Malaysian J Pathol 2015; 37(3) : 227 – 232

ORIGINAL ARTICLE

Molecular detection of the New Delhi metallo-β-lactamase-1 gene in Enterobacteriaceae isolates in a tertiary medical centre

Nor Zanariah ZAINOL ABIDIN *DrPath (UKM),* Anita SULONG *DrPath (UKM),* Hanafiah ALFIZAH *PhD (UKM),* Najihan Abdul Samat MUTTAQILLAH *DrPath (UKM)* and Chuan Hun DING *DrPath (UKM)*

Department of Medical Microbiology & Immunology, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur, Malaysia

Abstract

Background: New Delhi metallo-β-lactamase-1 (NDM-1) is a relatively recent carbapenemase enzyme that inactivates all β-lactam antibiotics with the exception of aztreonam. This study aims to ascertain the baseline prevalence and antibiotic susceptibility patterns of NDM-1-producing Enterobacteriaceae in a tertiary medical center in Malaysia. *Methods:* Over a period of one year, all Enterobacteriaceae isolates from all clinical specimens with reduced susceptibility to at least one carbapenem and resistance to at least one third generation cephalosporin were subjected to antibiotic susceptibility testing by disk diffusion and molecular detection of the NDM-1 gene by single-target PCR followed by gel electrophoresis. *Results:* A total of 13,098 Enterobacteriaceae isolates were screened and 63 (0.48%) had reduced susceptibility to at least one carbapenem. Of this 63, 18 (29%) were NDM-1-positive. Of this 18, 16 (89.0%) were *Klebsiella pneumoniae*, one (5.5%) was *Escherichia coli* and one (5.5%) was *Klebsiella ornithinolytica*. Reduced susceptibility to at least one aminoglycoside was seen in 17 (94%) of the NDM-1-positive isolates. All 18 (100%) had reduced susceptibility to ertapenem and were resistant to all the second and third generation cephalosporin antibiotics tested. *Conclusion:* The prevalence of NDM-1-producing Enterobacteriaceae among all the Enterobacteriaceae isolates in our institution is low (0.14%) and screening for the NDM-1 gene is best performed using ertapenem-impregnated disks.

Keywords: carbapenemase; Enterobacteriaceae; metallo-β-lactamase; NDM-1

INTRODUCTION

New Delhi metallo-β-lactamase-1 (NDM-1) was first described in 2008 in a 59-year-old Swedish patient after he was hospitalised in New Delhi, India for a urinary tract infection.[1] The majority of bacteria with the NDM-1 gene (bla_{NDM-1}) harbour other resistant genes and thus few antimicrobials are available for treating its infections. Many NDM-1-producing Enterobacteriaceae remain susceptible to tigecycline and colistin, and to a lesser extent fosfomycin.[2,3]

Besides *Klebsiella pneumoniae,* other microorganisms that have been found to harbour bla_{NDM-1} are other Enterobacteriaceae such as *Escherichia coli, Enterobacter cloacae, Klebsiella oxytoca, Citrobacter freundii, Morganella morganii,* and *Proteus*.[1,2,4,5] *bla*NDM-1 also has been transmitted to non-fermentative organisms such as *Acinetobacter* spp. and *Pseudomonas aeruginosa*.[6]

Screening for NDM-1 production is done by testing for non-susceptibility to carbapenems and demonstrating resistance to at least one third generation cephalosporin. The phenotypic confirmatory test for carbapenemase production known as the modified Hodge test is more useful for detecting KPC-type than NDM-type carbapenemases, with detection sensitivities exceeding 90% for the former but only 11% for the latter.[7] Thus, isolates with non-susceptibility to a carbapenem should be subjected to molecular detection of the bla_{NDM-1} gene.

The aim of our study was to ascertain the baseline prevalence and antibiograms of NDM-1 producing Enterobacteriaceae in our medical center, because a high prevalence would have

Address for correspondence and reprint requests: Dr. Anita Sulong, Department of Medical Microbiology & Immunology, 16th Floor, Pre-clinical Building, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel: +603-91455402. Email: dranita@ppukm.ukm.edu.my

therapeutic implications. To our knowledge, there are no published data from Malaysia specifically addressing the prevalence of *bla*$_{NDM-1}$ in a healthcare institution.

