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Microbiological Study on Nosocomial *Acinetobacter baumannii* Infection with Molecular Characterization of Metallo Beta- Lactamases

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Abstract

Acinetobacter baumannii is a nosocomial pathogen which the World Health Organization ' s considered number one critical priority pathogen. It has become a growing problem in hospitals as a predominant multi-drug resistant that left clinicians with limited treatment options. Its main mechanisms for β-lactam resistance are the production of carbapenems especially Amber class D β-lactamases followed by B β-lactamases. NDM-1 which is an example of the later poses a major health concern particularly in the light of its spread through population. To precede our study, 74 *Acinetobacter baumannii* isolates were collected from hospital laboratories during the period from July 2017 till June 2018. Antimicrobial susceptibility testing was done by Kirby-Bauer Disc Diffusion method (KBDD) and Minimum Inhibitory Concentration (MIC) was detected using E-test method. CRAB ones were tested by both of Modified Hodge Test (MHT) and Imipenem EDTA Combined Disc Test (CDT) to detect metallo βeta-lactams (MBL) producers. Then existence of NDM-1 gene was further identified. All of the 74 *Acinetobacter baumannii* isolates were found to be multi-drug resistant (MDR). 36 of them (48.65%) were resulted as carbapenem resistant and 27 (36.49%) were metallo βeta-lactams (MBL) producers. 12/27 (44.44%) MBL poses NDM-1 gene in the first detection procedure while the other negative 15 ones the percentage of NDM-1 was found to be 10/15 (66.67%) when using different primer. PCR products were then verified by DNA sequencing. The final consensus sequences were analyzed and submitted to NCBI GenBank data base, representing accession numbers are JF838352.1, MK682768.1 and MN251667.1. The alignments showed similarity ranged from 94%-96.4% amino acids identity. We concluded that detection of antibiotic resistant *Acinetobacter baumannii* revealed that multi-drug resistance arises dramatically due to the indiscriminate use of antibiotics and the poor applying of infection control precautions. These results emphasize the importance of implementation and comply both of antibiotic and infection control policies.

Keywords: *Acinetobacter baumannii*; Antibiotics; Mortality; Patients; Amino acids

Introduction

Nosocomial or hospital acquired infections that occur in both developed and developing countries resulting in morbidity and mortality of millions of patients annually are of important wide ranging concern in medical field [1-3]. They can affect patients in any type of settings either where they receive care or after discharge, furthermore, they include occupational infections among medical staff [4].

Acinetobacter baumannii is considered the World Health Organization ' s number one critical priority pathogen especially carbapenem producing and multi-drug resistant isolates. The reason is the limited available effective antimicrobial agents and the association of these resistant strains to poor prognosis [5,6]. *A. baumannii* can cause a wide range of infections including bacteremia, meningitis, urinary tract, blood-stream infections, surgical wound infections and ventilator associated pneumonia [7]. These infections are generally resistant to three or more groups of antibiotic families from quinolones, cephalosporins, β-lactams, aminoglycosides to carbapenems [8]. Due to its ability to accept exogenous genetic material and overexpress endogenous resistance genes, it has quickly resulted in the appearance of the MDR phenotype within multiple clonal lineages. In addition to antibiotic resistance, the ability to adhere and produce biofilm on both biotic and abiotic surfaces has been shown to be a virulence factor in many clinical isolates [9].

The main mechanisms of *Acinetobacter baumannii* for β lactam resistance are the production of carbapenemases especially Amber class D β-lactamases followed by B β-lactamases. *NDM-1,* a pattern of B β-lactamases and of high incidence is on focus of attention globally [10]. This international urgent need for NDM-1 producers' detection is due to: it has been identified in not only a single species but also in unrelated species indicating that it can spread at unpredictable rate, it is present also in both of *K. pneumoniae* and *E-coli* which are typical major nosocomial and community pathogens, and it is capable to transfer among groups as a result of over population, lack of basic sanitation, tropical climate and poor control of antibiotic use [11].

