International Journal Dental and Medical Sciences Research
Volume 5, Issue 2, Mar - Apr 2023 pp 505-512 www.ijdmsrjournal.com ISSN: 2582-6018

Phenotypic and Genotypic detection of Carbapenemase producing Gram Negative Bacilli

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Date of Submission: 20-03-2023 Date of Acceptance: 30-03-2023

ABSTRACT

(i) **Background & objectives**

Carbapenem-resistant Gram-negative bacilli (CRGNBs) cause serious infections resulting in increasedtreatmentcost,prolongedhospitalization, andmortalityrate.Carbapenem resistance (CR) occurs either due to production of plasmid - mediated carbapenemases (most common mechanism, CP-GNB) and/or by chromosomal mechanisms expressed as reducedexpressionand/ormutationsofporins[1].

Since, there is no single detection method ideal for all situations,Phenotypicdetectionofcarbapenemresistanceinconcertwithmolecularassayis therecommendedstrategy to facilitate an accurate detection and characterization of the CP-GNB for an appropriatemechanismtargetedantibiotictherapyin infectedpatients [2,3,4].

(ii) **Methods**: A prospectivestudy was done on 53 clinical CRGNB isolates, to evaluateandcomparetheperformancesofthephenotypicmethods,Vitek2and modified Carbapenemase Inhibition (mCIM) and EDTA Carbapenemase Inhibition (eCIM)[5]. Thegenotypicdetectionofthecarbapenemasegenes amongthe CRGNBisolates was done by an in-house developed Loop mediated amplification assay (CARB LAMP), using Calcein for detection of the product.

(iii) **Results**: mCIM/eCIM versus Vitek 2 showed 94% sensitivity, 67% specificity, 98% positive predictivevalue(PPV)and40%negativepredictivevalue(NPV).mCIM/eCIMversusLAMPshowed 92% sensitivity,50%specificity,96%PPVand33%NPV.Vitek2versusLAMPshowed92%sensitivity,67%specificity,98%PPVand33%NPV.

(iv) **Interpretation & conclusions**

Overall, the results indicated that Vitek 2 followed by the CARB LAMP for carbapenemaseencodinggeneswouldbeanappropriateand rapid diagnosticapproachforapromptand targeted therapy against CP-GNB infections.

Key words: Carbapenemase genes, Vitek 2,eCIM/mCIM, LAMP assay, CRGNB, Impermeability

I. INTRODUCTION:

Carbapenemantibiotics(doripenem,ertapenem,imipenemandmeropenem)arebroadspectrumdrugsandusuallyreservedforseverelife-threateninginfections.GNB,suchastheEnterobacterales, Pseudomonas and Acinetobacter species, have developed carbapenemresistance(CR)whichlimitsoptionsfortreatinginfectionsduetotheseorganisms [6].

CR may either be due to diminished expression or loss of the outer membrane porin & reducedpermeability, overexpression of Efflux pumps and Enzyme-mediated resistance due to theproductionofcarbapenemaseswhichmodifytheantibioticsbyhydrolysisofthebeta-lactammolecule. This type of antimicrobial resistance, especially when mediated by transferablecarbapenemase-encodinggenes,isspreadingrapidlycausingseriousoutbreaksanddramaticallylimitingtreatmentoptions [6]. SeveralCarbapenemaseencoding genes, of global importance, have been described to date but the most common genesconferring CR among the GNBs include bla_KPC (Klebsiella pneumoniae

International Journal Dental and Medical Sciences Research
Volume 5, Issue 2, Mar - Apr 2023 pp 505-512 www.ijdmsrjournal.com ISSN: 2582-6018

carbapenemase)(Ambler class A), bla_NDM (New Delhi metallo-β-lactamase-type 1) (Ambler class B),bla_OXA-48-like &bla_OXA-23 (oxacillinase)(Ambler class D), bla_VIM (Verona integronencodedmetallo-β-lactamase)andbla_IMP(imipenemasemetallo-β-lactamase)(AmblerclassB)[7].

Accurate detection of CP-GNB and the encoding genes in the clinical microbiology laboratoryisimportantso as tofacilitateappropriatemechanismdirectedantibiotictherapyin the infectedpatients.