MATERIALS AND METHODS

Study design and population

This cross sectional study was conducted in Universiti Kebangsaan Malaysia Medical Center (UKM Medical Center) over a period of one year. All Enterobacteriaceae isolates from all clinical specimens with reduced susceptibility (testing intermediate or resistant) to at least one carbapenem during the study period were screened. When the same bacterial isolate with an identical antibiogram was isolated more than once from a given patient, only the first isolate was included in the study.

Speciation of Enterobacteriaceae

Identification was done using API 20E strips (bioMerieux, France) according to the product insert.

Screening for carbapenem resistance

Antimicrobial susceptibility testing was carried out on all confirmed Enterobacteriaceae isolates by disk diffusion. A direct colony suspension incubated overnight with an inoculum equivalent to a 0.5 McFarland standard in cation-adjusted Mueller Hinton broth was prepared. The inoculum was then plated on 100-mm plates containing Mueller Hinton agar. The antibiotic disks used for each isolate were ampicillin (10 μg), cefuroxime (30 μg), gentamicin (10 μg), ceftazidime (30 μg), amoxicillin-clavulanate (20/10 μg), cefotaxime (30 μg), piperacillin-tazobactam (100/10 μg), cefepime (30 μg), ertapenem (10 μg), imipenem (10 μg), meropenem (10 μg), doripenem (10 μg), amikacin (30 μg), ciprofloxacin (5 μg) and chloramphenicol (30 μg). Each 100-mm Mueller Hinton agar plate held a maximum of five antibiotics and was incubated in ambient air at 35±2 °C for 16-18 hours. The susceptibility results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) Document M100-S21.[8] Isolates with reduced susceptibility to one or more carbapenems were subjected to molecular detection of *bla*$_{NDM-1}$.

Molecular detection of bla$_{NDM-1}$

Bacterial DNA was extracted using Genomic DNA Mini Kit (Geneaid Biotech Ltd, Taiwan). For each isolate, overnight bacterial colonies were transferred from MacConkey agar into 3.0 mL of trypticase-soy broth (TSB) in a sterile container. The inoculum was incubated in 37°C for 16-20 hours prior to DNA extraction in accordance to the extraction kit's protocol. The purity of the extracted DNA was determined with the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The DNAs were then stored at -20°C prior to PCR amplification. The primers used are listed in the Table 1 as described by Nordmann *et al*.[9]

The PCR reaction mixture for the detection of *bla*$_{NDM-1}$ consisted of 2.5 μL of 1X reaction buffer, 1.5 μL of 1.5 mM $MgCl_2$, 0.5 μL of 200 μM deoxynucleotide triphosphate, 0.75 μL of 0.3 μM each primer, 2.5 μL of DNA template, 0.4 μL of 2 U of Atlas Taq DNA polymerase and 16.1 μL of distilled H_2O. The DNA was amplified using iCycler Thermo Cycler (Bio-Rad Laboratories, USA). The amplification was carried out under the following cycling conditions: 10 minutes at 95°C; 36 cycles of amplification consisting of 30 seconds at 95°C, 40 seconds at 61.4°C, 50 seconds at 72°C; and 5 minutes at 72°C for the final extension. Five μL of the PCR product was mixed with 5 μL of loading dye. The DNA fragments were separated by electrophoresis at 90 V for 45 minutes in 1.5% agarose gel in 0.5X TBE buffer (First Base Laboratories Sdn. Bhd.). Amplified products were visualised under UV light after staining with GelRed dye.

