Scientific Research Publishing

Advances in Infectious Diseases, 2023, 13, 609-626
https://www.scirp.org/journal/aid
ISSN Online: 2164-2656
ISSN Print: 2164-2648

Evaluation of Disk Potentiation Test (DPT) and Double Disk Synergy Test (DDST) for The Detection of Metallo-β-Lactamases (MBLs) in Clinical Isolates of Bangladesh

Sumon Kumar Das[1,2], Afzal Sheikh[3,4*], Nikhat Ara[2], Suma Mita Biswas[2], Abhinandan Chowdhury[1], Fatimah Az Zahra[1], Chaman Ara Keya[1*]

[1]Department of Biochemistry & Microbiology, North South University, Dhaka, Bangladesh
[2]Department of Microbiology & Immunology, Evercare Hospital, Dhaka, Bangladesh
[3]Department of Biochemistry and Molecular Biology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Dhaka, Bangladesh
[4]Institute of Food Safety and Processing (IFSP), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Dhaka, Bangladesh
Email: *asheikh@bsmrau.edu.bd, *chaman.keya@northsouth.edu

How to cite this paper: Das, S.K., Sheikh, A., Ara, N., Biswas, S.M., Chowdhury, A., Zahra, F.A. and Keya, C.A. (2023) Evaluation of Disk Potentiation Test (DPT) and Double Disk Synergy Test (DDST) for The Detection of Metallo-β-Lactamases (MBLs) in Clinical Isolates of Bangladesh. *Advances in Infectious Diseases*, **13**, 609-626.
https://doi.org/10.4236/aid.2023.134050

Received: September 21, 2023
Accepted: December 15, 2023
Published: December 18, 2023

Abstract

Objective: Increasing the emergence of Metallo-β-lactamase (MBL) producing gram-negative bacteria and their dexterous horizontal transmission demands rapid and accurate detection. This study was conducted to determine a suitable method to promptly detect MBL-producing gram-negative bacteria. **Methods**: A total of 103 gram-negative bacteria were identified from various clinical samples at a tertiary care hospital in Dhaka city. MBL producers were detected by two phenotypic methods, the Disk Potentiation Test (DPT) and the Double Disk Synergy Test (DDST) based on β-lactam chelator combinations where EDTA/SMA has been used as an inhibitor and Imipenem, Ceftazidime as substrates. **Results**: 103 isolates which were identified as *Escherichia coli spp*, *Klebsiella spp*, *Pseudomonas spp*, *Acinetobacter spp*, *Proteus spp*, *Providencia spp* were found to be multidrug-resistant in antibiogram test. Isolates showed complete resistance (100%) to Imipenem, Meropenem, and Amoxiclav. The highest carbapenem-resistant etiological agents were *Acinetobacter spp* 40 (38.8%) followed by *Pseudomonas spp* 27 (26.2%), *Klebsiella spp* 26 (25.2%), *Escherichia coli* 8 (7.8%), *Proteus spp* 1 (1%) and *Providencia spp* 1 (1%). DPT method detected significantly ($p = 0.000009$) a higher number of MBL-producers (Imipenem with 0.5 M EDTA n = 61, 59.2% & Ceftazidime with 0.5 M EDTA n = 56, 54.4%) compared to the

DDST method (Imipenem - 0.5 M EDTA n = 43, 41.7%, Imipenem – SMA n = 38, 36.9% & Ceftazidime - 0.5 M EDTA n = 15, 14.6%). **Conclusion:** Pieces of evidence suggest that DPT is a more sensitive method than DDST and could be recommended for identifying MBL-producing bacteria in Bangladeshi hospitals for the proper management of patients, to reduce time constraints and treatment costs.

Keywords

Disk Potentiation Test (DPT), Double Disk Synergy Test (DDST), Metallo-β-Lactamase (MBL), Sodium Mercaptoacetate (SMA), and Ethylenediaminetetraacetic Acid (EDTA)

1. Introduction

Metallo-β-lactamases (MBLs) producing bacteria (*Enterobacteriaceae*) are of paramount global concern given limited therapeutic options, and untoward clinical outcomes. The horizontal transmission of carbapenemase genes mediated by mobile genetic elements (self-transmissible plasmids) carrying additional resistance elements confer resistance to various groups of antibiotics, resulting in multidrug resistance, including bacteria resistant to all available antibiotics [1] [2]. Due to their marked resistance to a wide range of antibiotics, infections caused by carbapenemase-producing *Enterobacteriaceae* are terribly difficult to manage. Moreover, *Enterobacteriaceae*, being a part of intestinal flora are easily spread and difficult to eliminate, especially in countries like Bangladesh with low levels of hygiene. The widespread use of antibiotics in humans and in the food chain and their spillover into the environment accelerate the selection and/or horizontal transfer of antibiotic-resistant genes or plasmids in a given bacterial population [3] [4]. Since MBLs are mostly transposon-and/or integron-encoded determinants, they can easily disseminate to other enterobacterial strains [5] [6] [7] [8].

It is conceivable that the primary source of these bacteria are hospitals and other healthcare settings where severe cases of bacterial infections are presented, and the volume of antibiotic use is high [9] [10]. Numerous studies have described and evaluated the performance of simple phenotypic tests for the specific detection of carbapenemase-producing strains by different groups of scientists all over the globe, with a marked endemicity according to enzyme type [10]. It appears that the effect of carbapenem-resistant *Enterobacteriaceae* (CRE) in Bangladesh and their impact on the environment are uncharted. To our knowledge, only one study for phenotypic detection of MBLs by DPT has been done in Bangladesh [11]. However, none of the researchers performed comparative studies among the MBL detection methods.

Therefore, practical and accurate phenotypic approaches are urgently

needed to detect obviously increasing carbapenemase producers among *Enterobacteriaceae* in the clinical laboratory or diagnostic laboratories lacking molecular identification setup. These assays may provide substantial information before the application of the more expensive and sophisticated molecular techniques [5] [12]. Furthermore, phenotypic methods will include all novel carbapenemase gene types [10] which "the predefined gene target" based PCR method cannot identify.

Considering the public health threat, the MBL producers pose and the rapid dissemination of MBL in bacteria, the systemic search for MBL detection methods based on β-lactam-chelator combinations seems praiseworthy which could perform well to identify all MBL-producing enterobacterial species in Bangladesh. Several phenotypic methods are used by researchers for the detection of the MBLs which are produced by gram-negative bacteria. Most of these methods are based on the ability of the metal chelator (EDTA) and sodium mercaptoacetate (SMA) to inhibit the enzyme activities [13] [14]. Therefore, the present study aimed to determine an accurate phenotypic method through comparative studies among the Disc Potentiation Test (DPT) and the Double Disc Synergy Test (DDST) to explore the incidence of multidrug-resistant MBL producers in Bangladesh along with antimicrobial-resistant patterns of these organisms.

2. Methods

2.1. Sample Collection

A total of 103 gram-negative bacteria isolated from various units of Evercare Hospital, previously called Apollo Hospital, Dhaka, were used for this study. These included 1) 13 isolates from outpatient (OPD) 2) 43 isolates from inpatients (IPD) 3) 47 isolates from the Intensive Care Unit (ICU). The ratio of infected males to females, as well as the infection rate in various age groups were recorded accordingly. Bacterial samples were isolated from different clinical specimens including Urine, Tracheal aspirate, Sputum, Blood, ET tube tips, Pus, Suction tips, Wound swabs, Foley catheter tips, Silicon catheters, and CVP tips. All experimental methods were carried out in accordance with guidelines and regulations approved by the "Research and Ethical Practice Committee" of Evercare Hospital, Dhaka, Bangladesh. Oral informed consent was obtained from the study subjects/patients or from their legal guardians and was approved by the "Research and Ethical Practice Committee" at Evercare Hospital, Dhaka, Bangladesh and the data were analyzed anonymously.

