Molecular characterization of multidrug-resistance in Gram-negative bacteria from the Peshawar teaching hospital, Pakistan

A. Masseron
L. Poirel
B. Jamil Ali
M. A. Syed
P. Nordmann

Follow this and additional works at: https://ecommons.aku.edu/pakistan_fhs_mc_med_pulm_critcare

Part of the Pulmonology Commons
Molecular characterization of multidrug-resistance in Gram-negative bacteria from the Peshawar teaching hospital, Pakistan

A. Masseron¹, L. Poirel¹,², B. Jamil Ali¹, M. A. Syed⁵ and P. Nordmann¹,²,³,6

¹) Medical and Molecular Microbiology, Section of Medicine, Faculty of Science and Medicine, 2) INSERM European Unit (IAME, France), University of Fribourg, Fribourg, 3) Swiss National Reference Centre for Emerging Antibiotic Resistance, University of Fribourg, Switzerland, 4) Section of Infectious Diseases, Department of Medicine, The Aga Khan University, Karachi, 5) Infectious Diseases Research Group, Department of Microbiology, University of Haripur, Khyber Pakhtunkhwa, Pakistan and 6) Institute for Microbiology, University of Lausanne and University Hospital Centre, Lausanne, Switzerland

Abstract

Extended-spectrum β-lactamases, carbapenemases, 16S rRNA methylases conferring pan-drug aminoglycoside resistance and colistin resistance were investigated among Gram-negative bacteria recovered from clinical samples (infections) from 200 individuals hospitalized at the Khyber Teaching Hospital of Peshawar, north Pakistan, from December 2017 to March 2018. Out of 65 isolates recovered, 19% were carbapenem resistant and 16% carried a blaNDM-1 gene, confirming the widespread distribution of NDM producers in this country. The association of the NDM carbapenem-resistance determinant, together with the extended-spectrum β-lactamase CTX-M-15 and 16S rRNA methylases, was frequent, explaining the multidrug-resistance pattern observed. All isolates remained susceptible to colistin.

Keywords: Carbapenemases, Extended spectrum β-lactamase, Pakistan, ST 215, ST 231, 16S rRNA methylases

Introduction

The increasing occurrence of resistance to carbapenems is a major issue for global public health. Resistance to carbapenems is often mediated by carbapenemases, with NDM-1 (New Delhi metallo-β-lactamase) being one of the most commonly identified carbapenemases worldwide, its main reservoir corresponding to the Indian subcontinent [1]. Accordingly, several studies reported a high occurrence of NDM-1 producers in India and Pakistan [2,3]. Most of the carbapenemase producers are co-resistant to other antibiotic families, so it is interesting to obtain further characterization about those co-resistance markers [4]. Our aim was to characterize the carbapenemases, extended-spectrum β-lactamases (ESBLs), 16S rRNA methylases conferring pan-aminoglycoside resistance, and colistin resistance (plasmid-mediated mcr genes) from a series of clinical isolates obtained in acute-care facilities from Peshawar, Pakistan.

Materials and methods

Bacterial isolates

A total of 200 samples were collected from 200 individuals between December 2017 and March 2018 at the Khyber Teaching Hospital of Peshawar, north Pakistan. Those samples included urine, blood, pus and broncheal lavage specimens. Samples were plated on URselect-4™ medium (Bio-Rad, Cressier, Switzerland) and identification was performed using the API-20E system (bioMérieux, La Balmes-Grottes, France). Antimicrobial susceptibility testing was performed using the disc diffusion method and interpreted according to the CLSI recommendations [5], except for colistin. Resistance to colistin was evaluated first by using the Rapid Polymyxin test.
[6], and then by determination of MIC values of colistin by broth microdilution, as recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.euCAST.org). Carbenemase and ESBL activities were detected using the Carba NP test [7] and the ESBL NP test [8], respectively.

