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Detection of carbapenem resistant bacteria (CRB) in Egypt

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ABSTRACT: The emergence of resistant bacteria has become a worldwide threat. Multidrug resistant bacteria are globally spread. Several studies were performed to detect new resistant organisms and also the genes which are responsible for their resistance. Carbapenem resistance is considered the most dangerous resistance. In this study, we detect the presence of carbapenem resistant bacteria (CRB) in Egypt. This may cause un-treatable epidemic if its organization is neglected. This study distinguished the pathogens that are carbapenemase producing due to the presence of bla-NDM gene. The results detected the presence of CRB stains such as *Klebsiella* sp., *Pseudomonas* sp., *Citrobacter* sp., *Enterobacter* sp., *Acinetobacter* sp. and *E. coli*. As a result from this study, it is now proved that there are CRB in Egypt, thus it must be given a great consideration and must be managed.

Keywords: Carbapenem resistance; Multidrug resistant bacteria; NDM (New Delhi Metallo-β-lactamase); Nosocomial infection; Metallo-β-lactamases.

1. INTRODUCTION

Antibiotics have a pivotal role in the treatment of several diseases of humans and animals in the past few years. As thousands or millions of lives had been saved by using antibiotics through the world. However, with antibiotic abuse pathogens become more resistant and changed into weapons against mankind [1, 2]. The infection of multidrug-resistant (MDR) pathogens becomes a threat to the public health, thus there is seriously a risk to the health of animal and humans. β-lactamase is considered as the most common drug-resistance mechanism [3]. It has the ability to hydrolyze the C–N bond of lactam ring and inactivate the antibiotics [4].

β-lactamases have two main subcategories; (a) serine-b-lactamases (SBLs) and (b) metallo-β-lactamases (MBLs) [5]. MBLs are more dangerous to humans and pose an increasing health risk [6]. Pathogens with MBLs have the ability to degrade penicillin, cephalosporin, carbapenems, and aztreonam [7, 8].

While Enterobacteriaceae are normally found in the intestines, its extended-spectrum β-lactamases (ESBLs) represent a challenge to the international medical community. These species include *Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*) and *Enterobacter cloacae* (*E. cloacae*) [9]. On the other hand, carbapenems are therefore are considered the last treatment option for serious infections as it can

resist many β-lactamase enzymes caused by ESBLs-producing Enterobacteriaceae [10]. The overuse of these antibiotics may lead to the development of carbapenem resistance and may contribute to the emergence of carbapenem-resistant *Enterobacteriaceae*. Carbapenem-resistant Enterobacteriaceae (CRE) can inactivate carbapenem by the production of carbapenemase enzymes. NDM (New Delhi Metallo-β-lactamase) is considered as one of the most clinically important carbapenmases. It is commonly found in *K. pneumoniae* isolates that have been associated with serious nosocomial infections. It is reported that *K. pneumoniae* contain NDM found in different countries. For instance NDM-1 was firstly identified in India. Moreover, it is reported in Asia, Australia, Europe, North America and Turkey [11]. Additionally, it had been previously reported in the Middle East and Europe [12].

Several molecular investigations had been utilized to characterize NDM-positive bacteria and the characterization of the plasmids containing blaNDM genes. The blaNDM has been found both in a wide range of species and genera of Gram-negative bacteria such as *K. pneumoniae, E. coli* and *Acinetobacter baumannii* (*A. baumannii*) [13-15]. Several studies reported that bacteria may contain plasmids of different sizes (30-50 kb) encoding NDM gene that is located on the chromosome, and although transferable to other types in vitro [16-24]. Furthermore, the usage of anti-cancer drugs may promote the spread of NDM-positive isolates [25].

