ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in
Zeinah R. Hameed Al-Sultani et al /J. Pharm. Sci. & Res. Vol. 11(3), 2019, 869-878

Detection of NDM-1 in Cabapenem-Resistant *Klebsiella pneumoniae*

***Zeinah R. Hameed Al-Sultani, *Hadi R. Rasheed Al-Taai**
** Department of Biology, , College of Science, Diyala University /Iraq*

Abstract

Two hundred fifty clinical samples were collected from patients suffering different infections, distributed among (165) urine , (37) sputum, (29) burns, (19) wound. Morphological, Biochemical tests, Microscopic test were used to identification bacterial isolates, and conform by using VITEK2 system, the result showed that (85) isolates of *Klebsiella* spp. included(78) *K. pneumoniae*. (5) *K. oxytoca* and (2) isolates of *K. planticola.* 14 isolates of carbapenem resistant *Klebsiella pneumoniae* also identified by Polymerase chain reaction technique (PCR) for the detection of 16-23S rRNA gene, results indicated that the 14 isolates have the 16-23S rRNA gene.

Antibiotic susceptibility test for *K. pneumoniae* isolates were performed against 6 antibiotic using disk diffusion method, the result showed Imipenem(14.1%), Meropenem(17.7%), (Gentamycin (41.02%), Tobramycin (39.74%), Tetracycline. (55.12%), and Tigecyclin (39.74%).

Two methods used to detect production of M*β*Ls, the first method is Combine EDTA Disk Test (CEDT), results indicated that 9 isolates(64.28%) produced MβLs, and five isolates (35.71 %) gave negative result. The second method is Modified Hodge Test (MHT), results indicated that 10 isolates (71.42.%) gave positive results and 4 isolates gave negative results(28.57 %)

Minimum Inhibitory Concentration (MIC) for Carbapenem resistant *K.pneumoniae* was done by VITEK 2 system. The result showed that the MIC rang between (2- 64 µg/ml) for Amikacin, Minocycline. Gentamycin, Ciprofloxacin (0.25- 4µg/ml) (1-16 µg/ml) respectively. Imipenem , Meropenem (16-8 µg/ml). Piperacillin Piperacillin / Tazobactam 128 >=µg/m. Ticarcillin and Ticarcillin/ Clavulanic acid (128 >=µg/ml). Tobramycin (1-16µg/ml). finally Trimethoprim /Sulfamethoxazole (20-320 µg/ml).

PCR used for the detection of bla_{NDM-1} gene in 14 isolates of Carbapenem- resistant *Klebsiella pneumoniae* by used primer for the bla_{NDM-1} , results indicated that 13 isolates have the bla_{NDM-1} gene.

The inhibitory effect of two nanoparticales materials (Tio_2 and Zno) was study towards the growth and ability of bacteria to produce metallo - β - lactamase enzymes for 9 isolates of Carbapenem resistant *Klebsiella pneumoniae*. Results indicated that the MIC for Zno size 50nm ranged between (325 - 1300 µg/ml) and the MIC for Tio_2 size 25nm ranged between (650 -2600µm/ ml). The results of nanoparticales affection of Zno and Tio_2 on the production of metallo- β- lactamase enzymes was equal for the two nanoparticales , 5 isolate from 9 isolates lost the ability of MβL production (55.55%)

INTRODUCTION

Klebsiella pneumoniae causes a wide range of diseases, including pneumonia, urinary tract infections, wound injuries, bacteriaemia, etc. It is the third most common cause of hospital acquire diseases , so it is a highly health-threatening bacteria (1). β-lactam antibiotics include four groups: penicillins, Cephalosporins, Monobactam and Carbapenem (2,3). Carbapenem antibiotics is one of the β-lactam antibiotics groups, which is the last resort to treat many infections that cause by gram negative (Multidrug resistant MDR) bacteria, which has the greatest effectiveness of β-lactam family (4,5). The most common mechanism for resistance to external killing by some elements, including antibiotics, and survival for longer is the production of enzymes (6), the most importantly of these enzymes is the β-lactamase, which include Extended spectrum β-lactamase and Carbapenemase enzymes, which are produced by many bacterial species, especially *Enterobacteriaceae* family members such as *E. coli, Serratia spp., Klebsiella spp, Salmonella* (7,8). β-lactamase enzymes divided into two family serine β-lactamase and Metallo β- lactamase. According to Ambler's classification these enzymes are divided into four groups (A, C, D) belong to the serine β- lactamase family and the B-class enzymes belong to the Metallo β-lactamase family, Carbapenemase ezymes belong to A, B, and D classes (9). Metallo β-lactamase need zinc ion or other heavy metals essential for their effectiveness. Metallo β-lactamase enzymes able to hydrolyze all β-lactame antibiotic except Monobactam antibiotics (10). The most important types of class B and the most common in the *Enterobacteriaceae* family are VIM, IMP and NDM enzymes (11,12). NDM-1 enzyme is encoded by a gene bla_{NDM-1} or NDM-1gene. This gene encodes 269 amino acid. This enzyme analyzes all β-lactam antibiotics and cannot be inhibited by clavulonic acid or Sulbactam (13).The gene bla_{NDM-1} is associated with other genes that gain bacteriae resistant to antibiotics including Erythromycin, Ciprofloxacillin, Rifampicin and Chloramphenicol. It is also associated with the genetic elements responsible for the flow pumps and Extended Spectrum enzyme type CMY-4. These genetic connections make the bla_{NDM-1} gene very dangerous (14). Bla_{NDM-1} gene encodes by plasmid, facilitating its transmission between different bacterial strains (15). The emergence of multiple antibiotic resistance by *K.pneumoniae*. has led to a lot of treatment failure(16,17). Recently, some nano materials have been used and their industry has evolved, with great efforts being made to develop. these substances have a significant effect on bacterial cell components (17), where studies have shown the effectiveness of these substances against bacteria and bacteria failure in nanomaterials resistance compared to antibiotics that developed different mechanisms to resist them (18). And these nanomaterials are zinc oxide, titanium oxide and other materials for nanoparticles such as copper, cobalt and silicon that have high efficacy against microorganisms(19).

This work aimed to study the distribution of bla_{NDM-1} gene in clinical isolates of *K. pneumoniae* isolates from Iraqi patients in Baghdad medical hospitals and the effect of nanoparticales against resistant *K.pneumoniae*

MATERIALS AND METHODS

Sample Collection and Identification

Two hundred fifty samples from four clinical sources were collected including 165 urine, 37 sputum samples, 29 smears of burns and19 smears of wounds. Period between October 2017 to February 2018. Identification bacterial isolates depended on the culture characteristics, microscopic, biochemical tests, also used the VITEC 2 system for diagnosis *Klebsiella pneumoniae* , and use polymerase chain reaction technique (PCR) for the defection of 16-23SrRNA gene.

Standard antimicrobial susceptibility testing

All isolates were tested using 6 antibiotics (Imipenem, Meropenem Gentamycin, Tobramycin, Tetracyclin, Tigecyclin) using the Kirby-Bauer method (20). Minimum Inhibitory Concentration (MIC) of the Carbapenem-resistant *K. pneumoniae* isolates was determination by used VITEK 2 system.

Carbapenemase production

Modified Hodge Test (MHT)

Modified Hodge Test (MHT) was used to detect the ability of *K. pneumoniae* isolates to produce carbapenemase enzyme(21). This test was conducted according to (22) with some modification (not use *E.coli* ATCC 25922 but use *E.coli* isolate that susceptible to carbapenem antibiotics).