### Molecular analysis
A series of acquired resistance genes were searched by PCR, including those encoding ESBLs (blaTEM, blaSHV and blaCTX-M-like genes), carbenemases (blaKPC, blaNDM, blaVIM, blaIMP, blaOXA-48, blaOXA-181), 16S rRNA methylases genes (armA, rmtA to rmtH and npmA), and mcr-like genes (mcr-1 to mcr-8) [9–14]. The obtained amplicons were sent for sequencing (Microsynth®, Balgach, Switzerland).

### Clonal diversity
Clonal relationship of the isolates was evaluated by pulsed-field gel electrophoresis (PFGE) [15]. Total DNA from Escherichia coli, Klebsiella pneumoniae and Enterobacter spp. were digested using the XbaI enzyme (New England Biolabs, Ipswich, MA, USA). The generated fragments were separated by PFGE using a CHEF-DR III System (Bio-Rad) creating a unique PFGE profile for each clonal strain. Multilocus sequencing typing was performed for pan-drug aminoglycoside-resistant strains belonging to the species E. coli (strain no. 31) and K. pneumoniae (strain no. 62) [16]. Sequence types (STs) were investigated using the online databases (http://genomicepidemiology.org/).

### Results
A total of 65 Gram-negative bacteria were recovered including 46 E. coli, 10 Enterobacter spp. (9 Enterobacter cloacae, 1 Enterobacter sakazakii), 6 K. pneumoniae, 2 Alcaligenes faecalis and 1 Citrobacter freundii.

Thirty-eight of the 65 Gram-negative bacteria produced a carbenemase, as assessed by the results of the Rapid Carba NP test, whereas 32 of those 65 isolates produced an ESBL according to the results of the ESBL NP test. Among the 46 E. coli isolates, 12 produced NDM-1, 5 co-produced NDM-1 and CTX-M-15, 2 co-produced NDM-1 and OXA-181, 2 isolates co-producing OXA-181 and CTX-M-15, a single isolate producing OXA-48. Twelve E. coli isolates produced CTX-M-15 without any carbenemase associated. A single blaNDM-1-positive E. coli isolate produced the 16S rRNA methylase RmtB.

Among the six K. pneumoniae isolates, one isolate co-produced NDM-1 and CTX-M-15, another co-produced NDM-1, OXA-181 and CTX-M-15, and a single isolate co-produced NDM-1, OXA-232 and CTX-M-15 together with the 16S rRNA methylase RmtB (see Table 1).

The ten Enterobacter spp. isolates (nine Enterobacter cloacae, one Enterobacter sakazakii) co-produced NDM-1 and CTX-M-15.

Two Alcaligenes faecalis isolates were identified, both producing a carbenemase VIM-4.

The single C. freundii isolate did not produce any carbenemase but was positive for CTX-M-15. None of the isolates was resistant to colistin. PFGE analysis showed a very high clonal diversity for all those isolates producing a carbenemase, except among Enterobacter spp. with a single clone being identified among six patients (EC-1) that may have resulted from an outbreak.

By using MLST, we showed that the blaNDM-1 and rmtB-positive E. coli clone belonged to ST215 (strain no. 31) and that the multidrug-resistant K. pneumoniae isolate belonged to ST231 (strain no. 62).

### Discussion
Here we evaluated the occurrence of multidrug resistance from infecting strains (not colonizers) from individuals hospitalized at the Khyber Teaching Hospital, Peshawar, north Pakistan from November 2017 to March 2018. A rate of 19% of carbapenem-resistant Enterobacteriaceae (38/200) was identified, among which 16% harboured the blaNDM-1 gene. This overall rate of 16% of NDM producers is in accordance with the estimated rate of NDM producers in Enterobacteriaceae in Pakistan (8.7%–18.5%). Actually, 18.5% stool samples were found to contain blaNDM-1-positive strains (mostly E. coli and Enterobacter cloacae) in military hospitals of Rawalpindi (Combined Military Hospitals and Military Hospital at Rawalpindi) [17]. 8.7% of the isolates were NDM-1 positive (K. pneumoniae, E. coli, Pseudomonas aeruginosa) from two tertiary-care hospitals (Pakistan Institute of Medical Science, Islamabad and Mayo Hospital, Lahore) [18] and 14.6% of the strains carried blaNDM-1 (E. coli, Enterobacter cloacae, Pseudomonas putida and K. pneumoniae from three children’s hospitals (The Children’s Hospital, Islamabad, The Children’s Hospital Multan and Nishtar Hospital, Multan) [19].