In this prospective study, thephenotypic methods, Vitek2and mCIM/eCIM and the genotypicdetectionofthecarbapenemasegenesamongtheCRGNBisolatesbythe CARB LAMPassaywas performedandevaluated on 53 CRGNBs.

II. MATERIALS&METHODS

This study was conducted by the Department of Microbiology, Kamineni Academy of Medical Sciences and Research Centre, Hyderabad, Telangana, India, between 2022. Well characterized GNB isolates from clinical specimen, were tested for their antimicrobial susceptibilityprofiles on the Vitek 2 system (bioMerieux, France) using the AST 280,281,405,406 cards. Fifty three (53) GNB isolates that weredetectedasCarbapenemresistant, defined as per the recommended strategy [4],wereincludedinthisstudy(TableI)These were 33 Enterobacterales (CRE), including E. coli(12), Klebsiellapneumoniae (17), Enterobacter cloacae complex (2) Providencia rettegeri (1)Serratiamarcescens (1) and 20 non-fermenters, including Pseudomonas aeruginosa (8), Acinetobacter baumaniicomplex (12). All the CRE isolates were screened by mCIM & eCIM to detect carbapenemaseproduction while the non-fermenters were tested only by mCIM as per CLSI recommendations[5].TheLAMPassaytodetectthemostcommoncarbapenemasegenes,bla$_{NDM}$,bla$_{KPC}$,bla$_{OXA23}$,bla$_{OXA-48\ like}$,bla$_{VIM}$ &bla$_{IMP}$wasdevelopedinhouse, at Huwell Lifesciences, Hyderabad[8]. However, Sybr green was replaced by Calcein dye for visual detection of the LAMP product.

III. RESULTS

Inalltherewere53CRGNBs;Enterobacteraleswere 33/53(62.3%)andthenon-fermenterswere 20/53 (37.7%) (Table I). On the Vitek 2, the carbapenem resistance was displayed eitherasCarbapenemaseproducers20/53(38%),Carbapenemase+Impermeability30/53(57%)oronlyimpermeability3/53(5%).TheresultsofthemCIM/eCIMweredocumentedasmetallo-beta- lactamase producers (MBLs) 40/53 (75%), serine enzyme producers 9/53 (17%) ornegative 4/53 (8%) for production of both the enzymes. Tables IIa, IIb,IIc show the results of the mCIM/eCIM assays alongwith the Vitek 2 results. By the CARB LAMP assay, Carbapenemase genes were detected among47/53(89%)isolates.NDMonlywasdetected in15/53(28%),OXA-23in2/53(4%),OXA-48likein4/53(8%)andtherestoftheisolates,26/53(49%)hadacombinationofthesegenes.The remaining isolates 6/53 (11%) were negative for all the genes. None of the isolates hadKPC,VIMorIMPgenes. Figure(i)showstheresultsoftheCARBLAMPassayon 5isolates.Figure (ii) shows the distribution of the CR genes among the 53 CR GNB isolates. Thespectrum oftheCarbapenemasegenesinassociationwithMBLs&Serineenzymesamongthe53CRGNBs is shown in figure (iii).

In Figure (iv) a comparison of the performance characteristics of all the 3 assays is shown.mCIM/eCIM versus Vitek 2 showed 94% sensitivity, 67% specificity, 98% positive predictivevalue(PPV)and40%negativepredictivevalue(NPV).mCIM/eCIMversusLAMPshowed92%sensitivity,50%specificity,96%PPVand33%NPV. Vitek2versusLAMPshowed92%sensitivity,67%specificity,98%PPVand33%NPV.

IV. DISCUSSION

In 2017, the World Health Organization (WHO) published a list of resistant bacteria against which there is an urgent need to develop new antibiotics [9]. Critical priority bacteria included carbapenem-resistant Enterobacterales (CRE). These bacteria are among the most common pathogens associated with severe infections, such as sepsis, pneumonia, urinary tract, and intra-abdominal infections, having, which along with the current COVID-19 pandemic are having a major impact, both clinically and economically.

The CP GNBs are categorized as Priority 1-critical pathogens by the WHO since the resistancetocarbapenemantibioticsisamajorandon-goingpublichealthproblemglobally[9].