2.2. Identification of Gram-Negative Bacteria

All these specimens were inoculated on appropriate culture media (MacConkey agar, Blood agar, Chocolate agar and only for urine samples HiCrome UTI agar was used) and incubated for 24 to 48 hours at 37˚C. After incubation, organisms were identified by standard microbiological procedures (Colonial morphology, Gram stain appearance, oxidase test, triple sugar iron test, motility test, urease

test, citrate test and indole test).

2.3. Antimicrobial Susceptibility Testing

Antibiotic susceptibility tests were performed using the Kirby-Bauer disc diffusion techniques according to CLSI guidelines [15]. Inoculates were prepared by suspending the isolates in normal saline equal to the turbidity of 0.5 McFarland turbidity standard and applied on Mueller-Hinton Agar plates. All antibiotic discs were obtained from Oxoid, UK. Antibiotic discs were used depending on the types of microorganisms and specimens (Amoxiclav 30 mcg, Ceftriaxone 30 mcg, Cefepime 30 mcg, Ceftazidime 30 mcg, Imipenem 10 mcg, Meropenem 10 mcg, Polymyxin 300 mcg, Amikacin 30 mcg, Gentamicin 10 mcg, Netilmicin 30 mcg, Tetracycline 30 mcg, Tigecycline 15 mcg, Ciprofloxacin 5 mcg, Levofloxacin 5 mcg, Cotrimoxazole 25 mcg). These discs were incubated along with controls for 18 - 24 hours at 37˚C aerobically.

In the present study, *Escherichia coli* ATCC 25922 was used for quality control of the antimicrobial tests. Isolated gram-negative bacteria were subjected to an array of antibiotics and the results were interpreted as resistant or sensitive according to criteria set by the Clinical and Laboratory Standards Institute (CLSI). Gram-negative isolates with Imipenem (IPM) resistance were tested for detection of MBL by DPT and DDST method. A total number of 103 gram-negative bacteria showed carbapenem resistance in routine culture.

2.4. Double Disc Synergy Test (DDST)

The bacterial isolates were sub-cultured on MacConkey agar media and incubated overnight at 37˚C. After overnight incubation, 2 - 3 isolated colonies of the organism were picked up from the subculture plate by a sterile inoculating wire loop and suspended in 3 ml sterile peptone water in a screw-capped test tube. The test strain was adjusted to the McFarland 0.5 standard. Within 15 minutes of adjustment of the density of the organism, streaking on Mueller-Hinton agar was done using a sterile cotton swab.

One disc containing Ceftazidime (CAZ) (30 mcg) and two IPM (10 mcg) was placed on the plate. The distance between every CAZ/IPM disc was kept at about 4 cm from center to center [16]. Two blank discs with 10 µl of 0.5 M EDTA added were placed near the CAZ/IPM disc, within a center-to-center distance of 2 cm. A sodium mercaptoacetate (SMA) (Metallo-β-lactamase SMA Eiken; Eiken Chemical Co., Ltd., Tokyo, Japan) disc was placed near the IPM disc respectively disc distance edge to edge 10 mm. The agar plate was incubated at 37˚C overnight [14] [17].

In the Double Disc Synergy Test (DDST) with 0.5 M EDTA, the enhancement of synergistic growth inhibition zones between the CAZ/IPM disc towards the disc containing 0.5 M EDTA was considered positive for MBLs [16]. The presence of a synergistic inhibition zone of IPM with greater than 5 mm enlargement with the SMA disc side, was interpreted as positive [14].

2.5. Disc Potentiation Test (DPT)

The bacterial isolates were sub-cultured on MacConkey agar media and after an overnight incubation at 37˚C, 2 - 3 isolated colonies of the organisms were suspended in 3 ml sterile peptone water. The test strain was adjusted to the McFarland 0.5 standard. Within 15 minutes of adjustment of the density of the organism, streaking on Mueller-Hinton agar was done using a sterile cotton swab.

Disc Potentiation Test (DPT) was done as per Yong *et al*. using IPM-EDTA [18] [19]. The test depends on comparing the zones given by disc containing IPM with or without EDTA modified the test by using two 10 mcg IPM disc and two 30 mcg Ceftazidime (CAZ) disc [19] [20]. A zone diameter difference between the IPM/CAZ and IPM/CAZ + 0.5 M EDTA disc with greater (>) than 7 mm was interpreted as a positive result.

Two discs containing 30 mcg CAZ and two discs containing 10 mcg IPM were placed on the plates. The distance between every CAZ/IPM disc was kept at about 4 cm from center to center. 10 µl of 0.5 M EDTA was added to one CAZ/IPM disc. The plate was incubated at 37˚C overnight. In this study, DPT with 0.5 M EDTA, the enlargement of the diameter of the growth inhibitory zone around CAZ/IPM + 0.5 M EDTA disc with greater than 7 mm, compared to CAZ/IPM alone was considered positive for MBLs.

2.6. Statistical Analysis

For the statistical analyses, data were entered into an Excel spreadsheet and analyzed using the statistical software SPSS version 20.0 (IBM; Chicago, IL, USA) for Windows. Bivariate correlation test was performed. A p-value of <0.05 was considered as statistically significant.

3. Results

3.1. Bacterial Isolates from All Age-Groups Showed 100% Resistance to Amoxiclav, and Carbapenems while "61 - 75" Age-Group Showed Highest Number of Resistant Isolates

A total of 103 gram-negative strains were isolated from different clinical specimens. All these bacteria isolated from the patients were subjected to an antibiogram test. Antibiotic susceptibility profile (n = 103) was analyzed for the six isolated bacterial spps. All 103 isolates showed 100% resistance to Imipenem, Meropenem, and Amoxiclav. We also investigated the distribution of resistant and susceptible isolates among male and female individuals. Results represented that the number of resistant isolates distribution was significantly high in male patients compared to the females (**Table 1**).

The percentage of bacterial isolates from male and female patient groups that showed 100% resistance to Imipenem, Meropenem, and Amoxiclav were 70% and 30% respectively (**Figure 1(a)**).

We also observed that *Escherichia coli spp*, *Klebsiella spp*, *Pseudomonas spp*, *Acinetobacter spp*, *Proteus spp*, and *Providencia spp*. showed a significantly higher

Table 1. Antibiotic susceptibility profiles of different carbapenem-resistant *Enterobacteriaceae* (CRE, n = 103). All 103 isolates showed 100% resistance to Imipenem, Meropenem, and Amoxiclav. The antibiotic susceptibility profile was analyzed for *Escherichia coli spp*, *Klebsiella spp*, *Pseudomonas spp*, *Acinetobacter spp*, *Proteus spp*, and *Providencia spp* (n = 103) against antibiotics using the Kirby-Bauer disc diffusion techniques according to CLSI guidelines. The distribution of resistant and susceptible isolates among males and females was also analyzed. Results represented that the number of resistant isolates distribution was significantly high in male patients compared to the females.

