D test: A simple test with big implication for *Staphylococcus aureus*
Macrolide-Lincosamide-Streptogramin B Resistance Pattern

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**ABSTRACT**

D test is a simple disc diffusion test giving high throughput results. It is used to study the macrolide lincosamide streptogramin resistance (MLSb), both constitutive and inducible as well as macrolide streptogramin resistance (MSb) in *Staphylococcus aureus*. In this test, erythromycin (macrolide) and clindamycin (lincosamide derivative) discs are placed adjacent to each other over the Mueller Hinton agar medium inoculated with the test organism. The growth of the organism up to the edges of the disc, flattening of the clindamycin zone (D test positive) near the erythromycin disc (resistant) and susceptible to both antibiotics implicate that the organism is having constitutive MLSb (CMLSb), inducible MLSb (IMLSb) and no resistance respectively. Further, the organism susceptible to clindamycin without any flattening of the zone (D test negative) near clindamycin disc (resistant) implicates that the organism is having macrolide streptogramin resistance (MSb). The test is performed in the same MHA plate in which the antibiotic sensitivity test is being done, taking into consideration that the discs are placed adjacent to each other maintaining the distance. Since clindamycin and streptogramin are among the few drugs of choice in the treatment of methicillin resistant *S. aureus* (MRSA) infections, knowing the resistance to these antibiotics is imperative.

**Keywords:** Resistance, erythromycin, clindamycin, streptogramin, *Staphylococcus aureus*.

**INTRODUCTION**

Macrolide, lincosamide and type B streptogramin (MLS) are chemically distinct antibiotic having similar target site and mode of action.¹² They all have a narrow spectrum of activity against Gram positive cocci especially staphylococci, streptococci and enterococci. Three mechanisms account for acquired resistance to these MLS antibiotics and they are modification of the target of the antibiotics, active efflux of the antibiotics and inactivation of the antibiotics. Target site modification is the most common mechanism of acquired resistance to MLS antibiotics in staphylococci. A single alteration in 23S rRNA confers broad cross-resistance to macrolides, lincosamides, and streptogramin B-type antibiotics and hence known as macrolide lincosamide streptogramin B resistance (MLSb resistance).³ MLSb resistance can be either constitutive MLSb (CMLSb) or inducible MLSb (IMLSb).⁴ MLSb resistance phenotype accounts for nearly all of the resistant clinical isolates. In staphylococci, the prevalence of this resistance phenotype in hospital settings is between 15 and 45%, but generalization cannot be made because of important local variations.⁵ Active efflux of antibiotic, less frequently encountered mode of acquired resistance is mediated by an ATP-dependent pump mediated by msrA.⁶ Inactivation of antibiotic yet another mode does not confer cross resistance⁷ and has limited value.

Macrolides consist of 14-, 15-, and 16- membered lactone ring macrolides. Erythromycin, oleandomycin, clarithromycin, dirithromycin and roxythromycin are macrolides having 14- membered lactone ring, Spiramycin, jasomycin, midecamycin, kitasamycin and rokitamycin are having 16-membered lactone ring and Azithromycin is having 15-membered lactone ring (also called azalide structure).

Clindamycin is a derivative of lincomycin, the lincosamide antibiotic that inhibits protein synthesis by the target modification. Clindamycin is a useful antibiotic for the treatment of skin and soft tissue infection, and infections caused by *Staphylococcus* spp. especially methicillin resistant *S. aureus* (MRSA). Clindamycin has excellent tissue and bone penetration, and accumulates in abscesses. Good oral absorption and no requisition of renal dosing adjustment make it an important therapeutic agent.⁸

Streptogramin antibiotic consists of at least 2 structurally unrelated molecules: group A (M) streptogramins (macrolactones) and group B (S) streptogramins. Pristinamycin and virginiamycin are naturally occurring streptogramins, whose use in clinical practice has been limited due to their complex and irregular
composition, and insolubility. Streptogramins A and B act synergistically and the mixture of the two compounds is more powerful than the individual components in inhibiting protein synthesis. Group A or group B compound alone has a moderate bacteriostatic activity, whereas the combination of the two exhibit strong bacteriostatic activity and often bactericidal activity. Streptogramins are effective in the treatment of vancomycin resistant Staphylococcus aureus (VRSA) and vancomycin resistant enterococci (VRE).