CPGNBsaredefinedasclinicalorsurveillancecultureyieldingE.coli,Klebsiellaspp.,andEnterobacter spp., other Enterobacterales, and Pseudomonas or Acinetobacter positive forknown carbapenemase gene or positive on phenotypic test for carbapenemase. Any othergenera that test

International Journal Dental and Medical Sciences Research
Volume 5, Issue 2, Mar - Apr 2023 pp 505-512 www.ijdmsrjournal.com ISSN: 2582-6018

positive for carbapenemase should be classified as confirmed .[4]

Enterobacterales, P. aeruginosa and A. baumanii showing resistance to at least one of the carbapenems (meropenem or imipenem) are called CRE, CRPA and CRAB respectively [1].

Early detection of these resistant pathogens is important for early diagnosis and appropriate management[7].Phenotypic antimicrobial susceptibility testing performed by the clinical laboratory on isolates obtained on culture can reveal CR in CRGNBs and is considered the gold standard.

On the Vitek 2, the CR is displayed either as only Carbapenemaseproducers,Carbapenemase+Impermeabilityoronlyimpermeability.Impermeabilityamongthe GNBsisduetoanoverexpressionoftheefluxpumpsormutationsintheporinchannels.InsuchCRGNBswithloneimpermeability,nocarbapenemasegenenortheenzymes will be present. However, in our study, there was one Enterobacter aerogenes isolate withimpermeability alone but was positive for the MBL and NDM+OXA-48 like genes, which were probably not expressed initially, but were induced during mCIM/eCIM test. Isolates with such discrepant resistance mechanisms may have to be confirmed by sequence analysis for the genes. Another discrepant result was with the 4 CRGNBisolatesshowing a combination of carbapenemase production andimpermeability.Thoughtheenzymeswerepresent,therewerenogenesdetectedbythe CARB LAMPassay.TheprobablepresenceofotherCRgenes, thatarenotincludedontheLAMPpanel,cannotberuledoutintheseisolates.

To confirm the production of carbapenemases and/or the presence of additional resistance mechanism, a variety of biochemical assays and/or gene-based diagnostics are available.

CP GNBs can be detected phenotypically using biochemical assays such as the Carba NP®, Blue Carba®, and Carba® tests, however these methods fail to detect other resistance mechanisms such as porin loss and efflux pump[5]. The hydrolysis of the substrate imipenem or meropenem by these phenotypic methods determines the presence of carbapenemases in the GNB isolates.

However, due to the indiscriminate results of the Carba NP, the CLSI recommended the phenotypic procedure, mCIM/eCIM[5]. This test isperformed as an additional test for the detection and differentiation of MBLs (Ambler class B)andtheSerineenzymes(AmblerclassA,CandD). The27theditionofCLSIM100described mCIMonly for Enterobacterales.Thiswaslatermodifiedtodetectcarbapenemaseseven in the non-fermenters. Since the mCIM cannot distinguish between serine and metallo-carbapenemases, it was further modified as eCIM with the addition of EDTA and has beenendorsed in the CLSI M100-S28 supplement in 2018[10]. eCIM accuratelydistinguishes between serine and metallo-carbapenemases but is recommended only for theEnterobacterales that are mCIM positive and not as a standalone test. In case of the non-fermenters, only mCIM should beperformed [5].

In the present study, one P. aeruginosa isolate (Carbapenemase + impermeability)and one A. baumanii isolate (Carbapenemase only), as per the VITEK result, were negative for the enzymes but the genes, NDM+OXA23+ OXA-48 & NDM +OXA 23weredetectedbytheLAMPassay.Thisdiscrepancyisprobablyduetonon-expressionofthe genes duringtheCIMassays. The presence of a gene does not always mean that an organism is carbapenem resistant as it depends on the amount of expression of the resistance gene.

Genotype methods do not rely on indicators of resistance like hydrolysis, but rather identify the genes known to express resistance. A number of molecular testing systems are now approved for use to identify enzyme-mediated resistance [11]. Molecular techniques that use the principle of conventional polymerase chain reaction (PCR) or real-time qualitative PCR are used for the identification of carbapenemase encoding genes [1]. Generally, the commercially available rapid molecular tests can target and detect the most common five carbapenemases (KPC, IMP, VIM, NDM and OXA-48 like variants) encoding genes, with a short turn- around time of less than 24hours and with a sensitivity of 80-100%. Although themolecular methods provide rapid results, they are not readily available in all laboratories since they are expensive in terms of infrastructure, equipment and trainedmanpower.

