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Phenotypic Identification and Molecular Detection of bla (NDM-1) Gene in Multidrug Resistant Gram Negative Bacilli in Sohag University Hospital

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Abstract

Background: Carbapenemase-producing Enterobacteriaceae (CPE) represents a worldwide public health problem. One of the most important emerging resistance traits corresponds to the production of the carbapenem-hydrolysing β-lactamases, which confer resistance to almost all β-lactams

Objectives: to determine the occurance of bla (NDM-1) gene among clinical isolates of multidrug resistant gram negative bacilli in Sohag University Hospital.

Methodology: This study was prospectively conducted over a period of 24 months between Februray 2017 and January 2019, at Sohag university hospital. This study included 150 isolates obtained from various clinical specimens from patients admitted to different department in Sohag University Hospital with age ranged from 1 to 81 years old.

Results: This study included 150 patients were infected by multi drug resistant gram negative bacilli, 100 of patients were resistant to carbapenem group (66.6%) and 50 patients were sensitive to carbapenem group (33.3%). There was no significant difference between the two groups regarding age (p value=0.782). Similarly, there was no significant difference between the two groups regarding sex distribution (p value=0.636). Most of cases was surgical site infection (34%) followed by respiratory tract infection (33.6) with no significant difference between groups regarding diagnosis (p value=0.25). In most of cases type of organism is *klebsiella pneumonia* (33 cases) 26 of them was positive NDM-1 gene followed by *Escherichia coli* (24 cases) 14 of them was positive NDM-1 gene. There is significant difference between groups regarding organism (p value=0.03).

Conclusion: Carbapenem resistant enterobacteriacae are spreading in our locality and Egypt.

Keywords: Phenotyping, Bla (NDM-1) Gene, Gram Negative Bacilli

Introduction

Carbapenemase-producing Enterobacteriaceae (CPE) represents a worldwide public health problem. Carbapenemase-producing Enterobacteriaceae (CPE) isolates have been increasingly identified worldwide. Multidrug resistance is now emerging increasingly in Enterobacteriaceae among nosocomial and community-acquired infections [1]. One of the most important emerging resistance traits corresponds to the production of the carbapenem-hydrolysing β-lactamases, which confer resistance to almost all β-lactams. Carbapenems are a class of lactam antibiotics with a broad spectrum of antibiotic activity and have a chemical structure rendering them resistant to most of the lactam antibiotics. [2]. Carbapenems are used as the last resort for treatment of infections caused by Multi Drug Resistant (MDR) Gram Negative Bacteria (GNBs). [3].

Resistance to carbapenems mostly develops due to production of carbapenemases which hydrolyze carbapenems as well as other lactams. Some of these carbapenemases include Imipenemase,

Oxacillinase-48, Veron Integron Metallo lactamase, Klebsiella pneumoniae carbapenemases and New Delhi Metallo (NDM) lactamase which are encoded by blaIMP, blaOXA-48, blaVIM, blaKPC and blaNDM-1 genes [4]. NDM-1 lactamase is the latest threat to medical fraternity. It is the novel metallo lactamase which hydrolyzes all the lactam antibiotics except Aztreonam. It was first reported in 2009 in *Klebsiella pneumoniae* and *E. coli* recovered from a Swedish patient of Indian origin who was previously admitted to a hospital in New Delhi, India [5].

Thereafter it was reported from India as well as other parts of the world [6, 7]. *K. pneumoniae* and *E. coli* are the most common NDM-1 producers. NDM-1 producing *K. pneumoniae* have emerged as important pathogen causing wide range of infections both in community as well as hospital settings [8].

Patients and methods

Patients: This study was prospectively conducted over a period of 24 months between February 2017 and January 2019, at Sohag University Hospital. This study included 150 isolates obtained from various clinical specimens (blood, urine, sputum, pus and pleural aspirate) from patients admitted to different department in Sohag University Hospital with age ranged from 1 to 81 years old.

Methods:

Patient data collection

Fresh samples were collected soon after collection of data from patients (150 specimens). The following data from all patients were recorded at admission including; age, gender, word, diagnosis, duration of hospitalization and antibiotic intake**.**

Clinical specimens and clinical laboratory work:

Types of clinical samples specimens (blood, urine, sputum, pus and pleural aspirate).

All samples were taken under complete aseptic condition in sterile container under aseptic precautions according to standard protocol for each sample. These samples were transported to Clinical Pathology Department, Microbiology Unit within 2 hrs. of collection.

Laboratory investigations :

A. Isolation of Gram negative bacilli

All samples were cultured on MacConcky agar, at 37 °C and incubated for 24 – 48 hrs.