These three antibiotics though are structurally different their mode of action is similar working in the same site during protein synthesis. Cross resistance among these antibiotics is due to modification of drug target. Erythromycin and other macrolides bind reversibly to 50S ribosomal subunit and methylate ribosomal protein in the 23S ribosomal RNA. Such rRNA methylation leads to conformational change in ribosome resulting into co-resistance between macrolides, lincosamide and streptogramin due to their common target of action. Therefore, erythromycin mediated methylase confers resistance to lincosamide and streptogramin in the presence of erythromycin. Clindamycin and streptogramin do not induce methylase. In the absence of erythromycin to induce the enzyme, organisms appear susceptible to these antibiotics.

**RESISTANCE TO MACROLIDE, LINCOSAMIDE AND STREPTOGRAMIN**

Resistance of bacteria against these antibiotics may be intrinsic or acquired. Gram negative bacteria like members of Enterobacteriaceae family, Pseudomonas spp. and Acinetobacter spp. are intrinsically resistant to MLS antibiotics due to the impermeability of the bacterial cell membrane. However in the gastrointestinal tract (GIT) infection the MIC is achieved in the range of 2-256 μg/ml, hence can be used in the infection occurred in the GIT.

Three mechanisms that account for the acquired resistance among bacteria against these antibiotics are target modification, active efflux of the antibiotic and inactivation of antibiotics.

**Target modification:** Single alteration in 23S rRNA confers broad cross resistance to macrolide, lincosamide and streptogramin B antibiotics. erm genes [erm(A), erm(B) and erm(C)] encoded methylase enzyme, methylate the ribosome at 23S thus target of the antibiotic is altered. As a result antibiotic cannot act upon the target and resistance is observed.

**Active efflux of antibiotics:** There are antibiotic resistance genes encoding for transport of proteins (efflux). They do not modify the antibiotic or the antibiotic target, rather pump (efflux) the antibiotics out of the cell or the cellular membrane such that intracellular concentration becomes low and ribosomes are free from the antibiotics.

Macrolide and streptogramin resistant msr(A), macrolide efflux mef(A) in Streptococcus Pyogenes and mef(E) in S. pneumonia; and virginiamycin factor A Vga(A) and vga(B) in staphylococci are three different efflux systems that have been described in gram positive cocci.

msr(A), msr(B) [also msr(A') and msr(B')] are different from mef genes in the aspect that they confer resistance to both macrolide and streptogramin B whereas the later confer efflux of macrolide only. A lincomycin specific efflux pump encoded in lmr(A) has been described in Streptomyces lincolnensis.

**Inactivation of antibiotics:** There are arrays of genes encoding for the enzymes that inactivate the antibiotics. There is no cross resistance when the mode of action is by inactivation of antibiotics. In the members of Enterobacteriaceae and in S. aureus, macrolide inactivation occurs by ErmA and ErmB enzymes that hydrolyze the lactone ring of the macrocyclic nucleus and also phosphotransferase [type I mph(A) and type II mph(A)] inactivate the macrolide. lin(A) gene conferring resistance only to lincosamide has been detected in S. aureus, S. haemolyticus, S. epidermidis, S. cohnii and S. hominis. Similarly lin(A') has been reported in S. aureus, S. epidermidis and S. cohnii. vgb gene in staphylococci encoding lactonase is capable of cleaving macrolactone of streptogramin B. Similarly vat(A) and vat(B) genes encoding acetyltransferases inactivate streptogramin A.

