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Original Article

Inducible and constitutive clindamycin resistance in *Staphylococcus aureus* in a northeastern Indian tertiary care hospital

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Abstract

Introduction: *Staphylococcus aureus* is one of the most common pyogenic bacteria. They are notorious for developing prompt resistance to newer antimicrobials. With increasing incidence of methicillin-resistant *S. aureus* (MRSA) isolates, the treatment options are also becoming limited. Clindamycin is an excellent drug for skin and soft tissue infections, but resistance mediated by the inducible phenotype ($iMLS_B$) leads to *in vivo* therapeutic failure even though there may be *in vitro* susceptibility. The double disk approximation test (D-test) can reliably detect the presence of such isolates. This study was aimed to detect and report the prevalence of the $iMLS_B$ phenotype in NEIGRIHMS, a tertiary care center in Northeast India.

Methodology: A total of 243 consecutive isolates were subjected to routine identification tests followed by antimicrobial sensitivity testing. Erythromycin-resistant isolates were tested for inducible resistance phenotype by the D-test.

Results: Among strains tested, 95 (39%) were erythromycin resistant. Twenty-six (10.7%) isolates were D-test positive ($iMLS_B$ phenotype), 41 (16.88%) were constitutively resistant ($cMLS_B$ phenotype), and 28 isolates (11.52%) were found to be negative by D-test. The incidence of both inducible and constitutive phenotypes was higher in MRSA isolates compared to methicillin-sensitive *S. aureus* (MSSA) isolates.

Conclusions: This study revealed a moderate prevalence of the inducible clindamycin phenotype in the staphylococcal isolates tested. Clinical microbiology laboratories in areas of high MRSA prevalence should consider performing the D-test routinely. This will help prevent prescription of drug(s) whose therapeutic efficacy is doubtful.

Key words: D-test; inducible clindamycin resistance; erythromycin; MRSA; *Staphylococcus aureus.*

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Introduction

Staphylococcus aureus is one of the most common pyogenic bacteria infecting humans. It is known for acquiring antimicrobial resistance promptly after introduction of new antibiotics [1]. Emergence of methicillin resistance in *S. aureus* has left very few therapeutic alternatives. The macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics serves as one such alternative, with clindamycin being the preferred agent due to its excellent pharmacokinetic properties [2]. Clindamycin is considered an useful alternative drug in penicillin-allergic patients for treatment of skin and soft tissue infections caused by *S. aureus*. It accumulates in abscesses and no renal dosage adjustments are required. It has excellent tissue penetration except into the central nervous system, where it does not cross the blood-brain barrier, even in the presence of inflamed meninges [3]. Good oral absorption makes it an attractive option for outpatient prescription or as a follow-up drug after intravenous therapy [1,4]. This permits an early transition to outpatient management of the susceptible infection without the complication of continued intravenous access [5]. Clindamycin is not impeded by a high bacterial burden at infection sites, and at the same time, it also inhibits production of certain toxins and virulence factors in *Staphylococcus* spp.

However, widespread use of MLS_B antibiotics has led to an increase in the number of staphylococcal strains acquiring resistance to these antibiotics [2,6]. Resistance to the MLS_B family of antibiotics falls into three different mechanisms: target site modification, enzymic antibiotic inactivation and impermeability or macrolide efflux pumps [7]. Macrolide resistance due to ribosomal target modification affects the activities of both macrolides and clindamycin; it is mediated by erythromycin ribosomal methylases encoded by *ermA*/*ermC* genes. Such resistance may be inducible or constitutive [8,9]. Macrolide resistance due to active efflux is encoded by the macrolide-

streptogramin resistance (*msrA*) gene in *Staphylococcus* spp. This energy-dependent pump effectively expels macrolides from the bacterial cell before they can bind to their target site on the ribosome [10]. It results in resistance to macrolides and streptogramin B antibiotics, but not to lincosamides (MS phenotype). Clindamycin is active against such isolates and there is no risk of therapy failure in such cases [9].

