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In vitro Detection of Antibacterial Activity of Glycyrrhizic Acid Nanoparticle against ESBL Producing *Klebsiella pneumoniae* strains

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EXTENDED-spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* strains can present resistance to many antibiotic groups due to resistant genes. This study conducted to detect and identify multi-drug resistant (MDR), ESBL producing *K. pneumoniae* strains from different clinical samples with detection and sequencing of both Temoneira (TEM) and sulfhydryl variable (SHV) genes and using Glycyrrhizic acid nanoparticle as an antimicrobial agent for ESBL producing *K. pneumoniae* strains. One hundred and fifty clinical specimens were processed. ESBL producing *K. pneumoniae* strains were detected by double disk synergy test. TEM and SHV genes responsible for MDR in *K. pneumoniae* were detected by polymerase chain reaction (PCR) and sequence alignment was done using DNA sequencing. The effect of different concentrations of Nano Glycyrrhizic acid was determined. *K. pneumoniae* was detected in 53.3% of the total collected samples (80/150). Seventy one percent (57/80) of them were found to be multi-drug resistant strains and 63% (36/57) also found to contain the ESBL enzymes. Males were highly infected than females. TEM gene was detected in 52.8% of the ESBL isolates while SHV gene was detected in 72.2%. Twenty Five percent of the ESBL producing *K. pneumoniae* was found to contain both TEM and SHV genes. Nucleic acid sequence alignment of both genes showed some mutations. Chloramphenicol was found to be the drug of choice to overcome ESBL producing *K. pneumoniae* with inhibition of 97.2%. The antibacterial activity of Nano Glycyrrhizic acid revealed that 10µg/ml was found to be the minimum bactericidal concentration (MBC) against ESBL producing *K. pneumoniae* isolates.

Keywords: *Klebsiella pneumoniae*, Glycyrrhizic acid, Multidrug-resistance.

Introduction

Klebsiella pneumoniae is considered as the main cause of nosocomial infections among Gram-negative bacteria**,** such as urinary tract, pneumonia, wound, septicemia and bloodstream infections (Arivett et al., 2015). It is very important to investigate the antimicrobial susceptibility pattern of *Klebsiella* in order to prevent the rapid spread of drug resistance (Namratha et al., 2015).

Extended-spectrum β-lactamases (ESBLs), multi-drug resistant *K. pneumoniae* contains virulence factors that cause treatment failure (Mustafa et al., 2017). ESBLs can hydrolyze β-lactam ring in β-lactam drugs by a nucleophilic attack (Papp-Wallace et al., 2011). The plasmids that encode the ESBL genes can also resist to Aminoglycosides (Vuotto et al., 2014).

The antibiotic treatments against *K. pneumoniae* infections contain Aminoglycosides group such as Gentamycin, Quinolones group and β-lactams group such as Carbapenems and Cephalosporins (Qureshi, 2015). Some resistant *K. pneumoniae* strains can form biofilm that resist against β-lactams, Carbapenems, Trimethoprim/Sulfamethoxazole, Aminoglycosides and Fluoroquinolones (Kumar et al., 2011).

Nanoscale particles and molecules are a potential alternative for treatment of disease based on their structure and size, which differ from traditional small-molecule drugs (Wagner et al., 2006). Several pharmaceutical companies have obtained approval from the US Food and Drug

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Administration (FDA) for the development of nanotechnology-based drugs. The global market for medical nanotechnology is expected to reach more than $3 billion (Sahoo et al., 2008).

Glycyrrhizic acid (GA) is obtained from the roots of licorice plants (Glycyrrhiza glabra). It is a triterpene glycoside which presents active pharmacological and antimicrobial activity (Jian-yuan et al., 2014). It was found that Glycyrrhetinic acid can be produced in the human body through metabolic processes. The pharmacological effect of GA is similar to Glycyrrhetinic acid (Yong, 2012). It was found that there are many useful secondary metabolites obtained such as alkaloids, saponins, and flavonoids due to the extraction of a Hydro-methanolic root (crude) from Glycyrrhiza glabra. All of these components are used as anti-bacterial and anti-oxidant ingredients (Sharma et al., 2013).