The LAMP assay is a novel molecular method for amplifying DNA and RNA (LAMP and RT-LAMP, respectively) with a high specificity, sensitivity, and simplicity as the assaydoesnotrequireexpensiveconsumables,sophisticatedinstrumentationnortrainedtechnicalpersonnel.Theresultsareobtainedin1hourandhencecanbeadoptedasa Pointofcaretest(POCT)fulfillingalltherequirementsofanASSUREDassay [12]. Though the LAMP is not marketed as a carbapenem detection assay, but research has shown the application of

this DNA amplification technology to identify CROs, including identification of specific carbapenemases in A. baumanii[11].

In the routine Microbiology laboratory, the CARB LAMP assay can be positioned andintegratedasaroutineassayoneveryGram-negativebacterialisolatethatmaybeconsideredclinicallysignificant.Theassaycanalsobedirectlyappliedonpositivebloodculturesw ithaGNB.

In this study, an inhouse developed LAMP technology(CARB LAMP) was used as a bench-side assay for the rapid detection of the most commoncarbapenemases among the CPGNBs[8].

Asshowninfigurei,theCPgeneswere detectedamong47/53(89%)isolatesby the LAMP assay. Six (11%) of the isolates were negative for all the genes. Amongthese,in2oftheisolatestheCRwasduetoimpermeability.Theremaining4/6isolateswere displayed ascarbapenemaseproducersontheVITEKandtheCIMassayswerepositiveforthe enzymes. Probably these 4 isolates had CR genes that were not included in the CARBLAMP panel, or the CR was due to an unknown resistance mechanism. As is the case with any molecular assay, the primary limitation of the CARB LAMP assay also is that only the known and most common genes were targeted and hencegenesencodingnovelcarbapenemaseswerepr obablymissed.

There are a few studies evaluating the phenotypic and the molecular assays for the detection ofCP GNBs [3]. However, there are no studies comparing the VITEK 2 analysis,CIMassaysandtheLAMPassayfortheCPgenes,aswasdoneinourstudy.Figureiiishows the comparisonbetweenthe3methodsusedinthestudy. Thoughthe cumulative sensitivityandthePPVwere high, the specificity and NPV were low probably due to different results on the 3 methods.Suchdiscrepantisolateshavetobefurtherstudiedbygenesequencingandorthe repertoire of the LAMP assay to target otherknowncarbapenemaseencodinggeneshas to be expanded. Also,studyingmorenumberof isolates would probably give more incite in the utility of the CARB LAMP assay as an additional tool, in the routinemicrobiologylaboratory.

Accurate detection and characterization of carbapenemase-producing GNB (CP-GNB) canfacilitateappropriatemechanismdirectedantibiotictherapyininfectedpatients[6]. Clinical laboratories should strive to identify and define carbapenem resistance in A. baumanii, P. aeruginosa, and Enterobacterales appropriately using available phenotypic or molecular based methods. Knowledge of the likely underlying resistance mechanism will permit clinicians to select the optimal antibiotic treatment for patients. These concerted efforts will enable the concept of precision medicine to be applied to the diagnosis and treatment of CROs with the intent to reduce the spread of resistance and improve patient outcomes [6].

Overall, the results of the present study indicate that Vitek 2 followed by the CARB LAMP assay for carbapenemaseencodinggeneswouldbeanappropriatediagnosticapproachforapromptandearlydetection of the CP GNBs. The identification of the specific genes would guide in appropriate mechanismbasedantibiotictherapy.ThoughthemCIM/eCIMhadgoodperformancecharacteristics,the resultswillbedelayedbyanother18-24hoursandhencemaynotbeclinicallyuseful.

Since the novel antibiotic therapies are based on targeting the specific CR gene, a simple and rapid bench side molecular assay, such as the CARB LAMP, is more appropriate.

Therapy of CROs requires early and accurate identification of the mechanism of resistance. Asisthecasewithanyotherdiagnosticassay(s),thereisnosingledetectionmethodthatisideal forcarbapenemasedetection.Henceitisrecommendedthatclinicallaboratoriesshouldu seacombination of both phenotypic and molecular methods for carbapenemase detection. They should choose the appropriate combination according to their hospital's economic strength, theavailability of the tests in their region and the type(s) of patients being treated and theinformationprovidedbythetest.Aspertheresultsofourstudyandtheneedforrapidresults,especially in the critically ill patients, the phenotypic Vitek 2 assay followed by the genotypic CARB LAMPassay for carbapenemase genes would be an appropriate and ideal approach for an earlydetectionandcharacterizationoftheCPGNBs. Thisstrategywillfacilitateanearly,specific andmechanismtargetedantibiotictherapyintheinfectedpatient(s).

