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Review Article

NDM-beta-lactamase-1: Where do we stand?

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Multidrug-resistant (MDR) Gram-negative bacilli (GNB) have been playing havoc in the field of nosocomial as well as community-acquired infections. Of particular concern are the carbapenem-resistant GNBs, belonging to Enterobacteriaceae and encoding for New Delhi metallo-beta-lactamase-1 (*NDM-1*) gene. These strains spread rapidly and horizontally in the population, thus exhibiting MDR traits as these can harbour several resistance encoding genes to almost all antimicrobial groups. Several predisposing factors are responsible towards its spread, *viz.* excessive antibiotic usage, improper aseptic conditions by healthcare workers, lack of awareness, abruptly discontinuing medication course, alternative medications and vector-borne factors contributing to the unchecked harbouring of these super bugs in India. Thus, a bugle call has already been sounded worldwide especially in India, where the country has taken serious cognizance to build up strategy *via* implementation of several national programs to combat antimicrobial resistance covering human, animal, agriculture and environmental aspects. As there is an exponential rise in variants of NDM-1 harbouring strains, molecular epidemiological investigations of these strains using genotyping techniques are of paramount importance for a better understanding of this rampant spread and curbing resistance thereafter. This review explores the urgent need to develop a cost-effective, rapid molecular assay, *viz.* the loop-mediated isothermal amplification method for field detection of MBL harbouring bacterial strains, especially NDM-1 and its variants, thus targeting specific carbapenemase genes at a grass root level even to the remote and rural regions of the country.

Key words Carbapenemase - enterobacteriaceae - Gram-negative bacilli - NDM-β-1 - susceptibility

Carbapenem-resistant *Enterobacteriaceae* (CRE) isolates have been found to display high resistance to broad-spectrum antimicrobials than non-CRE, implying carbapenem resistance might be linked with resistance to several other antibiotics as well. Since carbapenem are considered to be the last resort drugs of choice, the highly resistant CRE carrying the New Delhi metallo-β-lactamase-1 gene (NDM-1) can limit the therapeutic options[1,2]. Excessive antibiotic usage in health care, agriculture and veterinary care, has led to a selection pressure favouring the survival and spread of such resistant organisms, resulting in increased hospital stay, morbidity and mortality[3,4]. NDM-1 producers belonging to *Enterobacteriaceae* are mainly associated with urinary tract infections (UTI), peritonitis, septicaemia, pulmonary infections, tissue and other device–associated infections[5]. There are reports of the presence of NDM-1 in *Cedecea lapagei* isolated from the neonatal intensive care unit in Aligarh[6,7]. Studies have revealed widespread dissemination of bla_{NDM} variants through horizontal gene transfer among the Gram-negative bacilli (GNB) in developing countries

co-existing with other resistance markers[8]. As observed for other gut-colonizing multidrug-resistant (MDR) bacteria, which precede infections by NDM producers, their transmission mainly takes place *via* the oro-faecal route[9]. The most abundant carbapenemase producer globally is the *Klebsiella pneumoniae* carbapenemase (KPC) (bla_{KPC} gene), followed by the Verona integron-encoded metallo-ß-lactamase (VIM), NDM, Imipenemase (IMP) metallo-β-lactamase, and oxacillinase-48-type (OXA-48). These are all harboured by GNBs, *viz. K. pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Enterobacter cloacae*[10,11]. Aggressive acquirement of antimicrobial resistance (AMR) and harbouring of mobile genetic elements by members of *Enterobacteriaceae* and non-*Enterobacteriaceae* have greatly affected the community[12,13].