			Gender				Total	
			Male		Female			
Modes of Action	Antimicrobial Category	Antibiotics	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Cell wall synthesis inhibitors	Aminopenicillin	Amoxiclav (30 mcg)	-	72	-	31	-	103
	Extended-spectrum Cephalosporins	Ceftriaxone (30 mcg)	-	72	1	30	1	102
		Cefepime (30 mcg)	3	69	1	30	4	99
		Ceftazidime (30 mcg)	3	69	2	29	5	98
	Carbapenems	Imipenem (10 mcg)	-	72	-	31	-	103
		Meropenem (10 mcg)	-	72	-	31	-	103
Cell membrane	Polypeptide antibiotics	Polymyxin (300 mcg)	63	9	31	-	94	9
Protein synthesis inhibitors	Aminoglycosides	Amikacin (30 mcg)	8	64	3	28	11	92
		Gentamicin (10 mcg)	7	65	4	27	11	92
		Netilmicin(30 mcg)	6	66	3	28	9	94
	Tetracyclines	Tetracycline (30 mcg)	12	60	7	24	19	84
		Tigecycline (15 mcg)	55	17	28	3	83	20
Nucleic acid synthesis inhibitors	Fluoroquinolones	Ciprofloxacin (5 mcg)	2	70	2	29	4	99
		Levofloxacin (5 mcg)	4	68	2	29	6	97
Folate pathway inhibitors	Trimethoprim and Sulfamethoxazole	Cotrimoxazole (25 mcg)	9	63	5	26	14	89

distribution among male patient group compared to female patient group (**Figure 1(b)**). In addition, our observation of the distribution of resistant bacterial isolates (n = 103) among age group data revealed that the "61 - 75" age group showed the highest number of resistant isolates. The age group also represented the highest resistant isolates from ICU hospital units compared to OPD, and IPD units (**Figure 1(c)**). We performed a comparative analysis to investigate the level of significance among the five different tests and the statistical levels of significance by Pearson correlation has been shown in supplement **Table 1**. We also investigated if particular sample types are useful for testing the susceptibility of clinically isolated etiological agents with antibiotics for proper management of the patients.

We analyzed the sample-wise distribution of bacterial isolates and found that Urine, followed by Tracheal aspirate and Wound swab samples represented the highest number of carbapenem-resistant isolates (**Figure 2**. and **Table 2**).

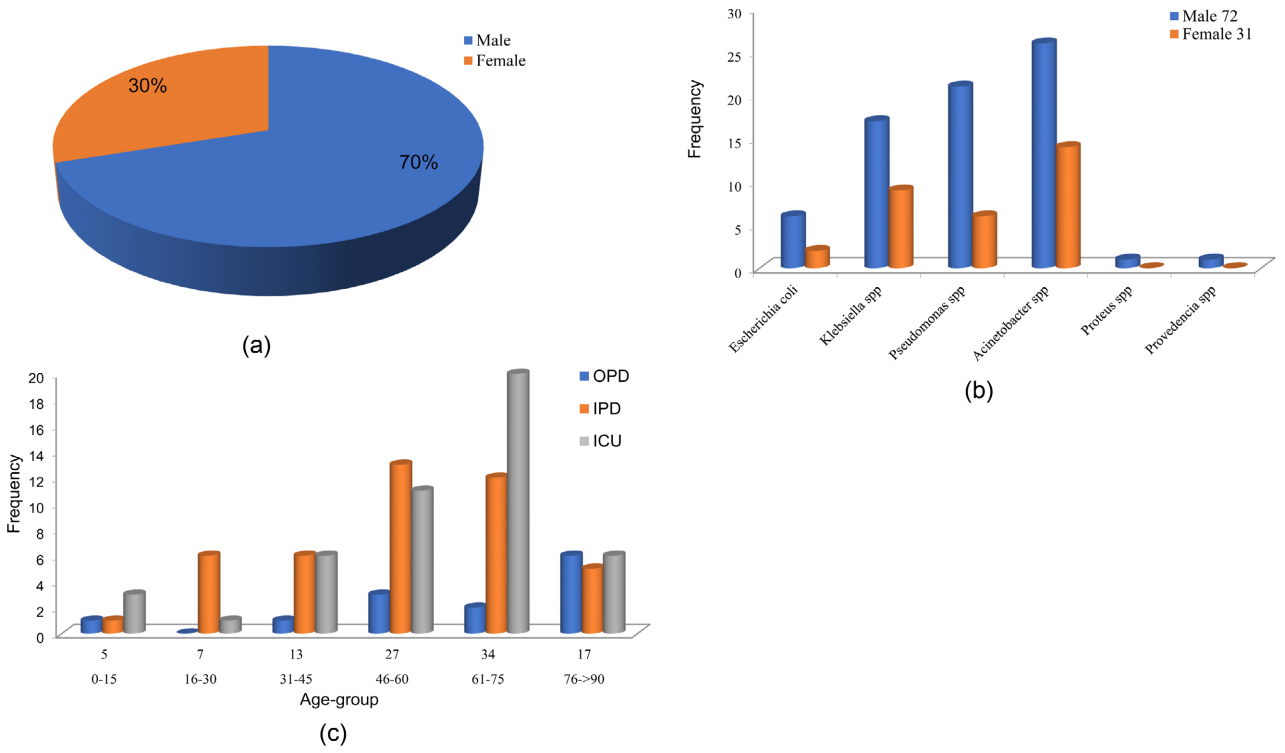

Figure 1. Bacterial isolates from all age groups showed 100% resistance to Amoxiclav, and Carbapenems while the "61 - 75" age group showed the highest number of resistant isolates. (a) Showing the percentage of males and females affected by CRE. (b) Frequency of clinically isolated etiological agents in clinical samples from various units in the hospital. (c) Showing the frequency of CRE isolated according to age group.

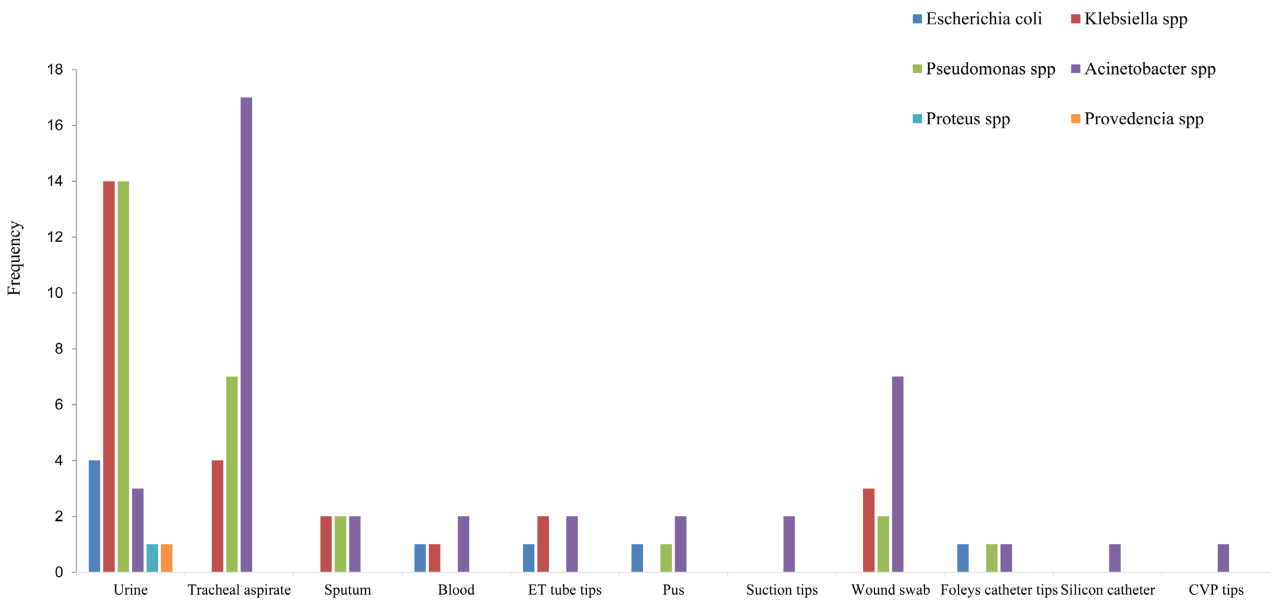

Figure 2. Sample-wise distribution spectrum of various gram-negative strains.