This study was aimed to detect and identify extended spectrum β-lactamase (ESBL) producing *K. pneumoniae* strains from two hospitals in Egypt. The study is concerned with the identification of specific genes in charge of resistance to a β-lactam antibiotic group and the detection of their sequences. In addition, the effects of some antibiotics from different groups were investigated to overcome ESBL producing *K. pneumoniae,* and also the effect of different concentrations of Glycyrrhizic acid nanoparticle for preventing and controlling ESBL producing *K. pneumoniae* strains.

Materials and Methods

Clinical specimens

One hundred and fifty different clinical specimens were included in this study (eight were from pus samples, thirty from blood samples, twenty from urine samples and ninety two from sputum samples). They were collected from different hospital departments; 120 samples from Intensive Care Unit, Coronary Care Unit, medical laboratory and Surgery department of El-Sadr Hospital and 30 samples from the medical laboratory of National Cancer Institute in Egypt from April 2016 to January 2017. The required documents were submitted and approved according to the committee guidelines showed by Central Directorate of Research and Health Development in Egypt. All samples were processed by standard methods (Cheesbrough, 2000 and Baveja, 2012).

Ages of patients ranged from 20 to 60 years old. Both male and female patients were enrolled in this study to investigate the relationship between age, gender and prevalence of infection with *K. pneumoniae*.

Microbiological analysis

Bacterial identification and confirmation by API 20E test

The samples were cultured on blood agar and MacConkey's agar media (Lab M, United Kingdom) and incubated at 37°C for 24h. All clinical isolates were examined morphologically for colony characteristics on agar media (Archana & Harsh, 2011). *K. pneumoniae* were isolated and purified on MacConkey's agar media. Biochemical standard procedures were used (Cruickshank, 1980). API-20E (BioMerieux, USA) specific test was used to confirm the presence of *K. pneumoniae*.

Antibiotic susceptibility test (disc diffusion)

The isolated *K. pneumoniae* strains were tested against 19 different antibiotics (belonging to nine groups) for their susceptibility. The antibiotic groups include the Penicillins group (Ampicillin 10µg, Piperacillin 100µg and Amoxicillin/ Clavulanic acid 20/10µg), Carbapenems group (Imipenem 10µg and Meropenem 10µg), Monobactam group (Aztreonam 30µg), Cephalosporins group (Cefaclor 30µg, Ceftazidime 30µg, Cefotaxime 30µg, Cefepime 30µg, Ceftriaxone 30µg and Cefoperazone 75µg), Quinolones group (Ciprofloxacin 5µg and Nalidixic acid 30µg), Aminoglycosides (Gentamycin 10µg and Amikacin 30µg), Rifampin 5µg, Chloramphenicol 30µg and Trimethoprim/ Sulphamathoxazole 1.25/23.7µg by Kirby- Bauer disc diffusion method on Mueller-Hinton agar medium (Oxoid, England) and interpreted using Clinical & Laboratory Standards Institute (CLSI) Guidelines (Freeman et al., 2014).

Antibiotic discs were placed on Muller-Hinton agar plates inoculated with 0.5 McFarland inoculum performed from overnight cultured isolates. Plates were incubated at 37ºC for 24h. Inhibition zone diameter was measured and compared to CLSI, 2014 criteria.