AMR menace prevails worldwide, especially among the carbapenem group of drugs. The Ministry of Health and Family Welfare, Government of India has been actively addressing AMR issues by launching several programs spanning the length and breadth of the country towards its containment. India is a member of the 10-Global Health Security Agenda (GHSA) steering group and the largest of 17 GHSA phase 1 countries supported by several international organizations to fight against AMR[14]. The Indian Council of Medical Research (ICMR) and National Centre for Disease Control (NCDC) have been working together on the five-year plans, implementing AMR surveillance, health-care associated infection control and reinforcing various laboratories. With the onset of the COVID-19 pandemic, treating potentially fatal secondary bacterial infections in COVID-19 patients became imperative. In such a situation the increased levels of antimicrobials released in wastewater from hospitals may have affected levels of antimicrobials in the environment at large. Thus, in such challenging times, antibiotic stewardship plays a crucial role[15].

The background of bla_{NDM-1} transmission

NDM-1-producing bacteria was first detected in India from a patient with UTI caused by carbapenem-resistant *K. pneumonia*[16]. What was alarming was that, the latter was found to be resistant to most of the antimicrobials[16]. Subsequently, reports of NDM-1 spread rapidly westward[17].

It was found that most of the isolates carrying the bla_{NDM-1} gene were on plasmids along with several other antibiotic resistance determinants, capable of transferring such massive resistance to the associated bacteria[18]. These code for resistance to all aminoglycosides, macrolides and sulphamethoxazole, thus converting resident bacterial isolates to multidrug-resistant ones[18]. Subsequently, bla_{NDM-1} and bla_{VIM-2} genes were found together in a strain of *P. aeruginosa* in West Bengal, displaying co-existence of different carbapenem resistance gene[19]. However, others have reported different reasons behind the background of NDM-1 dissemination, stating plasmids of different sizes with genetic signatures on either side of bla_{NDM-1} gene being responsible for its mobility and pathogenesis. Similar gene cassette carrying resistant traits were also found in a tertiary care hospital among northern India among other Gram-negative bacteria from the same patient[20,21]. These genes colonize together, thus spreading extensive resistance which is alarming as these bugs are usually related to causing life-threatening diseases[22-24].

Acquiring the NDM-1 gene and its proliferation: A multifaceted problem

Although studies related to NDM-1 have mostly been associated to the Indian subcontinent[1], the Balkan countries are considered as a secondary reservoir where NDM-producing bacteria have been isolated from patients following return after seeking medical benefits from abroad[9]. Karthikeyan *et al*[25] have reported detection of NDM-positive bacteria from the Middle East and North or Central Africa including Afghanisthan, Algeria, Cameroon, Egypt, Iraq, Israel, Kuwait, Lebanon, Morocco, the Sultanate of Oman and the United Arab Emirates[25]. The National Reference Laboratory of the Health Protection Agency, United Kingdom (UK) had investigated the rise in the unusual carbapenem-resistant isolates from UK patients[26]. These isolates of enterobacteriaceae showed NDM-1, but were negative for other known carbapenemase genes. All these cases had a history of travel to India or Pakistan within a brief span in common[26]. This calls for a routine detection and accurate reporting of NDM-1 in diagnostic laboratories, especially those from India. With the rampant dissemination of the NDM gene variants (NDM-2 to NDM-8) with varying capacity to cleave carbapenem drugs, molecular epidemiological investigations of these isolates especially from faecal specimen. The hospital sewage is a potential source of bla_{NDM} variants outbreaks which is of major concern[27]. Genotyping techniques are of paramount importance for a better understanding of their rampant spread and infection control[28].

Kumarswamy *et al*[29], have reported the prevalence of NDM-1 in enterobacteriaceae isolates from India, Pakistan and in the United Kingdom. Their study selectively identified NDM-1 isolates from Guwahati, Mumbai, Varanasi, Bengaluru, Pune, Kolkata, Hyderabad, Port Blair, and New Delhi in India, several cities in Pakistan and Dhaka in Bangladesh pointing to its rampant dissemination[29]. Similar cases of those having travelled to Asian countries and may have acquired infection while undergoing treatment are also available[2]. Distribution of AMR and colistin resistant pathogenic strains have also been isolated from Tamil Nadu, West Bengal, Punjab[30-32]. In a study conducted in south India, MDR *Enterobacteriaceae* colonizing the gut of adult rural population was observed indicating faecal carriage of AMR strains with a threat of rapid spread in the community[33].