Inducible resistance is expressed in the presence of strong inducers of methylase synthesis, such as 14 (erythromycin, clarithromycin, dirithromycin) and 15 (azithromycin)-membered macrolides. The 16-membered macrolides (josamycin, spiramycin), lincosamides (clindamycin), and type B streptogramin antibiotics appear active when tested by standard methods, as they are only weak inducers of methylase synthesis [11]. Strains with constitutive MLS_B resistance show *in vitro* resistance to all of these agents [4]. While strains that demonstrate constitutive resistance ($cMLS_B$) to clindamycin can normally be detected by standard susceptibility testing methods because they are resistant to both macrolides and lincosamides alike, the problem lies with inducible resistant ($iMLS_B$) strains. These strains are not readily detected by standard *in vitro* susceptibility testing methods, where they appear erythromycin resistant and clindamycin sensitive in routine laboratory tests, unless the tests include measures that result in induction of clindamycin resistance. In such cases, *in vivo* therapy with clindamycin may select *erm* mutants, leading to clinical therapeutic failure [2,10].

To detect $iMLS_B$ strains, there are special disk approximation tests that incorporate erythromycin induction of clindamycin resistance. These tests involve the placement of an erythromycin disk in close proximity to a disk containing clindamycin. As erythromycin diffuses through the agar, resistance to the lincosamide is induced, resulting in a flattening or blunting of the lincosamide zone of inhibition adjacent to the erythromycin disk, giving a "D" shape to the zone (D-zone effect). Each *S. aureus* isolate should be tested for inducible clindamycin resistance because the frequencies of the different resistance phenotypes vary widely between geographic regions and even between different hospitals [4].

To date, to the best of our knowledge, there have been no documented reports about this problem from the northeast region of India. The present study was therefore taken up in an attempt to investigate and detect the prevalence of erythromycin-induced clindamycin resistance among clinical isolates of *S. aureus* at NEIGRIHMS, a tertiary care hospital in Northeast India.

Methodology

The study was conducted between October 2012 and March 2013 in the Department of Microbiology at the NEIGRIHMS hospital in Shillong, India. A total of 243 consecutive, nonduplicate *S. aureus* isolates were prospectively recovered from clinical specimens such as pus, wound swabs, aspirates, blood, sterile fluids, catheters, and urine of patients with active infections. These isolates were biochemically confirmed as *S. aureus* by catalase, tube coagulase, latex agglutination tests (Plasmatec Labs, Bridport, UK) [12]. They were then subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MHA) plates (Hi-Media Labs, Mumbai, India). Methicillin resistance was determined by the disk diffusion method using a 30 μg cefoxitin disk. All susceptibility results were interpreted according to Clinical and Laborary Standards Institute (CLSI) guidelines [13]. The quality control for the erythromycin, clindamycin, and cefoxitin disks (Hi-Media Labs) was performed with *S. aureus* ATCC 25923. The results were interpreted with basic statistics and odds ratio analysis, and the p values were determined using MedCalc (version 12.7).

The isolates that turned out to be erythromycin resistant were further subjected to the double disk approximation test (D-test) as per CLSI guidelines for inducible clindamycin resistance. Herein, 0.5 McFarland's standard suspension of organisms was plated onto an MHA plate. An erythromycin disk (15 μg) and a clindamycin disk (2 μg) were placed 15 mm apart edge-to-edge on the MHA plate. Plates were analyzed after 18 hours of incubation at 35°C. Interpretation of zones of inhibition is indicated in Table 1.

Different phenotypes were shown when erythromycin (15 μg) and clindamycin (2 μg) disks were placed next to each other in the culture plate. They were interpreted as follows.

(1) **MS phenotype**: isolates resistant to erythromycin but sensitive to clindamycin with a circular zone of inhibition around clindamycin. This suggests resistance due to the *msrA*-coded active efflux pump mechanism.

(2) **$iMLS_B$ phenotype**: isolates resistant to erythromycin but sensitive to clindamycin showing flattening of zone of inhibition around clindamycin towards erythromycin disk producing a D-shaped

blunting. This suggests a resistance phenotype due to expression of *erm*-gene coded methylases.

(3) **cMLS$_B$ phenotype**: isolates resistant to both erythromycin and clindamycin with circular zone of inhibition if any around clindamycin. This suggests selection of *erm* gene mutants.