Sensitive *E.coli* was suspended with a turbidity similar to the McFarland 0.5 which is equivalent to 1.5 CFU / ml ^{8}10. Spread the suspention on Muller-Hinton agar plates by sterile cotton swab. removal of excess moisture by pressing on the walls of the tube from the inside and then distribution *E.coli* on all parts of the plates. Leave the plates at room temperature for 3 - 10 minutes and then placed on the center Meropenem or Ertapenem antibiotic. 3-4 colonies of each *K.pneumoniae* isolats were harvested by a sterile lobe and cultured in a straight line from the edge of the antithesis to the perimeter of the plate and the length should not be less than 20-25 mm. - Incubate the plates at 37 ° C for 19 hours. Read the result: *E.coli* growth is a positive result for the production of carbapenem enzymes. The lack of growth of *E. coli* has a negative effect on the production of carbapenzymes.

Combine EDTA Disk Test (CEDT).

The bacterial suspension of *K.pneumoniae* isolates under study was prepared . The suspension turbidity was equal to the McFarland 0.5, which is equivalent to 1.5 CFU / ml 810 and was cultured on a Müller-Hanton agar plate as indicated in (22). 4 µl of sterile EDTA solution added to disc of (Meropenem 10mg), EDTA-Meropenem were dried in the incubator and stored at -20 ° C. In a flask free of dehydrators until use (23). *Klebsiella pneumoiae* suspention spread onto Mueller Hinton agar plates using a sterile cotton swab. then Meropenem disk (10 µg) and Meropenem-EDTA was placed on test plates. The distance between discs is 20 mm.(24). Test plates incubation at 37 ° C for 24 hours, the results were read by measuring the areas of inhibition around the disks. Increasing the inhibitory area to 7 mm around the Meropenem-EDTA tablet compared with the Meropenem alone is a positive result of the bacteria and these bacteria are produced by the metallo beta lactamase MBL (25).

DNA extraction

DNA samples were extracted from *Klebsiella pneumoniae* according to manufacturer's instructions (using a bacterial genomic DNA extraction kit (Geneaid Biotech, Taiwan). The concentration and purity of DNA were measured by a nanodrop (BioDrop µLITE, BioDrop co., UK), while the DNA integrity was checked by a standard 0.8% (w/v) agarose gel electrophoresis with ethidium bromide, using a 1 kb ladder as a molecular weight marker (Cat #D-1040, Bioneer, Daejeon, South Korea). The isolated DNA was used as a template for PCR.

PCR design and amplification

PCR was designed for detection of 16-23SrRNA and bla_{NDM-1} using specific primers. The primers were obtained from Macrogen Company, Korea (**Table 1.)**

The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using Accu Power PCR premix (Bioneer, Daejeon, South Korea). The following program was applied in PCR thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Daejeon, South Korea).The amplification was began by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94˚C, annealingat 58˚C for16-23SrRNA gene and 50ºC for bla_{NDM-1} gene , and elongationat 72˚C, and was finalized with a final extension at 72°C for 10 min. Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris–Borate, pH 8.3), using a 100-bp ladder (Bioneer, Daejeon, South Korea) as a molecular weight marker. The PCR amplicons of two native isolates were commercially sequenced from forward terminiaccording to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea).

(Table 1): The specific primers' pairs designed to amplify two loci of *Klebsiella pneumonia*.

Primer	Sequence (5′-3′)	Amplicon size	AccessionNumber	Reference
16-23 S rRNA- F	ATTTGAAGAGGTTGCAAACGAT	132bp	CP027612.1 (2425935–2426067)	(26)
16-23 S rRNA- R	TTCACTCTGAAGTTTTCTTGTGTTC			
*bla*NDM-1-F	GGTTTGGCGATCTGGTTTTC	621bp	MF774796.1 (1686 – 2306)	(27)
*bla*NDM-1-R	CGGAATGGCTCATCACGATC			

DNA sequencing

The purified PCR products of two positive isolates of *bla NDM-1* and 16-23SrRNA gene were sequenced using the ABI capillary system (Macrogen Research, Seoul, Korea). Then, the sequences were compared using online BLAST software (http://www.ncbi.nlm. nih.gov/ BLAST/), and one isolate was confirmed as NDM-1 variant. So far, these sequence in the GenBank nucleotide database under accession number: LC412681.

Comprehensive phylogenetic tree construction

The observed PCR amplicons variants of bla_{NDM-1} for one isolate of *Klebsiella pneumoniae* (genetic locus B7 and B12) were compared with their neighbor homologous sequences using NCBI-BLASTnsuite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Then, the blast results of the observed variants were aligned and constructed using Clustal Omega and Simple phylogeny tools respectively (https://www.ebi.ac.uk/Tools/msa/clustalo/). A full inclusive tree, including the observed variant, was visualized as polar cladogram (Fig. 8) and a fish eye platform (Fig. 9) using Figtree tool (http://tree.bio.ed.ac.uk/software/figtree/). The observed sequences of each classified phylogenetic species-group in the comprehensive tree were colored appropriately.

Nanoparticles

The nutrient broth dilution method was used to quantitative estimation of the inhibitory effect of nanoparticles made from M K Impex corp (28,29) with some modifications.

- Zinc oxide and Titanium dioxide nanoparticales were present at specific concentrations starting from 5200 µg / ml as a primary concentration starting with the dilution series (5.07, 10.15, 20.3, 40.6, 81.25, 162.5, 325, 650, 1300, 2600).
- Add 4.9 ml of nutrient broth to all tubes and then add 5 ml of initial concentration (5200) µg / ml to tube 1. Mix well by moving up and down 6-8 times. Thus, tube 1 at 2600 µg / ml .
- Transfer 5 mL from tube 1 to tube 2 and mix well, thus reducing the concentration to 1300 µg / ml. Repeat the process up to tube 10 and then pull 5 ml of it .the concentration (5.07, 10.15, 20.3, 40.6, 81.25, 162.5, 325, 650).
- Add 0.1 ml of 24-hour bacterial suspension to each tube. Only the broth and the bacterial suspension were added to tube 11 and prepared a positive control.
- Only add nutrient broth and nanoparticles to tube 12 and prepare a negative control.
- Incubate the tubes at 37 ° C for 24 hours. Check for growth of bacteria by notice the turbidity in tubes using the eye and then observe the MIC.
- The effect of nanoparticles on the ability of bacteria to produce MβL enzymes was examined by taking a smear by cotton swab from the sub-MIC bacterial suspension and used (Combine EDTA Disk Test (CEDT)method.

RESULT AND DISCUSSION

A total of 250 samples were collected from patient with different infections, both sexes and different ages. The samples were collected from hospitals in the city of medicine (Baghdad Hospital, Martyr Ghazi Hariri Hospital, Burns and Wounds Hospital, Central Educational Laboratories) and Al-Shaheed Al-Sadr General Hospital, of which 165 were urine samples, 37 samples of burns, 29 samples of burns and 19 samples of wounds. Positive samples were 181 samples and 72.4%. The number of negative samples was 69 samples and 27.6% did not produce growth on the agricultural medium. Species based on sex were divided into 65 male samples and 185 female samples.