The following media were prepared according to the manufacturer instructions or according to as described below. The pH was adjusted to 7.4 and all media were sterilized by autoclaving at 121°C for 20 min. unless otherwise was mentioned [9].

B. Identification of Gram negative bacilli :

1. **Macroscopic appearance of colonies**:
   On MacConky agar pink colonies suspect lactose fermenters and pale colonies suspect non lactose fermanters.
2. **Microscopic examination of Gram stained colonies:**

By Gram stain isolates appear as pink rods.

VITEC 2 Compact identification kits is used to confirm the identification of the isolates and for antibiotic susceptibility test.

C. Determination of antibiotic susceptibility pattern of isolated organisms:

1. **Antibiotic susceptibility test by disc diffusion method:**

The susceptibility of the bacterial isolates to carbapenems agents was tested by standard Kirby-Bauer disc diffusion method on Mueller- Hinton agar (Oxoid)

2. **Antibiotic susceptibility test by VITEK® 2 compact system:**

(VITEK 2 system platform next generation, bioMerieux USA)

The VITEK 2 Antimicrobial Susceptibility Tests (AST) are intended for use with the VITEK 2 Systems for the automated quantitative or qualitative susceptibility testing of isolated colonies.

Principle of the Procedure [10].

The AST card for VITEK 2 Systems is an automated test methodology based on the MIC technique reported by MacLowry, Marsh and Gerlach. The AST card is essentially a miniaturized and abbreviated version of the doubling dilution technique for MICs determined by the microdilution method[11].

Each AST card contains 64 micro wells. A control well containing only microbiological culture medium is resident on all cards, with the remaining wells containing premeasured amounts of specific antimicrobials combined with culture medium[12].

The organism suspension to be tested must be diluted to a standardized concentration in 0.45% saline before being used to rehydrate the antimicrobial medium within the card[13].

The card is then filled, sealed, and placed into the instrument incubator/reader, either automatically (as with VITEK 2 60 or VITEK 2 XL) or manually (as with VITEK 2 Compact) [14].

The instrument monitors the growth of each well in the card over a defined period of time (up to 24 hrs. for bacteria or up to 36 hours for yeast). At the completion of the incubation cycle, MIC values (or test results, as appropriate) are determined for each antimicrobial contained on the card [14].

Phenotypic identification of Carbapenemases Combined Disk Test:

Principle:

The test organisms were inoculated on plates with Mueller Hinton agar as recommended by CLSI (2018). Two disks 10 ug Imipenem and Imipenem / EDTA (10 ug + 750 ug) were placed at a distanced of 25 mm, center to center, on the plate

Interpretation:

The inhibition zones of the Imipenem and Imipenem / EDTA disks were compared after 16 to 18 hrs. of incubations at 35C. A zone diameter difference of Imipenem and Imipenem / EDTA disks of ≥ 5mm was considered as Carpabenemase positive.

Fig (1). Double synergy test

Molecular investigations:

1. **Polymerase Chain Reaction (PCR):-**

Conventional PCR was performed for all strains to detect MDR Gram Negative Bacilli and to examine the genetic relatedness of isolates with bla NDM-1genes mutations.

A. Bacterial DNA isolation:-

By the use of QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA)

Sample treatment and DNA extraction:- (according to manufacture instruction)

1- 400 µ of **Genomic Lysis Buffer** were added to 200 µ of the shaken overnight nutrient broth culture into a **Zymo-Spin ™ IIC Column** in a **Collection tube** was centrifuged for

one minute at 10,000 rpm. The **Collection Tube** was discarded with the flow through.

2- **The Zymo-Spin ™ IIC Column** was transferred carefully to a new collection tube , then 200 µ of **DNA Pre-Wash Buffer** was added to the **Spin Column** without wetting the rim and mixed by pulse-vortexing for 15 seconds, then centrifuged at 10,000 rpm for one minute.
3- 500 µ of **DNA wash buffer** was added to the **Spin Column** without wetting the rim. Then centrifuged after closing the cap at 10,000 rpm for one minute.
4- **The spin column** was transferred to a clean microcentrifuge tube and 50 µ **DNA Elution Buffer** was added to the **Spin Column**. Then incubited for 2-5 min at room temp to increase the DNA yield, and then centrifuged at top speed for 30 seconds to elute the DNA. The eluted DNA was used immediately for molecular based application also" stored at $\leq$ -20 ^{0} C for future use".

B. Master Mix preparation:-

- Catalog No. BIO-25044: 200 × 50 µ reactions 4 × 1.25 mL (Thermo Fisher Scientific,Waltha, MA).
- bla NDM-1 gene, nucleotide sequence as follow:

Forwad(3'-GGGCAGTCGCTTCCAACGGT)
Reverse(5'-GTAGTGCTCAGTGTCGGCAT)

C .Amplification :-

Amplification of the sample according to the following directions (Amplification cycle profile), by using a Biometra Thermal Cyclar-T Gradient Software PCR system version 4.