Results

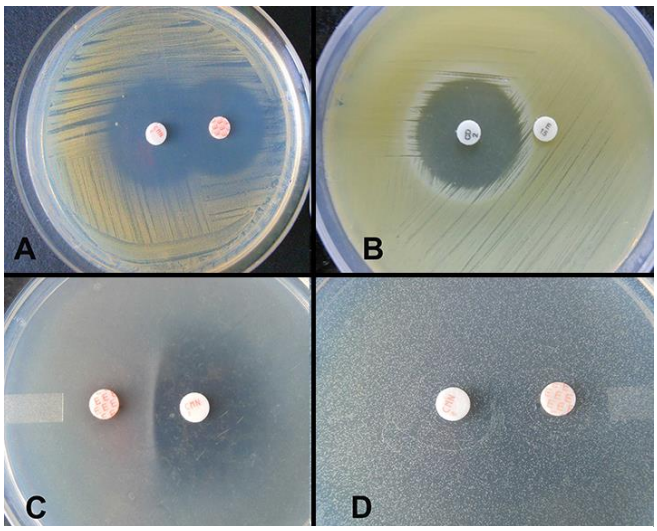
A total of 243 *S. aureus* isolates were tested for susceptibility to erythromycin and other antibiotics. Among them, 113 were methicillin-sensitive *S. aureus* (MSSA) and 130 were methicillin-resistant *S. aureus* (MRSA). Among all isolates tested, 95 strains (39%) were found to be resistant to erythromycin. These isolates were then subjected to the D-test, which revealed that 41 isolates (2 MSSA, 39 MRSA) were resistant to both erythromycin and clindamycin, indicating constitutive cMLS$_B$ phenotype. Fifty-four isolates were susceptible to clindamycin. Among these, 28 isolates (17 MSSA, 11 MRSA) were D-test negative, indicating MS phenotype, and 26 isolates (6 MSSA, 20 MRSA) showed a positive D-test, indicating inducible MLS$_B$ phenotype (Table 2).

Figure 1 shows the different phenotypes observed.

Twenty-six of 243 isolates were found to be resistant to clindamycin, based on D-test findings, which would have been otherwise missed in regular Kirby-Bauer disk diffusion susceptibility testing (Table 3).

Among all 130 MRSA isolates tested, 20 (15.38%) isolates were inducible clindamycin resistant whereas 39 (30%) isolates had the constitutive cMLS$_B$ phenotype. Of the 113 MSSA isolates tested, 6 (5.31%) had the iMLS$_B$ phenotype and 2 (1.77%) had the cMLS$_B$ phenotype. A total of 11 (8.47%) MRSA and 17 (15.05%) MSSA isolates were D-test negative, indicating truly clindamycin-susceptible MS phenotype. This study showed a predilection for the MRSA isolates to develop inducible resistance to clindamycin. This may further lead to *in vitro* and *in vivo* conversion of inducible to constitutive MLS$_B$ resistance in staphylococci and, subsequently, their spontaneous selection during clindamycin therapy, as

Figure 1. Interpretation of different phenotypes



(A) ER–S, CL–S; **(B)** ER–R, CL–S (MS phenotype) D-test –ve; **(C)** ER–R, CL–S (iMLS$_B$) D-test +ve; **(D)** ER–R, CL–R (cMLS$_B$).
ER: erythromycin; CL: clindamycin; R: resistant; S: sensitive; MLS$_B$: macrolide-lincosamide-streptogramin B antibiotics

Table 1. Interpretation of zones of inhibition

Drug	Sensitive	Intermediate	Resistant
Erythromycin (15 µg)	≥ 23 mm	14-22 mm	≤ 13 mm
Clindamycin (2 µg)	≥ 21 mm	15-20 mm	≤ 14 mm

Table 2. Breakup of tested clinical isolates

Phenotype	D-test	MRSA (%)	MSSA (%)	Total (%)	P value	Odds ratio	Z value	Confidence intervals (95%)
ER–S, CL–S		60 (46.15)	88 (77.87)	148 (60.90)	< 0.0001	0.2435	4.924	0.139–0.427
ER–R, CL–R	(cMLS$_B$)	39 (30.0)	2 (1.77)	41 (16.88)	< 0.0001	23.78	4.290	5.592–101.17
ER–R, CL–S	Negative (MS)	11 (8.47)	17 (15.05)	28 (11.52)	0.1133	0.5220	1.583	0.233–1.167
ER–R, CL–S	Positive (iMLS$_B$)	20 (15.38)	6 (5.31)	26 (10.70)	0.0153	3.2424	2.426	1.253–8.387
Total		**130 (53.5)**	**113 (46.5)**	**243**				

ER: erythromycin; CL: clindamycin; S: sensitive; R: resistant; MLS$_B$: macrolide-lincosamide-streptogramin B antibiotic; cMLS$_B$: constitutive MLS$_B$-resistant phenotype; iMLS$_B$: inducible MLS$_B$-resistant phenotype; MRSA: methicillin-resistant *Staphylococcus aureus*; MSSA: methicillin-sensitive *Staphylococcus aureus*

Table 3. The distribution pattern for the three phenotypes among all isolates tested

Types	Isolates showing growth	Percentage
Inducible clindamycin resistance	26/243	(10.69%)
Constitutive clindamycin resistance	41/243	(16.87%)
Susceptible MS phenotype	28/243	(11.52%)

shown in a previous study [7].