85 positive isolates of *Klebsiella* spp. 34% of the total samples of. 52 isolates of *E. coli* and 20.8% of total samples. 36 isolates from *Pseudomonas* bacteria and 14.4% of total samples.8 isolates of *Enterobacter* bacteria 3.2% of total samples. 78 isolates and 91.76% were identical to *Klebsiella pneumoniae*, while 5 isolates (5.88%) were identical to *Klebsiella oxytoca* and two isolates (2.35%) were identical to *Klebsiella planticola*. These results showed that *Klebsiella pneumoniae* isolates were the most common Compared with other strains of *Klebsiella* spp. . These results are consistent with the findings of (30) the percentage of *Klebsiella pnemoniae* was 90%, whereas the percentage of *Klebsiella oxytoca* 10% was also consistent with the study of (31) the percentage of *pneumoniae Klebsiella* 89.28%. The results were close to that of (32), which identified 83% of *Klebsiella* isolates of *Klebsiella* type, while (33) reported that the percentage of *pneumoniae Klebsiella* was 29.1%.

Molecular detection of of 16-23SrRNA gene

Fourteen isolates of carbapenim-resistant *Klebsiella pneumoiae* were identified using PCR. The results showed that all the isolates under study were carry 16-23SrRNA gene (100%) *Klebsiella pneumoniae*. These results were close to that of (34) as the number of isolates carrying the 16- 23S rRNA (33) isolates out of 40 isolats (82.5%), but did not agree to (35) in Egypt, where the number of isolates carrying the gene 16 isolates out of 27 isolates (59.25%).

Antibiotics susceptibility test

Six antibiotic disks were used in this study included two types of carbapenen antibiotics Imipenem(14.1%), Meropenem(17.7%). These results were close to the findings of the researchers (36), where they found that the resistance to Imipenem antibiotic was 13.9%, these antibiotics showed a higher efficacy than all other beta lactam antibiotics because they are relatively modern antimicrobial. The enzyme NDM-1 plays an important role in the resistance of certain strains of *K.pneumoniae* to the carbapenim antibiotics and other antimicrobial agents (37) and the ability of these bacteria to produce *Klebsiella pneumoniae* carbapenemases (KPC) enzymes, which are encoded by gene first detected in *K.pneumoniae* and (38). The resistance ratio of Gentamycin 41.02%. The results are consistent with the findings of the researcher (39). Which referred that 45.65% of *K.pneumoniae* were resistance to Gentamycin. Resistance percentage for Tobramycin is 39.74%, while (40) pointed that all isolates are sensitive to this antibiotic. The antibiotic inhibitors of this group to

modify the active site in the molecules of the antibiotic, making them less familiarity of the link in the path of the composition of RNA Thus discouraging (41).

Tetracycline resistance was 55.12%, while Tigecycline resistance was 39.74%. These results were relatively close to the findings of(42) in Iraq, where the resistance percentage of Tetracycline was 34.37%. Minimum Inhibitory Concentration (MIC) for Carbapenem resistant *K.pneumoniae* isolates was done by VITEK 2 system. The result showed that the MIC rang between (2- 64 µg/ml) for Amikacin, Minocycline. Gentamycin, Ciprofloxacin (0.25-4µg/ml) (1-16 µg/ml) respectively. Imipenem , Meropenem (16-8 µg/ml). Piperacillin Piperacillin / Tazobactam 128 >=µg/m. Ticarcillin and Ticarcillin/ Clavulanic acid (128 >=µg/ml). Tobramycin (1-16µg/ml). finally Trimethoprim /Sulfamethoxazole (20-320 µg/ml).

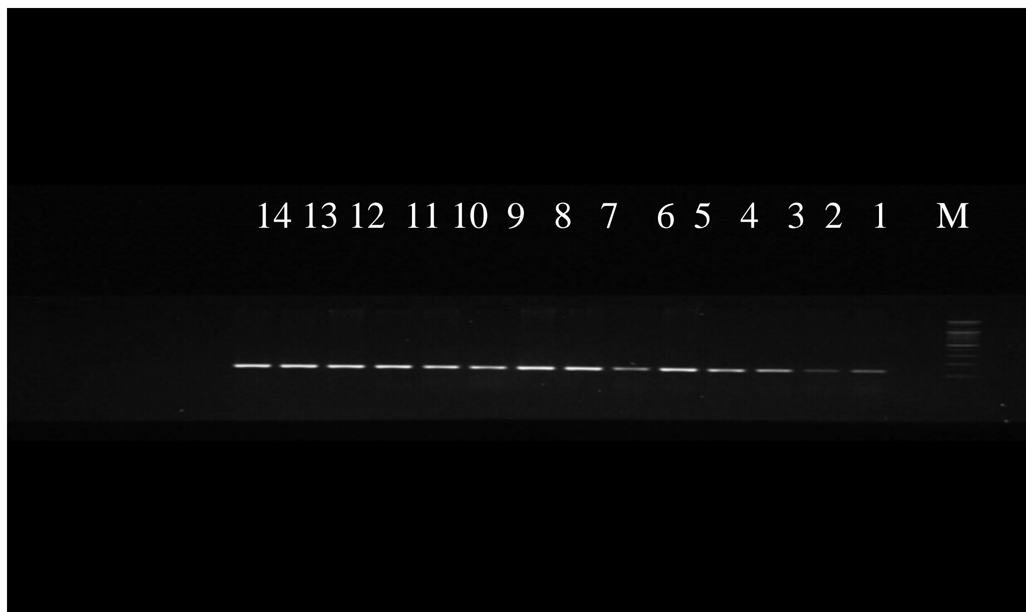

Fig 1: Agarose gel electrophoresis in 1% for 16-12SrRNAgene product show positive results(1- 14). Ethidium bromide stain (0.5%), Amplicon size (130), DNA Ladder (100bp), the electric current at 70 volt for 60min.

Table 1: resistance percentage for Antibiotics susceptibility test

Antibiotics	Percentage %
Imipenem	**14.1**
Meropenem	**17.7**
Gentamycin	**41.02**
Tobramycin	**39.74**
Tetracycline	**55.12**
Tiegecyclin	**39.74**

Detection of Carbapenemase enzymes

Several methods was used for phenotypic detection of MβLs The Combined EDTA Disk Test, one of the simplest methods of phenotypic detection of MβLs. The results of this study showed that 9 isolates out of 14 isolates of *K.pneumoniae* (64%) gave a positive result for this test. This percentage was relatively close to that of (43), where percentage of *K.pneumoniae* gave positive result was (71.9% - 50%). This means that the MβLs make the bacteria resistant to a wide range of β-lactam, due to the ability of these enzymes to analyze β-lactam antibiotics (44). And the second method is Modified Hodge Test(MHT). This test was used to investigate the ability of *K.pneumonia* isolates to produce carbapenimase enzymes. The results of this study showed that 10 isolates out of 14 isolates resistant to the carbapenem antibiotics (71.42%) gave a positive result of this test and 4 isolates(K17, K26, K91 and K180) gave a negative result (28.58%). While(45) that all his isolates showed a positive result (100%). (46) in Baghdad pointed that 5 isolates out of 53 isolates (9.43%) gave a positive result for this test. (34) reported that the number of isolates that gave a positive result for this test was 7 isolates out of 40 isolates (21.21%).as shown in Fig.2

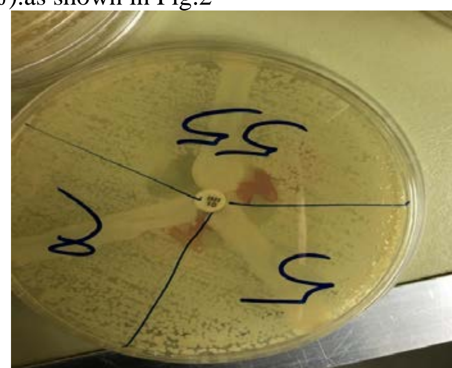

Fig 2: Modified Hodge Test (MHT)