Research Methodology

This study was carried out in Microbiology and Immunology department, Faculty of Pharmacy for Girls , Al Azhar University and Medical Ain Shams Research Institute (MASRI, Faculty of Medicine, Ain-

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Shams University on seventy four isolates that were collected from hospital laboratories for inpatients admitted to Urology, Surgery, pediatric, orthopedics, GIT disorders, pediatric intensive care unit and Neonatal intensive care unit departments in addition to adult Intensive care and open heart units after 48-72 hrs. of hospitalization from two tertiary care hospitals in Cairo and one private tertiary care hospital in Giza during the period from July 2017 till June 2018.

Detection of *Acinetobacter baumannii*

All samples were inoculated onto both blood and MacConkey agar plates and incubated at 37°C for 24-48 hrs. For blood culture bottles, it was incubated aerobically at 37°C for up to 7days and the bottles were examined daily for evidence of bacterial growth such as haemolysis, gas production or turbidity above the red cell line then sub-culture was done on blood and MacConkey agar plates. The resultant colonies were identified morphologically and biochemically through Gram stain, motility and sugar fermentation test mediums. The isolates were then confirmed as *Acinetobacter baumannii* using the Vitek 2 system (GNI-system) (BioMerieux vitek system Inc., Hazelwood, MO,USA).

Antimicrobial susceptibility testing

Acinetobacter baumannii isolates were tested for antimicrobial susceptibility by standardized disk diffusion technique using Kirby-Bauer method according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI, 2013) [12]. A total number of 17 antimicrobial discs were used representing different types of antimicrobial groups which were Amikacin 30μg, Amoxicillin clavulanic acid 20 /10 μg, Ceftriaxone 30 μg, Tetracycline 30 μg, Azithromycin 15 μg, Cefepime 30 μg, Cefotaxime 30 μg, Ceftazidime 30 μg, Ciprofloxacin 5 μg, Gentamycin 10 μg, Imipenem 10 μg, Levofloxacin 5 μg, Meropenem 10 μg, Nitrofurantoin 300 μg, Tigecycline 15 μg, Colistin 10 μg and Tri meth prim + sulphamethoxazole 1.25/23.7 μg. These antimicrobial discs were chosen as they are the most commonly used in the three hospitals. Multi-drug resistant (MDR) strains are well-defined as non-susceptible to at least one agent in three or more antimicrobial categories; while extensively drug resistant (XDR) are defined as resistant to all standard antimicrobial agents except colistin and tigecycline. But for Pan drug resistant ones, they were defined as resistant to polymyxin B and/or colistin in addition to the resistance to all agents of all antimicrobial groups.

Determination of MIC by E test

MICs by E-test were performed on Mueller-Hinton agar plates at 37°C for 18-24 hrs. The intersection of the lower part of the ellipse shaped growth inhibition area with the test strip indicates the MIC value. The breakpoint values of each antibiotic were further compared with the criteria recommended by CLSI in 2015 [13] for Acinetobacter species.

Detection of Carbapenem resistant *Acinetobacter baumannii* (CRAB)

MICs by E-test were performed on Mueller-Hinton agar plates at 37°C for 18-24 hrs. The intersection of the lower part of the ellipse shaped growth inhibition area with the test strip indicates the MIC value. The breakpoint values of each antibiotic were further compared with the criteria recommended [13] for *Acinetobacter* species.

Detection of Carbapenem resistant *Acinetobacter baumannii* (CRAB)

A). Modified Hodge Test (MHT): Meropenem resistant strains were subjected to Modified Hodge Test for detection of carbapenemase. An overnight culture suspension of *E-coli* ATCC 25922 was adjusted to 0.5 McFarland standard solution, then inoculated using sterile cotton swab on surface of Muller-Hinton agar plate. After drying (10 μg) imipenem disc (Oxoid, UK) was placed at the centre of the plate and the test strain was streaked from the edge of the disc to the periphery of the plate in the four different directions. The plate was incubated over night at 37°C. The presence of a cloverleaf shaped zone of inhibition due to carbapenemase production was considered positive result.