Of all isolates tested, 60.9% were sensitive to both erythromycin and clindamycin. Among all the *Staphylococcus* isolates, 16.87% had the constitutive resistance phenotype, 10.69% had the inducible resistance phenotype, and 11.52% isolates were truly clindamycin sensitive. Overall, 35.48% of MRSA and 73.91% of MSSA isolates that exhibited erythromycin-resistant and clindamycin-susceptible phenotypes did not demonstrate $iMLS_B$ resistance and can therefore be appropriately reported as clindamycin susceptible. The drug has been found to be more susceptible to MSSA isolates than to MRSA isolates.

When MRSA and MSSA strains among *S. aureus* were compared, inducible clindamycin resistance was determined to be 3.24 times (odds ratio) more common, and constitutive clindamycin resistance was determined to be 23.7 times more common, respectively, in MRSA against MSSA isolates, whereas the MS phenotype was 1.91 times more common in MSSA compared to MRSA isolates.

Discussion

In the context of increase in resistance and emergence of multidrug-resistant organisms, accurate antimicrobial susceptibility data of an isolate is crucial for appropriate therapy decisions. Empirical outpatient treatment options for staphylococcal infections have become more limited as concerns about the prevalence of MRSA have increased [1,14]. Among the options available for MRSA and MSSA infections, clindamycin has evoked much interest. Clindamycin is a very good alternative because of its excellent pharmacokinetic properties [2,15]. However, resistance to clindamycin is highly variable, and the incidence of constitutive and inducible MLS_B-resistant phenotypes varies by geographic region and even between hospitals [4]. Since there are no studies that report the presence of inducible clindamycin resistance in Northeast India, this present study was undertaken with the intention to detect and report the prevalence of the $iMLS_B$ phenotype in NEIGRIHMS hospital, a tertiary care health center of the region.

Resistance to the MLS_B group of antibiotics is mediated by three different mechanisms: target site modification, active efflux pump mediation, and enzymatic inactivation. The first two are much more commonly encountered. Active efflux pump is mediated due to expression of the *msrA* gene. Such isolates are resistant to erythromycin and streptogramin B antibiotics but retain sensitivity to lincosamides. The target site modification mechanism is mediated by methylases expressed by *erm* genes. This can be either inducible or constitutive. Resistance is induced by the binding of a macrolide to upstream translational attenuator sequences, leading to changes in mRNA secondary structure, exposure of the ribosomal binding site, and expression of the multi-allele plasmid-borne erythromycin ribosomal methylase (*erm*) gene that causes the production of the methylase enzymes [5]. These enzymes cause methylation of the A2058 residue, located in the conserved domain V of the 23S rRNA component of the 50S ribosomal subunit, which leads to cross-resistance and the formation of the inducible phenotype ($iMLS_B$) of the resistance pattern [16,17]. Alterations in these 5' upstream sequences including deletions, duplications, and other mutations, lead to constitutive expression of the methylase gene and constitutive MLS_B resistance [4]. It is possible for mutations that will transform inducible ($iMLS_B$) resistant strains to the constitutive phenotype ($cMLS_B$) without the presence of a macrolide inducer to occur spontaneously as well. The concern is that this change in expression might get selected in the midst of therapy with a lincosamide. Recent evidence also proves that constitutive resistance to clindamycin in *S. aureus* prevents the inhibition of toxin production and fails to inhibit growth [10]. Interestingly, the streptogramin B and A combination quinupristin-dalfopristin appears to retain its activity against $cMLS_B$ strains of Staphyloccci, although the presence of the $cMLS_B$ phenotype changes the agents activity from bactericidal to bacteriostatic [18]. Treatment of infections by the $iMLS_B$ and $cMLS_B$ resistant phenotypes with clindamycin will result in failure.