Detection of K. pneumoniae strains producing ESBL enzymes (ESBL screening)

The detection of ESBL producing *K. pneumoniae* strains was performed by using the Double Disk Synergy Test (DDST) as described

by Jailer et al. (1988). Mueller Hinton agar plates were inoculated with a standardized inoculum of *K. pneumoniae*. Augmentin (20µg Amoxicillin and 10µg Clavulanic acid) disc was placed at the center of the inoculated plate. Three antibiotics from Cephalosporins 3rd generation group (Cefotaxime 30µg, Ceftriaxone 30µg, Ceftazidime 30µg) and one Monobactam (Aztreonam 30µg) discs were placed at 20mm distance from Augmentin disc. Plates were incubated at 37^{0}C for 24h. Positive results are indicated as an enhancing zone around the three combined antibiotics (Onur & Durak, 2009).

Genotypic characterization

Preparation of genomic DNA and PCR procedures

Total bacterial DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, USA). Template DNA of *K. pneumoniae* was prepared from freshly cultured isolates by culturing on 5ml brain heart broth medium at 37^{0}C for 24h. After centrifugation for 10min at 7500rpm, the precipitate was resuspended in 180µl ATL buffer (tissue lysis) and 20µl proteinase K was added. After incubation at 56°C for 15min and centrifugation for 1min, 200µl buffer AL was added and further incubated at 70°C for 10min, 200µl ethanol 100% were added, then centrifuged for 1min. All microcentrifuge tubes were transferred to spin column, then centrifuged for 1min and discarded the tube containing the filtrate. At the last, 200µl AE (elution buffer) was added, incubated for 1min at room temperature and centrifuged for 1min. Extracted DNA was stored at -20ºC until PCR was performed (Rushdy et al., 2007 modified).

Genotypic detection of bla genes by polymerase chain reaction (PCR)

Molecular detection of bla_{TEM} and bla_{SHV} were performed by using PCR analysis. Specific primers were designed for the detection of these genes (Metabion International AG, Germany) as shown in Table 1.

PCR amplification was performed in 25µl master mix 2X concentration (5µl of 10X PCR buffer, 1.5mM $MgCl_2$, 400µM dNTP, 1 unit Taq DNA polymerase), 2µl of each primer 10pmol/µl (Promega, USA), 5µl of the DNA extracted in a total volume of 50µl with sterile H_2O DEPC treated. The cycling conditions for detection of bla_{TEM} and bla_{SHV} were done by Applied Biosystems Veriti 96-well Thermal Cycler (Puspanadan et al., 2013). The PCR products were subsequently loaded onto 1.5% agarose gel (vivantis, USA) and electrophoresis was performed in 1 X TBE buffer at 100V for about 30min. The gels were then stained with 2µl ethidium bromide 10mg/ml (Sigma, USA). DNA bands were visualized (UVPdual- intensity transilluminator, model: TM-20) at wavelength 312Nm and photographed by UVP-gel documentary system (Rushdy et al., 2007).

Sequencing of DNA fragment

Sequencing was done using "ABI 3730xl DNA sequencer" and Sequence Analysis Software v3.1 in GATC Company, Germany. The sequences and homology of the two genes were done by Basic Local Alignment Search Tool (BLAST) and BLAST nucleotide (BLASTN 2.2.13) software and compared to GenBank database.

Preparation of Glycyrrhizic acid nanoparticle

One mg Glycyrrhizic acid (GA) solution (Xi'an fujie pharmaceutical Co, China) was added to 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (Sigma-Aldrich, USA) and N-hydroxysuccinimide (Sigma-Aldrich, USA), they were dissolved in dimethylformamide (Amresco, USA). 2% chitosan was mixed with acetic acid and precipitated using acetone, then washed with 60% ethanol and ether. Then vacuum drying was done for the final product (Cheng et al., 2014). Nano Glycyrrhizic acid was scanned by scanning electron microscope.

TABLE 1. The sequences of the specific primers used to amplify regions of bla_{TEM} and bla_{SHV} genes.