While all the bacteria isolated were found to be resistant to antibiotics Imipenem, Meropenem, and Amoxiclav, the highest carbapenem-resistant etiological agents isolated was *Acinetobacter spp* 40 (38.8%) followed by *Pseudomonas spp*

27 (26.2%), *Klebsiella spp* 26 (25.2%), *Escherichia coli* 8 (7.8%), *Proteus spp* 1 (1%) and *Providencia spp* 1 (1%) (Table 3).

Our investigation on the susceptibility of bacterial isolates (n = 103) from all age groups to antibiotics (Amoxiclav, Ceftriaxone, Cefepime, Ceftazidime, Imipenem, Meropenem, Polymyxin, Amikacin, Gentamicin, Netilmicin, Tetracycline, Tigecycline, Ciprofloxacin, Levofloxacin, and Cotrimoxazole) (Table 4).

Table 2. Sample-wise distribution spectrum of various (CRE) bacterial isolates. Individual bacterial isolates (n = 103) from Urine, Tracheal aspirate, Sputum, Blood, ET tube tips, Pus, Suction tips, Wound swab, Foleys catheter tips, Silicon catheter, and CVP tips were treated against commonly used antimicrobial agents. *Escherichia coli spp, Klebsiella spp, Pseudomonas spp, Acinetobacter spp, Proteus spp, and Providencia spp* showed resistance to antimicrobial agents.

Antimicrobial agents	Clinical samples																					
	Urine		Tracheal aspirate		Sputum		Blood		ET tube tips		Pus		Suction tips		Wound swab		Foleys catheter tips		Silicon catheter		CVP tips	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
Amoxiclav (30 mcg)	-	37	-	28	-	6	-	4	-	5	-	4	-	2	-	12	-	3	-	1	-	1
Ceftriaxone (30 mcg)	-	37	-	28	-	6	-	4	1	4	-	4	-	2	-	12	-	3	-	1	-	1
Cefepime (30 mcg)	1	36	3	25	-	6	-	4	-	5	-	4	-	2	-	12	-	3	-	1	-	1
Ceftazidime (30 mcg)	1	36	3	25	-	6	-	4	1	4	-	4	-	2	-	12	-	3	-	1	-	1
Imipenem (10 mcg)	-	37	-	28	-	6	-	4	-	5	-	4	-	2	-	12	-	3	-	1	-	1
Meropenem (10 mcg)	-	37	-	28	-	6	-	4	-	5	-	4	-	2	-	12	-	3	-	1	-	1
Polymyxin (300 mcg)	31	6	26	2	6	-	4	-	5	-	4	-	2	-	12	-	3	-	-	1	1	-
Amikacin (30 mcg)	4	33	2	26	-	6	1	3	-	5	2	2	-	2	1	11	1	2	-	1	-	1
Gentamicin (10 mcg)	6	31	1	27	-	6	1	3	1	4	1	3	-	2	-	12	1	2	-	1	-	1
Netilmicin (30 mcg)	2	35	1	27	-	6	1	3	1	4	2	2	1	1	-	12	1	2	-	1	-	1
Tetracycline (30 mcg)	8	29	5	23	1	5	1	3	2	3	-	4	-	2	2	10	-	3	-	1	-	1
Tigecycline (15 mcg)	28	9	22	6	4	2	4	-	5	-	3	1	2	-	10	2	3	-	1	-	1	-
Ciprofloxacin (5 mcg)	2	35	1	27	-	6	-	4	-	5	-	4	-	2	1	11	-	3	-	1	-	1
Levofloxacin (5 mcg)	2	35	2	26	1	5	-	4	-	5	-	4	-	2	1	11	-	3	-	1	-	1
Cotrimoxazole (25 mcg)	2	35	5	23	1	5	1	3	1	4	-	4	-	2	3	9	-	3	1	-	-	1

Table 3. Distribution spectrum of resistant and susceptible CRE (n = 103) isolates among various bacterial spp against other commonly used antimicrobial agents. *Klebsiella spp*, and *Acinetobacter spp* showed the highest resistant isolated followed by *Pseudomonas spp*, *Escherichia coli spp*, *proteus spp*, and *Providencia spp*.

Antimicrobial agents	Gram-negative antibiotic-resistant organism											
	Escherichia coli		*Klebsiella spp*		*Pseudomonas spp*		*Acinetobacter spp*		*Proteus spp*		*Providencia spp*	
	S	R	S	R	S	R	S	R	S	R	S	R
Amoxiclav (30 mcg)	-	8	-	26	-	27	-	40	-	1	-	1
Ceftriaxone (30 mcg)	-	8	-	26	-	27	1	39	-	1	-	1
Cefepime (30 mcg)	-	8	-	26	4	23	-	40	-	1	-	1
Ceftazidime (30 mcg)	-	8	-	26	4	23	1	39	-	1	-	1
Imipenem (10 mcg)	-	8	-	26	-	27	-	40	-	1	-	1
Meropenem (10 mcg)	-	8	-	26	-	27	-	40	-	1	-	1
Polymyxin (300 mcg)	8	-	24	2	23	4	38	2	1	-	-	1
Amikacin (30 mcg)	4	4	1	25	5	22	1	39	-	1	-	1
Gentamicin (10 mcg)	3	5	4	22	2	25	2	38	-	1	-	1
Netilmicin (30 mcg)	2	6	-	26	4	23	3	37	-	1	-	1
Tetracycline (30 mcg)	-	8	10	16	7	20	2	38	-	1	-	1
Tigecycline (15 mcg)	8	-	24	2	12	15	38	2	-	1	1	-
Ciprofloxacin (5 mcg)	-	8	1	25	3	24	-	40	-	1	-	1
Levofloxacin (5 mcg)	-	8	1	25	3	24	2	38	-	1	-	1
Cotrimoxazole (25 mcg)	1	7	1	25	2	25	10	30	-	1	-	1

3.2. The DPT Testing Method Is More Sensitive and Detected Higher Number of MBL Producers Compared to the DDST

Among the five different tests performed by DPT and DDST methods compared in this study, of 103 clinical isolates, 61 (59.2%) isolates showed sensitivity by disc potentiation test (DPT) method using imipenem with 0.5 M EDTA and 56 (54.4%) isolates ceftazidime with 0.5 M EDTA. In the DPT test method using imipenem with 0.5 M EDTA, the highest resistance to carbapenem strains carrying MBL enzyme found in IPD patients was 67% (29 out of 43) followed by ICU 53.2% (25 out of 47) and OPD 53.8% (07 out of 13) (**Figure 3(a)**).

