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Original Article

Emergence of carbapenemase-producing urinary isolates at a tertiary care hospital in Dhaka, Bangladesh

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ABSTRACT

Objectives: A growing incidence of pathogens producing carbapenemases has been observed in many countries including Bangladesh. The present study was carried out to determine the presence of carbapenemase producers among uropathogens.

Materials and Methods: A total of 138 Gram-negative uropathogens were isolated and identified by conventional methods and were screened for carbapenemase production using imipenem discs. Phenotypic identification of carbapenemase production was done by the double disc synergy test, combined disc assay, and modified Hodge test. The minimum inhibitory concentration of imipenem was determined by the agar dilution method. Genes encoding *bla*NDM-1, *bla*IMP, *bla*VIM, *bla*KPC and *bla*OXA-48/*bla*OXA-181 were identified by polymerase chain reaction.

Results: Twenty (14.49%) imipenem resistant strains were detected among 138 Gram-negative uropathogens. The most common isolates were *Escherichia coli* and *Klebsiella* spp. Among 20 imipenem resistant strains, 16 (80%) carbapenemase producers were detected by polymerase chain reaction, 13 (65%) by double disc synergy, 15 (75%) by combined disc assay, and seven (35%) by modified Hodge test. The *bla*NDM-1 gene was most prevalent (55%), followed by *bla*OXA-48/OXA-181, *bla*KPC (20%), *bla*VIM (15%), and *bla*IMP (10%). More than one carbapenemase gene was present in nine (45%) of the isolates. The minimum inhibitory concentration of imipenem of the carbapenemase producers ranged from ≥128 μg/mL to 4 μg/mL. Overall, carbapenemase encoding genes were detected in 11.6% (16/138) of the studied Gram-negative uropathogens. All (100%) of the carbapenemase-producing organisms were resistant to all tested antibiotics apart from colistin.

Conclusion: The study shows a significant rate of urinary isolates were carbapenemase producers, including a high prevalence of *bla*NDM-1, in Bangladesh.

1. Introduction

Carbapenems are the mainstay of therapy for patients with serious and life threatening infections caused by Enterobacteriaceae, which produce extended spectrum β-lactamases (ESBL) [1]. However, the widespread emergence and dissemination of carbapenem resistance in this family has resulted in a public health crisis [2]. This resistance is mediated by production of carbapenemases [either serine based carbapenemase or metallo-β-lactamases (MBLs)], by modification of membrane permeability (i.e., porin loss) or by production of carbapenem hydrolyzing β-lactamases (i.e., hyperproduction of AmpC β-lactamase, certain ESBLs with increased capacity to hydrolyze carbapenems) and upregulation of efflux pumps [3]. Carbapenem-hydrolyzing β-lactamases, which belong to Ambler classes A, B, and D have been reported worldwide among Enterobacteriaceae. The most clinically significant ones are KPC-type (class A); IMP, VIM, and NDM-1 types (class B); and OXA-48 (class D). The genes encoding them are located on mobile genetic elements, which allow them to spread [1,4]. Class A and D enzymes have a serine-based hydrolytic mechanism for cleaving the β-lactam ring in antibiotics. MBLs, by contrast, are class B carbapenemases containing zinc at the active site [5]. Carbapenemase-producing pathogens are considered a serious nuisance as they have the ability to hydrolyze penicillins, cephalosporins, and monobactams as well as carbapenems [6].

Conflicts of interest: none.

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Under such circumstances of extreme resistance towards antibiotics, the pathogens remain susceptible only to colistin and tigecycline combination therapy [7]. However, with its increased use, we may actually trigger resistant mechanisms against these combinations leading to the end of the current era of pharmacopoeia [8]. The objective of our study was to isolate and study the occurrence of *bla*NDM-1 and other carbapenemase genes among urinary isolates in Bangladesh, along with the antimicrobial resistance patterns of these organisms. This knowledge will greatly help reduce the morbidity and mortality rates associated with urinary tract infections caused by these organisms.

2. Materials and methods

2.1. Bacterial isolates

The study was conducted after obtaining due approval from the institutional ethical committee. From January to December 2014, a total of 138 consecutive nonduplicate (one isolate per patient) Gram-negative isolates recovered from urine cultures of patients with clinically suspected urinary tract infection in the Department of Microbiology of Dhaka Medical College, Dhaka were included in the study. Bacterial identification was performed by routine conventional microbial cultures and biochemical tests using standard recommended techniques [9].