Table (1): Showing the temperature, duration of DNA amplification reactions.

Steps	Temperature	Time	Cycle No
Initial Denaturation	**95 °C**	**15 min**	**1X**
Denaturation **Annealing** **Elongation**	**94 °C** **52 °C** **72 °C**	**1 min** **1 min** **1 min**	**40X**
Extension	**72 °C**	**10 min**	**1X**

Agarose gel electrophoresis:-

The PCR products were separated by electrophoresis in a 2% agarose gel.

DNA Sequencing:

PCR products were purified and sequenced (Macrogen, Korea). Previously published sequences of NDM-1 isolates retrieved from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov) were used as the reference sequence (Gene bank accession number NG_049326). Nucleotide sequence analysis was performed with BLAST sequence algorithms and sequences were aligned using Clustal W on geneious software.

Statistical method:

- Statistical package for social sciences (IBM-SPSS), version 24 IBM-Chicago, USA (May 2016) was used for statistical data analysis.
- Data expressed as mean, standard deviation (SD), number and percentage. Mean and standard deviation were used as descriptive value for quantitative data, while number and percentage were used to describe qualitative data.
- Student t test was used to compare the means between two groups, and one-way analysis of variance (ANOVA) test was used to compare means of more than two groups. Mann Whitney test was used instead of

Student t test in case of non-parametric data.

- Pearson Chi square was used to compare percentages of qualitative data, and Fisher's Exact test was used for non-parametric data.
- For all these tests, the level of significance (P-value) can be explained as:
  - No significance P > 0.05
  - Significance P < 0.05
  - High significance P < 0.001.

Results

This study included 150 patients were infected by MDR-GNB, 100 of patients were resistant to carbapenem group and 50 patients were sensitive to carbapenem group. There was 100 resistant cases with percentage of 66.7% and the remaining cases were sensitive with percentage of 33.3%. The mean age of the study population was similar between the two groups, being around 32.6 years old among resistant cases and 31.5 years old among sensitive cases, with non significant difference between the two groups. Similarly, there was non significant difference between the two groups regarding sex distribution, as around 60% of cases in both groups were males. The study sample was collected from three departments, medical department with percentage of 26 %, pediatric department with percentage of 37 % and surgery department with percentage of 37 %. It also shows that the type of infection with either Hospital Aquired Infection (HAI) with percentage of 74 % or Community Aquired Infection (CAI) with percentage of 26 %. The mean duration of hospitalization is 12.92 ± 9.30 days with median 10 days.

The diagnosis of the studied population differs as it was distributed as follow: 34 % were diagnosed as Surgical Site Infection (SSI), 33 % diagnosed as respiratory infection, 8 % diagnosed as GIT infection, 3 % diagnosed as Pyrexia Of Unknown Infection (PUO), 3 % as dehydration, 2 % for each of Congestive Heart Disease (CHD), convulsion and stroke and 13 % for the remaining diagnosis. Most of the study sample lived with good outcome with percentage of 82 %. 25 % of the sample of the study population were sputum, 24 % were pus, 23 % were urine, 21 % were blood and 7 % for stool and pleural fluid. 33 % of the study population were *Klebsiella pneumonia*, 24 % were *Escherichia coli*, 16 % were *Pseudomonus aeruginosa*, 14 % were *Enterobacter cloacae* and 13 % were other organisms. Figure (1) show that more than half of the study population were positive for NDM-1 gene with percentage of 53 %.

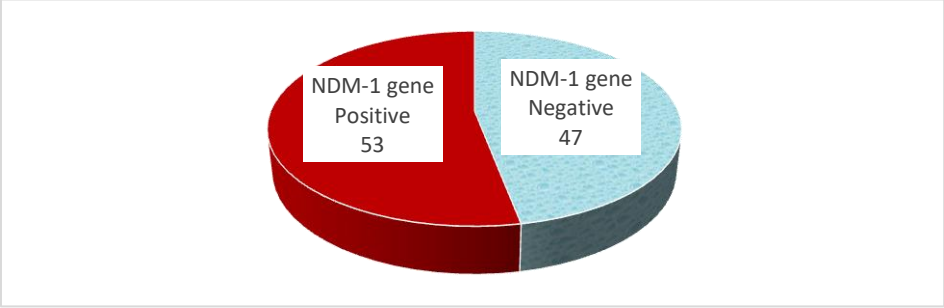

Figure 2. Distribution of studied population according to presence of NDM-1 gene.