The inflated number of carbapenem-resistant bacterial strains carrying MBL enzyme were found in age groups 0 - 15 years (80%, 4 out of 5) followed by 76 - > 90 years (76%, 13 out of 17) (**Figure 3(b)**). The proportion of males infected with MBL enzyme-producing resistant bacteria was higher than the proportion of females (44 out of 72) and (17 out of 31) respectively (**Figure 3(c)**). The highest percentage of MBL-producing strain was *Escherichia coli* (100%, 8 out of 8) (**Figure 3(d)**). Using ceftazidime with 0.5 M EDTA, the excessively high resistant strains to carbapenem carrying MBL enzyme were found in ICU patients (27 out of 47) followed by IPD (25 out of 43) and OPD (04 out of 13) (**Figure 3(a)**)

Table 4. Distribution spectrum of resistant bacterial CRE isolates (n = 103) among different age groups against antimicrobial agents using the Kirby-Bauer disc diffusion techniques. Bacterial isolates from all age groups showed 100% resistance to Amoxiclav (30 mcg), Imipenem (10 mcg), and Meropenem (10 mcg) while the "61 - 75" age group showed the highest number of resistant isolates.

Antimicrobial agents	Age group											
	0 - 15		16 - 30		31 - 45		46 - 60		61 - 75		76- > 90	
	S	R	S	R	S	R	S	R	S	R	S	R
Amoxiclav (30 mcg)	-	5	-	7	-	13	-	27	-	34	-	17
Ceftriaxone (30 mcg)	-	5	-	7	-	13	1	26	-	34	-	17
Cefepime (30 mcg)	-	5	-	7	2	11	-	27	1	33	1	16
Ceftazidime (30 mcg)	-	5	-	7	2	11	1	26	1	33	1	16
Imipenem (10 mcg)	-	5	-	7	-	13	-	27	-	34	-	17
Meropenem (10 mcg)	-	5	-	7	-	13	-	27	-	34	-	17
Polymyxin (300 mcg)	5	-	6	1	13	-	25	2	29	5	16	1
Amikacin (30 mcg)	-	5	1	6	3	10	2	25	2	32	3	14
Gentamicin (10 mcg)	-	5	2	5	3	10	2	25	1	33	3	14
Netilmicin (30 mcg)	1	4	2	5	3	10	2	25	1	33	-	17
Tetracycline (30 mcg)	1	4	1	6	3	10	6	21	6	28	2	15
Tigecycline (15 mcg)	5	-	6	1	11	2	20	7	26	8	15	2
Ciprofloxacin (5 mcg)	-	5	1	6	3	10	-	27	-	34	-	17
Levofloxacin (5 mcg)	-	5	1	6	3	10	1	26	-	34	1	16
Cotrimoxazole (25 mcg)	2	3	1	6	3	10	4	23	1	33	3	14

and, the highest distribution was found in the age group "61 - 75" years (19 out of 34) (**Figure 3(b)**). The proportion of males infected with MBL enzyme-producing resistance bacterial strains was higher than the proportion of females (37 out of 72) and (19 out of 31) respectively (**Figure 3(c)**). The highest number of MBL-producing bacterial strains was *Klebsiella spp* (15 out of 26) followed by *Escherichia coli* (4 out of 8) (**Figure 3(d)**).

In the DDST method using imipenem and 0.5 M EDTA 43 (41.7%) isolates showed sensitive patterns followed by 38 (36.9%) imipenem and SMA and 15 (14.6%) isolates showed susceptibility to ceftazidime and 0.5 M EDTA. In this test method, using imipenem and 0.5 M EDTA, the highest resistant bacterial isolates to carbapenem strains carrying MBL enzyme were found in ICU patients (20 out of 47) followed by IPD (18 out of 43) and OPD (05 out of 13) (**Figure 3(a)**). The highest carbapenem-resistant bacterial isolates carrying MBL-enzyme were found in the age group 61-75 years (17 out of 34) (**Figure 3(b)**) and the proportion of males infected with MBL enzyme-producing resistant bacterial strains was higher than the proportion of female (30 out of 72) and (13 out of 31) respectively (**Figure 3(c)**). The highest percentage of MBL-producing bacterial strains was *Escherichia coli* (100%, 8 out of 8) (**Figure 3(d)**). On the other hand,

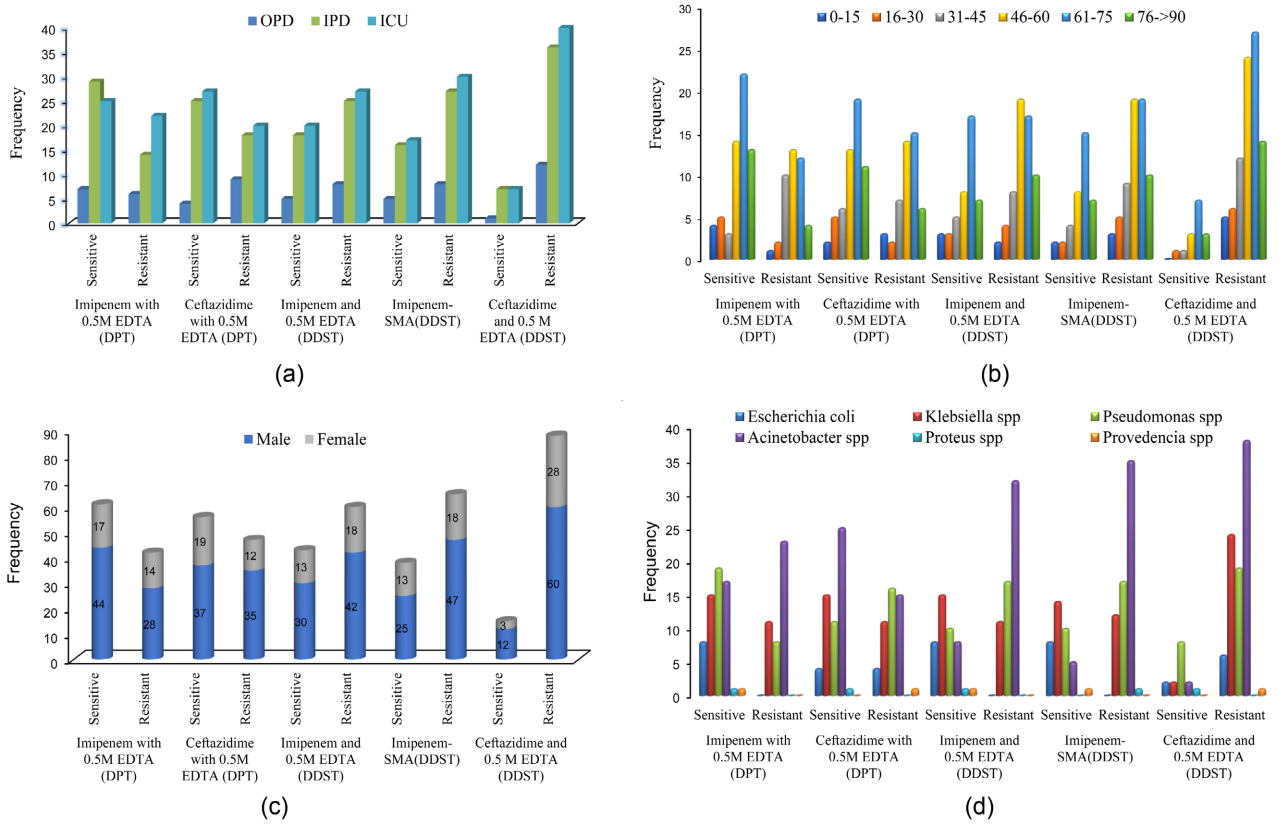

Figure 3. DPT testing method is more sensitive and detects a higher number of MBL producers compared to the DDST. (a) Number of MBL enzyme screenings using different inhibitors in various unit of the hospital. (b) Showing the frequency of MBL enzyme isolated according to age group (c) Number of MBL enzyme screening using different inhibitors affected by gender (d) Showing the frequency of MBL enzyme screening in different gram-negative strains.