2.2. Antimicrobial susceptibility testing

All bacterial isolates were subjected to antimicrobial susceptibility testing using the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) [10]. *Escherichia coli* ATCC 25922 was used as the quality control strain. The antibiotics tested were as follows (potency in μg/disc): ceftazidime (30), ceftriaxone (30), imipenem (10), meropenem (10), cefotaxime (30), cefoxitin (30), amoxiclav (20/10), ciprofloxacin (5), gentamicin (10), co-trimoxazole (1.25/23.75), azithromycin (15), nitrofurantoin (300), and colistin sulfate (10).

2.3. Screening for carbapenemases by the disc diffusion technique

Screening for carbapenem-resistance was determined using the Kirby–Bauer disc diffusion method with a 10 μg imipenem disc. Three to five well isolated colonies of test organisms were emulsified into 3 mL of sterile normal saline. The turbidity of the suspension was compared with the 0.5 McFarland turbidity standard and the suspension was incubated on Mueller–Hinton agar plates at 37°C for 24 hours. An inhibition zone of $\leq$ 19 mm diameter around the imipenem disc was considered resistant, 20–22 mm indicated intermediate, and $\geq$ 23 mm was considered sensitive [10].

2.4. Phenotypic detection of carbapenemase producers

All the isolates showing reduced susceptibility to imipenem (zone diameter ˂19 mm) were tested for carbapenemase production using the modified Hodge test. Briefly, a lawn culture (0.5 McFarland) of *E. coli* 25922 was streaked on a Mueller–Hinton agar plate. A 10 μg meropenem disc was placed in the center of the agar plate. The test isolates were streaked in a straight line from the edge of the disc to the edge of the plate and were incubated overnight. A positive test was indicated by a cloverleaf-like indentation at the intersection of the test organism and the standard strain, within the zone of inhibition of the carbapenem antibiotic [10]. The detection of MBL production was performed by the double-disc synergy (DDS) test and combined disc (CD) assay as described previously [11,12].

2.5. Detection of carbapenemase producers by the minimum inhibitory concentration of imipenem

The minimum inhibitory concentration (MIC) of imipenem against all carbapenemase producers was determined using the agar dilution method according to CLSI guidelines. For detection of carbapenem resistance among Enterobacteriaceae, an MIC of imipenem $\geq$ 4 μg/mL was considered resistant, 2 μg/mL intermediate, and $\leq$ 1 μg/mL sensitive [10]. For preparation of the imipenem stock solution, a vial of a 500 mg base of commercially available imipenem injection was added to 50 mL distilled water to a concentration of 10 mg/mL. For each plate, 50 mL Mueller–Hinton medium was prepared and impregnated with 5 μL, 10 μL, 20 μL, 40 μL, 80 μL, 160 μL, 320 μL, 640 μL, or 1280 μL of the imipenem stock solution to achieve concentrations of 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, 128 μg/mL, or 256 μg/mL per plate, respectively.

2.6. Molecular characterization of carbapenem resistance genes

Polymerase chain reaction (PCR)-based detection of Ambler class B MBLs (*bla*IMP, *bla*VIM and *bla*NDM-1), Ambler class D (*bla*OXA-48/OXA-181), and serine carbapenemases (*bla*KPC) was carried out on imipenem resistant isolates. Coexistence of carbapenemase encoding genes, namely, NDM-1, VIM, IMP, OXA-48/OXA-181 and KPC were also evaluated by PCR. Genomic DNA was extracted by the boiling method. The following pairs of previously used primers were used to yield PCR products: for *bla*NDM-1: ACC GCC TGG ACC GAT GAC CA (forward), GCC AAA GTT GGG CGC GGT TG (reverse); for *bla*IMP: GGA ATA GAG TGG CTT AAY TCT C (forward), CCA AAC YAC TAS GTT ATC T (reverse); for *bla*VIM: GAT GGT TTG GTC GCA TA (forward), CGA ATG CGC AGC ACC AG (reverse); for *bla*KPC: CGT CTA GTT CTG CTG TCT TG (forward), CTT GTC ATC CTT GTT AGG CG (reverse); for *bla*OXA-48: GCG TGG TTA AGG ATG AAC AC (forward), CAT CAA GTT CAA CCC AAC CG (reverse); and for *bla*OXA-181: ATG CGT GTA TTA GCC TTA TCG (forward), AAC TAC AAG CGC ATC GAG CA (reverse) [13–16]. The following cycling parameters were used: initial denaturation at 95°C for 10 minutes, then 30 cycles of denaturation at 95°C for 1 minute, annealing at 63°C (for *bla*NDM-1 and *bla*OXA-48), 52°C (for *bla*IMP and *bla*VIM), or 55°C (for *bla*OXA-181) for 45 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under UV light.