B). Imipenem EDTA Combined Disc Test (CDT): The test isolate was inoculated onto a plate of Muller-Hinton agar. Two (10 μg) imipenem discs (Oxoid, UK) were placed on the plate and appropriate amount of 10 μl of EDTA solution was added to one of them. The inhibition zones of both were compared after 16-18 hrs. of incubation at 37°C. Increase in inhibition zone with the imipenem and EDTA disc >7mm than imipenem disc alone was considered as metallo beta-lactamase positive.

Identification of *NDM 1* gene existence

DNA was extracted from bacterial isolates using bacteria DNA preparation kit supplied from Zymo Research, USA protocol. PCR reactions were performed in a final volume 30 μl and PCR mixture composed of 15μl PCR master mix ready to use (Dream taq ™ Green PCR Master Mix (2X) 200rxn (Sigma, USA)), 1.5μl Forward primer, 1.5μl Reverse primer and2μl Nuclease free water. Reaction mixtures were mixed gently then centrifuged for 5 sec. and placed in a cold rack before adding 10 μl of DNA extract. The thermal cycler (Gene Amp PCR 9700, Applied biosystem, USA) was programmed with the following cycling profiling 10 min at 94°C; 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52°C, and 50 s at 72°C; and 5 min at 72°C for the final extension. DNA fragments were visualized after electrophoresis in 2% agarose gel. The isolates in which *NDM-1* gene was not detected were further examined using the primers of *Manchanda* et al. method [14] and the thermal cycler (Gene Amp PCR 9700, Applied biosystem, USA) was programmed with the following cycling profiling as denaturation of DNA at 94°C for 3 min, followed by 30 cycles of denaturation, annealing and extension at 94°C, 60°C and 72°C, respectively, for 30 s each. The final extension step was performed for 3 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel. PCR products were then purified using Montage 96 well purification kit protocol for verification by DNA sequencing using the 3100 Applied Biosystems® gene analyser and the resulting sequences were compared with those available on GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Results

Culture results on bacteriological media for conventional identification and biochemical reactions done revealed that the 74 collected isolates were of Acinetobacter species. Those isolates were confirmed genotypically using the Vitek 2 system (GNI system) (BioMerieuxvitek system Inc., Hazelwood, MO, USA) that was carried out by the aid of Ain-shams specialized hospital laboratories.

74 *Acinetobacter baumannii* clinical isolates (43 from males and 31from females) were collected in our study with high frequent distri-

bution among ICU wards (57/74) followed by surgical wards (10/74) and finally non-surgical ones (7/74). *A. baumannii* were more abundant among blood specimens (n=25), urine (n=15), sputum (n=12), wound (n=9), Endotracheal tube swab (n=6), Aspirate (n=4), umbilical catheter swab, wound drain swab and central venous line swab (n=1) respectively. Regarding age, it was observed that the majority of *A. baumannii* infected patients were of the working age as 35 (47.30%) lied in the range of 25-60 years old, 20 (27.03%) were above sixty years old and 11 (14.86%) were less than one month while only 8 (10.81%) lied in the range of 1month-24 years. Moreover distribution of age groups of *A. baumannii* infected patients among various types of wards of the hospitals show a highly significant differences as shown in following Table 1.