using imipenem and SMA, the highest number of bacterial isolates showing resistance to carbapenem-carrying MBL-enzyme were found in ICU patients (17 out of 47) followed by IPD (16 out of 43) and OPD (05 out of 13) (**Figure 3(a)**), and the highest distribution of carbapenem-resistant isolates carrying MBL-enzyme were found in age-group .. years (15 out of 34) (**Figure 3(b)**) while the proportion of males patients infected with MBL enzyme-producing bacterial isolates was higher than of female (25 out of 72) and (13 out of 31) respectively (**Figure 3(c)**). The highest number of MBL-producing bacterial strains was *Escherichia coli* (8 out of 8) (**Figure 3(d)**). Using ceftazidime and 0.5 M EDTA, the highest number of bacterial isolates showing resistance to carbapenem strains MBL-enzyme was found in ICU patients (07 out of 47) followed by IPD (07 out of 43) and OPD (01 out of 13) (**Figure 3(a)**). The highest distribution of carbapenem-resistant bacterial isolates carrying MBL-enzyme was observed in the age group 61-75 years (07 out of 34) and the proportion of males infected with MBL-enzyme-producing resistant bacterial strains was higher than that of females (12 out of 72) and (03 out of 31) respectively (**Figure 3(b)** and **Figure 3(c)** respectively), while the highest number of MBL-producing bacterial strain was *Pseudomonas spp* (8 out of 27) (**Figure 3(d)**).

4. Discussion

The global spread of carbapenemase-producing bacteria underlined the necessity of appropriate diagnostic methods available in all clinical laboratories to monitor their emergence. Special attention needs to be given to the developing country like Bangladesh, where sheer negligence leads to a higher risk of spread. Not only negligence, the availability of proper detection strategies for MBL-producing gram-negative bacteria leads to otherwise expensive detection systems. Therefore, a cheaper, readily available and proper detection method for MBL-producing gram-negative bacteria, especially in peripheral hospitals is necessary. Acquired MBLs in gram-negative bacteria are becoming an emerging resistant determinant worldwide [11]. To address this rising resistant determinant, we have investigated the prevalence and the distribution of MBL producers among the imipenem-resistant isolates following standard methods of isolation and identification. Even though detection using PCR a simple and straightforward approach is gaining momentum worldwide [21], it comes with a considerable expense and is usually not affordable and adaptable by most hospitals and clinics in Bangladesh. In contrast to traditional molecular-genetic techniques, which are only able to detect known genes, phenotypic detection of carbapenemase activity is also able to detect novel carbapenemases. Hence, for the detection of MBL-producing *Enterobacteriaceae*, DDST and DPT methods were used, as these two methods are accessible and affordable in most of the local hospitals.

Our study suggests males are more susceptible compared to females to the resistant *Enterobacteriaceae* no matter which location in Bangladesh they belong to, while the leading affected age group was "61 - 75" years of age, the most probable cause can be due to the weakened immune system at this age. A concerning issue was the highest number of bacterial isolates detected of the age group "61 - 75" in ICU unit of the Hospital (**Figure 1**) although, the total number of bacterial isolates in OPD and IPD units were collectively higher than that of ICU unit.

Reduced susceptibility of the MBL-producing gram-negative bacteria to imipenem has been frequently reported [22] [23]. Interestingly, many of these reports have documented only moderate resistance to Imipenem [23] [24]. The present study showed highsest levels (100%) of Imipenem and Meropenem resistance for all the gram-negative bacteria tested (**Table 1**). In addition, our analysis results on the multi-drug resistance (100%) represented that the highest number of resistant isolates were obtained from Tracheal aspirate followed by Urine and Wound swabs. However, the results were not affected the sample types of the host (**Figure 2** and **Table 2**) origin. A significant increase in the percentage of *Acinetobacter spp* isolates (62.5%, 25 out of 40) Ceftazidime with 0.5 M EDTA (DPT) and (42.5%, 17 out of 40) Imipenem with 0.5 M EDTA (DPT) MBL detection was observed in the present study (**Figure 3(d)**) which is in contrast to the 83.87% MBL-producer was found in combined disc assays and (100%) MBL-producer *A. baumannii* by PCR test were previously reported in

another local study, reflecting the evolving scenario in Bangladesh. Our study demonstrated that all MBL-producing isolates were multidrug-resistant. Selective pressure and/or the simultaneous presence of other drug-resistant genes such as gene cassettes or other resistance mechanisms might be the reason for the co-resistance [11]. The present study gave significant indications that DPT (combined disc test) using EDTA as an inhibitor and Imipenem as the substrate can be successfully used for the identification of MBL enzymes among the bacteria of the family *Enterobacteriaceae*. Although acquired MBL enzymes are frequently found in *Pseudomonas* spp. and *Acinetobacter* spp. This study suggests that the existence of MBL in the species of *E. coli* and *K. pneumoniae* might be due to plasmid-mediated horizontal transfer that occur continuously among gram-negative bacilli, as reported previously [11].

The carbapenem-resistant strains with no MBL noticeable by the DPT test in this study may possess other enzymes mediating carbapenem resistance, such as OXA-type lactamases (class D) or AmpC b-lactamases, and/or other mechanisms such as outer-membrane permeability and efflux mechanisms [20] that were yet left to check. Therefore, our research signifies that the identification of MBL-producing gram-negative bacteria by DPT is a better method compared to DDST (**Figure 3**) in identifying the spread of multiple antibiotic-resistant organisms. This finding will allow the development of containment measures, leading to broader impacts in reducing their transmission to the communities in Bangladesh. Moreover, our results highlight a handful of issues related to the prevalence and characteristics of MBL-producing gram-negative bacteria in hospitals in Dhaka, a potential public health threat. Apart from the detection method, alarming fact about the rate of exposure of different age, sex groups, locations and specific sections in the hospital was brought to light.

The high incidence of MBLs-producers among carbapenem, and amoxiclav-resistant gram-negative micro-organisms obtained in this study, demands necessary implementations of strict infection control, rearranging the patient's management including the detection methods to overcome the therapeutic challenges, reduce hospitalization-time constraints and treatment cost. Early detection of multi-drug resistance, implementation of strict antimicrobial policies and infection control programs by hospitals may avoid the rapid dissemination of these organisms. This investigation gives an apprehensive critique to the dynamic detection of MBL-producing bacterial infection and will be of interest to the scientists, researchers, physicians working in hospitals, and microbiologists to understand, resistant mechanisms, controlling infection, as well as to government policymakers to set up a guideline for managing hospitals.