Molecular detection of bla_{NDM-1} gene

Polymerase chain reaction (PCR) assay for detection of bla_{NDM-1} gene was performed for all 14 resistant isolates. (92.85%) of the resistant *Klebsiella pneumoniae* isolates

were positive for bla_{NDM-1} gene. These results are consistent with (47) in Iran, where the number of isolates carrying the bla_{NDM-1} are 27 of 29 isolates were resistant to carbapenim (93.1%). These results were also close to (45) in Baghdad where all 20 isolates were carrying bla_{NDM-1} (100%), while (48) in Turkey found that the percentage of isolates carrying this gene is 20.4%. The isolalt that gave negative result (kp11) may be due to the fact that bla_{NDM-1} is not the gene that responsible for showing the resistance characteristic, many studies suggest that the bla_{-vim} - and bla_{imp-1} gene is a widespread MβL gene in *Enterobacteriaceae* bacteria (49,50,51).

DNA Sequencing

1. DNA Sequencing of 130 PCR amplicons of *Klebsiella pneumonia* isolates

The PCR amplicons of two native isolates were commercially sequenced from forward termini according to instruction manuals of the sequencing company. Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local specimens with the retrieved DNA sequences of *K. pneumoniae* (GenBank acc.CP027612.1), the exact position and other details of the retrieved PCR fragmentswere identified (Fig.4). Included within this genetic fragment, a tRNA encoding gene was found, namely AM475_12260, which occupies 76 nucleotides in length.

Fig 2: Agarose gel electrophoresis in 1% for *bla NDM-1*gene product show positive results(1-2-3-4-5-6-7-8-9-10-12-13- 14) and (11)gave negative resulte. Ethidium bromide stain (0.5%), Amplicon size (621bp), DNA Ladder (100bp), the electric current at 70 volt for 60min.

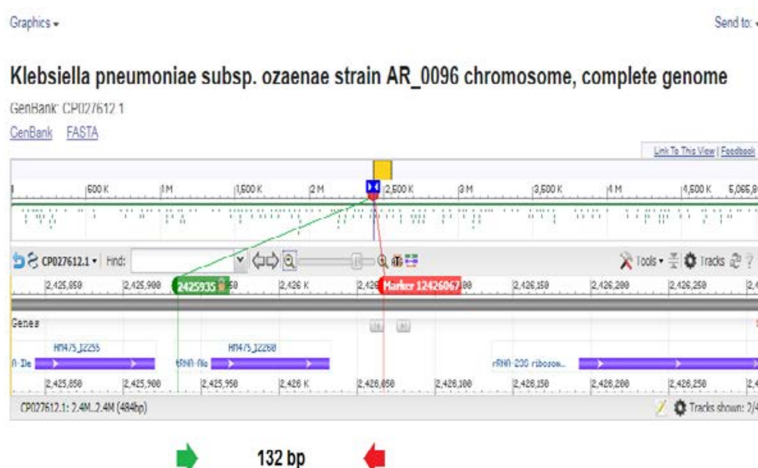

Fig. 4. The exact position of the studied 132bpamplicon within AM475_12260gene in *Klebsiella pneumoniae* sequences (accno.CP027612.1). The green arrow refers to the starting point of this amplicon while the red arrow refers to its end point.

Table 2. The position and length of the PCR amplicon used to amplify AM475_12260gene in*Klebsiellapneumoniae*sequences. The amplified sequence was extended from 2425935into 2426067of the NCBI reference DNA sequence (GenBank acc. no. CP027612.1).The grey colored regions refer to forward and reverse primers respectively.

Amplicon	Referring locus sequences (5′ - 3′)	Length
	ATTTGAAGAGGTTGCAAACGATGGGGCTATAGCTCAGCTGGGAGAGCGCCTGCTTT GCACGCAGGAGGTCTGCGGTTCGATCCCGCATAGCTCCACCATCTTTACTGCGAAC ACAAGAAAACTTCAGAGTGAA	132bp

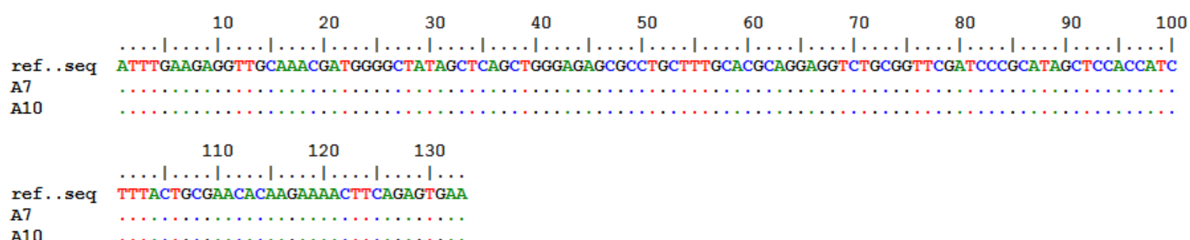

Fig. 5.DNA sequences alignment of the observed local strains with their corresponding reference sequences of the 132bp amplicon of *Klebsiella pneumoniae* sp. sequences. The symbol "ref" refers to the NCBI referring sequence.

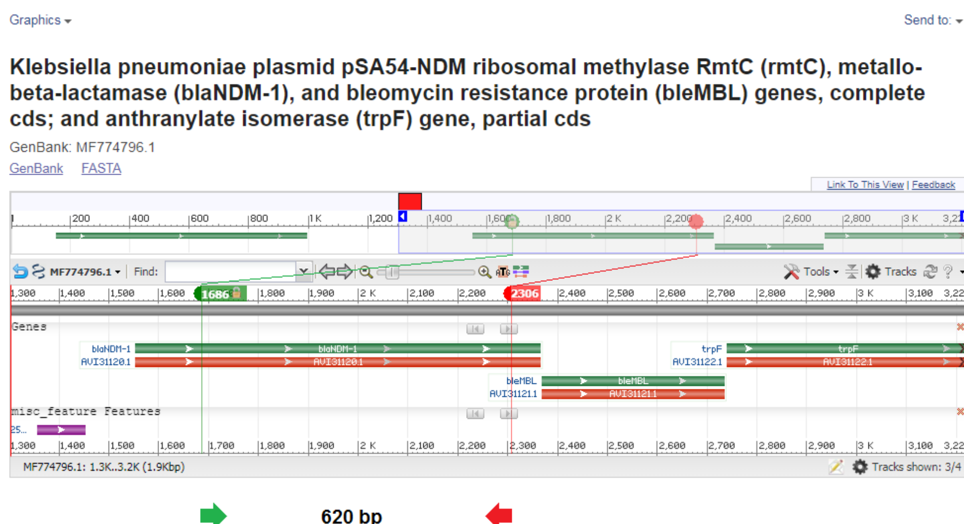

Fig.6. The exact position of the studied 621bpampliconwithin *blaNDM*-1gene in *Klebsiellapneumoniae* sequences (acc no.MF774796.1). The green arrow refers to the starting point of this amplicon while the red arrow refers to its end point.

After positioning the sequences of AM475_12260gene in *K. pneumoniae* sequences sp., the details of its sequences was highlighted (Table 2).