Several human (improper antibiotic stewardship and infection control, human migration) and bacterial factors (nosocomial spread of bla_{NDM} gene) have consequently led to the rapid spread of this gene[34,35](Figure).

Drinking water quality, defective sewage system, vector–mediated *viz.* house flies, uncontrolled circulation of antibiotics are other major factors promoting the spread of these resistance microbes in a healthy population. Furthermore, there is a tendency to switch to alternative medication particularly the Indian traditional medicinal system such as the Ayurveda, Siddha and Unani having yielded successful results worldwide especially in remote and rural areas[36].

Routine laboratory diagnosis of NDM

Currently, the NDM-1 class of carbapenemase is of clinical concern as it shares 32.4 per cent amino acid match with VIM-1/VIM-2, on the other hand minute identity match with other metallo-β-lactamases (MBLs). NDM-1 binds strongly to most cephalosporins compared to VIM-2. NDM-1 effectively hydrolyzes broad range of β-lactams including penicillin, cephalosporin and carbapenem and just sparing monobactams like other MBL[9].

Screening of carbapenem-resistant Gram-negative bacilli (GNBs) carrying *NDM-1* gene

Currently, detection of NDM producers is based on preliminary screening using the antibiotic susceptibility test (AST)[9]. Susceptibility to NDM-1 is usually by disc diffusion method in accordance with Clinical and Laboratory Standard Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the British Society for Antimicrobial Chemotherapy (BSAC) guidelines as done in practice- and/or determination of minimum inhibitory concentration (MIC) by manual or automated methods[37-39]. It is noteworthy that there might be certain differences in MIC values for isolates depending on the reference used to interpret antibiograms. Susceptibility to carbapenems are observed for some NDM producers and additional tests for carbapenemase detection are needed to identify them accurately[9]. Different chromogenic plate methods are used for performing AST and identification of carbapenemase producers which are detailed as:

Chromogenic plate method: Carbapenemase producers are presumptively identified using chromogenic plates useful for direct screening of high-risk asymptomatic carriers *viz*. stool samples. This cost-effective, rapid and simple detection of carbapenemase producers is vital for effective infection management control interventions and also in preventing outbreaks of nosocomial infections by these organisms. Different chromogenic media for detection of NDM-1 producers are available *viz*. ChromID ESBL and CHROMagar KPC media alongwith acceptable specificity and sensitivities ranging from 53-100 per cent[40]. However, its disadvantage being chromogenic media is not reliable detection for all types of carbapenemase producing isolates and requires confirmation tests[41]. Screening stool specimen of patients hospitalized for carbapenemase producers is done using screening culture media, *viz*. CHROMagar media and ChromID ESBL which is a bit time-consuming before the actual status of the patient is known[42-46]. This is of primordial significance as these NDM-1 carrying enterobacteriaceae isolates mainly inhabit the gut region.

Several other routinely and commercially available automated systems which used to identify and detect NDM-1 carbapenemase-producing isolates include the Vitek 2 automated system (France). Studies evaluating the accuracy of these platforms in the detection of carbapenemase activity have shown appropriate sensitivities but inadequate specificities, which leads to the requirement of confirmatory tests[40,45].