Acknowledgements

This work was partially supported by the personal fund of Dr. N.Ara in Evercare Hospital Dhaka, Bangladesh.

All authors contributed to this research work from their responsibility to the

society. We are grateful to the patients for providing samples and for their participation in our research.

Declaration Statement

Ethics Approval and Consent to Participate

All experimental procedures were carried out in accordance with guidelines and regulations, approved by the "Research and Ethical Practice Committee" of Evercare Hospital, Dhaka, Bangladesh. Oral informed consent was obtained from the study subjects/patients or from their legal guardians and was approved by the "Research and Ethical Practice Committee" of Evercare Hospital, Dhaka, Bangladesh.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Authors' Contributions

Conceptualization: SKD, CAK, and NA; Experiment: SKD and SMB; Data analysis and interpretation: SKD and AS; Preparation of Figures and Tables: SKD, AS; Methodology: SKD, CAK. AC, and FZ; Writing—original draft: SKD and AS; Writing—review & editing: AS, CAK, and NA.

Competing Interests

The authors declare that there are no conflicts of interest.

References

[1] Hrabák, J., Chudáčková, E. and Papagiannitsis, C.C. (2014) Detection of Carbapenemases in Enterobacteriaceae: A Challenge for Diagnostic Microbiological Laboratories. *Clinical Microbiology and Infection*, **20**, 839-853. https://doi.org/10.1111/1469-0691.12678

[2] Kumarasamy, K.K., Toleman, M.A., Walsh, T.R., Bagaria, J., Butt, F., Balakrishnan, R., Woodford, N., *et al.* (2010) Emergence of a New Antibiotic Resistance Mechanism in India, Pakistan, and the UK: A Molecular, Biological, and Epidemiological Study. *The Lancet Infectious Diseases*, **10**, 597-602. https://doi.org/10.1016/S1473-3099(10)70143-2

[3] Andersson, D.I. and Hughes, D. (2014) Microbiological Effects of Sublethal Levels of Antibiotics. *Nature Reviews Microbiology*, **12**, 465-478. https://doi.org/10.1038/nrmicro3270

[4] Islam, M.A., Islam, M., Hasan, R., Hossain, M.I., Nabi, A., Rahman, M., Faruque, S.M., *et al.* (2017) Environmental Spread of New Delhi Metallo-β-Lactamase-1-Producing Multidrug-Resistant Bacteria in Dhaka, Bangladesh. *Applied and Environmental Microbiology*, **83**, e00793-17.

https://doi.org/10.1128/AEM.00793-17

[5] Tsakris, A., Poulou, A., Pournaras, S., Voulgari, E., Vrioni, G., Themeli-Digalaki, K., Sofianou, D., *et al*. (2010) A Simple Phenotypic Method for the Differentiation of Metallo-β-Lactamases and Class A KPC Carbapenemases in Enterobacteriaceae Clinical Isolates. *Journal of Antimicrobial Chemotherapy*, **65**, 1664-1671. https://doi.org/10.1093/jac/dkq210

[6] Queenan, A.M. and Bush, K. (2007) Carbapenemases: The Versatile β-Lactamases. *Clinical Microbiology Reviews*, **20**, 440-458. https://doi.org/10.1128/CMR.00001-07

[7] Naas, T., Cuzon, G., Villegas, M.V., Lartigue, M.F., Quinn, J.P. and Nordmann, P. (2008) Genetic Structures at the Origin of Acquisition of the β-Lactamase Bla KPC Gene. *Antimicrobial Agents and Chemotherapy*, **52**, 1257-1263. https://doi.org/10.1128/AAC.01451-07

[8] Ikonomidis, A., Tokatlidou, D., Kristo, I., Sofianou, D., Tsakris, A., Mantzana, P., Maniatis, A.N., et al. (2005) Outbreaks in Distinct Regions Due to a Single *Klebsiella pneumoniae* Clone Carrying a *bla*$_{\text{VIM-1}}$ Metallo-β-Lactamase Gene. *Journal of Clinical Microbiology*, **43**, 5344-5347. https://doi.org/10.1128/JCM.43.10.5344-5347.2005

[9] Picão, R.C., Cardoso, J.P., Campana, E.H., Nicoletti, A.G., Petrolini, F.V., Assis, D.M., Gales, A.C., *et al*. (2013) The Route of Antimicrobial Resistance from the Hospital Effluent to the Environment: Focus on the Occurrence of KPC-Producing *Aeromonas* spp. and Enterobacteriaceae in Sewage. *Diagnostic Microbiology and Infectious Disease*, **76**, 80-85. https://doi.org/10.1016/j.diagmicrobio.2013.02.001

[10] Goic-Barisic, I., Hrenovic, J., Kovacic, A. and Musić, M.Š. (2016) Emergence of Oxacillinases in Environmental Carbapenem-Resistant *Acinetobacter baumannii* Associated with Clinical Isolates. *Microbial Drug Resistance*, **22**, 559-563. https://doi.org/10.1089/mdr.2015.0275

[11] Farzana, R., Shamsuzzaman, S.M. and Mamun, K.Z. (2013) Isolation and Molecular Characterization of New Delhi Metallo-β-Lactamase-1 Producing Superbug in Bangladesh. *The Journal of Infection in Developing Countries*, **7**, 161-168. https://doi.org/10.3855/jidc.2493

[12] Franklin, C., Liolios, L. and Peleg, A.Y. (2006) Phenotypic Detection of Carbapenem-Susceptible Metallo-β-Lactamase-Producing Gram-Negative Bacilli in the Clinical Laboratory. *Journal of Clinical Microbiology*, **44**, 3139-3144. https://doi.org/10.1128/JCM.00879-06

[13] Prakash, M.R., Veena, M., Sharma, A., Basavaraj, K.N. and Viswanath, G. (2012) The Prospective Evaluation of Four Convenient Methods for Detecting MBLs in the Clinical Isolates. *Journal of Clinical & Diagnostic Research*, **6**, 1196-1199.

[14] Notake, S., Matsuda, M., Tamai, K., Yanagisawa, H., Hiramatsu, K. and Kikuchi, K. (2013) Detection of IMP Metallo-β-Lactamase in Carbapenem-Nonsusceptible Enterobacteriaceae and Non-Glucose-Fermenting Gram-Negative Rods by Immunochromatography Assay. *Journal of Clinical Microbiology*, **51**, 1762-1768. https://doi.org/10.1128/JCM.00234-13

[15] Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A. and Iqbal, M. (2011) Detection and Antibiotic Susceptibility Pattern of Biofilm Producing Gram Positive and Gram Negative Bacteria Isolated from a Tertiary Care Hospital of Pakistan. *Malaysian Journal of Microbiology*, **7**, 57-60. https://doi.org/10.21161/mjm.25410

[16] Arakawa, Y., Shibata, N., Shibayama, K., Kurokawa, H., Yagi, T., Fujiwara, H. and Goto, M. (2000) Convenient Test for Screening Metallo-β-Lactamase-Producing Gram-Negative Bacteria by Using Thiol Compounds. *Journal of Clinical Microbi-*

ology, **38**, 40-43. https://doi.org/10.1128/JCM.38.1.40-43.2000

[17] Shibata, N., Doi, Y., Yamane, K., Yagi, T., Kurokawa, H., Shibayama, K., Arakawa, Y., *et al*. (2003) PCR Typing of Genetic Determinants for Metallo-β-Lactamases and Integrases Carried by Gram-Negative Bacteria Isolated in Japan, with Focus on the Class 3 Integron. *Journal of Clinical Microbiology*, **41**, 5407-5413. https://doi.org/10.1128/JCM.41.12.5407-5413.2003