2.7. Statistical analysis

Data were analyzed using Excel (2007) software (Microsoft, Redmond, WA, USA).

3. Results

Twenty of the total 138 Gram-negative urinary isolates (14.49%) were found to be imipenem resistant by the disk diffusion test and by the MIC of imipenem. Thirteen *E. coli,* five *Klebsiella* spp., one *Citrobacter* spp. and one *Pseudomonas* spp. were isolated from the imipenem-resistant organisms. The MIC of imipenem of these isolates ranged from $\geq$ 128 μg/mL to 4 μg/mL. Among 20 imipenem-resistant strains, 16 (80%) carbapenemase producers were detected by PCR. Eleven (84.62%) of the 13 imipenem-resistant *E. coli,* four (80%) of the five *Klebsiella* spp., and the only *Citrobacter* spp. were

positive for carbapenemase-encoding genes by PCR. The one (100%) imipenem resistant *Pseudomonas* spp. was negative for carbapenemase-encoding genes. Out of 20 imipenem-resistant strains, 11 (55%) were positive for *bla*NDM-1, 8 (40%) for *bla*OXA-48/*bla*OXA-181, 4 (20%) for *bla*KPC, 3 (15%) for *bla*VIM, and 2 (10%) for *bla*IMP (Table 1).

Representative PCR amplified NDM-1, VIM, IMP, OXA-48/OXA-181, and KPC genes are shown in Fig. 1.

Among the carbapenemase-producing organisms, nine (45%) contained two or more carbapenemase genes, seven (35%) contained a single carbapenemase gene, and four (20%) had no carbapenemase gene. Among the 16 carbapenemase producers, *bla*NDM-1 + *bla*OXA-48/OXA-181 was the predominant combination, which was present in four (25%) of the isolates (Table 2).

The DDS test, CD assay, and modified Hodge test detected 13 (65%), 15 (75%), and seven (35%) carbapenemase producers, respectively, among the 20 imipenem-resistant isolates. Out of the four negative amplified PCR products, three were positive by the DDS test and all were positive by CD assay and modified Hodge test. Considering PCR as the gold standard, the sensitivity of the DDS test, CD assay, and MHT test were 62.5%, 68.75%, and 18.75%, respectively. All (100%) of the carbapenemase-producing organisms showed resistance to other β-lactam antibiotics, aminoglycosides and quinolones tested but were found to be sensitive to colistin.

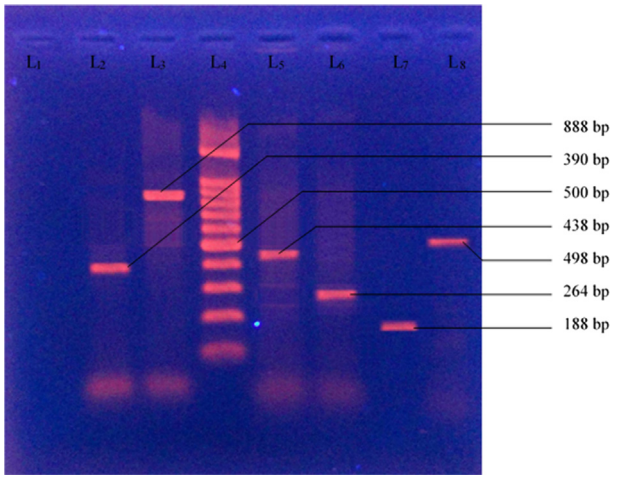

Fig. 1. Photograph of amplified NDM-1, OXA-48, OXA-181, KPC, VIM and IMP genes. Lane 4: 100-bp DNA ladder; Lane 2: amplified DNA of 390 bp for the VIM gene; Lane 3: 888 bp for the OXA-181 gene; Lane 5: 438 bp for the OXA-48 gene; Lane 6: 264 bp for the NDM-1 gene; Lane 7: 188 bp for the IMP gene; Lane 8: and 498 bp for KPC gene; Lane 1: negative control (*Escherichia coli* ATCC 25922).

Table 2
Distribution of *bla*VIM, *bla*IMP, *bla*NDM-1, *bla*OXA-48/OXA-181, *bla*KPC among imipenem-resistant organisms.