Result of phenotypic identification of carbapenemase

Screening for the occurance of carbapenemase was accomplished by phenoltypic identification using carbapenem-

ase combined disk test in accordance of CLSI 2018 guidelines. It showed that 100 % of resistant cases were positive carbapenemase combined disk test which mean prescence of carbapenemase.

Relation between patient's characteristics and NDM-1 gene

The majority of cases were males (62 with percentage of 62 %), 29 of them were positive NDM-1 gene with percentage of 46.77% and 33 of them were negative NDM-1 gene with percentage of 53.23 %. There is non-significant difference between groups regarding gender of the patients (p value = 0.11 "N.S"). Figure (2) show that most of cases lived (82 with percentage of 82 %), 45 of them were positive NDM-1 gene with percentage of 54.88 % and 37 of them were negative NDM-1 gene with percentage of 45.12%.There is non-significant difference between groups regarding gender of the patients (p value = 0.42 "N.S").

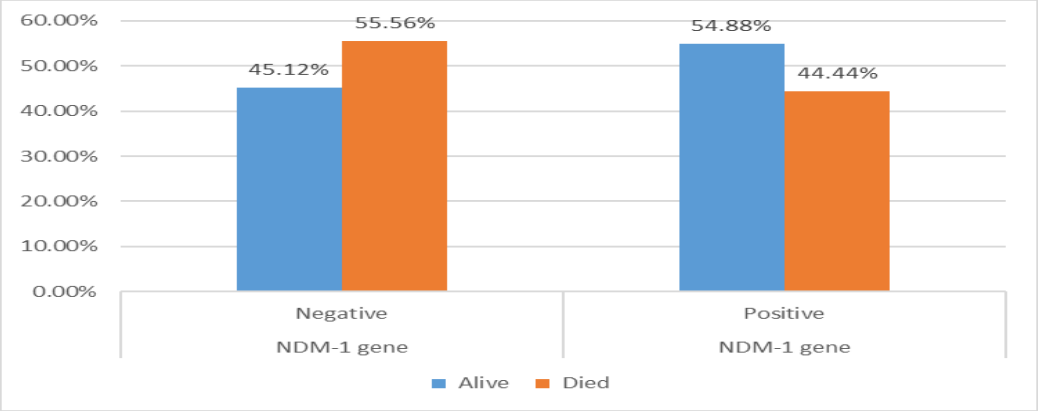

Figure 3. Relation between patient's outcome and NDM-1 gene

Relation between ward and type of infection and NDM-1

1st group of cases were in the medical department (26 with percentage of 26 %), 11 of them were positive NDM-1 gene with percentage of 42.31 % and 15 of them were negative NDM-1 gene with percentage of 57.69 %. It shows that the 2nd group of cases were in the pediatric department (37 with percentage of 37 %), 26 of them were positive NDM-1 gene with percentage of 70.27 % and 11 of them were negative NDM-1 gene with percentage of 29.73 %. It also shows that the 3rd group of cases were in the surgery department (37 with percentage of 37 %), 16 of them were positive NDM-1 gene with percentage of 43.24 % and 21 of them were negative NDM-1 gene with percentage of 56.76 %. There is significant difference between groups regarding distribution of the patients (p vaule = 0.03 "S"). Most of cases had HAI type of infection (74 with percentage of 74 %), 37 of them were positive NDM-1 gene with percentage of 50 % and 37 of them were negative NDM-1 gene with percentage of 50 %.There is non-significant difference between groups regarding gender of the patients (p value = 0.31 "N.S").

