ORIGINAL ARTICLE

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

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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 bla_{NDM-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
Original Submission: 7 June 2019; **Revised Submission:** 12 September 2019; **Accepted:** 29 September 2019
Article published online: 11 October 2019

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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 URIselect-4™ medium (Bio-Rad, Cressier, Switzerland) and identification was performed using the API-20E system (bioMérieux, La Balme-les-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

https://doi.org/10.1016/j.nmni.2019.100605

[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). Carbapenemase 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 (bla_{TEM}-, bla_{SHV}- and bla_{CTX-M}-like genes), carbapenemases (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA-48}, $bla_{OXA-181}$), 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 *Xba*I 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 carbapenemase, 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 carbapenemase associated. A single bla_{NDM-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 carbapenemase VIM-4.

The single *C. freundii* isolate did not produce any carbapenemase 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 carbapenemase, 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 bla_{NDM-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 bla_{NDM-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 bla_{NDM-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 bla_{NDM-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 carbapenemase genes (bla_{NDM-1} and $bla_{OXA-181}$), as observed previously in Singapore [20], Nigeria [21] and Romania [22].

In our study, co-resistance to multiple antibiotics was commonly observed among those carbapenem-resistant isolates, with 60% of them harbouring at least two other resistance genes (ESBL, or 16S rRNA methylase gene). A single

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	Ofloxine	*Alcaligenes faecalis*	VIM-4	None	None	None
3	Urine	Batkhela	Unknown	*Citrobacter freundii*	None	CTX-M-15	None	EsC-7
4	Pus	Peshawar	Ciprofloxacin	*Enterobacter cloacae*	NDM-1	None	None	EC-1
5	Urine	Peshawar	Unknown	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
6	Urine	Kohat	Unknown	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
7	Blood	Unknown	Cefixime	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
8	Urine	Mardan	Ofloxine	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
9	Urine	Afghanistan	Unknown	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
10	Urine	Afghanistan	Unknown	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	EC-1
11	Urine	Peshawar	Levofloxacin	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	None
12	Urine	Peshawar	Levofloxacin	*Enterobacter cloacae*	NDM-1	CTX-M-15	None	None
13	Urine	Peshawar	Unknown	*Enterobacter sakazakii*	NDM-1	CTX-M-15	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	*Escherichia coli*	NDM-1	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	Unknown	*Escherichia coli*	NDM-1	None	None	None
20	Pus	Peshawar	Unknown	*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	Unknown	*Escherichia coli*	NDM-1	None	None	None
24	Urine	Peshawar	Unknown	*Escherichia coli*	NDM-1	None	None	None
25	Unknown	Peshawar	Cefixime	*Escherichia coli*	NDM-1	None	None	None
26	Blood	Mardan	Unknown	*Escherichia coli*	NDM-1	CTX-M-15	None	None
27	Blood	Peshawar	Unknown	*Escherichia coli*	NDM-1	CTX-M-15	None	EsC-15
28	Urine	Peshawar	Unknown	*Escherichia coli*	NDM-1	CTX-M-15	None	None
29	Urine	Peshawar	Unknown	*Escherichia coli*	NDM-1	CTX-M-15	None	None
30	Urine	Peshawar	Unknown	*Escherichia coli*	NDM-1	CTX-M-15	None	None
31	Unknown	Unknown	Unknown	*Escherichia coli*	NDM-1	None	rmtB	ST 215
32	Urine	Peshawar	Ciprofloxacin	*Escherichia coli*	NDM-1; OXA-181	None	None	EsC-10
33	Urine	Peshawar	Unknown	*Escherichia coli*	NDM-1; OXA-181	None	None	None
34	Urine	Peshawar	Ciprofloxacin	*Escherichia coli*	OXA-48	None	None	EsC-1
35	Blood	Peshawar	Ciprofloxacin	*Escherichia coli*	OXA-181	CTX-M-15	None	EsC-5
36	Blood	Peshawar	Unknown	*Escherichia coli*	OXA-181	CTX-M-15	None	EsC-1
37	Blood	Kohat	Unknown	*Escherichia coli*	None	CTX-M-15	None	EsC-14
38	Pus	Dir	Unknown	*Escherichia coli*	None	CTX-M-15	None	EsC-2
39	Pus	Peshawar	Ofloxine	*Escherichia coli*	None	CTX-M-15	None	EsC-2
40	Urine	Peshawar	Amoxicillin-Clavulanate	*Escherichia coli*	None	CTX-M-15	None	EsC-2
41	Urine	Peshawar	Levofloxacin	*Escherichia coli*	None	CTX-M-15	None	EsC-2
42	Bronchial lavage	Peshawar	Levofloxacin	*Escherichia coli*	None	CTX-M-15	None	EsC-3
43	Pus	Peshawar	Cefixime	*Escherichia coli*	None	CTX-M-15	None	EsC-3
44	Urine	Peshawar	Unknown	*Escherichia coli*	None	CTX-M-15	None	EsC-3
45	Urine	Kohat	Unknown	*Escherichia coli*	None	CTX-M-15	None	EsC-3
46	Urine	Peshawar	Ciprofloxacin	*Escherichia coli*	None	CTX-M-15	None	EsC-9
47	Urine	Peshawar	Levofloxacin	*Escherichia coli*	None	CTX-M-15	None	EsC-12
48	Urine	Afghanistan	Levofloxacin	*Escherichia coli*	None	CTX-M-15	None	None
49	Blood	Peshawar	Cefixime	*Escherichia coli*	None	None	None	EsC-16
50	Blood	Unknown	Cefixime	*Escherichia coli*	None	None	None	EsC-13
51	Pus	Afghanistan	Unknown	*Escherichia coli*	None	None	None	EsC-11
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	Cefixime	*Escherichia coli*	None	None	None	EsC-4
57	Urine	Peshawar	Unknown	*Escherichia coli*	None	None	None	EsC-4
58	Urine	Peshawar	Unknown	*Escherichia coli*	None	None	None	EsC-6
59	Urine	Peshawar	Unknown	*Escherichia coli*	None	None	None	EsC-8
60	Blood	Peshawar	Cefixime	*Klebsiella pneumoniae*	NDM-1	CTX-M-15	None	KP-1
61	Pus	Dir	Amoxicillin	*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
63	Pus	Peshawar	Ciprofloxacin	*Klebsiella pneumoniae*	None	None	None	KP-2
64	Urine	Noweshehra	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.

K. pneumoniae isolate carried two carbapenemase genes, namely bla_{NDM-1} and $bla_{OXA-232}$, along with the ESBL-encoding gene $bla_{CTX-M-15}$ 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-

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 bla_{VIM-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.

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