The multiplicity and complexity of MLS resistance phenotypes of bacteria observed today are largely due to the recent detection of new mechanisms of resistance mainly the inactivation of antibiotics. However, these new mechanisms have a limited importance in practical point of view due to their low incidences. Inactivation of lincosamide has been reported in 2 % of S. aureus and 4-8 % in coagulase negative Staphylococcus (CoNS). Less than 5 % of S. aureus inactivate streptogramin antibiotics. This is in contrast to that MLS resistance conferring nearly all the resistance observed among the clinical isolates which accounts for 15-45 % of resistance among S. aureus isolated from hospital settings. Erythromycin resistance in MRSA has been reported to be higher than 90 % in numerous countries. However, generalization is difficult due to the importance of local variation.

**Macrolide-lincosamide-streptogramin B (MLSb) resistance:** Cross resistance occurring between
macrolide, lincosamide and streptogramin B also known as Macrolide-lincosamide-streptogramin B resistance is an acquired resistance encoded in erythromycin methylase (erm) genes. Three distinct methylase genes erm(A), erm(B) and erm(C) have been detected in staphylococci. Expression of these methylase genes is controlled by translational attenuation.

MLS$_B$ resistance in _S. aureus_ may be constitutive or inducible. When the expression is constitutive, the organisms are resistant to all macrolides, lincosamides and type B streptogramin antibiotics. In contrary, when the resistance expression is inducible, the organisms are resistant to 14- and 15-membered macrolides; and are sensitive to 16 membered macrolide, lincosamide and streptogramin B in the absence of inducer erythromycin. Since, 14- and 15-membered macrolides are effective inducers of methylase synthetase, methylase is produced only in the presence of an inducer (erythromycin). Azithromycin, the 15-membered macrolide also induce resistance in clindamycin. Strains with inducible resistance are resistant to erythromycin and appear susceptible to clindamycin and streptogramin B in the absence of inducer the erythromycin. They are resistant to these antibiotics in the presence of inducer.

**Erythromycin ribosome methylase gene:** Till 1999, 22 classes of rRNA methylase (erm) genes had been reported. Twenty one classes contained the identified and characterized erm genes and in 22$^{nd}$ class contained all unclassified and uncharacterized genes. In 2009, 33 classes of erm genes have been reported. Of those, only 9 classes [erm(A), (B), (C), (F), (G), (Q), (T), (Y), erm(33)] have been identified in _S. aureus_. The most prevalent genes encoding the methylase in _S. aureus_ have been designated erm(A), erm(B), and erm(C). Of these three too, erm(A) and erm(C) are the most common ones and erm(B) is found in the _Staphylococcus_ isolates from animal origin. erm(A) and erm(C) genes are located in chromosome and plasmid respectively. The distribution of erm(A) and erm(C) is often species specific. Rarely occurring erm(B) gene is located in transposon of _S. aureus_.

**Genetic basis of MLS$_B$ resistance:** erm genes code for MLS$_B$ resistance irrespective of their constitutive or inducible nature of resistance. The methylase enzyme produced by erm gene methylates the 23S ribosomal RNA, specifically adenine 2058 in 23S rRNA. The methylation alters the conformation of ribosome leading to resistance to macrolide. The erm mediated methylase produced by erythromycin resistant _S. aureus_ is also responsible for cross resistance to clindamycin and streptogramin due to their common site and mode of action.

The inducible or constitutive expression of resistance is not related to class of erm gene. It solely depends on the regulatory region sequence present upstream of the methylase structural gene. The regulation of expression of MLS$_B$ resistance occurs by translation attenuation, where translation of methylase encoding genes occurs depending on the presence of inducer. Two point mutations in the control region convert the inducibly resistant strain to constitutively resistant strain irrespective of the presence or absence of the inducer.

**Macrolide-streptogramin B (MS$_B$) resistance:** Staphylococci that exhibit resistance to 14- and 15-membered ring macrolide and streptogramin B but are sensitive to 16 membered ring macrolide and lincosamide are said to have MS$_B$ resistance. MS$_B$ resistant staphylococci harbor macrolide streptogramin resistance (msr) gene or a similar gene that encodes an ATP dependent efflux pump mechanism. MS$_B$ resistant strains remain Clindamycin susceptible in disc diffusion test.