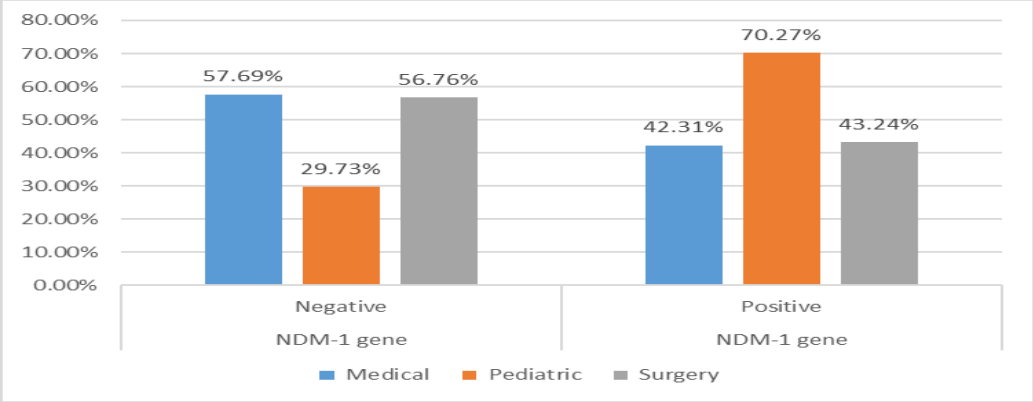

Figure 4. Relation between Ward and NDM-1 gene

Relation between diagnosis and NDM-1 gene

Most of cases were SSI (34 with percentage of 34 %), 13 of them were positive NDM-1 gene with percentage of 38.24 % and 21 of them were negative NDM-1 gene with percentage of 61.76 %. It shows that the other group of cases had respiratory infection (33 with percentage of 33 %), 20 of them were positive NDM-1 gene with percentage of 60.61 % and 13 of them were negative NDM-1 gene with percentage of 39.39 %. It also shows that some of cases had GIT infection (8 with percentage of 8 %), 7 of them were positive NDM-1 gene with percentage of 87-.5% and 1 of them were negative NDM-1 gene with percentage of 12.5 %. There is non-significant difference between groups regarding diagnosis of the patients (p value = 0.25 "N.S").

Relation between site of infection and NDM-1 gene

At most of cases the site of infection was pulmonary tract (27 with percentage of 27 %), 15 of them were positive NDM-1 gene with percentage of 55.56% and 12 of them were negative NDM-1 gene with percentage of 44.44 %. It shows that in other cases the site of infection was wound (24 with percentage of 24 %), 9 of them were positive NDM-1 gene with percentage of 37.5 % and 15 of them were negative NDM-1 gene with percentage of 62.5 %. It also shows that in other cases the site of infection was urinary tract (23 with percentage of 23 %), 10 of them were positive NDM-1 gene with percentage of 43.48 % and 13 of them were negative NDM-1 gene with percentage of 56.52 %. It shows that in other cases the site of infection was blood (21 with percentage of 21%), 15 of them were positive NDM-1 gene with percentage of 71.43% and 6 of them were negative NDM-1 gene with percentage of 28.57 %. It shows that in other cases the site of infection was G-IT (5 with percentage of 5 %), 4 of them were positive NDM-1 gene with percentage of 80% and 1 of them were negative NDM-1 gene with percentage of 20 %. There is non-significant difference between groups regarding site of infection (p value = 0.11 "N.S").

Relation between type of sample and NDM-1 gene

In most of cases the sample was sputum (25 with percentage of 25 %), 14 of them were positive NDM-1 gene with percentage of 56 % and 11 of them were negative NDM-1 gene with percentage of 44 %. It shows that in other cases sample was pus (24 with percentage of 24 %), 9 of them were positive NDM-1 gene with percentage of 37.5 % and 15 of them were negative NDM-1 gene with percentage of 62.5 %. It also shows that in other cases sample was urine (23 with percentage of 23 %), 10 of them were positive NDM-1 gene with percentage of 43.48%

and 13 of them were negative NDM-1 gene with percentage of 56.52 %. It shows that in other cases the sa-mple was blood (21 with percentage of 21 %), 15 of them were positive NDM-1 gene with percentage of 71.43 % and 6 of them were negative NDM-1 gene with percentage of 28.57 %. It shows that in some cases the sample was stool (5 with percentage of 5 %), 4 of them were positive NDM-1 gene with percentage of 80 % and 1 of them were negative NDM-1 gene with percentage of 20%. Finally, it shows that in some cases the sample was pleural fluid (2 with percentage of 2 %), 1 of them were positive NDM-1 gene with percentage of 50 % and 1 of them were negative NDM-1 gene with percentage of 50 %. There is non-significant difference between groups regarding sample taken (p value = 0.18 "N.S").

Table 2. Relation between type of sample and NDM-1 gene

Variable	NDM-1 gene Negative	NDM-1 gene Positive	P value
Sputum	11 (44.00%)	14 (56.00%)	0.18
Pus	15 (62.50%)	9 (37.50%)	
Urine	13 (56.52%)	10 (43.48%)	
Blood	6 (28.57%)	15 (71.43%)	
Stool	1 (20.00%)	4 (80.00%)	
Pleural fluid	1 (50.00%)	1 (50.00%)	

Relation between organism and NDM-1 gene

In most of cases the organism was *Klebsiella pneumoniae* (33 with percentage of 33 %), 26 of them were positive NDM-1 gene with percentage of 78.79% and 7 of them were negative NDM-1 gene with percentage of 21.21 %. It shows that in other cases the organism was *Escherichia coli* (24 with percentage of 24 %), 14 of them were positive NDM-1 gene with percentage of 58.33 % and 10 of them were negative NDM-1 gene with percentage of 41.67 %. It also shows that in other cases the organism was *Pseudomonus aeruginosa* (16 with percentage of 16 %), 6 of them were positive NDM-1 gene with percentage of 37.5 % and 10 of them were negative NDM-1 gene with percentage of 62.5 %. It shows that in other cases the organism was *Enterobacter cloacae* (14 with percentage of 14 %), 4 of them were positive NDM-1 gene with percentage of 28.57 % and 10 of them were negative NDM-1 gene with percentage of 71.43 %. There is significant difference between groups regarding organism (p value = 0.03 "S").