Confirmatory detection of carbapenemase activity among the screened Gram-negative bacilli (GNBs)

Different techniques are available for identification of carbapenemase producers based

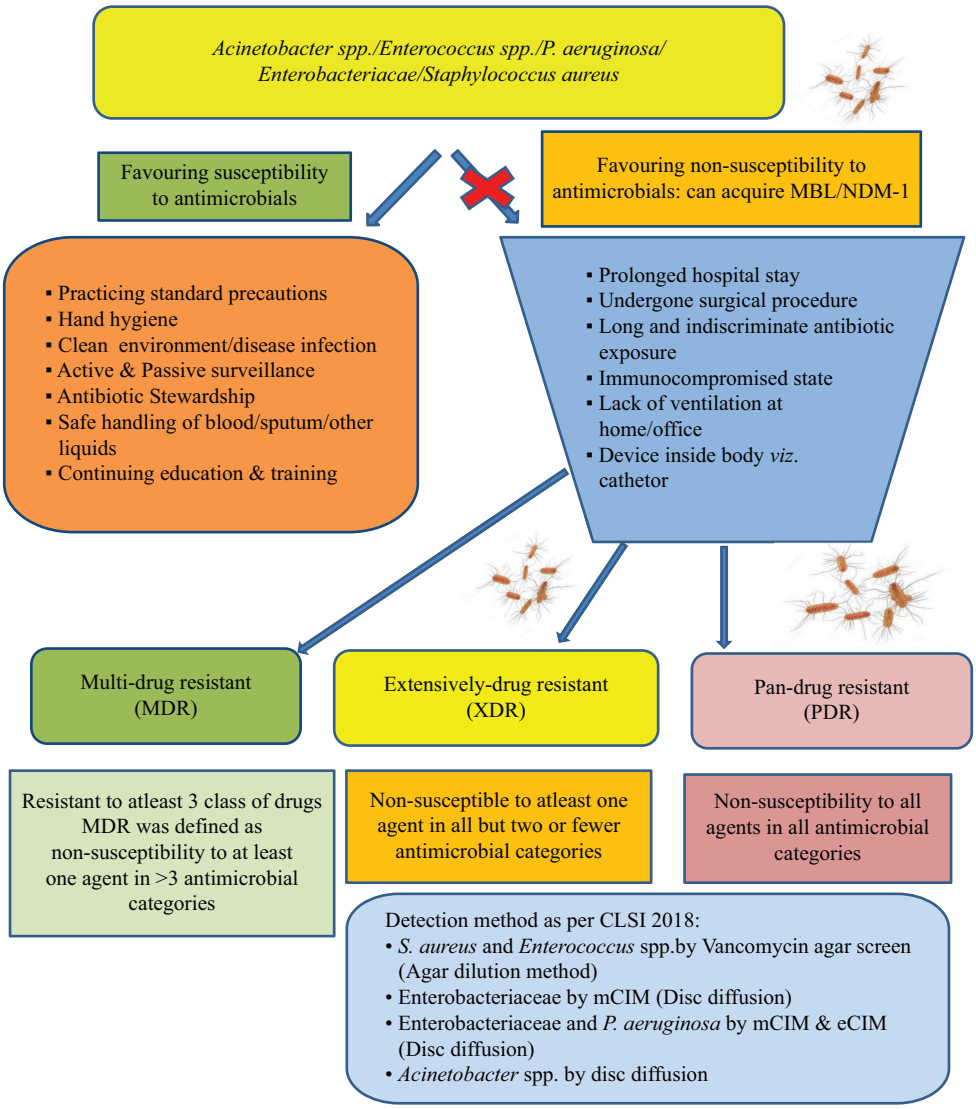

Figure. Flow chart showing screening of Gram-negative bacteria resistant to carbapenem drugs.

on several phenotypic and genotypic methods[41]. The phenotypic methods are based on inhibitors or breaking down of carbapenem drugs or else on spectrophotometric approach, whereas genotypic detection is much more precise based on polymerase chain reaction (PCR)/real-time PCR (RT-PCR)/ DNA sequencing/micro array based[10,18,42]. However, phenotypic methods are much simpler, less time consuming and cheap. Phenotypic assays are designed based on the following principles:

Inhibition of metallo-carbapenemase: These phenotypic tests rely on the combined effect of discs *viz.* meropenem alongwith ethylenediamine-tetraacetic acid (EDTA), phenylboronic acid (PBA)/ both EDTA and PBA for differentiation of class A and B enzymes or dipicolinic acid (DPA) to specifically inhibit metallo-carbapenemase activity[46,47]. Considering that PBA inhibits other β-lactamases as well, AmpC β-lactamases, cloxacillin (an AmpC inhibitor without activity against KPC and other class A carbapenemases) is also used routinely to differentiate between AmpC and KPC production[48,49] as it was found that strains harbouring both KPC and MBL could be detected using both EDTA and PBA in a single disk. Thus, ruling out of other common mechanisms for carbapenem resistance can indirectly help the laboratory to diagnose the presence of NDM-1 by virtue of exclusion[50].

Combined disc test (CDT): Studies evaluating CDT in comparison to other molecular tests have shown

high sensitivities and specificities (90-100%) with reference to bla_{NDM-1}, bla_{KPC}, bla_{VIM}, and bla_{IMP} in carbapenemase-producing isolates from Hyderabad[41,51].

Double disk synergy test (DDSTs): This method uses Mg-EDTA to detect MBL-producing strains, including NDM-1 producers[41,52]. This method has demonstrated good sensitivities (100%) and specificity of 91.0 per cent. However, this method also reports certain shortfalls, such as test outcome interpretation is subjective requiring a qualified personnel[53,54].

Gradient diffusion strips: For detecting MBL and KPC various gradient diffusion strips formats have been designed, which separately detect MBL and KPC. The E-test (epsilometer test) MBL using imipenem and imipenem-EDTA was simple for their detection, except in two cases (*E. cloacae* D and *K. pneumoniae*), in which interpretation of the results was not possible because the imipenem MICs were too low[40,55-57]. The sensitivity of E-test MBL for bla_{NDM-1} positive isolates was 66.7 per cent and specificity was 100 per cent. However, gradient diffusion strips are quite expensive[58].

Detection based on carbapenem hydrolysis

The cloverleaf method [modified Hodge test, (MHT)]: According to Aguirre-Quinonero and coworkers, MHT is still being used for carbapenemase detection following CLSI guidelines[41,59]. However, as a result of the poor performance of the Hodge test displaying ambiguous results, modifications of the assay such as the addition of ZnSO4[45] or cloxacillin[58] to the agar plate was done. Pasteran and co-workers have reported inclusion of Triton X-100 (a non-ionic surfactant) to improve NDM-1 carbapenemases detection, as the compound solubilizes membrane lipoproteins and consequently membrane-bound carbapenemases[60]. This latter version was known as the Triton Hodge Test (THT)[61,62]. The MHT did not show good results in detecting NDM-producing isolates (merely 20 and 32.5% sensitivity for meropenem and ertapenem, respectively). On the other hand, sensitivities of the THT were 100 per cent with ertapenem and 92.5 per cent with meropenem for the latter MBL producing strains[60].

Colorimetric assays

Assays displaying colour change due H^+ ion concentration in the media has been exploited that is, *p*H based detection method or colorimetric assays including *p*H indicators *viz.* phenol red for Carba NP[41] or bromothymol blue for Blue-CARBA.

Carba NP: This assay was initiated by Nordmann *et al*[63] and named thereafter as Carba NP. This assay has reported specificity of 100 per cent and sensitivity between 90 and 100 per cent in the detection of carbapenemases[58,60]. This assay was thus used for identifying bla_{NDM-4}, bla_{NDM-5}, bla_{NDM-7} from rectal and stool swabs from a neonatal intensive care unit in Aligarh, India[64].

Blue-CARBA: The Blue-CARBA which is a modification of the Carba NP yields similar results as the Carba NP[9,60,61,63]. The Blue-CARBA is an inexpensive test with 93.3 per cent sensitivity and 100 per cent specific detecting any type of carbapenemase of enterobacteriaceae including NDM-1 producers[49,65].