The alignment results of both sequenced samples revealed the absenceof any SNP, thus, both samples did not exert any noticeable variation(s)(Fig.5).

DNA Sequencing of 621 bp PCR amplicons of *Klebsiella pneumonia* isolates

The PCR amplicons of two native isolates were commercially sequenced from forward terminiaccording to instruction manuals of the sequencing company . Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local specimens with the retrieved DNA sequences of *K. pneumoniae* (GenBank acc.MF774796.1), the exact position and other details of the retrieved PCR fragments were identified (Fig.6).

After positioning the sequences of *bla*$_{NDM-1}$ gene in *K. pneumoniae* sequences sp., the details of its sequences was highlighted

It was found that this genetic fragment is included within a metallobeta-lactamase encoding genetic portion. The later protein consists of 270 amino acids, only 206 of them were encoded by this amplicon (Table 3).

Table 3.The amino acid sequences of metallo-beta lactamase that encoded by the amplified *blaNDM*-1 gene in*Klebsiellapneumoniae* sequences.The grey colored regions refer to the encoded amino acids from the studied 621 bp*blaNDM*-1 amplicon.

Amplicon	Referring locus sequences (5′ - 3′)	Length
blaNDM-1	MELPNIMHPVAKLSTALAAALMLSGCMPGEIRPTIGQQMETGDQRFGDLVFR QLAPNVWQHTSYLDMPGFGAVASNGLIVRDGGRVLVVDTAWTDDQTAQILN WIKQEINLPVALAVVTHAHQDKMGGMDALHAAGIATYANALSNQLAPQEGM VAAQHSLTFAANGWVEPATAPNFGPLKVFYPGPGHTSDNITVGIDGTDIAFGG CLIKDSKAKSLGNLGDADTEHYAASARAFGAAFPKASMIVMSHSAPDSRAAIT HTARMADKLR	270bp

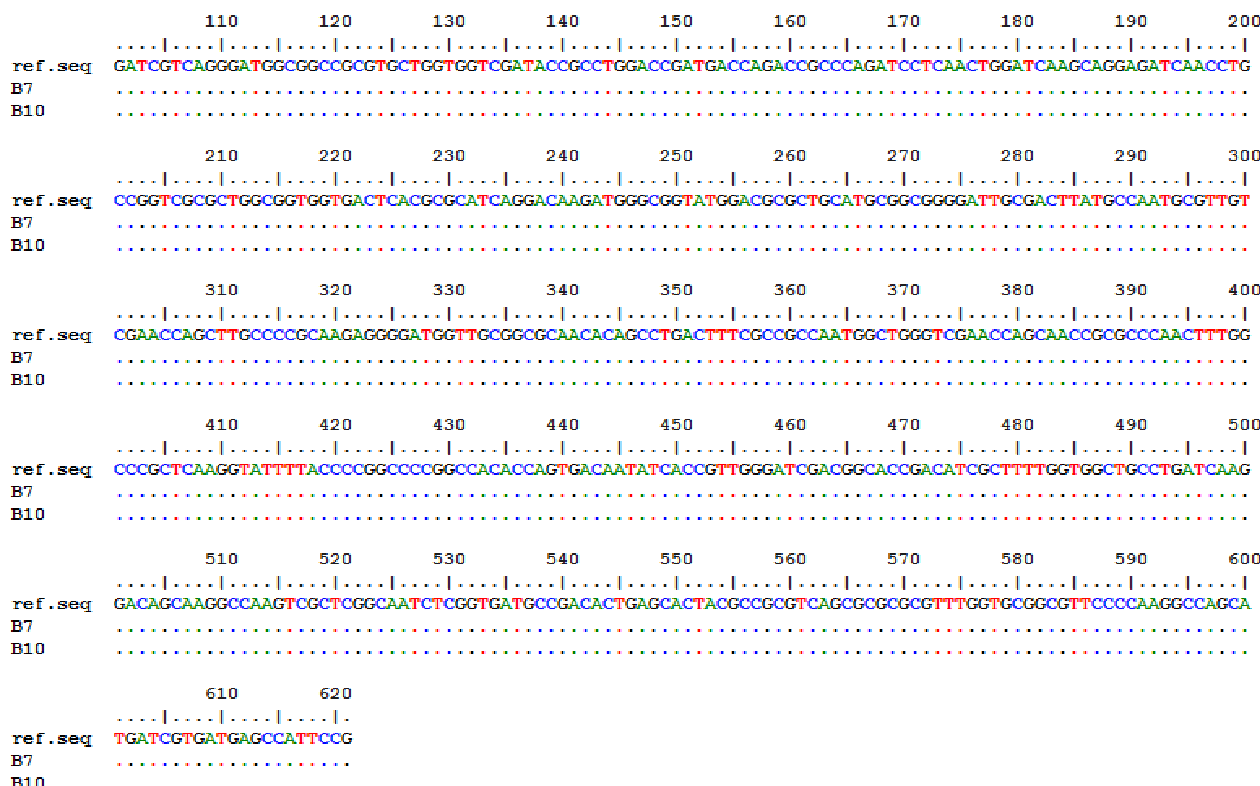

Fig. 7.DNA sequences alignment of the observed local strains with their corresponding reference sequences of the 621bpamplicon of *Klebsiellapneumoniae* sp. sequences. The symbol “ref” refers to the NCBI referring sequence.

The alignment results of both sequenced samples revealed the absence of any SNP, thus, both samples did not exert any noticeable variation(s) (Fig. 7).

Comprehensive phylogenetic tree construction

The current constructed comprehensive tree indicated the presence of at least eighteen species allover scanned of *bla*$_{NDM-1}$ variants sequence – related species. The total number of the aligned nucleic acid sequences, in the current*blaNDM-1* variants based comprehensive tree was 100. In relation to both *blaNDM-1* variants, the comprehensive involved organisms were included; *Klebsiella pneumoniae, Klebsiella michiganesis, Pnatoea agglomerans,Citrobacter freundii,Morganella morganii,Acinetobacterbuamanni,Acinetobacter sp.,Serratia marcescens ,Escherichia coli, Enterobacter sp., Enterobacter cloacae., Enterobacter ludwigii, Enterobacter hormaechei, Enterobacter xiangfangensis, Chryseobacterium indologenes, Stenotrophomonas maltophilia, Salmonella enterica, Proteus mirabilis,* and *Raoultella ornithinolytica* of bacterial species. It was found that both studied *blaNDM-1* variants were occupied a distinctive position within the tree. Despite the absence of any known mutation, both studied *blaNDM-1* variants were occupied a unique characterization within the current constructedphylogenetic tree. This fact is obviously observed in the current constructed comprehensive phylogenetic tree as both variants positioned near seven variable strains of*Klebsiella pneumoniae*in the deposited referring sequences. Regarding the current classified position of the currently constructed tree, the highly association between *Klebsiella pneumoniae* and *Escherichia coli* was suggested from the constructed cladogram in Fig.8. Whereas Fig. 9 was provided another suggestion for such association in terms of the apparent priority for *Klebsiella pneumoniae*then by*Escherichia coli*strains. Followed *Klebsiella pneumoniae*, it was found that occupied a very close position regarding the acc. no.