RESULTS

Reduced carbapenem susceptibility

A total of 13,098 Enterobactericeae isolates were screened. Out of this, 63 (0.48%) non-repetitive Enterobacteriaceae isolates had reduced susceptibility to at least one carbapenem. Of the

TABLE 1: Primer sequences used for detection of *bla*$_{NDM-1}$

bla$_{NDM-1}$ primer	Sequence (5' – 3')	Amplicon size (base pairs)
Forward	GGT TTG GCG ATC TGG TTT TC	621
Reverse	CGG AAT GGC TCA TCA CGA TC	

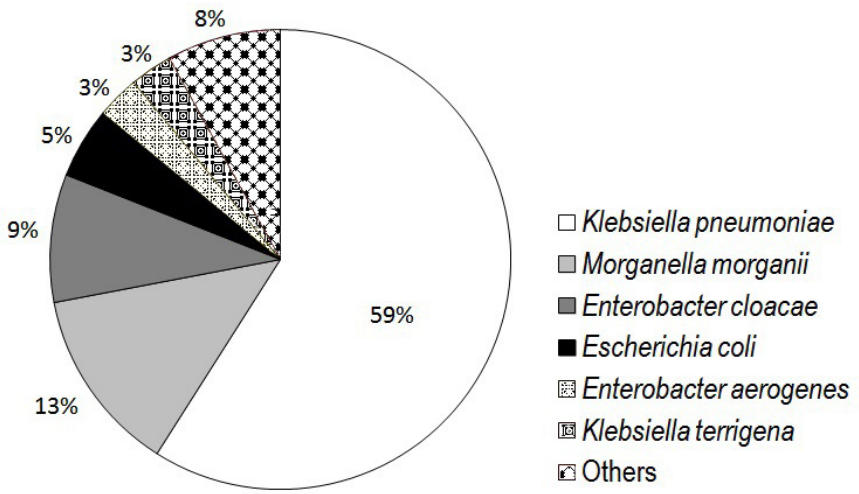

FIG. 1: Enterobacteriaceae isolates with reduced susceptibility to at least one carbapenem(%)

63, *Klebsiella pneumoniae* was the most common Enterobacteriaceae with reduced carbapenem susceptibility, accounting for 37 (59%), followed by *Morganella morganii* (n=8; 13%), *Enterobacter cloacae* (n=6; 9%), *Escherichia coli* (n=3; 5%), *Enterobacter aerogenes* (n=2; 3%) and *Klebsiella terrigena* (n=2; 3%), as illustrated in Figure 1. The remaining five (8%) were identified as *Citrobacter freundii, Enterobacter sakazakii, Enterobacter asburiae*, *Klebsiella ornithinolytica* and *Pantoea* species.

Molecular detection of bla_{NDM-1}

All 63 isolates were subjected to single-target PCR followed by gel electrophoresis (Figure 2). Eighteen (29%) were positive for bla_{NDM-1}.

Distribution of bla_{NDM-1}

As illustrated in Figure 3, 16 (89%) of the 18 isolates positive for bla_{NDM-1} were *Klebsiella pneumoniae*, followed by one (5.5%) *Escherichia coli* isolate and one (5.5%) *Klebsiella ornithinolytica* isolate.

Antibiotic susceptibility patterns of bla_{NDM-1}-positive isolates

As presented in Figure 4, all 18 (100%) isolates with bla_{NDM-1} were resistant to all second and third generation cephalosporin antibiotics. Cefepime, a fourth generation cephalosporin, had a resistance rate of 94%. One hundred percent resistance was observed for ampicillin and amoxicillin-clavulanate. With regards to

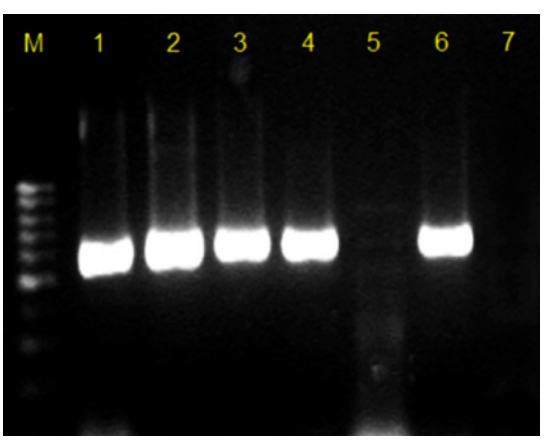

FIG. 2: PCR products from the molecular detection of NDM-1 on agarose gel. Lane M is a 100-base-pair DNA molecular weight ladder, lane 1 is the positive control (621 base pairs) and lane 7 is the negative control. Each lane from 2 to 6 contains DNA from a different Enterobacteriaceae isolate. Samples in lanes 2, 3, 4 and 6 are positive for the NDM-1 gene.

