then stored in a deep freeze. The 25 µl volume was prepared, which contained 12.5 µl of a 2X Master mix (Vazyme, China), 0.5 µl of both forward and reverse (10µM) primers, 2 µl DNA with a concentration of 200 ng/µl, and completed with nuclease-free water. The program was as displayed in Table 1. Gel electrophoresis was performed using 1.5% agarose (Sigma, Germany). The agarose was dissolved in TBE buffer, after it dissolved completely, ethidium bromide (EtBr) was added to the gel and 100bp ladder was added to the well and visualized with UV documentation system (Alpha Biotec, China) and photographed using a camera [11,12].

Table 1 : PCR Protocol.

Phase	Temperature (℃)	Time	No. of Cycles
Initial Denaturation	95	3 min	1
Denaturation	95	15 sec	30
Annealing	60	15 sec	
Extension	72	1 min	
Final Extension	72	5 min	1

Cefoxitin Disk Diffusion

Methicillin resistance was detected by cefoxitin disk diffusion. Isolates showing diameters ≥ 22 were sensitive, whereas isolates showing diameters ≤ 21 were resistant [6].

Antibiotic susceptibility testing

It was carried out using the standard sensitivity test. They included: gentamicin, Erythromycin, clindamycin, Ciprofloxacin, Levofloxacin, Rifampicin, and Tetracycline. A prepared bacterial suspension was adjusted to a no. 0.5 McFarland and inoculated on Muller-Hinton agar (Liofilchem, Italy). Then, disks were placed with forceps, and plates were incubated for 18-24h at 35-37 ºC. Following incubation, diameters were measured with a ruler. The results were interpreted according to CLSI [7].

ICR Detection

ICR was detected with the d-test. Both antibiotics were tested singly and together. A bacterial suspension of each isolate was adjusted to a no. 0.5 McFarland and inoculated on Muller-Hinton agar. Then, both antibiotics were placed 15-26 mm apart using forceps. Then, the plates were incubated for 16-18h at 35-37 ºC. Flattening of the inhibition zone around clindamycin proximal to Erythromycin indicates ICR. Isolates showing a d-zone were considered positive. On the other hand, the sensitive inhibition zone around clindamycin was considered negative. Additionally, any hazy growth noticed in the zone indicates ICR [7-9].

2. Results

Bacterial isolation

The bacteria were isolated from burns and wounds and were tested for catalase, oxidase, and coagulase. The results revealed 100% positive for catalase, 84.16% positive for coagulase, and 100% negative for oxidase.

PCR Study

The results of the current study reported that all eighty-five isolates were positive (100%) via detection of the species-specific SA442 fragment.

Antibiotic sensitivity test

The antibiotic sensitivity results of 85 isolates were as follows: gentamicin, 11 resistant, 5 intermediate, and 69 sensitive. For Levofloxacin, 6 of them exhibited resistance, 8 exhibited intermediate resistance, and 71 were sensitive; for Ciprofloxacin, 15 were resistant, one intermediate, and 69 were sensitive; for Tetracycline, 9 were resistant, 40 intermediate, and 36 were sensitive, for Rifampicin, 7 resistant, 7 intermediate, and 71 were sensitive. Clindamycin and Erythromycin are displayed in Table 2.