Antimicrobial susceptibility of *A. baumannii* isolates

The results of antimicrobial susceptibility of *A. baumannii* clinical isolates revealed the dramatic rise in antibiotic resistance as all of the 74 isolates were found to be multi-drug resistant (MDR). Results are demonstrated in Figures 1-4. Our results showed that there was a striking resistance which in some wards reaches the complete resistance phase towards the empirical antibiotics that are used according to both national and international antibiotic policies guidelines. These antibiotics were mainly augmentin, ceftriaxone and cefepime. For ICU wards, all of augmentin, cefepime, cefotaxime and ceftriaxone were the most resistant antibiotics 56/57 (98.25%) that appeared to be near complete resistance followed by ceftazidime 53/57(92.98%) and trimethoprim + sulphamethoxazole 52/57 (91.23%), then gentamycin 45/57 (78.95%) and azithromycin44/57 (77.19%). In case of surgical wards, augmentin, cefotaxime, ceftazidime and ceftriaxone were completely resistant followed by both of cefepime and trimethoprim + sulphamethoxazole that their resistances were the same 9/10 (90%) then gentamycin 7/10 (70%) and all of Meropenem, ciprofloxacin and tetracycline were of the same resistance which was 6/10 (60%) and finally levofloxacin 5/10 (50%). But for non-surgical wards, complete resistance to all of augmentin, cefepime, cefotaxime and trimethoprim + sulphamethoxazole were resulted. Very high resistance to all of azithromycin, ceftazidime and ceftriaxone were also found as were 6/7 (85.71%) and moderate resistance to all of gentamycin, levofloxacin and tetracycline were also resulted 4/7 (57.14%).

Detection of MIC

A. baumannii isolates were tested for Minimum Inhibitory concentrations (MICs) using E-Test. High resistance was found towards cephalosporin's antibiotic group .Regarding tigecycline and colistin, their moderate sensitivity were found to be higher than that resulted by susceptibility testing using Kirby-Baur method. This is shown in Table 2.

Detection of metallo-beta lactamase production

The seventy four phenotypic carbapeneme resistant *A. baumannii* isolates results for metallo-Beta lactamase production were 36 (48.65%) and 27 (36.49%) by applying Modified Hodge Test (MHT) and Imipenem EDTA Combined Disc Test (CDT) for them respectively. Upon revising the data and the sensitivity pattern of the thirty-six positive MHT isolates, we found that nine isolates were only meropenem resistant and the rest of isolates (twenty seven ones which are the positive isolates for EDTA (CDT)) were of both resistance to meropenem and imipenem.

Detection of NDM-1 gene existence

The Carbapenem resistant 27 *A. baumannii* Producing metallo beta-lactam isolates results for the detection of New Delhi Metallo beta lactamase-1 gene *(NDM-1)* existence after first PCR round according to protocol showed 12 (44.44%) positive cases. Positive PCR results showed a band at 621bp which refers to *NDM-1* gene. The other negative 15 isolates underwent a second PCR round according to Martín-Aspas et al. [14] protocol using different primer sequences. The percentage of *NDM-1* positive cases were 10/15 (66.67%) with a band at 475bp which indicates the *NDM-1* gene existence. Five isolates were still untypeable after using both protocols.

In this study we find high significant correlation between the sensitivity pattern of tigecycline and amikacin of the carbapenem resistant *Acinetobacter baumannii* isolates and NDM-1 gene existence. P value results were 0.014 and 0.046 for the two antibiotics respectively.

Randomly chosen positive PCR products of both protocols were verified by DNA sequencing. The final consensus sequences were analysed and submitted to NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST) data base under the accession numbers (JF838352.1), (MK682768.1) and (MN251667.1). The alignments showed similarity ranged from 94% to 96.4% amino acids identity.

Discussion

Multi-drug resistant *Acinetobacter baumannii* is an important noso-

Table 1. Frequency of *A. baumannii* infected patients among age groups within wards of hospitals.