**Macrolide streptogramin resistance gene:** In _S. aureus_, the MS$_B$ resistance is conferred by the macrolide streptogramin resistance msr(A) gene. This is the most prevalent gene conferring MS$_B$ resistance. Another gene conferring MS$_B$ resistance is msr(B) which has not been reported yet. The msr(B) gene homologous to msr(A) is significantly shorter than the msr(A) gene sequence which is roughly half the size of msr(A). Recently in 2009, msr(B) along with msr(SA), msr(SA') have been included in msr(A) gene.

**Genetics of MS$_B$ resistance:** The msr(A) gene encodes for a hydrophilic ATP binding protein, Mrsa that functions as a drug efflux pump, an ATP dependent process. Mrsa protein belonging to ATP binding cassette (ABC) transporters super family exports antibiotics across the cell membrane. msr(A) gene expression is regulated by translational attenuation and removal of the control region of the gene leads to constitutive expression of msr(A).

**EPIDEMIOLOGY**

In 2 hospitals in the USA (Chicago) occurrence of CMLS$_B$ resistance has been stated to be much higher among MRSA (84 % and 82 %) compared to that among methicillin sensitive _S. aureus_, MSSA (3 % and 18 %). In the same hospitals, the incidence of IMLSB resistance has been reported to be low (7 and 12 %) among MRSA and among MSSA (20 % and 19 %). However, in another US hospital MSSA isolates (34 %) has been reported to be almost three times more likely to have IMLSB resistance compared to MRSA isolates (11 %).
another report from Atlanta USA 32 % of S. aureus isolates had IMLS₉ and 13.7 % had CMLS₉ resistance in a collection of S. aureus strains from Center of Disease Control and prevention and project, and Rockefeller University, USA.14 Association of MRSA with IMLS₉ resistance has been put forward by Maple et al.26 They have stated that clindamycin resistance emerge readily a common event in MRSA.

In Spain Significantly higher prevalence of IMLS₉ than CMLS₉ resistance among S. aureus has been reported.27 In a European study from 24 university hospitals, majority of the macrolide resistant MSSA strains were CMLS₉ phenotype, whereas IMLS₉ resistance was predominant among MSSA.28 Similar higher occurrence of IMLS₉ resistance among MSSA has been reported in Birmingham.29 On the contrary, in Greece Higher prevalence of CMLS₉ (60 %) followed by IMLS₉ (35 %) and clindamycin susceptible phenotype (5 %) has been reported in S. aureus.30 Similarly, in Turkey a higher occurrence of CMLS₉ resistance in MRSA (44.2 % versus 24.4 %) and IMLS₉ in MSSA, (14.8 % versus 4.5 %) has been reported.31 Comparatively higher occurrence of CMLS₉ resistance in MRSA has been put forward in a Turkish study.15

In Nepali context, Mohapatra et al have reported association of CMLS₉ and IMLS₉ with MRSA.32 In similar Nepali study, MLS₉ resistance was found associated with MRSA (97.6 %).33 MRSA having CMLS₉ resistance has been stated to be 94.7 % and 100 % of the IMLS₉ resistant isolates were MRSA.33

In USA (Atlanta), 8.5% of the S. aureus exhibited MS₉ resistance.14 In another report from two US hospitals (Chicago), quite a low occurrence of MS₉ among MRSA and MSSA has been reported.4 O’Sullivan et al. have stated that MS₉ resistance occurs less commonly than IMLS₉ but they have also stated that the resistance pattern show great geographical variation.34 On the contrary, Merino-Diaz et al. have reported that in Spain MS₉ resistance was the most common resistance type comprising of 7.2 % in S. aureus of the erythromycin-resistant strains. In the same study, the occurrence of IMLS₉ resistance has been reported to be higher (5.2 %) than rate of CMLS₉ resistance (1.7 %) in S. aureus.35 In a Turkish study Azap et al. have reported that MS₉ resistance was found among MSSA. Again in another Turkish study, almost equal occurrence of MS₉ resistance among MRSA and MSSA has been reported.31

In Nepal, no association of MS₉ resistance with MSSA or MRSA has been reported.35 MS₉ resistance was found in small frequency that occurred mostly among MSSA and heterogeneous MRSA.33