It is reported that NDM-positive isolates are isolated from 40 countries covering all continents except South America and Antarctica. This global transmission of NDM involves both strain spread and gene spread. The dominant mechanism of dissemination is the gene spread. The epidemiology of NDM may possibly increase as the blaNDM-1 gene has the ability to transmit other bacterial strains with known epidemic or pandemic potential such as *E. coli* [26-30]. Moreover, it is remarkably noted that KPC (*Klebsiella pneumoniae* carbapenemase) has spread globally because of its dissemination [31].

It may be clear that the virulence of NDM-positive isolates may lead to further spread and expand its infection and colonization to encompass animals. It is really reported in a study in the USA, which documented isolation of NDM-positive *E. coli* from companion animals [32].

The aim of this study was initially to identify the presence and epidemic threat of CRB in Egypt. Ultimately, we aim to identify the problem of carbapenem resistance. Moreover, some bioinformatic analyses to evaluate the evolution and transformation of the resistance gene.

2. MATERIALS AND METHODS

2.1. Materials

In order to detect the presence of CRB in Egypt, 50 samples were clinically isolated from hospitals of Alexandria governorate, Egypt. These samples were isolated and inoculated on nutrient broth medium, blood and MacConkey agars (Oxoid, England). Antibiogram was performed by using antibiotic discs (Oxoid, England). The CRB isolates were biochemically identified. Moreover the protein contents of these isolates were extracted and purified, the partially purified proteins were analyzed by Gel Electrophoresis. Chemicals of electrophoresis are provided by (sigma chemicals, Egypt). Some phylogenetic and Bioinformatics analysis was performed with the help of NCBI (The National Center for Biotechnology Information) [33] and EBI (The European Bioinformatics Institute) [34].

2.2. Methods

2.2.1. Sample collection

Several samples were isolated from patients of Alexandria governorate hospitals, private hospitals and also private labs. These samples were from different infection sites and fluids such as urine, CVC (Central

Venous Catheter), sputum, wound and nasopharyngeal swabs. The isolates were identified by MALDI- TOF [35]. Bacterial isolates were isolated and inoculated as mentioned by El-Malek et al. [36], briefly, sterile swabs were used to transfer samples to broth tubes directly and these tubes were incubated for 24 hours at 37^{o}C. then these tubes were re-inoculated onto blood and MacConkey agars for another 24 hours at 37^{o}C.

2.2.2. Antibiogram analysis of the isolated samples

After incubation for 37^{o}C, 22 bacterial isolates were obtained. These isolates were tested for their antibiotic susceptibility using several antibiotic discs known by (CLSI, 2017) [37]. This assay is performed on Mueller-Hinton agar with 0.5 MacFarland concentration of bacteria incubated at 37^{o}C overnight. The total antibiotic susceptibility data are provided in supplementary sheets.

2.2.3. Selection and identification of carbapenem resistant bacterial isolates

The carbapenem resistant isolates that have the ability to resist (Imipenem, Meropenem and Ertapenem) are chosen. The biochemical identification is the utilized method for their identification.

2.2.4. Protein precipitation and purification

The protein precipitation was done as previously mentioned by Kelly et al. [38]. The harvesting of cells was performed in mid-exponential phase by centrifugation for 5 min at 10.000 rpm at 4^{o}C. The pellets were re-suspended in 1 ml (phenol/guanidine isothiocyanate; Invitrogen) per 100 mg cells. Thus protein samples were extracted. Moreover, the partially purified proteins were used for the next step of gel electrophoresis.

2.2.5. Phylogenetic and bioinformatics

Several analyses were performed on data provided from NCBI and EBI for bacteria similar to those bacteria under investigation in our study.

3. RESULTS AND DISCUSSION

3.1. Antibiogram analysis of the isolated bacteria

The antibiotic sensitivity analysis that was performed on the 22 isolate results in 9 isolates are carbapenem resistant bacteria. These bacteria represent 41% of the total number of isolates under investigations (Fig. 1).