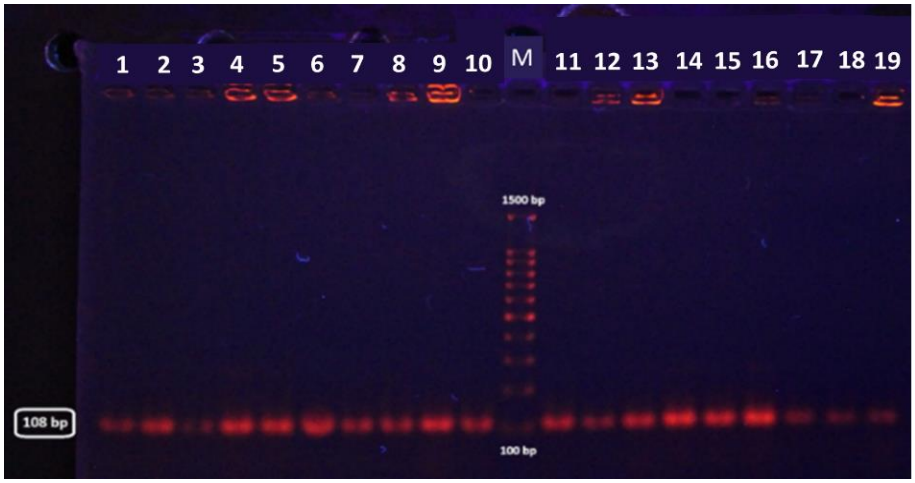

Figure 1: Visualization of *Sa442* gene by 1.5% agarose gel analysis stained with EtBr and run at a voltage 150 for 30 minutes. The shown bands are representative of PCR products of 108 bp. The bacterial isolates are labelled at the top of the gel while M represents for 100 bp Ladder.

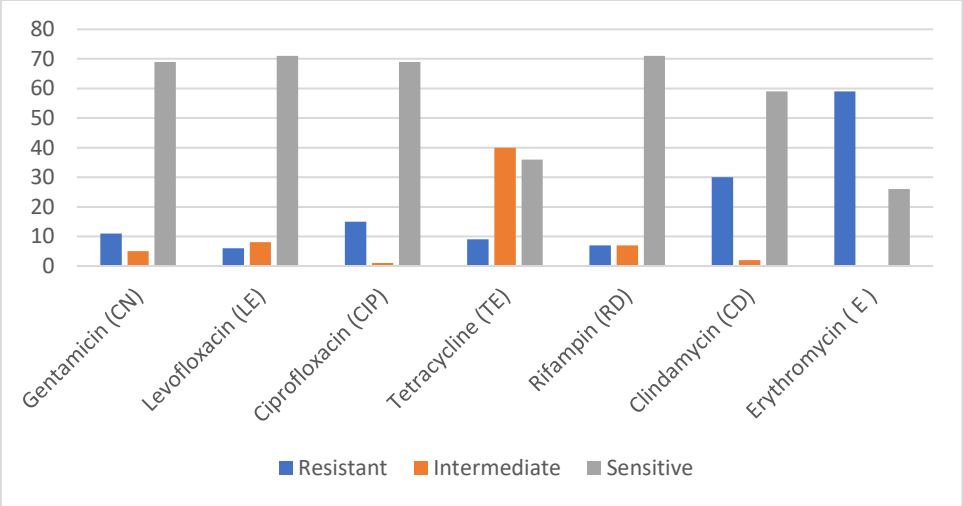

Figure 2: Antibiotic sensitivity results.

MRSA susceptibility to Erythromycin and clindamycin

Eighty-five isolates were tested for ICR using a d-test. The results were interpreted based on the observed phenotype for each isolate. Different MLSb phenotypes among isolates have been characterized. Seven phenotypes were noticed in our study, as shown in Table 3. The results showed 32.94% of isolates exhibiting inducible resistance, of which 21.18% showed d-phenotype where a flattened zone was noticed around clindamycin, and 11.76% showed d+ phenotype where easily distinguishable colonies were found in the flattened edge of the d-zone around clindamycin. These phenotypes were considered positive. Other phenotypes were considered negative due to the absence of d-zone. Constitutive resistance was detected in 32.94%, of which 24.71% showed the HD phenotype, where growth was noticed around antibiotics with a flattened, internal hazy zone, and 8.24% of them showed R phenotype, where growth was seen around both antibiotics.

The MS_B and non-MLS_B phenotypes, each were detected in 3.53%. MS_B showed a resistant zone around Erythromycin and a sensitive zone around clindamycin. Conversely, non-MLS_B showed a sensitive zone around Erythromycin and a resistant zone around clindamycin. Finally, 27.06% showed zones around both antibiotics, showing a sensitive phenotype.