AP018572.1. This positioning of such isolates wasn't an unusual event since *Escherichia coli* species have belonged to the same enteric family of bacteria (52), and it was found to occupy a close relation with many *Klebsiella pneumoniae* counterparts (53). However, other bacterial species were followed these phylogenetic association accordingly. It's worth mentioning that there is a relatively equal relation amongst the presented bacterial species with each other regarding the *blaNDM-1* gene-based tree. This entitles a universal presence for such genetic fragment in almost all phylogenetically represented bacterial species. though no mutation(s) as observed in both variants, the present PCR-sequencing- comprehensive tree construction strategy has provided an additional unquestionable answer concerning the guaranteed identity of the classically identified isolates. This notion provided a further inclusive indication about the identity of these local studied isolates.

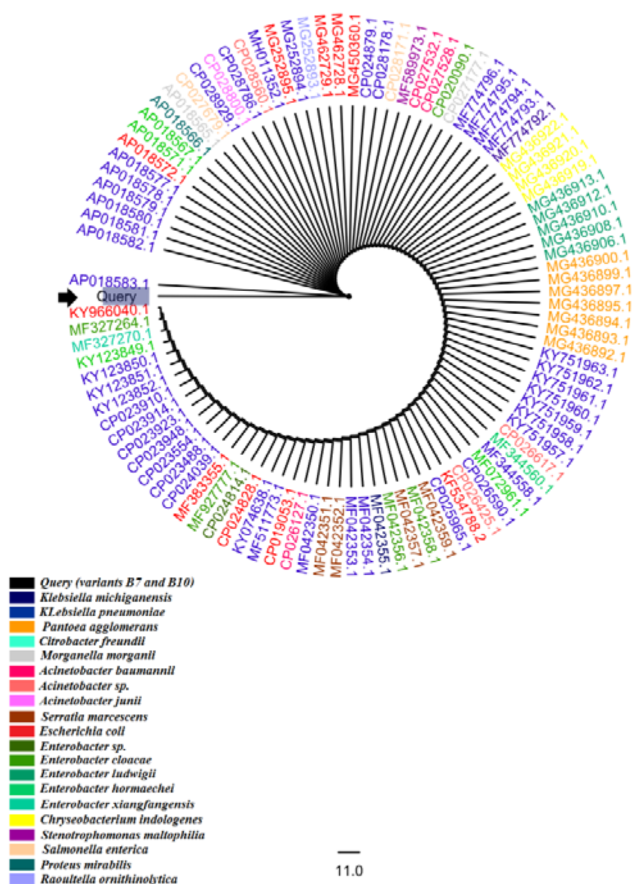

Fig. 8. The comprehensive cladogram phylogenetic tree of the 620bp variants of *blaNDM-1*(*B7-B12*)genetic fragment of *Klebsiella pneumoniae*local isolates. Both black square color and arrow refer to the sequenced two variants, while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species.The number "11.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

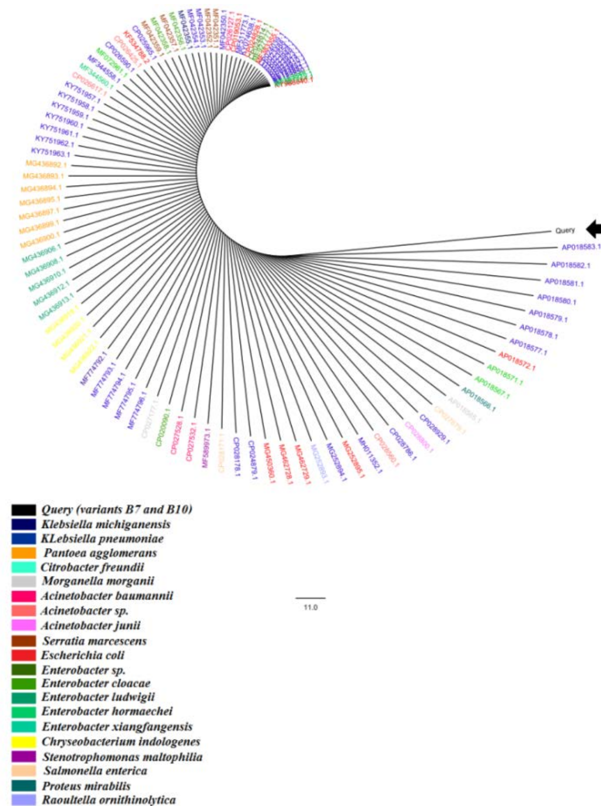

Fig. 9. The comprehensive fish eye phylogenetic tree of the 620bp variants of *blaNDM-1*(*B7-B12*)genetic fragment of *Klebsiella pneumoniae*local isolates. Both black square color and arrow refer to the sequenced two variants, while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to Genbank acc. no. of each referring species.The number "11.0" at the bottom of the tree refers to the degree of scale range among the comprehensive tree categorized organisms.

Nanoparticales

The minimum inhibitory concentration MIC value for ZnO nanoparticles

The minimum inhibitory concentration of 50 nm zinc oxide nanoparticles ranged between (1300-325 µg / m). There are several proposed mechanisms that explain the antimicrobial affect of zinc oxide, such as the synthesis of hydrogen peroxide (H2O2) on the surface of zinc oxide, which inhibits bacterial cell growth (54). Or the release of the Zn^{+2} ion, which can break down the fat and proteins of the bacterial cell membrane, leading to leakage of internal cell components and death(55).

The minimum inhibitory concentration of titanium dioxide

The minimal inhibitory concentration of 25nm titanium dioxide nanoparticles ranged between (2600-650 µg / ml). Antimicrobial affect of TiO2 may be achieved through TiO2 surface reaction with water. After exposure of TiO2 nanoparticles to ultraviolet radiation, free radicals such as OH, O2, HO2 and H2O2, which is a powerful oxidizing force, affect and kill bacteria(56).

The phenotypic detection of metallo-β-lactamase enzymes after the use of nanoparticles

After the use of Zno and Tio2 nanoparticles on 9 isolates that resistant to carbapenem antibiotics, a combined EDTA disk test for subMIC concentrations of both Tio_2 and Zno nanoparticles was used to investigate the nanoparticales affected on the ability of bacteria to produce the metallo beta-lactamase enzyme. The results indicated that 5 isolates out of 9 isolates (Kp2, Kp7, Kp9, 10,Kp12) lost their ability to produce MβL enzymes by Tio_2 and by percentage (55,55%), the inhibiting diameter of the Meropenem-EDTA was increased by 7 mm from the diameter of the inhibition zone of Meropenem alone and 4 isolates (Kp1, Kp5, Kp6, Kp 14) gave negative results and not affected by nanoparticles, as they retained their ability to produce the MβL enzymes even after treatment with nanoparticles. The results of Zno indicated that 5 isolates (55.55%) gave positive results (Kp2, Kp6, Kp7, Kp9, Kp10) and 4 isolates gave negative results (Kp 1, Kp5, Kp12,Kp14).

CONCLUSION

The study had shown the distribution of NDM-1 enzyme in *K. pneumoniae* isolates among patients suffering different diseases. *K. pneumoniae* isolates showed high resistance against various antibiotic groups, especially beta lactam antibiotics. The ability of these enzymes to analyze Carbapenem as well as their ability to analyze a wide range of antibiotics belonging to the group of beta lactam. High accuracy in the diagnosis of *Klebsiella pneumonia* by detecting the the present of 16-23SrRNA using polymerase chain reaction technique. Effect of nanoparticles of zinc oxide and titanium oxides in inhibiting the growth of *Klebsiella pneumoniae* bacteria as well as their effect in the production of MβL enzymes.