Table 3. Relation between organism and NDM-1 gene

Variable	NDM-1 gene Negative	NDM-1 gene Positive	P value
Klebsiella pneumonia	7 (21.21%)	26 (78.79%)	**0.03**
Escherichia coli	10 (41.67%)	14 (58.33%)	
Pseudomonus aeruginosa	10 (62.50%)	6 (37.50%)	
Enterobacter cloacae	10 (71.43%)	4 (28.57%)	
Acinetobacter baumannii	1 (33.33%)	2 (66.67%)	
Klebsiella oxytoca	1 (50.00%)	1 (50.00%)	
Salmonella enterica	2 (100%)	0	
Chryseobacterium indologenes	1 (100%)	0	
Enterobacter aerogenes	1 (100%)	0	
Enterobacter gergoviae	1 (100%)	0	
Morganella morganii	1 (100%)	0	
Pasteurella canis	1 (100%)	0	
Proteus vulgaris	1 (100%)	0	

Percentage of NDM-1 gene according to type of sample and organism

Klebsiella pneumonia organism was found in sputum samples as 8 of them NDM-1 gene positive with percentage of 27.27 % and 3 samples NDM-1 gene negative with percentage of 72.73 %. It (*Klebsiella pneumonia*) was also found in pus samples with 2 of them NDM-1 gene positive with percentage of 66.67 % and 1 samples NDM-1 gene negative with percentage of 33.33 %. *Klebsiella pneumonia* organism was found in urine samples that was 1 only with NDM-1 gene positive with percentage of 100 %. In blood samples *Klebsiella pneumonia* was found with 13 of them NDM-1 gene positive with percentage of 81.25 % and 3 samples NDM-1 gene negative with percentage of 18.75 %. *Klebsiella pneumonia* organism was found in stool samples that was 1 only with NDM-1 gene positive with percentage of 100 %. *Klebsiella pneumonia* organism was found in pleural fluid samples that was 1 only with NDM-1 gene positive with percentage of 100 %.

Escherichia coli organism was found in sputum samples as 2 of them NDM-1 gene positive with percentage of 33.33 % and 4 samples NDM-1 gene negative with percentage of 66.67 %. It (*Escherichia coli*) was also found in pus samples with 2 of them NDM-1 gene positive with percentage of 66.67 % and 1 samples NDM-1 gene negative with percentage of 33.33 %. *Escherichia coli* organism was found in urine samples that was 7 only with NDM-1 gene positive with percentage of 63.64 %. In blood samples *Escherichia coli* was found zero samples. *Escherichia coli* organism was found in stool samples that was 3 only with NDM-1 gene positive with percentage of 75 %. *Escherichia coli* organism was found in pleural fluid samples in zero sample.

Pseudomonus aeruginosa organism was found in sputum samples as 1 of them NDM-1 gene positive with percentage of 25 % and 3 samples NDM-1 gene negative with percentage of 75 %. It (*Pseudomonus aeruginosa*) was also found in pus samples with 3 of them NDM-1 gene positive with percentage of 75% and 1 samples NDM-1 gene negative with percentage of 25 %. *Pseudomonus aeruginosa* organism was found in urine samples that was 2 only with NDM-1 gene positive with percentage of 28.57 %. In blood samples *Pseudomonus aeruginosa* was found in zero samples. *Pseudomonus aeruginosa* organism was found in stool samples in zero samples.

Enterobacter cloacae organism was found in sputum samples that was 1 only with NDM-1 gene positive with percentage of 100 %. It (*Enterobacter cloacae*) was also found in pus samples with 2 of them NDM-1 gene positive with percentage of 16.67 % and 10 samples NDM-1 gene negative with percentage of 83.33 %. *Enterobacter cloacae* organism was not found in urine samples. *Enterobacter cloacae* organism was found in blood samples that was 1 only with NDM-1 gene positive with percentage of 100%. *Enterobacter cloacae* organism was not found in stool samples neither pleural fluid samples.

Acinetobacter baumannii organism was found in sputum samples with 2 of them NDM-1 gene positive with percentage of 66.67 % and 10 samples NDM-1 gene negative with percentage of 33.33 %. *Acinetobacter baumannii* organism was not found in pus, urine, blood, stool or pleural fluid samples.