Table 2: The percentage of clindamycin and erythromycin resistance.

Zone (Range mm)	%
Clindamycin (≤ 14 Resistant, 15–20 Intermediate, ≥ 21 Sensitive)	**35.29%, 2.35%, 69.41%**
Erythromycin (≤ 13 Resistant, 14–22 Intermediate, ≥ 23 Sensitive)	**69.41%, 0.00%, 30.58%**

Table 3: characteristics of noticed ICR combined with figure 3.

Clindamycin	Erythromycin	Inducible	Resistant	Description
R	R	HD	$cMLS_B$	Two zones around clindamycin antibiotic. External zone showed hazy growth to another zone. The hazy growth shown flattened.
R	R	R	$cMLS_B$	Absence of hazy growth extending to both antibiotics.
S	R	D	$iMLS_B$	Clear d- inhibition zone around clindamycin.
S	R	D+	$iMLS_B$	Clear d- inhibition zone around clindamycin, with small colonies.
S	R	Neg	MS_B	Inhibition zone around clindamycin.
S	S	S	no resistance	Inhibition zones around both antibiotics.
I/R	S	Clindamycin resistant, Erythromycin sensitive	non-MLS_B	Inhibition zone around Erythromycin and resistant zone around clindamycin.

*Abbreviations: $cMLS_B$: constitutive MLS_B, $iMLS_B$: inducible MLS_B

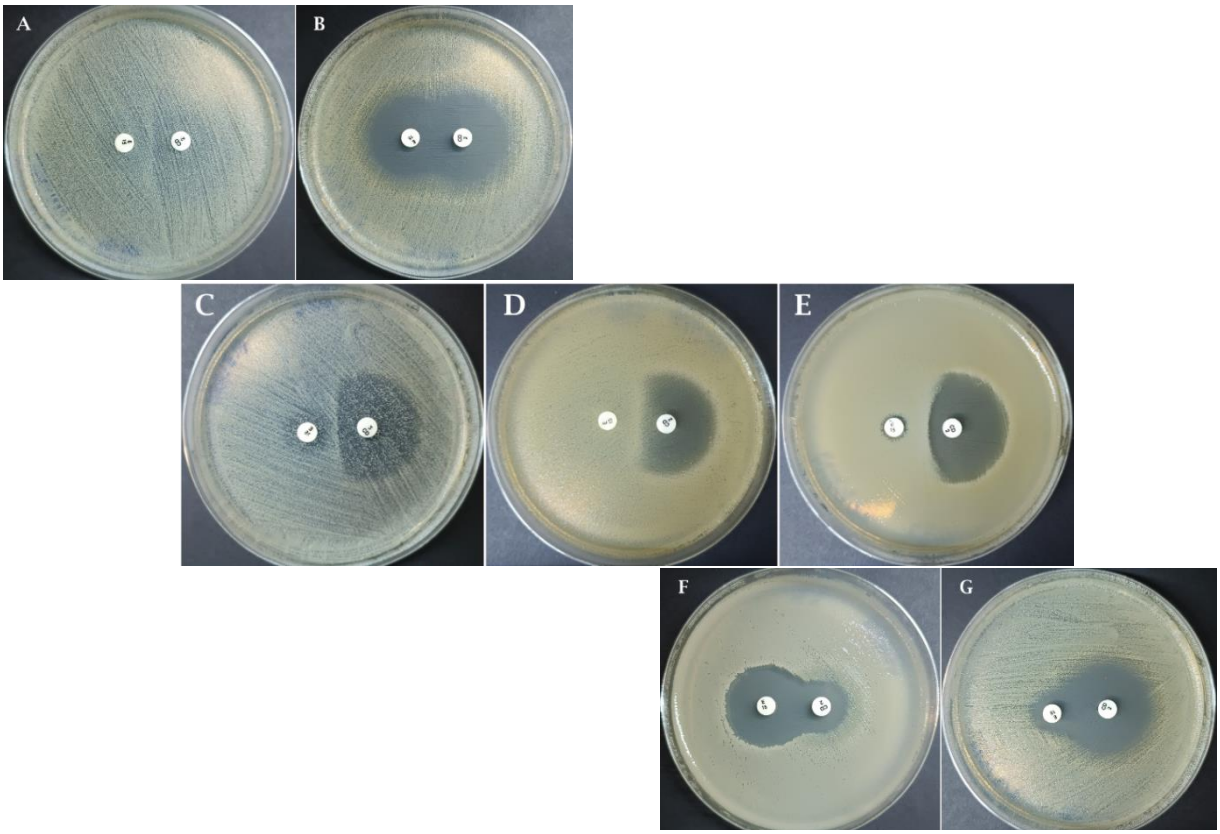

Figure 3: Isolates showing MLS_B. A: resistant, B: sensitive, C: HD, D: d+ phenotype, E: d, F: non MLS_B, G: MS_B .

3. Discussion

The emergence of MDR pathogens has led to an increased incidence of treatment failures. Such resistance has been recognized as one of the greatest challenges to human health in modern medicine. That's why accurate results are important for effective treatment and patient safety. They play a major role in mitigating antibiotic resistance. The recurrence of MRSA in patients and continuously changing resistance led to a renewed focus on whether clindamycin is suitable for such staphylococcal infections. This study aimed to report the prevalence of ICR in the Baghdad Teaching Hospital. The current study yielded 32.94%, which showed ICR. These findings are consistent with those of Ifediora *et al.,* found a prevalence of 17.7%, and Yacouba *et al.,* showing 36% ICR [13,14]. Furthermore, Jahanbakhshi *et al.* and Che Hamzah *et al.