Routine susceptibility testing can easily detect $cMLS_B$ phenotypes, but the real challenge lies in correctly identifying those $iMLS_B$ strains that are clindamycin sensitive *in vitro* but result in therapeutic

failure *in vivo*. This can be achieved by testing the isolates using the D-test in accordance with CLSI guidelines [13]. In this test, 14/15 membered macrolides (strong methylase inducers) and clindamycin disks are placed 15 mm apart edge-to-edge on a lawn culture of *Staphylococcus* spp. isolates and incubated overnight. Those strains that express the *erm* genes produce abundant methylases, induced by the erythromycin disk placed in the vicinity. This ensures that the strains that carry the $iMLS_B$ phenotype exhibit it *in vitro* as well. This precludes the prescription of clindamycin as a therapeutic drug in such scenarios. The benefit of routine D-testing is that we can clearly identify those strains that remain susceptible to clindamycin despite being resistant to macrolides. Isolates with the inducible resistance phenotype should be reported as clindamycin resistant [7]. On the other hand, negative result for inducible clindamycin resistance confirms clindamycin susceptibility and serves as a very good therapeutic option [2,13].

This study demonstrated a high percentage (39%, 95/243 isolates) of erythromycin resistance. Among the isolates, 26 (27.36%) tested positive for inducible clindamycin resistance by D-test while 41 (43.15%) isolates showed constitutive resistance, and 28 isolates (29.47%) showed true sensitivity to clindamycin (MS phenotype). Observations suggest that if the D-test had not been done, nearly a quarter of the erythromycin-resistant isolates would have been misidentified as clindamycin sensitive, resulting in therapeutic failure.

Almost half (48.14%) of erythromycin-resistant clindamycin-sensitive *S. aureus* isolates demonstrated inducible resistance. The present study revealed an incidence of 10.7% for the $iMLS_B$ phenotype among all *S. aureus* isolates tested (15.38% in MRSA and 5.31% in MSSA isolates, $p = 0.0153$). However, the incidence of the constitutive resistance phenotype was 16.88% among all *S. aureus* isolates, more in MRSA isolates (30%) compared to MSSA (1.77%) isolates ($p < 0.0001$). The incidence of the MS phenotype was 11.52% among the isolates tested (8.47% in MRSA and 15.05% in MSSA isolates).

Consequently, when a clinician is faced with an *S. aureus* isolate, the probability that this strain may be inducible MLS_B resistant is 10.7%. Provided that the strain is methicillin resistant, the possibility that the strain may be inducible MLS_B resistant is 3.24 times higher compared to a methicillin-sensitive strain. Constitutive MLS_B strains are 23 times more common in MRSA strains compared to MSSA strains. True clindamycin-sensitive strains (MS phenotype) are 1.91 times more common among MSSA compared to MRSA strains.

Reports on the pattern of MLS_B resistance among *Staphylococcus* spp. show wide variations. Some studies [4,6,19] have reported a high prevalence of the $iMLS_B$ phenotype, while others have indicated a much lower incidence [1,5,20] pattern. Different studies [2,3,5-7,20-22,23] have been indicating varying figures for $cMLS_B$ resistance patterns as well. True incidence pattern depends upon the patient population studied, geographic location, hospitals, and methicillin susceptibility.

In a study from Turkey, Yilmaz *et al*. [7] reported $iMLS_B$ at 19.81% in all *S. aureus* (24.4% MRSA and 14.8% MSSA) isolates. They found 25.3% strains carrying the $cMLS_B$ phenotype. Deotale *et al*. [2] reported an incidence of 14.5% for the inducible resistant phenotype and 3.6% for the constitutive resistant phenotype. Other studies by Schreckenberger *et al*. [21], Upadhya *et al*. [3], and Gade *et al*. [22] showed 15.88%, 20.2%, and 13.2% incidence of inducible clindamycin resistance, respectively. Reports by Azap *et al*. (4.6%) [20], Juyal *et al*. (7%) [5], and Ajantha *et al*. (7.2%) [1] demonstrated a low incidence pattern for $iMLS_B$ strains, whereas Fiebelkorn *et al*. (29%) [4], Levin *et al*. (27.5%) [19], and Gadepalli *et al*. (21%) [6] quoted high figures for $iMLS_B$ resistance.