Financial support &Sponsorship: nil
Conflicts of interest: none

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Table- I
Spectrum of Carbapenem resistant isolates(n=53)

S.no	Isolate	No.ofisolate	MICof Carbapenems	CommonMechanismsof CR
1	Enterobacterales	33	>4	Carbapenemase+++Impermeability+
2	A.baumanii	12	>8	Carbapenemase+++Impermeability+++

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3	**P.aeruginosa**	**8**	**>8 (Int. Res toErtapenem)**	**Carbapenemase+Impermeability+++**

Table- IIa
VITEK result of CRGNB-Carbapenemase production only(n=20)

Organism	**No.of isolates**	**Metallo-beta-lactamase**	**Serine producer**	**Noenzyme**
Escherichiacoli	1	1	-	-
Klebsiellapneumoniae	1	1	-	-
Serratiamarcescens	1	1	-	-
Providenciarettegeri	1	1	-	-
Acinetobacterbaumanii complex	12	10	1	1
Pseudomonasaeruginosa	4	4	-	-

Table- IIb:
VITEK result of CRGNB---Carbapenemase+imperme ability(n=30)

Organism	**No.of isolates**	**Metallo-beta-lactamase**	**Serine producer**	**Noenzyme**
Escherichiacoli	11	9	2	-
Klebsiellapneumoniae	16	8	8	-
Enterobactercloacaecomplex	1	1	-	-
Pseudomonasaeruginosa	2	1	-	1

Table- IIIc:
VITE K result of CRGNB---Impermeability only(n=3)

Organism	**No. ofisolates**	**Metallo-beta-lactamase**	**Serineproduction**	**Noenzyme**
Enterobactercloacaecomplex	1	1	-	-
Pseudomonasaeruginosa	2	1	-	1

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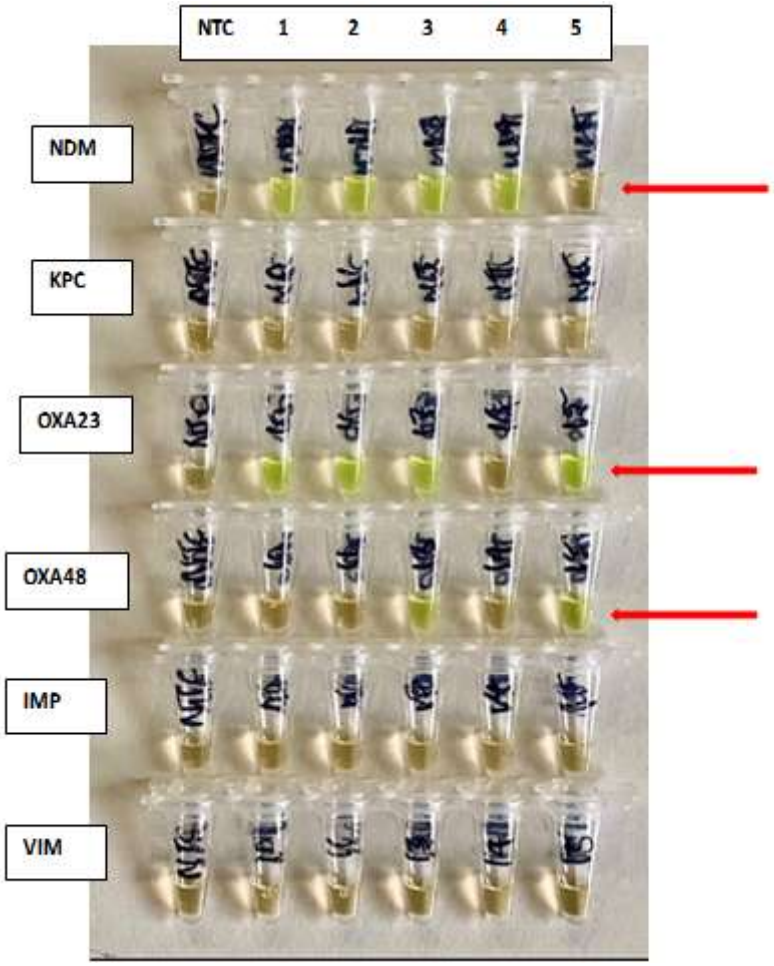
Volume 5, Issue 2, Mar - Apr 2023 pp 505-512 www.ijdmsrjournal.com ISSN: 2582-6018