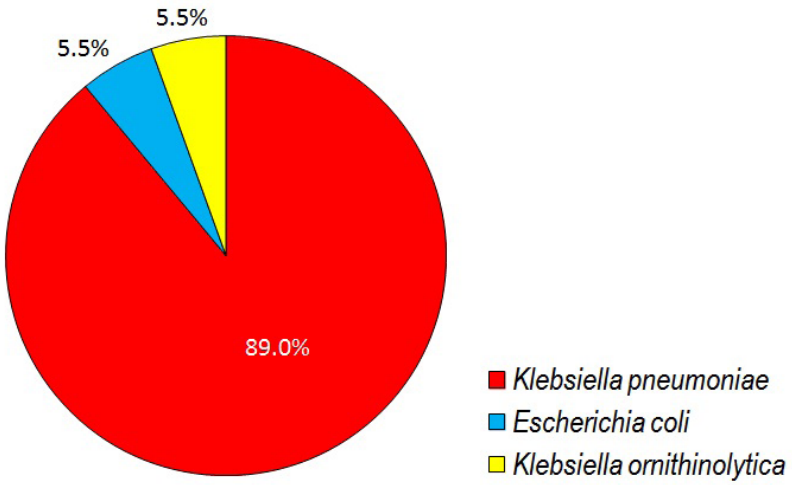

FIG. 3: Enterobacteriaceae isolates positive for bla_{NDM-1} (%)

aminoglycosides, gentamicin resistance was seen in 16 (89%) and reduced amikacin susceptibility in 17 (94%). Of the 17 isolates with reduced amikacin susceptibility, 13 were resistant and four had intermediate susceptibility. As for the carbapenems, reduced susceptibility towards ertapenem was highest (n=18; 100%), followed by doripenem (n=17; 94%), imipenem (n=16; 89%) and meropenem (n=16; 89%).