Ward 1	Age Groups										Chi-Square	
	< 1 Month		1 Month -24 Years		25 Years -60 Years		>60 Years		Total			
	N	%	N	%	N	%	N	%	N	%	X^2	P-value
Urology	0	0.00	0	0.00	0	0.00	1	5.00	1	1.35	141.854	<0.001*
Surgery	0	0.00	0	0.00	5	14.29	1	5.00	6	8.11		
Adult ICU	0	0.00	1	12.50	24	68.57	14	70.00	39	52.70		
Paediatric ICU(PICU)	0	0.00	7	87.50	0	0.00	0	0.00	7	9.46		
Neonatal ICU (NICU)	10	90.91	0	0.00	0	0.00	0	0.00	10	13.51		
Gastrointestinal tract disorder (GIT)	0	0.00	0	0.00	4	11.43	2	10.00	6	8.11		
Orthopaedic	0	0.00	0	0.00	1	2.86	1	5.00	2	2.70		
Paediatric	1	9.09	0	0.00	0	0.00	0	0.00	1	1.35		
Unit ward	0	0.00	0	0.00	1	2.86	1	5.00	2	2.70		
Total	11	100.00	8	100.00	35	100.00	20	100.00	74	100.00		

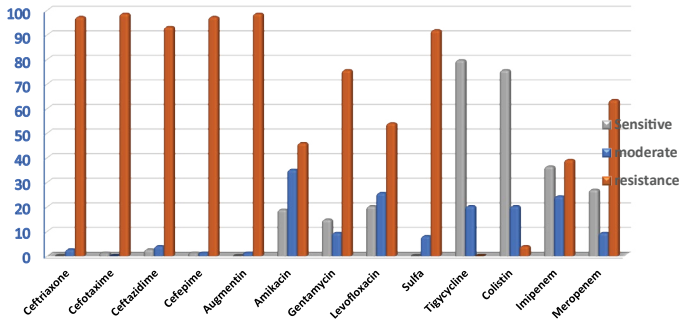

Figure 1. Frequency of antibiotic sensitivity pattern of collected *A. baumannii* Isolates.

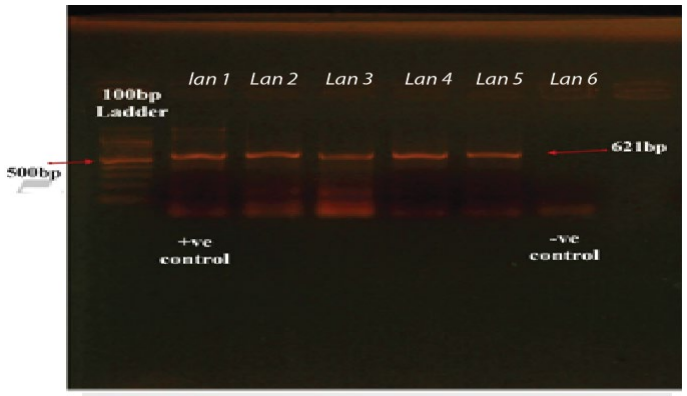

Figure 2. Shows the results of PCR amplification of *NDM-1* gene**.** The first lane (from the left) shows the DNA100 bp ladder. Lane 1 represents the positive control; lane 6 is of the negative control. Lanes 2, 3, 4 and 5 showed band at 621 bp which refers to *NDM-1* gene.

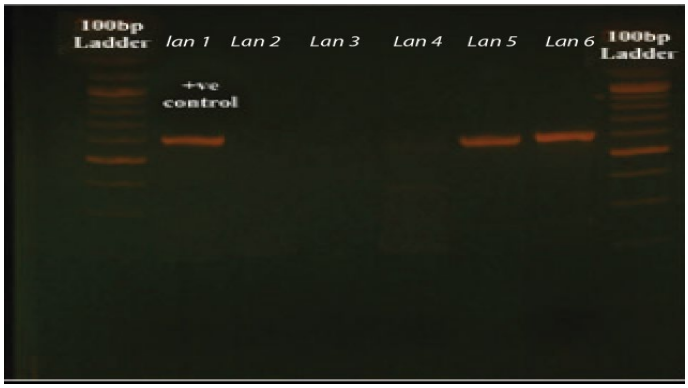

Figure 3. Shows the results of PCR amplification of *NDM-1* gene**.** The first and last lanes show the DNA100 bp ladder. Lane 1 represents the positive control. Lanes 2, 3 and 4 show the untypeable strains. Lanes 5 and 6 showed band at 621 bp which refers to *NDM-1* gene.