Carbapenem inhibition method (CIM): Initiated by Van der Zwaluw *et al*[66], the CIM method display high sensitivity (98-100%) and specificity of 100 per cent. This method has proved to be inexpensive and easy to interpret. Moreover, this assay has been modified and further recommended to be used for carbapenemase detection (mCIM) in enterobacteriaceae and *P. aeruginosa* in the 28th edition of CLSI[67]. If mCIM comes positive, then only eCIM (carbapenemase activity is inhibited in the presence of EDTA) is done to differentiate MBL[67].

Spectrometry techniques: Mass spectrometry (MS) is more often being used for the detection of carbapenemase-producing isolates.

MS: Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS is reliable for isolate identification of bacteria and fungi and is routinely in use in laboratories. This method is effective for detection of carbapenemase-producing Gram-negative bacteria especially NDM-1. Studies have found sensitivities ranging between 77 to 100 per cent and specificities from 94 to 100 per cent[66,68]. However, MALDI-TOF is time-consuming and costly and its operation requires trained microbiologists. This technique was based on detection of a carbapenem spectrum and of its main derivatives resulting from carbapenem hydrolysis[9].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS): The LC-MS/MS methods are analytical, reliable and sensitive. The chromatographic retention time, precursor ion mass, and product combine to impart a good analytical specificity. LC-MS/MS is the gold standard for small-molecule detection and quantitation[69,70].

Immunochromatographic assay: Detection of NDM-1 by immunochromatographic assay, using rat monoclonal antibodies are also being studied[63].

Molecular CP-carbapenem resistant Enterobacteriaceae (CRE) detection methods: Varying susceptibility patterns of isolates towards various carbapenem drug depends on the efficiency with which the drugs are hydrolyzed[42,43]. This variability in hydrolysis spectrum hampers identification. Phenotypic tests are good for screening the carbapenemases, but most of them are incapable of detecting the specific carbapenemase responsible for drug resistance. The World Health Organization (WHO) in an effort towards AMR surveillance and prevention program, through the Global Antimicrobial Resistance Surveillance System (GLASS) has emphasized on the reliability of phenotypic assays whereas, molecular testing could provide additional data regarding resistance profile and mechanisms. Molecular diagnostic assays require costly set-up, rapid, with high specificity and sensitivity for identifying the harboured resistance genes *viz*. $bla_{\text{NDM-1}}$,bla_{VIM} ,bla_{IMP}. Thus, depending on the laboratory capacity and prevalent AMR data, WHO has stratified molecular diagnostic testing to be performed depending on its complexity and cost effectiveness. Thus molecular diagnostics are available for laboratories with low capacity, especially belonging to the low- and middle-income countries the molecular methods recommended are either fully integrated and automated cartridge-operated PCR or the loop-mediated isothermal amplification (LAMP) devices and lateral flow assays requiring visual inspection for interpreting results. Whereas, high-capacity laboratories with good experience in molecular diagnostics might prefer complex diagnostic assays, such as microarrays technology and the whole genome sequencing (WGS) to analyses/interpret the raw data. However, WHO does not endorse nor validate any particular molecular method but relies on the phenotypic detection of resistance in isolates using antimicrobial agents by measuring the MIC which is the gold standard[71].

PCR allows for rapid identification of specific carbapenemase genes using primers and probes to conserve regions in the gene target for real-time assays, can be carried out by PCR. PCR being specific to a given gene, and can be further tailored to detect specific subgroups of a gene family[72]. As $bla_{\text{NDM-1}}$ encoded plasmids are rampantly spreading worldwide especially among members of *Enterobacteriaceae* faecal screening should be prioritized using PCR-based molecular screening techniques *viz*. multiplex PCR nucleic acid amplification test (NAAT) for efficient detection of faecal pathogens[73]. A multilocus sequence typing (MLST) can be performed to identify house-keeping genes *viz*. on plasmids in pathogens resistant to carbapenem drugs and displaying widespread resistance in the community[74]. Plasmid sizing is done using S1-nuclease pulsed-field gel electrophoresis (PFGE) and Southern blot is performed for identification of NDM-1 plasmid[44,73]. The specificity and sensitivity using qPCR of $bla_{\text{NDM-1}}$ has been reported as 98.4 and 100 per cent, respectively[35,42]. The primary limitation of PCR is that the only known genes can be targeted while, those encoding novel carbapenemase will be missed with molecular approaches[75].