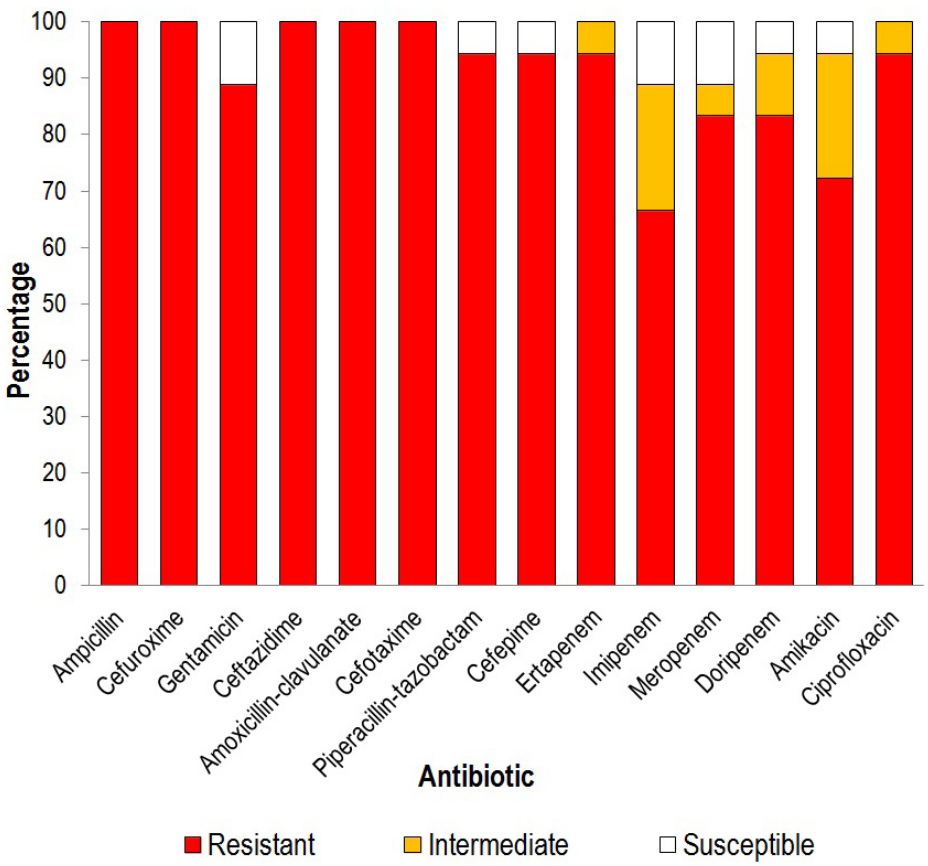

FIG. 4: Antibiotic susceptibility patterns of bla_{NDM-1}-positive Enterobacteriaceae isolates

DISCUSSION

Metallo-β-lactamase enzymes are named as such due to their requirement for a Zn^{2+} ion at the active site.[10] NDM-1 is not the earliest metallo-β-lactamase enzyme to be discovered because the first metallo-β-lactamase was extracted from *Bacillus cereus* and described in 1965.[11] Also, since NDM-1 was first described in 2008, other NDM variants have been discovered, the most recent one being NDM-14 in 2015.[12]

In this study, the most common (89%) Enterobacteriaceae harbouring $bla_{\text{NDM-1}}$ was *Klebsiella pneumoniae*, echoing findings of other investigators.[2] Including *Klebsiella ornithinolytica,* members of the *Klebsiella* genus made up nearly 95% of all NDM-1-positive isolates in our cohort. Thus, because of the high likelihood of NDM-1 expression, we propose that all *Klebsiella* spp. isolated from clinical specimens should be screened for this enzyme. Fortunately, the presumptive identification of *Klebsiella* spp. is relatively easy due to the mucoid appearance of their lactose-fermenting colonies on MacConkey agar, a culture medium widely used in many clinical bacteriology laboratories.

Among the four carbapenems tested, reduced susceptibility towards ertapenem was the most consistent finding, being present in all the NDM-1-postitive isolates. Thus we concur with Nordmann *et al* in recommending ertapenem as the most appropriate carbapenem for detecting NDM-1 producers.[9] Imipenem is less suitable as an NDM-1 screening tool, due to a detection sensitivity of 89%. With regards to meropenem, despite being recommended by the CLSI as an appropriate antibiotic to screen for carbapenamases, the NDM-1 detection sensitivity in our study was similar to that of imipenem (i.e. 89%). As for doripenem, its sensitivity in detecting NDM-1 was slightly better (i.e. 94%) than either imipenem or meropenem. Although the CLSI does not recommend using imipenem to screen for carbapenemases, there is no specific CLSI recommendation on using doripenem for this purpose.[7]

Eight Enterobacteriaceae isolates in this study with reduced imipenem susceptibility were *Morganella morgani* but they remained susceptible to other carbapenems. Although members of the bacterial tribe Proteae (e.g. *Morganella morganii, Providencia* spp. and *Proteus* spp.) may have an elevated MIC to imipenem by a mechanism other than carbapenemase,[7] we included *Morganella morganii* in our study because it is also an Enterobacteriaceae. Although we cannot rule out the presence of other carbapenemase enzymes in these *Morganella morganii* isolates, all of them were predictably negative for $bla_{\text{NDM-1}}$.