Factors affecting the prevalence of different resistance phenotype strains

The differences in the occurrence of CMLS₉, IMLS₉, MS₉ resistance among MRSA, and MSSA could be due to geographic variation.34 It has been stated that the incidence of resistance is highly variable with regard to the country, type of infections among the patients,21 geographical region and specific clones of MRSA may differ in different hospitals and regions.29 Further, Patel et al have stated that the prevalence of resistance phenotypes, and specific clones of MRSA may vary in different regions.29 The incidence of IMLS₉ resistance is important in a setting where clindamycin is prescribed empirically, and this incidence is known to differ between hospitals.4,15 Further, Maple et al have stated that clindamycin resistance emerge readily which is common in MRSA.26 Hence, local statistics are of crucial value for empiric therapy. Surveillance of incidence of macrolide resistance and the respective prevalence of the various resistance types should be done in each hospital and D test is the simple and highly indicative test for the purpose.

Methodology of D test

D test is a simple disc diffusion test where erythromycin and clindamycin discs are placed adjacent to each other on a lawn of the test organism. D test has a high throughput indicating different types of resistance phenotypes in a single test. This easy to read test can be done along with the antibiotic susceptibility test or even in the same plate hence does not require any extra energy, cost and effort.

For D test, guidelines of recent Clinical Laboratory Standard Institute (CLSI) 200736 should be followed. 5/6 colonies of the test isolate grown on blood agar is directly suspended in physiological saline (0.85% sodium chloride in distilled water) and is matched with 0.5 McFarland’s turbidity standard (1.5x10⁸ bacterial load of per ml). Within 15 minutes of the preparation of the bacterial suspension, it is inoculated onto a dried (37ºC for 30 minutes) Mueller Hinton agar (MHA) plate having a depth of 4 mm ± 0.5 mm and pH 7.3 ± 1. A sterile swab is dipped into the matced inoculum suspension and pressed against the inside of tube to express excess of the inoculum, and is inoculated onto MHA plate. The plate is allowed to stand on bench for 5 -10 minutes. Erythromycin (15 μg) and clindamycin (2 μg) antibiotics discs that have been stored at 2-8ºC and have been brought to room temperature are used. The antibiotic discs are placed over the inoculated MHA plate at a distance of 15 mm edge to edge, allowed to stand on bench for 30 minutes and then incubated at 35ºC for 18 hrs.26
**D TEST INTERPRETATION**

The susceptible phenotypes are susceptible to both erythromycin and clindamycin. Presence of flattening of clindamycin zone adjacent to erythromycin disc is a characteristic known as D zone and the isolate is referred to as D test positive.

Any test strain that is resistant to erythromycin and is D test positive is exhibiting IMLS$_B$ resistance and any strains that are resistant to both erythromycin and clindamycin are having CMLS$_B$ resistance. The genes encoding such resistance may carry either one of erm(A), erm(B) or erm(C) conferring methylation of adenine 2058 in 23S rRNA of ribosomal RNA.

D test also detects strains with macrolide-streptogramin B (MS$_B$) resistance. The strains which are resistant to erythromycin, susceptible to clindamycin and are D test negative (no flattening of clindamycin zone adjacent to erythromycin disc) are having MS$_B$ resistance. These strains are resistant to macrolide and streptogramin and are susceptible to clindamycin. Such resistance is encoded in macrolide streptogramin resistance (msr) genes, which are either msr(A) or msr(B) conferring active efflux of antibiotics such that intracellular concentration becomes low and ribosomes are free from the antibiotics. (Figure 1)

Steward, Raney, Morrell et al. have described two distinct phenotypes induction phenotypes and non-induction phenotypes. Induction phenotypes consists of two IMLS$_B$ resistance phenotypes namely D and D$^+$. Non-induction phenotypes consist of four phenotypes and are Neg (MS$_B$), HD (CMLS$_B$), R (CMLS$_B$) and S (susceptible) among the isolates of *S. aureus* (Table-1).