Primer		Nucleotide Sequences	No. Of primer (bp)	Tm ^{0}C	Product size (bp)
TEM	Forward	CATCGAGCTGGATCTCAACA	20	58^{0}C	478
	Reverse	TTGCCGGGAAGCTAGAGTAA	20	58^{0}C	
SHV	Forward	CTTTCCCATGATGAGCACCT	20	58^{0}C	606
	Reverse	GGGGTATCCCGCAGATAAAT	20	58^{0}C	

Screening for antibacterial activity of different concentrations of Glycyrrhizic acid nanoparticle, a standard inoculum of multi-drug resistant *K. pneumoniae* bacterial isolate was subcultured on Mueller-Hinton agar (Oxoid, UK) and incubated at 37°C for 24h. (Cappuccino & Sherman, 1995).

The lowest concentration of the antimicrobial agent which prevents the visible growth of a microorganism in a broth dilution susceptibility test is defined as minimal inhibitory concentration (MIC), while the minimal bactericidal concentration (MBC) was determined, after determining the results for the MIC, as the lowest concentration that achieved as 99.9% decrease in viable bacterial growth (Rushdy & Othman, 2011). Different dilutions of Glycyrrhizic acid nanoparticles were prepared in 1% Dimethyl sulfoxide (DMSO) (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100µg). Wells were made using sterile borer and were loaded with 0.45µl of each concentration, then placed in incubator at 37^0C overnight. The diameter of the inhibition zones was taken as a measure of the antibacterial activity (Othman & Hussein, 2015).

Two tubes (one contained 2ml brain heart broth, 20µl bacterial suspension and 50µl Glycyrrhizic acid nanoparticles while the other tube was the control without Glycyrrhizic acid nanoparticles) were incubated in duplicates at 37°C for 24h. Bacterial growth was measured on spectrophotometer (T80 UV/VIS Spectrometer, United Kingdom) by optical density at 600nm.

Results

Eighty isolates out of the 150 (53.3%) collected from two different hospitals were identified as *K. pneumoniae* in the period from April 2016 to January 2017. Four were isolated from pus, 15 from blood, 10 from urine and 51 from sputum samples. These isolates were first morphologically identified as large dome-shaped colonies on Blood, lactose fermenting mucoid colonies on MacConkey agar plates. Biochemical characteristics of these isolates were performed by analytical profile index (API 20E) where positivity for o-nitrophenyl-β-D-galactoside, Lysine Decarboxylase, Citrate Test, Urease Test, Voges-Proskauer Test and sugar (Glucose, Sorbitol, Mannitol,Rhamnose, Inositol, Sucrose, Arabinose, Melibiose and Amygdalin) fermentation. The clinical specimens collected from different hospital units are shown in Table 2.

TABLE 2. The frequency of *K. pneumoniae* in hospital units.

Hospital unit	Numbers	Percentage (%)
Surgery	16	20.0
Laboratory	39	48.75
Coronary Care	5	6.25
Intensive Care	20	25.0
Total	80	100

Fifty isolates (62%) out of 80 *Klebsiella pneumoniae* were from males and 30 (38%) were from females. Figure 1 shows the distribution of *Klebsiella pneumoniae* infection in different age groups and Fig. 2 shows the distribution of *Klebsiella pneumoniae* infection in relation to patient sex and type of specimen.

The antibiotic susceptibility testing of the eighty *K. pneumoniae* isolates showed high susceptibility to Carbapenems (Imipenem 88.75% and Meropenem 88.75), Chloramphenicol 96.25%, Aminoglycosides (Amikacin 68.75% and Gentamycin 73.75%), Trimethoprim/ Sulphamathoxazole 62.5% and Quinolones (Ciprofloxacin 60%). On the other hand, *K. pneumoniae* strains were found to be highly resistant to Penicillin group (Ampicillin 86.25%, Piperacillin 62.5% and Amoxicillin/ Clavulanic acid 81.25%), Cephalosporins group (Cefaclor85%, Ceftazidime 92.5% and Ceftriaxone 100%), Quinolones group (Nalidixic acid 68.75%) and Rifampin 100% (Fig. 3).