* reported that the prevalence of ICR was 15.9% and 46.7%, respectively [15,16]. However, numerous studies reported low ICR prevalence. For example, Abdelmawgoud *et al.,* and Rania *et al.,* reported only nine isolates exhibiting this phenotype [17,18]. During clindamycin treatment, ICR gives rise to spontaneously constitutively resistant mutants in vivo and in vitro, since results show clindamycin sensitivity, thus leading to treatment failure. Inducible resistance cannot be detected with the e-test and broth microdilution. The second notable phenotype in our study was 32.94% cMLSb. This result agrees with Hakim, who detected 54.1% cMLSB in the south of Iraq [19]. Furthermore, Flayyih and Mohammed found only two isolates showing cMLSb in Baghdad [20]. The third phenotype was 3.53 MS_B. This result is in agreement with Mahfouz, who also detected only two isolates in Egypt [21]. However, Hakim, who conducted a study in Basra, reported no isolates showing MS_B [19]. The sensitive phenotype was detected in 27.06%. They agree with Abdelmawgoud and Che Hamzah *et al., who* reported 23 and 18,

respectively [16,17]. Finally, non-MLS_B was detected in three isolates, representing 3.53% of the total isolates in our study. Since this phenotype does not indicate resistance, it received little attention. Only two studies mentioned this phenotype; one of them reported no isolates, and the other study was conducted in Nepal and reported 4.8% [22,23]. The variation in ICR prevalence in studies is due to geographical differences. MLS_B antibiotics are protein synthesis inhibitors. Although they have different structures, they share mechanisms of action by targeting the 50S ribosomal subunit. In staphylococci, resistance to clindamycin was primarily related to erythromycin ribosome methylase (*erm*) genes, which are *ermA* and *ermC*. On the other hand, MS_B is related to methionine sulfoxide reductase A (*msrA*) [24]. Additional research can be performed to find out if there is a relationship between ICR and methicillin resistance.

4. Conclusion

Routine susceptibility tests cannot detect ICR. Therefore, it is recommended to include d-test in laboratories as part of susceptibility tests. Reporting MRSA without checking for ICR may result in treatment failure.

5. Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

I would like to sincerely thank Mr. Ali A. Mussa and Mr. Muhammad Jadoo for their invaluable guidance and support.

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