On the other hand, the LAMP method is promising tool. It is simple, rapid, cost-effective, requiring no sophisticated instrument. It is a single tube amplification reaction requiring Bst DNA polymerase for strand displacement and DNA synthesis under isothermal conditions based on auto-cycling. The LAMP technology has been widely used in clinical diagnosis; field detection of MBL harbouring bacterial strains, qualitative and quantitative detection of epidemic bacteria, viruses, and parasites; as well as in fetal sex identification[76]. It is suggested that time demands requiring further research in the development of the LAMP assay as a molecular tools for the detection and confirmation of NDM-1 producers along with its variants and other MBLs. Thus, a simple and affordable detection assay at a genetic level would certainly help combat MBLs spread, with screening remote and rural areas.

On the other hand, various commercial PCR-based customised assays are also available *viz.* the Xpert Carba-R test and the hyperplex SuperBug ID[47,58,77]. These assays are specific and sensitive but costly. The controls used for culture-based tests, phenotypic assays and PCR for NDM-1 detection is usually *K. pneumoniae* ATCC BAA-1705 as positive control and *K. pneumoniae* ATCC BAA-1706 as negative control, however, other controls can be selected from in-house confirmed positive /negative strains confirmed by sequencing[78].

Microarray technology is another promising tool and can be paired with PCR-based target amplification. It utilizes probes that hybridize to DNA targets,

including resistance genes. Microarrays can be used to target and extract DNA from bacterial isolates or patient specimens and can handle hundreds of DNA targets, thus multiplexing with numerous carbapenemase genes, along with distinguishing between closely related variants[79-81].

The WGS is versatile and comprehensive with a capacity to recognize all resistance mechanisms across the bacterial genome. Apart from targeting various carbapenemases, it can also identify other contributors to resistance, such as porin loss, efflux genes, *etc*. The data generated in WGS can also help to elucidate the source in outbreak investigations by extracting information regarding the type of plasmid carrying resistance genes, evolutionary lineage of the isolate and its relatedness with other isolates[43,56]. Currently, WGS is expensive and requires qualified personnel for necessary data extraction and interpretation[82].

Other promising approaches worth exploring:

Using macrophage: The versatility of macrophages in response to environmental stimuli and their engagement largely in pathogenesis of numerous human diseases makes them a desired target cells to be considered suitable for future therapy. This combined with nano-biotechnology can offer advantage in improving therapy outcome[83].

Lytic phage proteins: Using lytic phages or else lytic phage proteins, a concept which originated in the beginning of the 20th century[84] for combating AMR holds promise in curbing its spread.

Reactive oxygen therapy (ROS): Targeted use of ROS to the site of infection in various forms has also been suggested as a potential alternative as ROS has antimicrobial activity towards pathogens including biofilm breakdown[85].

Conclusion

The extent of threat owing to the spread of pathogens producing NDM-1 belonging to *Enterobacteriaceae* worldwide is alarming. This is of serious concern in the Indian sub-continent, parts of Asia, Europe and America. The Indian government has taken cognizance of the situation thus creating public awareness against unhygienic practices by initiating cleanliness drive throughout the country.

The lack of a routine standardized phenotypic test for NDM variant detection, expensive molecular tests may have led to under-reporting. The efforts made by various national institutions *viz*. NCDC, ICMR and GHSA of India and other nations worldwide; along with other international organizations *viz*. the WHO can steer the world to a safer and securer environment.

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