Klebsiella oxytoca organism was found in urine samples which was only NDM-1 gene negative. *Klebsiella oxytoca* organism was not found in pus, spu-

tum, stool or pleural fluid samples. *Kle-bsiella oxytoca* organism was found in blood samples that was 1 only with NDM-1 gene positive with percentage of 100 %.

Salmonella enterica organism was not found in sputum, urine, stool or pleural fluid samples. It was found in pus and blood samples (only one for each) which was only NDM-1 gene negative.

Chryseobacterium indologenes was found in blood samples (only one) which was only NDM-1 gene negative. While *Enterobacter aerogenes* was found in pus samples (only one) which was only NDM-1 gene negative. *Enterobacter gergoviae* was found in urine samples (only one) which was only NDM-1 gene negative. *Morganella morganii* was found in urine samples (only one) which was only NDM-1 gene negative. *Pasteurella canis* was found in blood samples (only one) which was only NDM-1 gene negative. Proteus vulgaris was found in urine samples (only one) which was only NDM-1 gene negative.

Results of sequencing:

-Sequencing of bla NDM gene showed that samples 4 and 7 contain bla-NDM-5 genotype of bla NDM family. Summary of sequencing results are shown in table (3).

Table (3). Summary of blaNDM genotypes using sequencing analysis

Isolate number	blaNDM Genotype
1	blaNDM-1
2	blaNDM-1
3	blaNDM-1
4	blaNDM-5
5	blaNDM-1
6	blaNDM-1
7	blaNDM-5
8	blaNDM-1
9	blaNDM-1
10	blaNDM-1

Discussion

Regarding the age and gender of the studied population, we found that the mean age of the study population is 32.61±28.23 years with median 35 years. It also shows that 62 % of the samples were males. Our data was similar to [15], whose cases had a mean age of 32 years and around 64% were males. On the other hand, [16] revealed that the mean age of their cases was 54.9 years, and more than 75% of their cases were females; which is opposite to our study population. The study of [17], revealed only 4 positive NDM-1 gene cases, three of them were neonates (14-26 days) and one was old aged (60 years). This was somewhat different from the findings of [16] where 50% of their cases had UTI, followed by respiratory infections, and one case had wound infection, esophageal carcinoma or chronic kidney disease. In the study of, [18], 73.3% of their isolates were isolated from pus, 14.7% from urine and 12% from othe body fluids. Regarding the outcome of the studied population, we found that most of the study sample lived with good outcome with percentage of 82%. In the study of [16], two out of their 14 cases died, and the remaining 12 cases improved or had unknown fate. We found that 25 % of the sample of the study population were sputum, 24% were pus, 23% w-ere urine, 21% were blood and 7% for stool and pleural fluid. In the study of [17], samples were sputum in 2 cases (50%); blood and anal secretions in one case each. The study of [16] showed that in 50% of cases, the sample was

urine, sputum and/or tracheal aspirate and/or endotracheal tube in the other half, with only one case of wound secretions. Regarding the organism, we found that the organism was *Klebsiella pneum-oniae* in 33% of the study population, 24% were *Escherichia coli*, 16% were *Pseudomonus aeruginosa*, 14% were *Enterobacter cloacae* and 13% were distributed between the remaining organisms. According to [19], the most common organism was *Acinetobacter baumannii* (27%), followed by *Providencia rettgeri* (25.6%), then *Escherichia coli* and *Klebsiella pneumoniae* (13.5% each), *Pseudomonas aeruginosa* and *Enterobacter cloacae* (5.4% each), *Proteus vulgaris* (2 cases only) and lastly *Alcaligenes fecalis, Citrobacter freundii, Roultella ornitholytica, Pseudomonas putida* and *Burkholderia cepacia* (one case each).

According to [18], *Citrobacter* species predominated (24.13%) followed by E. coli (22.41%), *Pseudomonas* (20.68%), *Serratia* (14.65%), *Klebsiella* (7.75%), *Acinetobacter* (4.31%), Proteus (3.4-4%), *Providencia (*1.72), and *Aeromonas* (0.86%). On the other hand, all of the cases of [17] were *A. baumannii*. As regard to the presence of NDM-1 gene, we found that more than half of the study population were positive for NDM-1 gene with percentage of 53%. [18] found similar results to our study, and stated that the NDM-1 gene positivity among their cases was 57%. The study done by [19] found that the percentage of NDM-1 gene positivity was 46%, which is a little lower than out results. However, the study of [16], stated that the NDM-1 positivity was only 35% among their isolates (14 out of 55 cases). The study of [19] showed further less percentage as they detected a 29.8% positivity for NDM-1 gene among multidrug resistant bacteria in their study (53 cases). Our results shows that most of cases lived (82%), 54.9% of them were positive NDM-1 gene. The study of [17] found that the infection due to MDRGNB resolved in 58 of the 61 patients. The remaining three patients succumbed to death.