Figurei:

Results of CARBLAMP for 5isolates



NTC=No template/targetcontrol
Positive result - green color
Negative result–palecolor

Figure ii :

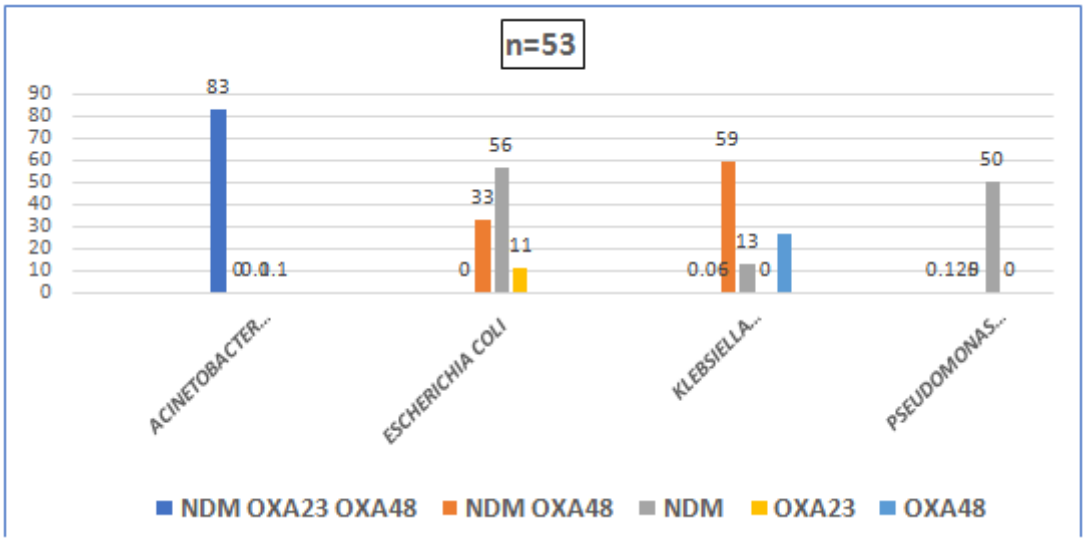


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Figure iii :
Carbapenemase encodinggenes & the associated enzymes

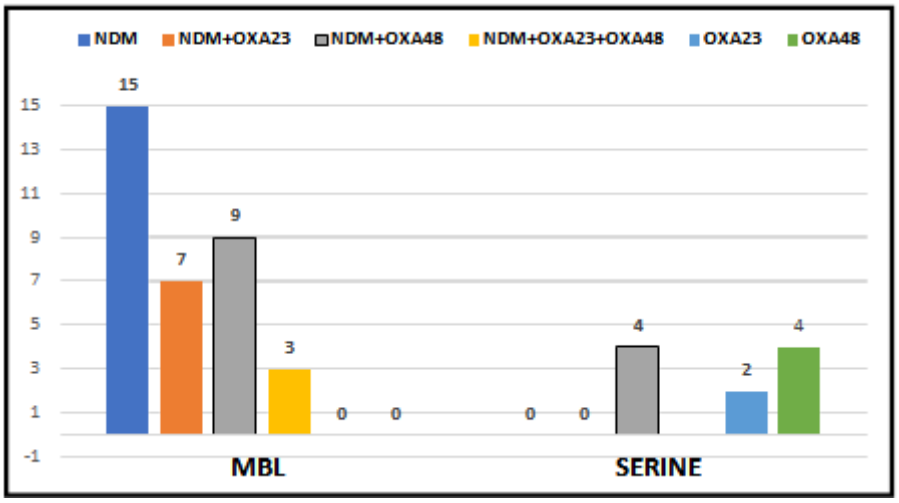

Figure iv :
Comparison of the Performance characteristics of the three methods(%).