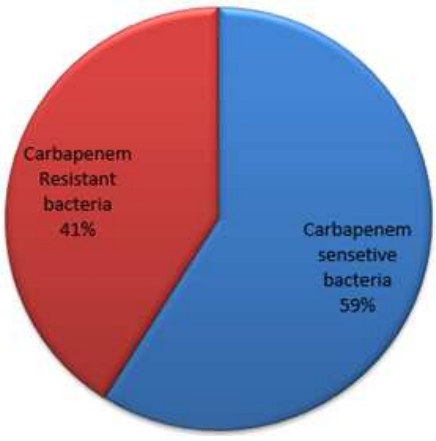

Figure 1. Presence of CRB and CSB.

The assay of antibiogram was done using 30 antibiotics. The overall measurement results are summarized in supplementary data. The results illustrate that 9 isolates are resistant to the entire carbapenem group. These results are presented in Table 1. The ability of these bacteria to resist carbapenems agrees with this of previous studies of Nordmann et al. and Miriagou et al. [39, 40]. From the results obtained, it is clear that strains No. 1, No. 3, No. 6, No. 10, No. 12, No. 16, No. 18, No. 19 and No. 22 are carbapenem resistant strains.

3.2. Biochemical identification of the selected CRB

The identification of the CRB is performed through biochemical methods [41]. The results are summarized in Table 2. From the table, it is observed that the bacterial isolates No. 1 is *Pseudomonas sp.*, No. 3 and No.22 are *Acinetobacter sp*, No. 6 is *Citrobacter sp.*, No. 10 is *E. coli*, No. 12 and No. 18 are *Klebsiella sp.*, while No. 16 and No. 19 are *Enterobacter sp.*

3.3. Total protein analysis of the selected CRB

The bacterial proteins that were extracted and purified from the selected isolates were analyzed by gel electrophoresis Fig. 2. It is remarkably observed that all the nine isolates contain a clear protein band on the molecular weight of 30KDa and this is what was previously observed by Wang et al. [42] besides, Bogaerts et al. that estimate that NDM gene plasmid has different sizes (30–50 kb) [16], who detected the peak of 29 KDa for bla NDM-1 (New Delhi Metallo-β-lactamase). This means that the carbapenem resistance exhibited by these isolates may be due to the presence of this band.

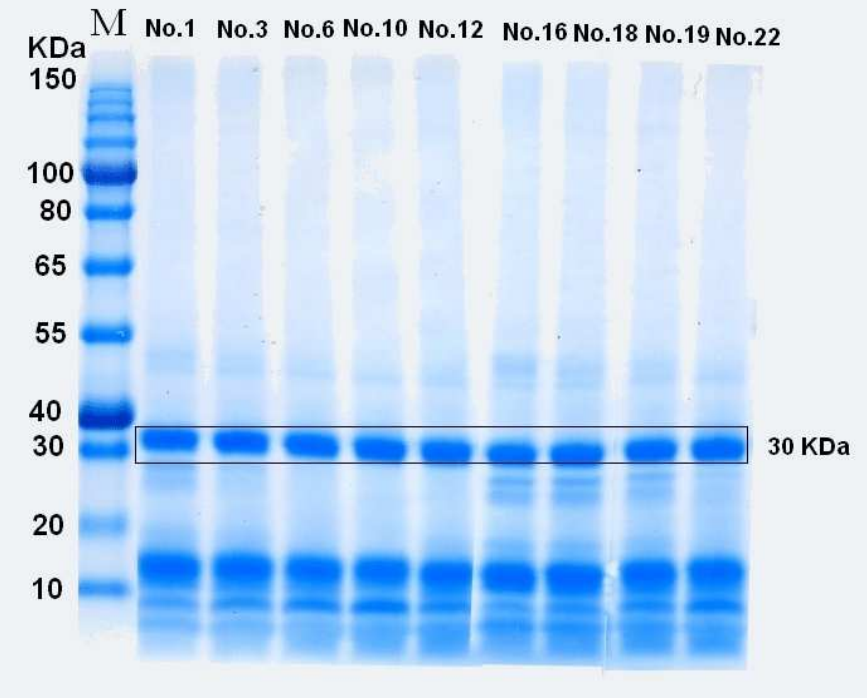

Figure 2. Gel electrophoresis sheet for the selected isolates.