Our results show that most of cases had HAI type of infection (74%), 50% of them were positive NDM-1 gene.

Regarding the relation between diagnosis and NDM-1 gene, our study show that the most common diagnosis was SSI (34%of cases; 38% of them were NDM-1 positive), respiratory infection (33%; with over 60.61% positive for NDM-1 gene). Some of the cases had GIT infection (8 cases, with 87.5% of them were positive for NDM-1 gene). There is non-significant difference between groups regarding diagnosis of the patients.

Regarding the relation between site of infection and NDM-1 gene, our study show that the first site of infection was pulmonary tract (27 cases; 55.56% of them were NDM-1 genel positive); followed by wound (24 cases; 37.5% were NDM-1 positive), then urinary tract (23 cases; 43.48% were NDM-1 gene positive), blood infections (21 cases; 71.43% positive NDM-1). GIT infections were seen in 5 cases; 80% of them were positive for NDM-1 gene. There is non-significant difference be-tween groups regarding site of infection (p vale = 0.11).

According to [18], the most common NDM-1 bacterial isolate in pus were *Escherichia coli* (65.38%) and *Citrobacter species* (39.29%). The predominant NDM-1 bacterial isolates in urine were also E. coli (26.92%) and *Klebsiella species* (22.22%). However, in other body fluids *P. aeruginosa* and *Citrobacter species* (30.0% each) were predominant NDM-1 iso-lates. Surprisingly, none of the isolates of *Providencia* and *Proteus* species were NDM-1 positive. In the study of [19], out of the 53 positive NDM-1 genel positive isolates, 19 were isolated from urine, followed by tissue (11), sputum (9), ET

secretion (6), pus and pleural fluid (2 isolates each), and lastly drain fluid, c-up tip, PICC tip and blood (one isolate each).

Regarding the relation between organism and NDM-1 gene, our study show that the commonest organism was *Klebsiella pneumoniae* (33%; 78.8% of them were positive NDM-1 gene), followed by *Escherichia coli* (24%; 58.33% of them were NDM-1 positive), then *Pseudomonus aeruginosa* (16%; with 37.5% of them were NDM-1 positive); with non significant relation between type of organism and NDM-1 positivity.

The study of [16] found that, out of the 14 cases with positive NDM-1 genel positivity, *K. pneumoniae* was the most common organism (7 cases; 50%), followed by *A. baumannii* (3 cases), *E. coli* (two cases), and *P. rettgeri* and *C. freundii* (one case each).

According to [18]; although the most common pathogen isolated in their study was Citrobacter species (24.13%), the prevalence of blaNDM-1 gene positivity was highest among E. coli (100%) isolates.

Regarding the distribution of studied population according to antibiotic resistance, our study shows that the most common antibiotic resistance in the studied population was Ampicillin resistance, where 100% of the organisms were resistant to it. This was followed by Cefazolin (99% resistant); then Ampicillin/Sulbactam and Cefoxitin (97% each), Piperacillin/Tazobactam (88%), Ciprofloxacin (54%), and Amikacin (36%). Regarding the distribution of studied population according to antibiotic resistance if NDM-1 gene is positive, our study shows antibiotic resistance in the studied population was 100% for Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefazolin, Ceftriaxone and Cefoxitin; and was 98.1% for Ceftazidime; 96.2% for Cefepime and Meropenem; 64.2% for Gentamicin and 35.85% for Amikacin; figires which are signifycantly worse compared to NDM-1 negative cases.

In the study of [17], all NDM-1-producing *A. baumannii* were resistant to all carbapenems, cephalosporins and b-lactamase inhibitor combinations tested, while 75% of them were sensitive to gentamicin, amikacin, ciprofloxacin, minocycline, tigecycline and colistin. In the study of [19], among the 53 NDM-1 positive isolates, a single *K. pneumoniae* isolate from urine was found to be resistant to colistin and was responsible for the death of the patient. 13/53 isolates (24.5%) were resistant to tigecycline of which 9 isolates were *K. pneumoniae*, 2 isolates were *P. aeruginosa* and one isolate each of *E. coli* and *A. faecalis*[20].

Conclusion

The incidence of NDM-1 gene positivity is very high among bacteria detected in our study, especially *Klebsiella* and *Escherichia coli* and this indicate that CRE are spreading in our locality and Egypt.

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