Constitutive clindamycin resistance in our study was determined in 16.8% of all *S. aureus* isolates, which is in agreement with the findings of Juyal *et al*. (14.64%) [5] and Gade *et al*. (12.4%) [22]. Many international studies reported a higher prevalence of the $cMLS_B$ phenotype, including those of Schreckenberger *et al*. (38.14%) [21], Azap *et al*. (33.3%) [20], Yilmaz *et al*. (25.36%) [7], and Gadepalli *et al*. (26.5%) [6]. Deotale *et al*. (3.6%) [2], Upadhya *et al*. (8%) [3], and Mittal *et al*. (6.15%) [23] all showed a low incidence pattern of the $cMLS_B$ phenotype. In the present study, both inducible and constitutive resistance was seen more often in MRSA compared to MSSA isolates. Most of the national and international study reports have had similar results. However, only Schreckenberger *et al*. [21] and Levin *et al*. [19] demonstrated higher percentages of inducible and constitutive clindamycin resistance in MSSA isolates compared to MRSA isolates.

Truly clindamycin-sensitive strains, which exhibit efflux pump-mediated resistance to macrolides (MS phenotype), represented 11.52% of all isolates tested in our study. This is in close agreement with Gadepalli *et al*. (12%) [6], Gade *et al*. (12%) [22], and Mittal *et al*. (15%) [23]. This fact implies that clindamycin can

be safely and effectively instituted as a therapeutic drug in such clinical scenarios despite macrolide resistance. All erythromycin-resistant isolates need not necessarily be resistant to clindamycin. Conversely, labeling all erythromycin-resistant isolates as clindamycin resistant and not reporting clindamycin resistance in the presence of the $iMLS_B$ phenotype will prevent the use of clindamycin as an effective therapy in situations where it is most likely to respond [21]. Early detection of inducible MLS_B resistance patterns saves time and resources for both the clinician and patient alike. Hence, clindamycin should never be used in treatment of infections with $iMLS_B$ strains, which will help to avoid therapeutic failure.

The bulk of data from available literature appear to support the concerns that have been raised over the use of clindamycin in $iMLS_B$ infections, especially those that are deep seated or have a large bacterial burden. In our study, we found moderate prevalence of inducible and constitutive resistant phenotypes among erythromycin-resistant *S. aureus* isolates. The incidence of inducible clindamycin resistance is important in health settings where the drug is used as an initial empirical therapy, and this is known to vary even between hospitals. It should be determined for each individual laboratory [20]. The prevalence may change over time with the emergence of strains with different sensitivity patterns, so periodic surveys should be performed if testing is not done routinely [9]. This is, to our knowledge, the first study from Northeast India that emphasizes the prevalence of inducible resistance in this region, the need for routine testing by laboratories using the D-test method, and the need for judicious prescription of the drug by clinicians.

Conclusions

In an era where we are experiencing an emergence of resistance to multiple antibiotics for treating infections, our armamentarium of drugs are depleting very quickly. Clinicians already have limited options to choose from. Judicious use of suitable general antibiotics should be promoted to treat common infections and the special antibacterial agents, those with an excellent sensitivity profile, should be reserved and used only as a last resort to treat critical infections. Simultaneously, appropriate therapeutic decisions are not possible without proper antibiotic susceptibility data. Clindamycin, a very good alternative for Gram-positive and anaerobic organisms, must not be misused. This is where the D-test becomes significant. The D-test is a simple, credible, easy to perform method to differentiate sensitive as well as resistant phenotypes of clindamycin.

Clinical microbiology laboratories, especially in areas with high rates of MRSA infections, should consider performing routine testing and reporting of inducible clindamycin resistance in *S. aureus* isolates. Clinicians should take note of this fact, and update and equip themselves with sound and practical knowledge for judicious use of the drug in their respective hospitals and geographical locations. They should prescribe the drug in clinical scenarios only when it is truly susceptible. This will ensure that clindamycin remains a viable and excellent antibiotic alternative for staphylococcal infections.

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Authors' contributions

Amit Banik was involved in concepts, design, definition of intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing, and was a manuscript review guarantor.

Annie Bakorlin Khyriem was involved in concepts, design, definition of intellectual content, literature search, clinical studies, data analysis, manuscript preparation, manuscript editing, and was a manuscript review guarantor. Jeetendra Gurung was involved in concepts, definition of intellectual content, clinical studies, experimental studies, data acquisition, and manuscript editing. Valarie Wihiwot Lyngdoh was involved in concepts, definition of intellectual content, experimental studies, data analysis, manuscript editing, and was a manuscript review guarantor.

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