comial pathogen associated with morbidity and mortality [15]**.** Two characteristics have been implicated in the importance of *A. baumannii* as a human pathogen: first, its ability to survive on animate and inanimate surfaces for long periods with the risk of prolonged endemic infections and second, its resistance to multiple antibiotics-including carbapenems- which complicates treatment [16]**.** The most frequent mechanisms of resistance are carbapenem-hydrolysing class D β-lactamases (CHDL) production, followed by class B metallo-β-lactamases (MBL) [17]**.** New Delhi metallo-β-lactamase-1(NDM-1) generated much global alarm and gained media notoriety being labelled as superbugs which had the reputation of being impossible to treat [18]**.**

The isolates were obtained from seventy -four patients comprising 43 male (58.10%) and 31female (41.90%). Similar to our results,

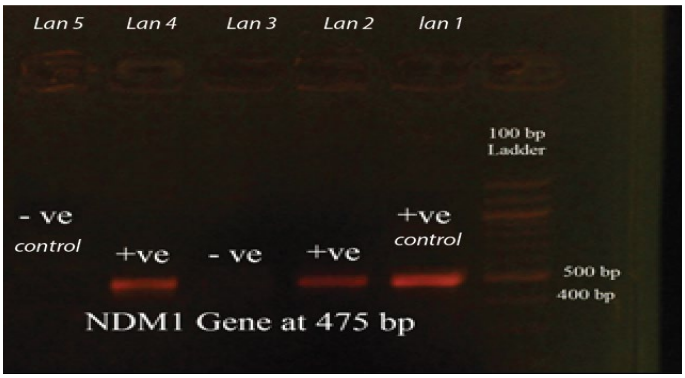

Figure 4. Shows PCR amplification of *NDM1* gene. The first lane from the right shows DNA ladder. Positive control was in lane 1 and negative control was in lane 5. Lanes 2 and 4 showed band at 475 bp which refers to *NDM1* gene while lane 3 represents one of the untypeable strains.

previous studies showed that *Acinetobacter baumannii* was more disseminated among males rather than females. Results of present study reported higher percentages of *A.baumannii* infections in ICUs wards (57/74) then surgical wards (10/74) followed by non-surgical (7/74). Alike high percentage rates in ICUs were reported in previous published data [19]. The majority of patients 35/74 (47.30%) were between 25-60 years old which indicates that infection is common in working age groups of patients. Similar age groups of patients were reported in other studies. *A.baumannii* positive cases were observed in clinical samples from patients more than 60 years old.

Our research demonstrates that highly resistant isolates were to augmentin 73 (98.65%), followed by cefotaxime 73 (98.65%), ceftriaxone 72 (97.30%), cefepime 72 (97.30%), ceftazidime 69(93.24%) and trimethoprim + sulphamethoxazole 65 (91.89%). Highly moderate resistance were correlated to azithromycin 57 (77.03%), gentamycin 56 (75.68%), meropenem 47 (63.51%), ciprofloxacin 45 (60.81%) and levofloxacin 40 (54.05). low moderate resistance was found in isolates of both amikacin 34 (45.95%) and imipenem 29 (39.19%). On the other hand high sensitivity was found to both tigecycline and colistin as it was 59 (79.73%) and 56 (75.68%) respectively. No isolates were resistant to tigecycline but only three were resistant to colistin. Similar finding were (94.91%) of isolates were resistant to augmentin, (94.35%) to ceftriaxone, (89.26%) to cefepime, (88.70%) to ceftazidime, while cefotaxime resistance was (83.61%), gentamicin resistance was (78.37%) and meropenem was (66.10%). Regarding amikacin and imipenem results; the resistance was found (76.83%) and (60.45%) respectively. Which showed high levels of resistance toward ceftazidime (90%), cefepime (81%), gentamicin (74%) and ciprofloxacin (67%) and not similar for imipenem (89%), meropenem (87%) and trimethoprim + sulphamethoxazole (69%). Also, Heydari, Farzad et al. [20] published data which were found to be similar to our study in case of ceftazidime (94.44%), cefepime (92.22%) and trimethoprim + sulphamethoxazole (91.11%) and different for ciprofloxacin whose resistant was (95.55%) and colistin that all isolates were sensitive.