REFERENCE

1- **Magill**, S. S.; Edwards, J. R.; Bamberg, W.; Beldavs, Z. G.; Dumyati, G.; Kainer, M.B.; Lynfield, R.; Maloney, M.; McAllister-Hollod, L.; Nadle, J.; Ray, S.M.; Thompson, D.L.; Wilson, L.E., and Fridkin, S.K.(2014). Multistate point-prevalence survey of health care–associated infections.N.Engl.J.Med., 370(13):1198–1208.

2- **Nordmann**, P.; Dortet, L. and Poirel, L. (2012a). Carbapenem resistance in *Enterobacteriaceae*: here is the storm!. Trends in Molecular Medicine, 18(5):263–272.

3- **Nordmann**, P.; Poirel, L. and Dortet, L. (2012b). Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg. Infect. Dis. 18(9): 1503–1507.

4- **Jeon**, J.H.; Lee, J.H.; Lee, J.J.; , Park, K.S.; Karim, A.M.; Lee, C.R.; Jeong, B.C. and Lee, S.H.(2015). Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. Int J Mol Sci., 16(5): 9654–9692.

5- **Mate**, H.; Devi, S.; Devi, M.; Damrolien, S.; Devi, N.L. and Devi, P.P. (2014): Prevalence of carbapenem resistance among Gram-negative bacteria in a tertiary care hospital in north-east India. IOSR Journal of Dental and Medical Sciences.; 13(12): 56–60.

6- **Neslihan**, G.; Sumru, C. and Emel Y. (2011).Virulence properties of extended spectrum β –lactamase producing *Klebsiella* spp in Meat samples. J.Food protection.,74(4):559-564.

7- **González**, R. A.C.; Gil, G. F.; Solórzano, R .M.; Cruz, G. J.; Puig, P. J.; Suárez, S. M. and Nieves, B. B.(2011). Outbreak of multiresistant and extended spectrum β-lactamase producing *Klebsiella pneumoniae* in a high risk neonatal unit. Rev Chilena Infectol., 28(1):28-34.

8- **Barguigua**, A.; El Otmani F, Talmi M, Bourjilat F, Haouzane F, Zerouali K, Timinouni M. (2011) . Characterization of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from community in Morocco. J Med Microbiol. (Abstract) .

9- **Nordman**, P. (2014). Carbpenemase-producing *Enterobacteriaceae*: overview of amajor puplic health challenge. Med Mal Infect.; 44:51-56.

10- **Palzkill**, T.(2013). Metallo-β-lactamase structure and function. Ann. N. Y. Acad. Sci., 1277: 91–104.

11-**Walsh**, T.R.; Toleman, M.A.; Poirel, L. and Nordmann, P. (2005) Metallo-β-lactamases: The quiet before the storm? Clin. Microbiol. Rev., 18(2) 306–325.

12-**Yong**, D.; Toleman, M.A.; Giske, C.G.; Cho, H.S.; Sundman, K.; Lee, K.; Walsh, T.R. (2009). Characterization of a new metallo-β-lactamase gene, *blaNDM-1*, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob. Agents Chemother., 53(12) 5046–5054.

13- **Abdul Ghafur**, K.(2010) . An obituary - On the death of antibiotics. *J. of the association of physicians of India*, 58(3):143-144.

14- **Moellering**, R.C.(2010). NDM-1: A cause for worldwide concern. *New Eng J Med.*, 363(25):2377-2379.

15-**Muir**, A. and abdweinbren, M.J. (2010). New Delhi metallo- beta-Lactamase: acautionary tale. J. Hosp. Infect. 75(3):239-240.

16-**Yu**, B.; Leung, K. M.; Guo, Q.; Lau, W. M. and Yang, J. (2011). Synthesis of Ag-TiO2 composite nano thin film for antimicrobial application. Nanotechnology. 22(11):1-9.

17- **Maurya**, A.; Chauhan, P.; Mishra, A. and Pandey, A. K. (2012). Surface Functionalization of TiO2 with plant extracts and their combined Antimicrobial Activities against E. faecalis and E. coli. Journal of Research updates in polymer Science., 1(1): 43-51.

18-**Roy**, A. S.; Parveen, A.; Koppalkar, A. R.; Ambika Prasad, M.V. N. (2010). Effect of nano-titanium Dioxide with different antibiotics against methicillin-resistant *Staphylococcus aureus*. Journal of Biomaterials and Nanobiotechnology.1(1):37-41.

19- **Thomas**, A.; Shailaja Raj, M. and Venkataramana, J. (2014). Antimicrobial activity of TiO2, Nanoparticles against microbial isolates causing dental plaques. International Journal of Bioassays, 3(06) 3106-3110.

20- **Lalitha**, M. K. (2004). Manual on Antimicrobinal Susceptibility Testing. Under the auspices of Indian Association of Medical Microbiologist.

21- **Solank**, R.; Vanjari, L.; Subramanian, S.; B .A.; E .N. and Lakshmi, V. (2014). Comparative Evaluation of Multiplex PCR and Routine Laboratory Phenotypic Methods for Detection of Carbapenemases among Gram Negative Bacilli. J Clin Diagn Res., 8(12): DC23–26.

22- **CLSI**. (2014) . Performance standards for antimicrobial susceptibility testing twenty-second informational supplement . M100-S24.Clinical Laboratory Standards Institute . 34 (1): 58-172.

23- **Samatha**, P. and Parveen,K.V. (2011). Prevalance of ESBL Ampc B-lactamase in Gram negative clinical isolates. Journals of bioscience and technology .24: 353- 357.

24- **Behera**, B.; Mathur, P.; Das, A.; Kapil, A.; and Sharma, V. (2008). An evaluation of four different phenotypic techniques for detection of metallo -lactamase producing *Pseudomonas aeruginosa*. Indian J Med Microbiol, 263: 233-237.

25- **Supriya**, U.; lay, R. and Amitabha, B. (2010). Presence of different B -lactamase classes among clinical isolates of *Pseudomonas aeruginosa* expressing AmpC -lactamase enzyme. J Infect Dev Ctries., 44: 239-242.

26-**Liu**, Y., Liu, C., Zheng, W., Zhang, X., Yu, J., Gao, Q., Hou, Y. & Huang, X.(2008). PCR detection of Klebsiellapneumoniae in infant formula basedon 16S–23S internal transcribed spacer. Int J Food Microbiol 125, 230–235.

27-**Nordmann** P, Poirel L, Carrër A, Toleman MA, Walsh TR. How To Detect NDM-1 Producers. Journal of Clinical Microbiology. 2011;49(2):718-721. doi:10.1128/JCM.01773-10.

28- **Saginur**, R.; Denis, M.S.; Ferris, W.; Aaron, S.D.; Chan, F.; Lee, C. and Ramotar, K. (2006). Multiple combination bactericidal testing of *Staphylococcal* Biofilms from implant-associated infections. Antimicrobial Agents Chemother, 50(1): 55-61.

29-**Amsterdam**, D. (1996). Susceptibility testing of antimicrobials in liquid media. In: Loman V., ed. Antibiotics in Laboratory Medicine, 4th ed. Williams and Wilkins, Baltimore, MD. p.52-111.

30- **Abd AL-Majed**, B.M.; AL- Talabany, S.S. and AL-Jobory, I.S.(2017). Comparative diagnostic study of *Klebsiella* usingApi20E

System and the device vitek2 and PCR. Kirkuk University Journal /Scientific Studies (KUJSS)., 12(1): 81-95.