Genotype	*n* (%)
NDM−1 + OXA−48/OXA−181 + KPC	1 (5)
NDM−1 + IMP + VIM	2 (10)
NDM−1 + OXA−48/OXA−181	4 (20)
NDM−1 + KPC	2 (10)
NDM−1	2 (10)
VIM	1 (5)
OXA−48/OXA−181	3 (15)
KPC	1 (5)

4. Discussion

The global emergence and dissemination of acquired carbapenemases among Gram-negative bacteria are considered a major public health problem. The carbapenemase-encoding genes are often located on plasmids along with other resistance genes, resulting in multidrug-resistant, extremely drug-resistant and pandrug-resistant bacteria [17]. Continuous monitoring and rapid detection of these virulent organisms may check their spread and play a vital role in infection control. To address this rising resistant determinant, we have observed the occurrence of carbapenemase-encoding genes among carbapenem-resistant Gram-negative uropathogens.

The present study identified 16 (80%) carbapenemase producers out of the 20 imipenem-resistant bacteria, which included 84.62% of the imipenem-resistant *E. coli* and 80% of the imipenem-resistant *Klebsiella* spp. Although acquired carbapenemase-encoding genes are frequently found in *Pseudomonas* spp. and *Acinetobacter* spp., the existence of carbapenemase-encoding genes in the species of *E. coli* and *Klebsiella pneumoniae* in this study suggests that plasmid-mediated horizontal transfer of carbapenemase-encoding genes occurs continuously among Gram-negative bacilli, as reported previously [2].

In the present study, out of 20 imipenem-resistant Gram-negative bacteria, 11 (55%) *bla*NDM-1 producers were detected by PCR, including seven (53.85%) of the imipenem-resistant *E. coli* and four (80%) of the imipenem-resistant *Klebsiella* spp. The prevalence of *bla*NDM-1 producers is increasing in Bangladesh, which is reflected by several studies conducted in Bangladesh [18,19]. In Indian studies, the prevalence of *bla*NDM-1 producers among carbapenem-resistant Enterobacteriaceae ranged between 31.2% and 91.6% [20]. An editorial by Abdul Ghafur highlights the widespread nonprescription use of antibiotics in this subcontinent, leading to huge selection pressure, and predicts that the NDM-1 problem is likely to get substantially worse in the foreseeable future [21]. This scenario is of great concern because there are few new anti−Gram-negative antibiotics in the pharmaceutical pipeline and none that are active against NDM-1 producers [7]. A study by Bushnell et al reported that urine was the most common specimen source and *K. pneumoniae* and *E. coli* were the most frequently detected bacteria detected in 60 cases of *bla*NDM-1 producing bacteria [22]. Rapid dissemination of *bla*NDM-1 producing

Table 1
Distribution of carbapenemase-encoding genes among different species of imipenem resistant organisms.

Imipenem-resistant organisms	Carbapenemase-encoding genes				
	NDM-1 (%)	VIM (%)	IMP (%)	OXA-48/OXA-181 (%)	KPC (%)
Escherichia coli ($n = 13$)	7 (53.85)	2 (15.38)	2 (15.38)	5 (38.46)	3 (23.08)
Klebsiella spp. ($n = 5$)	4 (80.00)	1 (20.00)	0 (0.00)	2 (40.00)	1 (20.00)
Citrobacter spp. ($n = 1$)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100.00)	0 (0.00)
Pseudomonas spp. ($n = 1$)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total ($n = 20$)	11 (55.00)	3 (15.00)	2 (10.00)	8 (40.00)	4 (20.00)

The total of last row is more than 100% as most of the isolates had two or more carbapenemase genes.

organisms might be facilitated by conditions such as overcrowding, over-the-counter availability of antibiotics, low levels of hygiene, and weak hospital antibiotic policies [23].

Among 20 imipenem-resistant isolates, four (20%) were positive for the *bla*KPC gene. So far there are no reports of *bla*KPC positive Enterobacteriaceae in Bangladesh. In the current study, three (23.08%) of the imipenem-resistant *E. coli* and one (20%) of the imipenem-resistant *Klebsiella* spp. were positive for the *bla*KPC gene. The highest prevalence of KPC producing organisms to date were identified mostly in the USA, Israel, and Greece [24]. Shanmugam et al in India reported that 31 (67.4%) of 46 carbapenem-resistant Enterobacteriaceae isolates harbored the *bla*KPC gene [25]. The *bla*KPC genes that encode KPCs are present on transferable plasmids and are flanked by transposable elements, thus allowing for the gene to move from plasmid to the bacterial chromosome and back [26]. The presence of this gene suggests the possibility of horizontal transmission, as this carbapenemase has been associated with mobile genetic elements (transposons), which can be transferred from one bacterium to another [27].