Table 1. Antibiotic sensitivity assay for the 9 carbapenem resistant bacteria.

Isolate	No.1	No.3	No.6	No.10	No.12	No.16	No.18	No.19	No.22
Source	Sputum	CVC	Axillary Swab	Urine	E.T.T	Groin swab	Axillary swab	Sputum	CVC
Antibiotics									
Amikacin	0.0	0.0	12.1	0.0	0.0	13.1	9.3	12.1	10.2
Amoxacillin-clavulanic	0.0	0.0	14.3	0.0	0.0	0.0	0.0	0.0	0.0
Ampicillin – sulbactam	0.0	0.0	0.0	0.0	0.0	6.3	0.0	11.5	0.0
Aztreonam	0.0	0.0	0.0	8.2	10.2	0.0	0.0	8.2	0.0
Cefadroxil	0.0	0.0	0.0	10.2	11.3	0.0	0.0	0.0	0.0
Cefepime	0.0	11.1	0.0	0.0	0.0	0.0	11.3	0.0	0.0
Cefoprazone	0.0	0.0	0.0	0.0	0.0	0.0	12.1	0.0	0.0
Cefoprazone- Sulbactam	0.0	0.0	0.0	8.1	0.0	0.0	0.0	0.0	0.0
Cefotaxime	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.1
Cefoxitin	0.0	0.0	0.0	0.0	8.1	0.0	8.1	9.0	0.0
Ceftazidime	0.0	0.0	14.1	0.0	0.0	0.0	0.0	0.0	0.0
Ceftriaxone	0.0	0.0	0.0	0.0	0.0	6.8	0.0	0.0	0.0
Cefuroxime sodium	0.0	0.0	13.2	9.1	0.0	0.0	0.0	0.0	0.0
Ciprofloxacin	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
Colistin	16.2	13.2	0.0	0.0	0.0	13.5	12.3	14.2	0.0
Doxycyclin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ertapenem	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gentamicin	0.0	0.0	10.2	11.2	10.0	0.0	10.3	0.0	0.0
Imipenem	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Levofloxacin	0.0	0.0	0.0	10.1	0.0	9.1	0.0	0.0	7.2
Line Zolid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Meropenem	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Minocyclin	0.0	0.0	11.0	0.0	0.0	0.0	0.0	0.0	0.0
Ofloxacin	0.0	10.6	12.0	0.0	11.2	0.0	0.0	0.0	0.0
Rifampicin	0.0	0.0	0.0	0.0	13.4	11.2	0.0	10.2	0.0
Tazobactam-Piperacillin	0.0	0.0	11.0	0.0	0.0	10.1	7.4	0.0	7.1
Teicoplanin	0.0	0.0	0.0	8.2	0.0	0.0	12.1	11.2	0.0
Tigecycline	11.4	0.0	12.3	0.0	8.1	0.0	0.0	10.1	0.0
Tobramycin	0.0	0.0	9.2	0.0	0.0	0.0	0.0	0.0	0.0
Vancomycin	8.2	0.0	0.0	10.2	0.0	10.3	0.0	10.2	11.2

Table 2. The biochemical identification of the isolates.