31-**AL-Mulla**, H. M. N. ; Mlkunein, A. K. and Shaukat , S.S. (2005). Isolation of *Klebsiella platicola* from clinical infections in Iraq. J. Iraqi for science, 46(1): 125-132.

32- **Jasim**, N.A.(2012). Genetics Detection of Metallic and Extended spectrum Beta- Lactamase production from *Klebsiella pneumoniae* Isolated from different clinical sources. MSc. Thesis. College of Science,

33- **Abdul Razzaq**, M.; Trad, J. and Al-Maamory, E. (2013). Genotyping and detection of some virulence genes of *Klebsiella pneumoniae* isolated from clinical cases. Med. J. of Babylon, 10 (2): 387-399.

34- **AL-Hashimi**, N. K. M.(2013). A bacteriology study for Multidrug Resistant *Klebsiella pneumoniae*. MSc.thesis. College of Science,

35-**Younis**, A.I. ; Elbialy, A.I.; Abo Remila, E.M. and Ammar, A.M. (2017). Molecular Detection of Genus *Klebsiella* and Genotypic Identification of *Klebsiella pneumoniae* and *Klebsiella oxytoca* by Duplex Polymerase Chain Reaction in Poultry. Global Veterinaria, 18 (3): 234-241.

36-**Manikandam**, C. and Amsath, A.(2013). Antibiotic susceptibility of bacterial strains isolated from wound infection patients in Pattukkottai, Tamilnadu India. Int.J.Curr.Microbiol.App.Sci., 2(6): 195-203.

37-**Tilak**, J.D. (2011). Bacterial Resistance to Antibiotics: A Growing Public Health Problem. Commentary; 8(1): 58-61.

38- **Yigit**, H.; Queenan, A.M.; Andeeson, E.J. and Domenech, S. (2001). Novel carbapenem- hydrolyzing Betalactamase, KPC1, from a carbapenem resistant strain of *Klepsiella pneumoniae*. Antimicrobial agents and chemotherapy, 45 (4): 1151-1161.

39-Bajelan, K. H. I.(2014). Biosynthesis of titanium oxide nanoparticles by Lactobacillus spp. and their activity against some bacterial isolates associated with recurrent urinary tract infection in a sample of Iraqi patients. MSC. Thesis, Al-MustansiriyahUniversity, Biology/College of Science.

40-**Al-Dulam**i, T. H. K.(2017). Study *Klebsiella pneumoniae* resistant to antibiotics by use VITEK system from clinical isolates. J. Babylon University, 25(4): 1298-1305.

41-**Llano-Sotelo**, B.; Azucena, E.F.; Korta , L.P.; Mobashery, S. and Chow, C.S. (2002). Aminoglycosides Modified by Resistance Enzymes Display Dimini shed Binding to the Bacterial Ribosomal Aminoacyl-tRNA Site. Chemistry & Biology, 9(4): 455-463.

42- **Aljanaby**, A.A.J. and Alhasan, A.H.A. (2016). Virulence factors and antibiotic susceptibility patterns of multidrug resistance *Klebsiella pneumoniae* isolated from different clinical infections. Afr.J. Microbiol. Res, 10(22): 829-843.

43- **Charan**, J.; Mulla, S. ; Ryavanki, S. and NareshKantharia, N. (2012). New Delhi Metallo – beta lactamase – 1 containing *Enterobacteriaceae*: Origin, Diagnosis, Treatment and Public health concern. *pan african medical journal*, 11(22):1-7.

44-**Wang** , J. F. and Chou, K.C. (2011) . Insights from Modeling the 3D Structure of New Delhi Metallo-b-Lactamse and Its Binding Interactions with Antibiotic Drugs. PLoS ONE */journal.pone*., 6(4):1-7.

45-**Hammoudi**, A. A.; Hussein, A. N. and Jebur M. S.(2016). Detection of blaNDM -Metallo-β-Lactamase Genes in *Klebsiella pneumonia* Strains Isolated From Burn Patients in Baghdad Hospitals. Medical Journal of Babylon; 13(4): 904 – 913.

46- **Rhumaid**, A.K. and Al-Mathkhury, H.J.F. (2015). Detection of blaKPCGene in Some Clinical *Klebsiella pneumoniae* Isolates in Baghdad. Iraqi Journal of Science, 56(4A) : 2853-2861.

47- **Hosseinzadeh**, Z., Ebrahim-Saraie, H. S., Sarvari, J., Mardaneh, J., Dehghani, B., Rokni-Hosseini, S. M. H., & Motamedifar, M. (2018). Emerge of blaNDM-1 and blaOXA-48-like harboring carbapenem-resistant Klebsiella pneumoniae isolates from hospitalized patients in southwestern Iran. *Journal of the Chinese Medical Association*, *81*(6), 536-540.

48-**Ulu**, A. C.; Gökmen, T. G.; Kibar, F.; Kurtaran, B.; Önlen, C.; Kuşçu, F.; İna, A. S.; Kömür, S.; Yaman, A.; Aksu, H. S. Z. and Taşova, Y. (2017).Molecular epidemiology of carbapenem-resistant Klebsiella pneumoniae at a Turkish centre: Is the increase of resistance a threat for Europe?. *Journal of global antimicrobial resistance*, *11*, 10-16.

49- **Galani**, I.; Reketsina, P.D., Hatzaki, D.; Plachouras, D.; Souli, M. and Giamarellou, H. (2008). Evolution of different Laboratory tests for the detection of metallo-*B*-Lactamase. Producing in *Enterobacteriaceas*. Journal of Antimicrobiol chemotherapy., 61(3):548-553.

50- **Nordman**, P. and poirel, L. (2002). Emerging carbapenem in Gram-Negative aerobes . clin .microbiol. Infect., 8(6):321-331.

51- **Peymani**, A.; Nahaei., M-R; FaraJania, s.; hasan. A., miralenian, a.; sohrabi, N. and. abbasi, L. (2011). High. Prevalence of metallo - B-Lactamases. Produce *Acinetobacter baumannii* in a Teaching Hospital in Tabriz, Iran. JPn. J Infect. Dis., 64(1): 69-71.

52-Kim D, Hong JS-J, Qiu Y, Nagarajan H, Seo J-H, Cho B-K, et al. (2012) Comparative Analysis of Regulatory Elements between *Escherichia coli* and *Klebsiella pneumoniae* by Genome-Wide Transcription Start Site Profiling. PLoS Genet; 8(8).

53-McClelland M, Florea L, Sanderson K, Clifton SW, Parkhill J, et al. (2000) Comparison of the Escherichia coli K-12 genome with sampled genomes of a Klebsiella pneumoniae and three salmonella enterica serovars, Typhimurium, Typhi and Paratyphi. Nucleic Acids Res 28: 4974–4986.

54-**Yamamoto**, O. (2011). Influence of particle size on the antibacterial activity of zinc oxide. Int. J. Inorg. Mater., 3(7) 643–646.

55-**Xie**, Y.; He, Y.; Irwin, P. L.; Jin, T. and Shi, X. (2011) Antibacterial activity and mechanism of action of zinc oxide nanoparticles against Campylobacter jejuni. Appl. Environ. Microbiol., 77(7): 2325–2331.

56- **Shiraishi**, K.; Koscki, H.; Tsurumoto, T.; Baba, K.; Naito, M.; Nakayama, K. and Shindo, H. (2008) Antimicrobial metal implant with a TiO2-conferred photocatalytic bactericidal effect against *Staphylococcus aureus*. Surf. Inter. Anal. 41(1): 17-21.