A point mutant analog of OXA-48, OXA-181, with similar carbapenemase activity, has been identified in strains from India and strains of Indian origin [16]. In the current study, out of 20 imipenem-resistant Gram-negative isolates, 8 (40%) were positive for *bla*OXA-181 and OXA-48 detected by PCR (using specific primer). These included five (38.46%) of the imipenem-resistant *E. coli,* two (40%) of the imipenem-resistant *Klebsiella* spp., and the one (100%) imipenem-resistant *Citrobacter* spp. PCR screening results need to be validated by sequencing to ascertain the presence of either OXA-48 or OXA-181, which was not performed in the present study, although OXA-181 is more common in the Indian subcontinent than OXA-48. In September 2012, the CDC reported isolation of *bla*OXA-181 positive *K. pneumoniae* in two patients from Bangladesh who were admitted to separate hospitals in Singapore within a short period [28]. A study by Castanheira et al in India reported that 10 of 39 (25.64%) carbapenem-resistant strains harbored the *bla*OXA-181 gene [16]. The findings in the present study revealed that *bla*OXA-48/OXA-181 carbapenemase-producing organisms appear to be an emerging cause of carbapenem resistance in Gram-negative bacteria in Bangladesh in addition to *bla*NDM-1-producing organisms.

Combinations of different genes in single strains were observed, with a combination of *bla*NDM-1 and *bla*OXA-48/*bla*OXA-181 being predominant (20%). A study by Khajuria et al in India reported 55% of carbapenemase-producing urinary isolates of *E. coli* co-harbored *bla*NDM-1 and *bla*OXA-48 [29]. Plasmids carrying the *bla*NDM-1 gene are diverse and can harbor a high number of resistance genes associated with other carbapenemase genes (OXA-48 types, VIM types), plasmid-mediated cephalosporinase genes, ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase), and rifampin (rifampin-modifying enzymes) and sulfamethoxazole resistance genes as sources of multidrug resistance and pandrug resistance [20].

The actual prevalence of carbapenemase producers among Gram-negative bacteria is still unknown because many countries that are likely to be their main reservoirs have not established any search protocol for their detection [30]. In the present study, 16.2% (16/138) of Gram-negative uropathogens showed the occurrence of carbapenemase encoding genes, which is almost four times higher than other data reported in Bangladesh, where only 4.8% isolates were found to be carbapenemase producers [31]. The discrepancy in the findings between the latter and present studies might be due the increased use of carbapenem in Dhaka, Bangladesh.

Imipenem discs were used for screening carbapenemase production. However, we observed that four (20%) imipenem-resistant strains showed negative amplification by PCR. Gram-negative bacteria have the capacity to elude the action of carbapenems through modification of outer membrane permeability (i.e. porin loss), upregulation of efflux systems, production of carbapenem-hydrolyzing β-lactamases (i.e. hyperproduction of AmpC β-lactamases, certain ESBLs with increased capacity to hydrolyze carbapenems) and production of carbapenemases (either serine based carbapenemases or MBLs) [3]. The plethora of imipenem resistance among the noncarbapenemase producers in this study might be due to resistance mechanisms other than carbapenemase production.

In addition to showing resistance to β-lactams, all the carbapenemase-producing organisms were highly resistant to most other antibiotic classes, including aminoglycosides and fluoroquinolones. The higher antibiotic resistance in the present study might be due to the fact that common antibiotics are sold over the counter in Bangladesh and anybody can buy them without a doctor's advice. By contrast, the coresistance of MBL producers to non-β-lactam antibiotics might be due to simultaneous presence of other drug resistance mechanisms in addition to MBL genes [32].

5. Conclusion

This study shows a significant rate of carbapenemase producing Gram-negative uropathogens. This is extremely worrisome, as dissemination of plasmids carrying resistant determinant genes from one species to another makes organisms refractory to the common antibiotics used in clinical practice. The need of the hour is a strong antimicrobial stewardship program, which is followed by all concerned doctors, with further emphasis on better, cost-effective, logical infection control measures to prevent the dissemination of these multidrug resistant bacteria.

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