[18] Yong, D., Lee, K., Yum, J.H., Shin, H.B., Rossolini, G.M. and Chong, Y. (2002) Imipenem-EDTA Disk Method for Differentiation of Metallo-β-Lactamase-Producing Clinical Isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *Journal of Clinical Microbiology*, **40**, 3798-3801. https://doi.org/10.1128/JCM.40.10.3798-3801.2002

[19] Galani, I., Rekatsina, P.D., Hatzaki, D., Plachouras, D., Souli, M. and Giamarellou, H. (2008) Evaluation of Different Laboratory Tests for the Detection of Metallo-β-Lactamase Production in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy*, **61**, 548-553. https://doi.org/10.1093/jac/dkm535

[20] Uma Karthika, R., Srinivasa Rao, R., Sahoo, S., Shashikala, P., Kanungo, R., Jayachandran, S. and Prashanth, K. (2009) Phenotypic and Genotypic Assays for Detecting the Prevalence of Metallo-β-Lactamases in Clinical Isolates of *Acinetobacter baumannii* from a South Indian Tertiary Care Hospital. *Journal of Medical Microbiology*, **58**, 430-435. https://doi.org/10.1099/jmm.0.002105-0

[21] Walsh, T.R., Toleman, M.A., Poirel, L. and Nordmann, P. (2005) Metallo-β-Lactamases: The Quiet before the Storm? *Clinical Microbiology Reviews*, **18**, 306-325. https://doi.org/10.1128/CMR.18.2.306-325.2005

[22] Joshi, S.G., Litake, G.M., Ghole, V.S. and Niphadkar, K.B. (2003) Plasmid-Borne Extended-Spectrum β-Lactamase in a Clinical Isolate of *Acinetobacter baumannii*. *Journal of Medical Microbiology*, **52**, 1125-1127. https://doi.org/10.1099/0022-1317-52-12-1125

[23] Sinha, M. and Srinivasa, H. (2007) Mechanisms of Resistance to Carbapenems in Meropenem-Resistant Acinetobacter Isolates from Clinical Samples. *Indian Journal of Medical Microbiology*, **25**, 121-125. https://doi.org/10.1016/S0255-0857(21)02169-1

[24] Taneja, N. and Sharma, M. (2003) Imipenem Resistance in Nonfermenters Causing Nosocomial Urinary Tract Infections. *Indian Journal of Medical Sciences*, **57**, 294-299.

Supplemental Information

Statistical analysis: For the statistical analyses, data were entered into an excel spreadsheet and analyzed using the statistical software SPSS version 20.0 (IBM; Chicago, IL, USA) for Windows was utilized. Bivariate correlation tests were performed. A p-value of < 0.05 was considered statistically significant.

Table S1. Statistical significance of cross-Metallo-β-lactamases (MBLs), evaluated through the Disc Potentiation Test (DPT); Double Disc Synergy Test (DDST) were analyzed by the Pearson correlation test. Significant cross- Metallo-β-lactamases (MBLs) were found between (a) Imipenem + 0.5 M EDTA (DPT) and Ceftazidime + 0.5 M EDTA (DPT) (P = 0.006); (b) Imipenem + 0.5 M EDTA (DPT) and Imipenem-0.5 M EDTA (DDST) (P = 0.000); (c) Imipenem + 0.5 M EDTA (DPT) and Imipenem-SMA (DDST) (P = 0.000); (d) Imipenem + 0.5 M EDTA (DPT) and Ceftazidime-0.5 M EDTA (DDST) (P = 0.019); (e) Ceftazidime + 0.5 M EDTA (DPT) and Imipenem + 0.5 M EDTA (DPT) (P = 0.006); (f) Ceftazidime + 0.5 M EDTA (DPT) and Imipenem-0.5 M EDTA (DDST) (P = 0.149); (g) Ceftazidime + 0.5 M EDTA (DPT) and Imipenem-SMA (DDST) (P = 0.077); (h) Ceftazidime + 0.5 M EDTA (DPT) and Ceftazidime-0.5 M EDTA (DDST) (P = 0.031); (i) Imipenem-0.5 M EDTA (DDST) and Imipenem + 0.5 M EDTA (DPT) (P = 0.000); (j) Imipenem-0.5 M EDTA (DDST) and Ceftazidime + 0.5 M EDTA (DPT) (P = 0.149); (k) Imipenem-0.5 M EDTA (DDST) and Imipenem-SMA (DDST) (P = 0.000); (l) Imipenem-0.5 M EDTA (DDST) and Ceftazidime-0.5 M EDTA (DDST) (P = 0.000); (m) Imipenem-SMA (DDST) and Imipenem + 0.5 M EDTA (DPT) (P = 0.000); (n) Imipenem-SMA (DDST) and Ceftazidime + 0.5 M EDTA (DPT) (P = 0.077); (o) Imipenem-SMA (DDST) and Imipenem-0.5 M EDTA (DDST) (P = 0.000); (p) Imipenem-SMA (DDST) and Ceftazidime-0.5 M EDTA (DDST) (P = 0.790); (q) Ceftazidime-0.5 M EDTA (DDST) and Imipenem + 0.5 M EDTA (DPT) (P = 0.019); (r) Ceftazidime-0.5 M EDTA (DDST) and Ceftazidime + 0.5 M EDTA (DPT) (P = 0.031); (s) Ceftazidime-0.5 M EDTA (DDST) and Imipenem-0.5 M EDTA (DDST) (P = 0.000) and (t) Ceftazidime-0.5 M EDTA (DDST) and Imipenem-SMA (DDST) (P = 0.790).

		Imipenem + 0.5 M EDTA (DPT)	Caftazidime + 0.5 M EDTA (DPT)	Imipenem-0.5 M EDTA (DDST)	Imipenem-SMA (DDST)	Ceftazidime-0.5 M EDTA (DDST)
Imipenem+ 0.5 M EDTA (DPT)	Pearson Correlation	1	0.271**	0.422**	0.471**	0.231*
	Sig. (2-tailed)		0.006	0.000	0.000	0.019
	N	103	103	103	103	103
Caftazidime + 0.5 M EDTA (DPT)	Pearson Correlation	0.271**	1	0.143	0.175	0.212*
	Sig. (2-tailed)	0.006		0.149	0.077	0.031
	N	103	103	103	103	103
Imipenem-0.5 M EDTA (DDST)	Pearson Correlation	0.422**	0.143	1	0.699**	0.376**
	Sig. (2-tailed)	0.000	0.149		0.000	0.000
	N	103	103	103	103	103
Imipenem-SMA (DDST)	Pearson Correlation	0.471**	0.175	0.699**	1	0.027

Continued

	Sig. (2-tailed)	0.000	0.077	0.000		0.790
	N	103	103	103	103	103
Ceftazidime-0.5 M EDTA (DDST)	Pearson Correlation	0.231*	0.212*	0.376**	0.027	1
	Sig. (2-tailed)	0.019	0.031	0.000	0.790	
	N	103	103	103	103	103

**Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). Disc Potentiation Test (DPT); Double Disc Synergy Test (DDST), sodium mercaptoacetate (SMA).