Interestingly, some strains were found to harbour two unrelated carbenemase genes (blaNDM-1 and blaOXA-181), as observed previously in Singapore [20], Nigeria [21] and Romania [22].

In our study, co-resistance to multiple antibiotics was commonly observed among those carbenem-resistant isolates, with 60% of them harbouring at least two other resistance genes (ESBL, or 16S rRNA methylase gene). A single
K. pneumoniae isolate carried two carbapenemase genes, namely blaNDM-1 and blaOXA-232, along with the ESBL-encoding gene blaCTX-M-15 and the 16S rRNA methylase gene rmtB. That isolate was an ST231 clone, already described in South-East Asia [23,24] and Switzerland [25], and associated with OXA-232. Among the 16S rRNA methylase genes, the methylase gene rmtB, rather than rmtF, was mostly identified here, which is not surprising owing to the spread of rmtB-positive strains in Pakistan [26]. Our study showed the frequent identification of the ESBL CTX-M-15 among those multidrug-resistant bacteria. It is possible that the spread of CTX-M-15 that is observed worldwide originated from the Indian subcontinent because we identified the first CTX-15 producers from India in 2001 [27]. Our study confirms the frequent association of those three important resistance markers that are NDM, ESBL of the CTX-

### TABLE 1. Information and genetic features of samples isolated in Peshawar teaching hospital, Pakistan

| Patient/strain no. | Site of isolation | Residence | Treatment | Strains | Carbapenemase | ESBL | 16S RNA methyltransferase | Clonality |
|--------------------|-------------------|-----------|-----------|---------|---------------|------|--------------------------|-----------|
| 1                  | Pus               | Peshawar  | Ciprofloxacin | Alcaligenes faecalis | VIM-4 | None | None | None |
| 2                  | Urine             | Mardan    | Ofloxacin   | Alcaligenes faecalis | VIM-4 | None | None | None |
| 3                  | Urine             | Bahki     | Ofloxacin   | Citrobacter freundii | None | None | None | None |
| 4                  | Pus               | Peshawar  | Ciprofloxacin | Enterobacter cloacae | NDM-1 | None | None | EC-1 |
| 5                  | Urine             | Unknown   | Unknown     | Enterobacter cloacae | NDM-1 | None | None | EC-1 |
| 6                  | Urine             | Mardan    | Unknown     | Enterobacter cloacae | NDM-1 | None | None | EC-1 |
| 7                  | Blood             | Unknown   | Unknown     | Citrobacter freundii | None | None | None | EC-1 |
| 8                  | Urine             | Mardan    | Ofloxacin   | Enterobacter cloacae | NDM-1 | None | None | EC-1 |
| 9                  | Urine             | Afghanistan | Unknown | Enterobacter cloacae | NDM-1 | None | None | EC-1 |
| 10                 | Urine             | Peshawar  | Levofloxacin | Enterobacter cloacae | NDM-1 | None | None | None |
| 11                 | Urine             | Peshawar  | Levofloxacin | Enterobacter cloacae | NDM-1 | None | None | None |
| 12                 | Urine             | Peshawar  | Levofloxacin | Enterobacter cloacae | NDM-1 | None | None | None |
| 13                 | Urine             | Peshawar  | Levofloxacin | Enterobacter sakazavii | NDM-1 | None | None | None |
| 14                 | Fluid             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 15                 | Pus               | Charsada  | Ciprofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 16                 | Pus               | Dir       | Amoxicillin-Clavulanate | Escherichia coli | None | None | None | None |
| 17                 | Pus               | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 18                 | Pus               | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 19                 | Pus               | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 20                 | Pus               | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 21                 | Urine             | Peshawar  | Ceftriaxone | Escherichia coli | NDM-1 | None | None | None |
| 22                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 23                 | Urine             | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 24                 | Urine             | Peshawar  | Levofloxacin | Escherichia coli | NDM-1 | None | None | None |
| 25                 | Unknown            | Peshawar  | Ceftriaxone | Escherichia coli | NDM-1 | None | None | None |
| 26                 | Blood             | Mardan    | Unknown     | Escherichia coli | NDM-1 | None | None | EC-1 |