Test	**Bacterial Isolates**								
	No.1	**No.3**	**No.6**	**No.10**	**No.12**	**No.16**	**No.18**	**No.19**	**No.22**
Morphological and physiological tests									
Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Gram reaction	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Motility	–	–	+	+	–	+	–	–	–
Biochemical tests									
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	+	–	–	–	–	–	–	–	–
Indole test	–	–	–	+	–	–	–	–	–
Methyl red test	–	–	+	+	–	–	–	–	–
Voges - prtoskaurer test	–	–	–	–	+	+	+	+	–
ONPG	–	–	+	+	+	+	+	+	–
Citrate Utilization	+	–	+	–	+	+	+	+	–
Nitrate reduction	+	–	+	+	+	+	+	+	–
H2S production	–	–	+	–	–	–	–	–	–
Urease production	–	–	+	–	–	–	–	–	–
Gelatin Hydrolysis	–	–	–	–	–	–	–	–	–
Organism	*Pseudomonas sp.*	*Acinetobacter sp.*	*Citrobacter sp.*	*E. coli*	*Klebsiella sp.*	*Enterobacter sp.*	*Klebsiella sp.*	*Enterobacter sp.*	*Acinetobacter sp.*

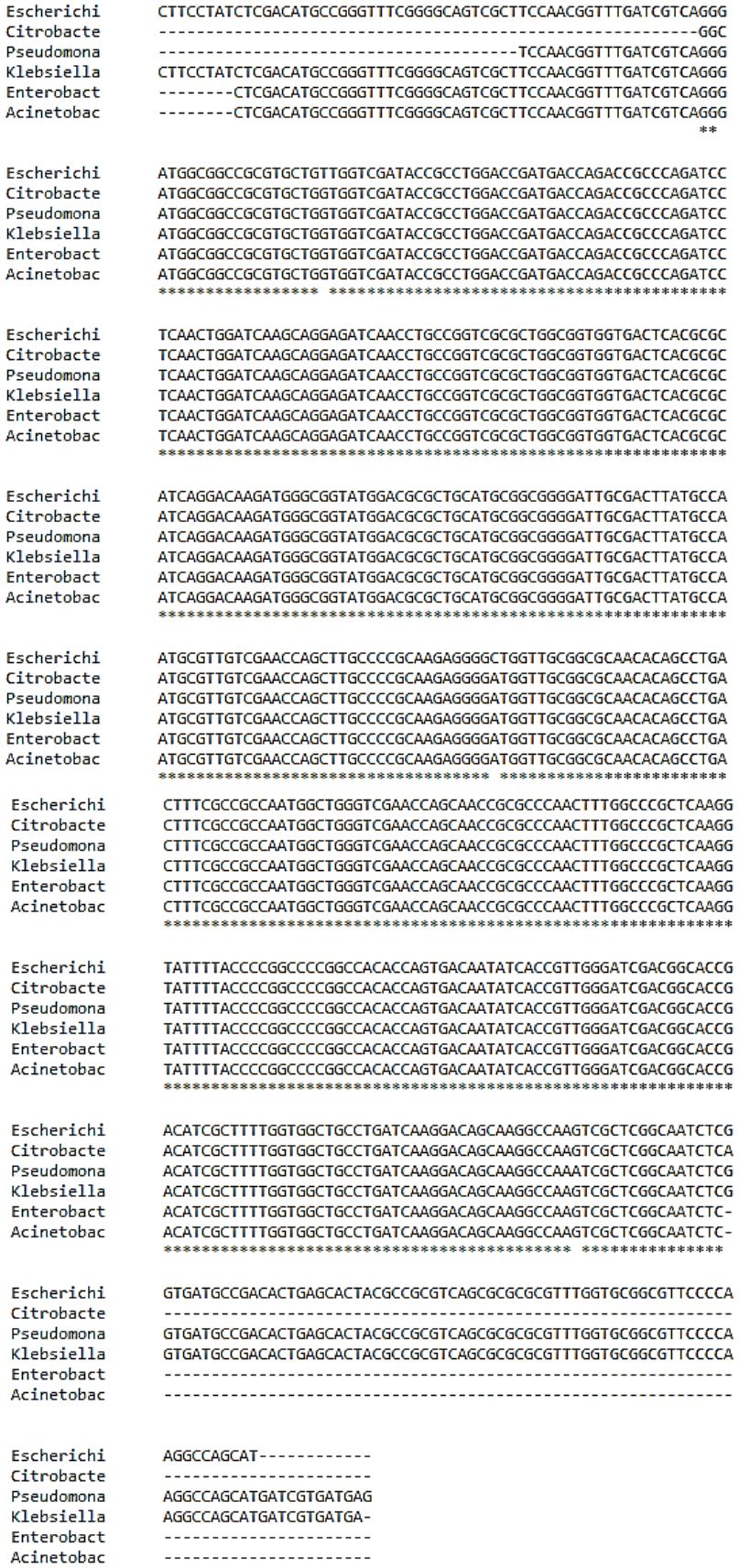

Figure 3. Multiple sequence alignment of blaNDM-1 in different bacterial isolates.

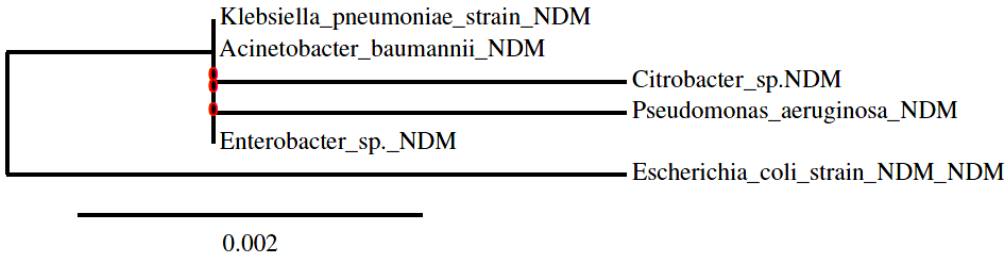

Figure 4. Phylogenetic tree of local *bla*-NDM positive bacteria by Phylogeny.fr [44] that used the maximum likelihood method to generate phylogenetic tree.

3.4. Phylogenetic and bioinformatics for similar NDM bacteria

The bioinformatics and phylogenetic analysis aims to illustrate the similarities between the isolates. These similarities are exhibited through the activity and also in gel electrophoresis, which may be due to the presence of the same gene for carbapenem resistance. This gene is widely known as blaNDM-1. The analysis shows that *Klebsiella sp.* (Accession: JX477134), *Pseudomonas sp.* (Accession: MF356396), *Citrobacter sp.* (Accession: KF284092), *Enterobacter sp.* (Accession: JN794562) *Acinetobacter sp.* (Accession: JN794560) and *E. coli* (Accession: KX495152) are very close. They all contain the blaNDM-1 gene [43] See Fig. 3.

Phylogenetic analysis for nucleotide sequence of NDM present in different isolates is illustrated by cladogram in Fig. 4. The tree generated by Genomic-NDM sequence alignment values also exhibits very high resolution. All the six strains are clustered into two groups. The five of isolates *Klebsiella sp., Citrobacter sp., Pseudomonas sp., Enterobacter sp.* and *Acinetobacter sp.* are clustered in one group as they are relatively close and away from *E. coli.* This group is analyzed as *Klebsiella sp., Enterobacter sp.* and *Acinetobacter sp.* are a closed sub- group of NDM- positive bacteria while *Citrobacter sp.* and *Pseudomonas sp.* represent another sub- group. On the other hand *E. coli* represents a different group of NDM-positive bacteria.

4. CONCLUSION

It is now evident that the spread of NDM provides just one example of how antibiotic resistance can rapidly disseminate internationally. The paucity of a new antibiotic development makes the antibiotic resistance a dangerous threat to public health. There are many calls from the World Health Organization (WHO) and the European Centre for Disease Prevention and Control (ECDC) for solving this problem. Overcoming these difficulties poses a major challenge, and international cooperation will be critical in controlling this global threat.

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Conflict of Interest: The author declares no conflict of interest.

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