E-test results confirmed the higher resistance in antibiotic sensitivity pattern. These resulted MICs results coincides with studies conducted [21,22].

Upon using Modified Hodge Test (MHT) to examine the carbapenem resistant isolates for Beta Lactamase production, 36 isolates out of 74 were found to be positive, comprising 48.65% of all isolates. This finding complies with [23] (55%), but doesn't match with results pub-

Table 2. The interpretation of MICs results by E-test method.

Antibiotic strip	Sensitive		Moderate		Resistant	
	N	%	N	%	N	%
TIG	55	74.32	19	25.68	0	0.00
COL	52	70.27	17	22.97	5	6.75
IPM	27	48.64	16	21.62	31	41.89
MEM	20	27.03	8	10.81	46	62.16
AK	15	20.27	25	33.78	34	45.94
AMC	0	0.00	1	1.35	73	98.65
FEP	0	1.35	2	2.70	72	97.30
CTX	1	1.35	1	1.35	72	97.30
CAZ	2	2.70	3	4.05	69	93.24
CRO	0	0.00	2	2.70	72	97.30
CN	11	14.86	9	12.16	54	72.97
LEV	15	20.27	19	25.68	40	54.05
SXT	0	0.00	6	8.11	68	91.89

*Amikacin (AK), Amoxicillin-clavulanic acid (AMC),Ceftriaxone (CRO), Cefepime (FEP), Cefotaxime (CTX). Ceftazidime (CAZ), Gentamycin(CN), Imipenem (IPM), Levofloxacin (LEV), Meropenem (MEM), Tigecycline (TIG), Colistin (COL) and Tri-methoprim + sulphamethoxazole(SXT).

lished by Yong et al. [24], which introduced 83.3% resistance. While upon using Imipenem EDTA Combined Disc Test (CDT) for detection of beta-lactamase production, only 27 isolates (36.49%) were positive. Other researchers [25] used both methods for detection of beta-lactamase production in their studies which matches our results.

In this study, two different primer sequences were used for the detection of New Delhi Metallo beta lactamase-1 gene *(NDM-1)* existence in the carbapenem resistant *A. baumannii* producing metallo beta- lactam isolates. The first PCR round was performed acc.to protocol. This protocol was also conducted in the studies applied. The negative isolates went through a second PCR round acc.to protocol [11-26] also use the same primer sequence for *NDM-1* identification.

Conclusion

The research also demonstrates that all positive *NDM-1* carbapenem resistant *A. baumannii* were positive for the Modified Hodge Test (MHT). These results were similar to previously published work data.

In our study, DNA sequencing was applied to randomly chosen positive PCR products of both protocols. Other researchers verify the existence of *NDM-1* in their studies also by DNA sequencing for the positive PCR products. The above data confirm the growing rate of carbapenem resistant *A. baumannii* which continues to be a dangerous nosocomial pathogen because of its high resistance to several antibiotic classes and ability to spread within hospitals environment.

Antibiotic policy should be formulated and updated according to the antibiogram results obtained in each hospital. Sensitive antibiotics as tigecycline and colistin should be used judiciously and within the antibiotic policy implemented. Primary caretakers should comply with the implemented antibiotic and infection control policies. This is not only done for the sake of protecting patients, but also for the preventing of the tremendous costs that arises intensely upon infection spread.

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