| 27                 | Blood             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-1 |
| 28                 | Urine             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-1 |
| 29                 | Urine             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-1 |
| 30                 | Urine             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-1 |
| 31                 | Unknown            | Unknown   | Unknown     | Escherichia coli | NDM-1 | None | None | None |
| 32                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1; OXA-181 | None | None | EsC-10 |
| 33                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1; OXA-181 | None | None | None |
| 34                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | OXA-48 | None | None | None |
| 35                 | Blood             | Peshawar  | Ciprofloxacin | Escherichia coli | OXA-181 | CTX-M-15 | None | EsC-9 |
| 36                 | Blood             | Peshawar  | Ciprofloxacin | Escherichia coli | OXA-181 | CTX-M-15 | None | EsC-9 |
| 37                 | Blood             | Mardan    | Unknown     | Escherichia coli | NDM-1 | None | None | EsC-2 |
| 38                 | Pus               | Dir       | Unknown     | Escherichia coli | NDM-1 | None | None | EsC-2 |
| 39                 | Pus               | Dir       | Unknown     | Escherichia coli | NDM-1 | None | None | EsC-2 |
| 40                 | Urine             | Peshawar  | Amoxicillin-Clavulanate | Escherichia coli | None | None | None | EsC-2 |
| 41                 | Urine             | Peshawar  | Levofloxacin | Escherichia coli | None | None | None | EsC-2 |
| 42                 | Branchial lavage  | Peshawar  | Levofloxacin | Escherichia coli | None | None | None | EsC-3 |
| 43                 | Pus               | Peshawar  | Ceftriaxone | Escherichia coli | None | None | None | EsC-3 |
| 44                 | Urine             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-3 |
| 45                 | Urine             | Peshawar  | Unknown     | Escherichia coli | NDM-1 | None | None | EC-3 |
| 46                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1 | None | None | EC-3 |
| 47                 | Urine             | Peshawar  | Ciprofloxacin | Escherichia coli | NDM-1 | None | None | EC-3 |
| 48                 | Urine             | Afghanistan | Levofloxacin | Escherichia coli | None | None | None | EC-3 |
| 49                 | Blood             | Peshawar  | Ceftriaxone | Escherichia coli | None | None | None | EC-3 |
| 50                 | Blood             | Unknown   | Unknown     | Escherichia coli | None | None | None | EC-3 |
| 51                 | Pus               | Afghanistan | Unknown | Escherichia coli | None | None | None | EC-3 |
| 52                 | Pus               | Charsada  | Ciprofloxacin | Escherichia coli | None | None | None | None |
| 53                 | Pus               | Peshawar  | Ciprofloxacin | Escherichia coli | None | None | None | None |
| 54                 | Pus               | Peshawar  | Linezolid    | Escherichia coli | None | None | None | EsC-18 |
| 55                 | Urine             | Afghanistan | Unknown | Escherichia coli | None | None | None | EsC-17 |
| 56                 | Urine             | Peshawar  | Ceftriaxone | Escherichia coli | None | None | None | EsC-4 |
| 57                 | Urine             | Peshawar  | Unknown     | Escherichia coli | None | None | None | EC-4 |
| 58                 | Urine             | Peshawar  | Unknown     | Escherichia coli | None | None | None | EC-4 |
| 59                 | Urine             | Peshawar  | Unknown     | Escherichia coli | None | None | None | EC-4 |
| 60                 | Blood             | Peshawar  | Ceftriaxone | Klebsiella pneumoniae | NDM-1; OXA-232; CTX-M-15 | None | None | KP-1 |
| 61                 | Pus               | Dir       | Amoxicillin-Clavulanate | Klebsiella pneumoniae | NDM-1; OXA-181 | None | None | KP-1 |
| 62                 | Blood             | Mardan    | Unknown     | Klebsiella pneumoniae | NDM-1; OXA-232; CTX-M-15 | rmtB | ST-231 | KP-1 |
| 63                 | Pus               | Peshawar  | Ciprofloxacin | Klebsiella pneumoniae | None | None | None | KP-2 |
| 64                 | Urine             | Nowshera  | Ciprofloxacin | Klebsiella pneumoniae | None | None | None | KP-2 |
| 65                 | Unknown            | Afghanistan | Levofloxacin | Klebsiella pneumoniae | None | None | None | None |