Among the NDM-1-positive bacteria, nearly 95% had lowered susceptibility to at least one aminoglycoside. This observation has therapeutic implications because some clinicians may administer an aminoglycoside together with an antibiotic from another class for synergistic activity. Thus, if the infection is due to an NDM-1 producer, the role for aminoglycosides would be marginal at best. Since the aminoglycoside prescribing practice in our institution for infections caused by gram-negative bacilli is limited to mainly gentamicin and amikacin, only these two aminoglycosides were studied even though there are other members in this antibiotic class (e.g. netilmicin and streptomycin). Susceptibility to colistin and tigecycline were not evaluated because interpretative breakpoints for susceptibility testing of these antibiotics against Enterobacteriaceae have not been published by the CLSI.[7]

Similar to other carbapenem-resistant Enterobacteriaceae, the importance of infection control in curbing the transmission of NDM-1-producing Enterobacteriaceae cannot be overemphasised. Essential practices such as early notification of positive cases to a dedicated infection control team, rapid initiation of effective contact precautions, carrier cohorting or isolation and the assignment of dedicated healthcare staff to care for infected/colonised patients should be adopted. As cultures of clinical specimens may only identify a small fraction of infected patients, screening (e.g. through rectal swab cultures) should be carried out to identify colonisation in epidemiologically linked contacts of patients known to be infected/colonised with NDM-1-producing Enterobacteriaceae or to detect new cases among patients nursed in high-risk settings (e.g. intensive care units or high dependency wards).

The limitation of the study is that we specifically looked only for NDM-1 and did not include its variants or other carbapenemase genes (e.g. bla_{KPC}). Thus, further studies to detect the mechanisms of resistance in the non-NDM-1 strains should be undertaken. Also, although we selected non-repetitive Enterobacteriaceae isolates from the same patient, we cannot discount that some of them could have been

the same isolate if cross infection had occurred between patients.

In conclusion, the prevalence of reduced carbapenem susceptibility to at least one carbapenem by disk diffusion in Enterobacteriaceae isolates in our institution was 0.48% and the prevalence of bla_{NDM-1} in the same Enterobacteriaceae population was 0.14%. We recommend ertapenem disk diffusion testing as the single most reliable method to screen for NDM-1 production and its detection by PCR is important because NDM-1-producing isolates also harbour many other resistance genes.

ACKNOWLEDGEMENT

We thank the Dean of the Faculty of Medicine, Universiti Kebangsaan Malaysia for his permission to conduct this study and to UKM Medical Centre for providing us with a research grant (FF-038-2012). We have no conflicts of interest to declare.

REFERENCES

1. Yong D, Toleman MA, Giske CG, *et al*. Characterization of a new metallo-b-lactamase gene, bla_{NDM-1}, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother. 2009; 53(12): 5046-54.
2. Kumarasamy KK, Toleman MA, Walsh TR, *et al*. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010; 10(9): 597-602.
3. Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. Int J Antimicrob Agents. 2011; 37(5): 415-9.
4. Kus JV, Tadros M, Simor A, *et al*. New Delhi metallo-β-lactamase-1: local acquisition in Ontario, Canada, and challenges in detection. CMAJ. 2011; 183(11): 1257-61.
5. Deshpande P, Rodrigues C, Shetty A, Kapadia F, Hedge A, Soman R. New Delhi Metallo-beta lactamase (NDM-1) in Enterobacteriaceae: treatment options with carbapenems compromised. J Assoc Physicians India. 2010; 58: 147-9.
6. Walsh TR, Weeks J, Livermore DM, Toleman MA. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect Dis. 2011; 11(5): 355-62.
7. CLSI. Performance standard for antimicrobial susceptibility testing; twenty-third informational supplement. CLSI document M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.
8. CLSI. Performance standard for antimicrobial susceptibility testing; twenty-first informational supplement. CLSI document M100-S21. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
9. Nordmann P, Poirel L, Carrër A, Toleman MA, Walsh TR. How to detect NDM-1 producers. J Clin Microbiol. 2011; 49(2): 718-21.
10. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 2010; 54(3): 969-76.
11. Sabath LD, Abraham EP. Zinc as a cofactor for cephalosporinase from *Bacillus cereus* 569. Biochem J. 1966; 98(1): 11C–13C.
12. Zou D, Huang Y, Zhao X, *et al*. A novel New Delhi metallo-β-lactamase variant, NDM-14, isolated in a Chinese Hospital possesses increased enzymatic activity against carbapenems. Antimicrob Agents Chemother. 2015; 59(4): 2450-3.