Debate over the use of clindamycin in IMLS$_B$ resistance phenotype infection

Clindamycin, one of the drugs of choice in the treatment of infections by homogeneous MRSA cannot be used for those exhibiting CMLS$_B$. MS$_B$ resistance phenotypes do not develop resistance to clindamycin during therapy. There is doubt in usefulness of clindamycin for the treatment of infections by homogeneous MRSA exhibiting IMLS$_B$. Although IMLS$_B$ resistance phenotype isolates appear susceptible to clindamycin in the absence of an inducing agent macrolide, there is widespread reluctance to prescribe clindamycin for treatment of patients with infections caused by such organisms due to the concerns that resistance to clindamycin will develop during therapy.

<table>
<thead>
<tr>
<th>Induction test phenotype</th>
<th>Resistance phenotype</th>
<th>Erythromycin result</th>
<th>Clindamycin result</th>
<th>Test description</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Inducible MLS$_B$</td>
<td>R</td>
<td>S</td>
<td>Blunted D shaped clindamycin inhibition zone adjacent to erythromycin disc</td>
</tr>
<tr>
<td>D$^+$</td>
<td>Inducible MLS$_B$</td>
<td>R</td>
<td>S</td>
<td>Blunted D shaped clindamycin inhibition zone near erythromycin disc and small colonies in the zone</td>
</tr>
<tr>
<td>Neg</td>
<td>MS$_B$</td>
<td>R</td>
<td>S</td>
<td>Clear inhibition zone around clindamycin disc</td>
</tr>
<tr>
<td>R</td>
<td>Constitutive MLS$_B$</td>
<td>R</td>
<td>R</td>
<td>Growth up to clindamycin and erythromycin discs</td>
</tr>
<tr>
<td>HD</td>
<td>Constitutive MLS$_B$</td>
<td>R</td>
<td>R</td>
<td>Double Clindamycin zones, one zone is light, hazy growth extending from clindamycin disc to second zone where the growth is heavy. The inner light zone exhibit flattened zone like in D phenotype</td>
</tr>
<tr>
<td>S</td>
<td>No resistance</td>
<td>S</td>
<td>S</td>
<td>Clear susceptible zone around clindamycin and erythromycin discs</td>
</tr>
</tbody>
</table>
Lewis et al. have recommended avoidance of clindamycin for the treatment of complicated infections having a high bacterial burden, such as abscesses or osteomyelitis. Clindamycin if used for treatment of a less severe IMLS$_B$ infection, the patient must be closely monitored for signs of treatment failure or relapse of infection. Non-IMLS$_B$ infections can be treated with clindamycin. Nevertheless, clindamycin is a frequent choice for treating some staphylococcal infections because it can be given orally and is well tolerated.

Conclusion

The sharp rise in staphylococcal infection all over the world and changing pattern of antimicrobial resistance including the emergence of MRSA have led to the use of clindamycin therapy in the treatment of staphylococcal infections. Increasing frequency of CMLS$_B$ resistance phenotype may be the reflection of the increased use of clindamycin in the treatment of staphylococcal infection. Occurrence of CMLS$_B$ and IMLS$_B$ resistance in MRSA and also in MSSA has made it necessary to perform D test in all $S. aureus$ isolates. Further, association of both CMLS$_B$ and IMLS$_B$ resistance with MRSA has also been reported. It has been suggested that IMLS$_B$ phenotypes determined by disk diffusion methods correlate well with genotypic test and the degree of correlation is so strong that disk diffusion results may be used to predict genotype.

Use of clindamycin in MRSA expressing IMLS$_B$, is a matter of debate due to its ability to develop clindamycin resistance in vitro and in vivo during clindamycin therapy. However, there are reports of successful clindamycin treatment of infection by MRSA expressing IMLS$_B$ resistance. Hence, D test should be included in routine susceptibility test of all $S. aureus$ isolates. Any $S. aureus$ isolate positive in D test (IMLS$_B$ resistance phenotype) should be reported as clindamycin resistant with a comment that the organism is presumed to be resistant based on the detection of inducible clindamycin resistance and clindamycin may still be effective in some patients.

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