Abbreviations: EC, Enterobacter cloacae clone X; ESBL, extended spectrum β-lactamase; EsC, Escherichia coli clone X; KP, Klebsiella pneumoniae clone X; ST, sequence type.
M-15 type and 16S rRNA methylases as a source of multidrug resistance. This combination complicates the treatment of infected patients.

We also identified two A. faecalis isolates producing a same VIM-4 metallo-β-lactamase. Occurrence of carbapenemase production in that bacterial species is rare. Only a single case of A. faecalis carrying blvIM-4 has been described in Gaza, Palestine [28]. Further work may identify the genetic basis of such acquisition of carbapenemase. Other reports about ESBL in clinical isolates of Alcaligenes species have been described in Malaysia [29], France [30] and Italy [31].

Finally, no resistance to colistin was observed whereas MCR-1-mediated colistin resistance isolates was observed recovered from E. coli in animals [32,33] and from a single patient [34] in Pakistan. Lack of colistin resistance among those multidrug-resistant isolates is good news as it would indicate the availability to use that drug for treating infected patients in this country.

**Funding**

This work was supported by the Swiss National Science Foundation (project FNS-31003A_163432) and by the University of Fribourg.

**Conflicts of interest**

None to declare.

**References**

[1] Dortet L, Poirel L, Nordmann P. Worldwide dissemination of the NDM-type carbapenemases in Gram-negative bacteria. Biomed Res Int 2014;2014:1–12.

[2] Kumarasamy KK, Tolemen MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, 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;10:597–602.

[3] Zahedi Bialvaei A, Samadi Kaayesh B, Husein A, Ghirardi S, et al. Prevalence of faecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media. J Antimicrob Chemother 2011;66:2288–94.

[4] Nahid F, Khan AA, Rehman S, Zahra R. Prevalence of metallo-β-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistan hospitals and implications for public health. J Infect Public Health 2013;6:487–93.

[5] Qamar MU, Nahid F, Walsh TR, Kamran R, Zahra R. Prevalence and clinical burden of NDM-1 positive infections in pediatric and neonatal patients in Pakistan. Pediatr Infect Dis J 2014;33:452–4.

[6] Balm MN, La MV, Krishnan P, Jureen R, Lin RTP, Teo JWP. Emergence of Klebsiella pneumoniae co-producing NDM-type and OXA-181 carbapenemases. Clin Microbiol Infect 2013;19:421–3.

[7] Uwaechue NS, Kieffer N, Iregbu KC, Nordmann P. First report of OXA-181 and NDM-1 from a clinical Klebsiella pneumoniae isolate from Nigeria. Int J Infect Dis 2017;61:1–2.

[8] Székely E, Damjanova J, Jánvári L, Vas KE, Molnar S, Bilcsa DV, et al. First description of blaNDM-1, blaOXA-48, blaOXA-181 producing Enterobacteriaceae strains in Romania. Int J Med Microbiol 2013;303:697–700.

[9] Abdul Momin MHF, Liakopoulos A, Phee LM, Wareham DW. Emergence and nosocomial spread of carbapenem-resistant OXA-232-producing Klebsiella pneumoniae in Brunei Darussalam. J Glob Antimicrob Resist 2017;9:96–9.

[10] Teo JWP, Kurup A, Lin RTP, Hsien KT. Emergence of clinical Klebsiella pneumoniae producing OXA-232 carbapenemase in Singapore. N Microbe N Infect 2013;1:13–5.

[11] Mancini S, Poirel L, Tritten M-L, Lienhard R, Bassi C, Nordmann P. Emergence of an MDR Klebsiella pneumoniae ST231 producing OXA-232 and RmpF in Switzerland. J Antimicrob Chemother 2018;73:821–3.

[12] Habeeb MA, Haque A, Nematzadeh S, Iversen A, Giske CG. High prevalence of 16S RNA methylase RmF among CTX-M extended-spectrum β-lactamase-producing Klebsiella pneumoniae from Islamabad, Pakistan. J Antimicrob Agents 2013;41:524–6.

[13] Karim A, Poirel L, Nagaranj S, Nordmann P. Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3-like) from India and gene
association with insertion sequence IS\text{Ecp1}. FEMS Microbiol Lett. 2001;201:237–41.

[28] Al Laham N, Chavda KD, Cienfuegos-Gallet AV, Kreiswirth BN, Chen L. Genomic characterization of VIM metallo-\(\beta\)-lactamase-producing \textit{Alcaligenes faecalis} from Gaza, Palestine. Antimicrob Agents Chemother 2017;61(11). pii:e01499-17.

[29] Puah SM, Puthucheary SD, Chua KH. First report of TEM-116 and OXA-10 extended-spectrum \(\beta\)-lactamase in clinical isolates of \textit{Alcaligenes} species from Kuala Lumpur, Malaysia. Jpn J Infect Dis 2019;72:266–9.

[30] Dubois V, Arpin C, Coulange L, Andre C, Noury P, Quentin C. TEM-21 extended-spectrum \(\beta\)-lactamase in a clinical isolate of \textit{Alcaligenes faecalis} from a nursing home. J Antimicrob Chemother 2006;57:368–9.

[31] Pereira M, Perilli M, Mantengoli E, Luzzaro F, Toniolo A, Rossolini GM, Amicosante G. PER-1 extended-spectrum \(\beta\)-lactamase production in an \textit{Alcaligenes faecalis} clinical isolate resistant to expanded-spectrum cephalosporins and monobactams from a hospital in Northern Italy. Microb Drug Resist 2000;6:85–90.

[32] Azam M, Ehsan I, Sajjad-Ur-Rahman, Saleemi MK, Javed MR, Mohsin M. Detection of the colistin resistance gene \textit{mcr-1} in avian pathogenic \textit{Escherichia coli} in Pakistan. J Glob Antimicrob Resist 2017;11:152–3.

[33] Lv J, Mohsin M, Lei S, Srinivas S, Wigar RT, Lin J, Feng Y. Discovery of a \textit{mcr-1}-bearing plasmid in commensal colistin-resistant \textit{Escherichia coli} from healthy broilers in Faisalabad, Pakistan. Virulence 2018;9:994–9.

[34] Mohsin M, Raza S, Roschanski N, Guenther S, Ali A, Schierack P. Description of the First \textit{Escherichia coli} clinical isolate harboring the colistin resistance gene \textit{mcr-1} from the Indian subcontinent. Antimicrob Agents Chemother 2016;61:1–2.