Seventy one percent (57/80) isolates of *K. pneumoniae* were found to be multi-drug resistant strains and 63% (36/57) also found to contain the ESBL enzymes, so they were tested by double disk synergy test for the production of extended-spectrum β-lactamases (Table 3 and Fig. 4). The figure illustrates double-disk synergy test for amoxicillin/clavulanic acid 20/10µg, ceftazidime 30µg, cefotaxime 30µg, ceftriaxone 30µg and aztreonam 30µg.

Genotypic analysis for bla_{TEM} and bla_{SHV} genes by polymerase chain reaction. The 36 multi-drug resistant *K. pneumoniae* isolates which were ESBL positive subjected to the detection of TEM and SHV genes. Genotypic characterization by PCR revealed presence of bla_{TEM} and bla_{SHV} genes at 478bp and 606bp, respectively (Fig. 5 and 6).

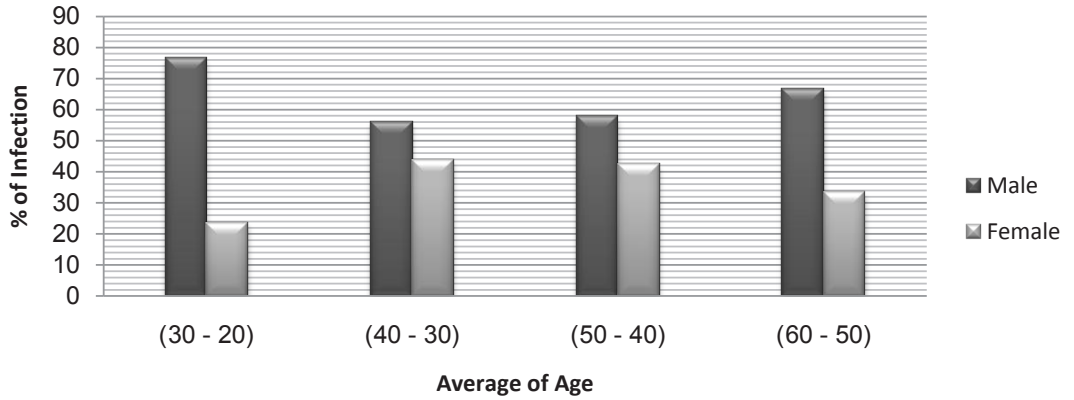

Fig. 1. Prevalence of *K. pneumoniae* in clinical samples with co-relation to patient age.

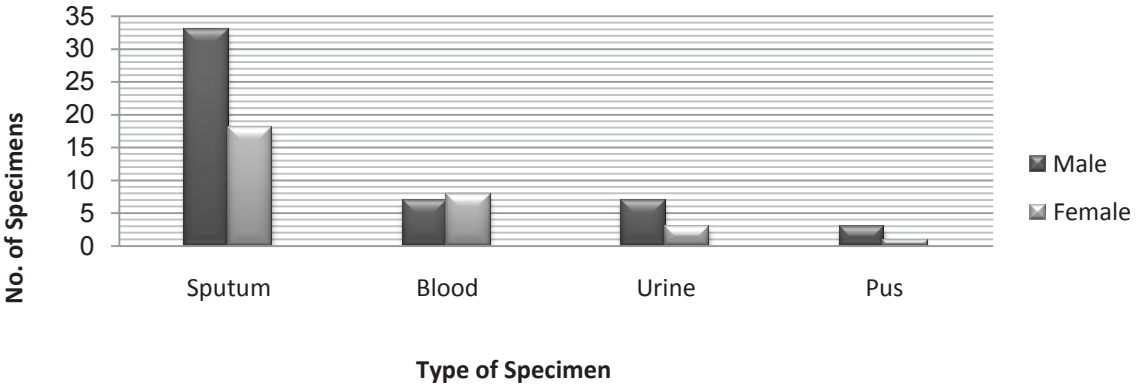

Fig. 2. Prevalence of *K. pneumonie* in clinical samples with co-relation to patient sex and type of specimen.

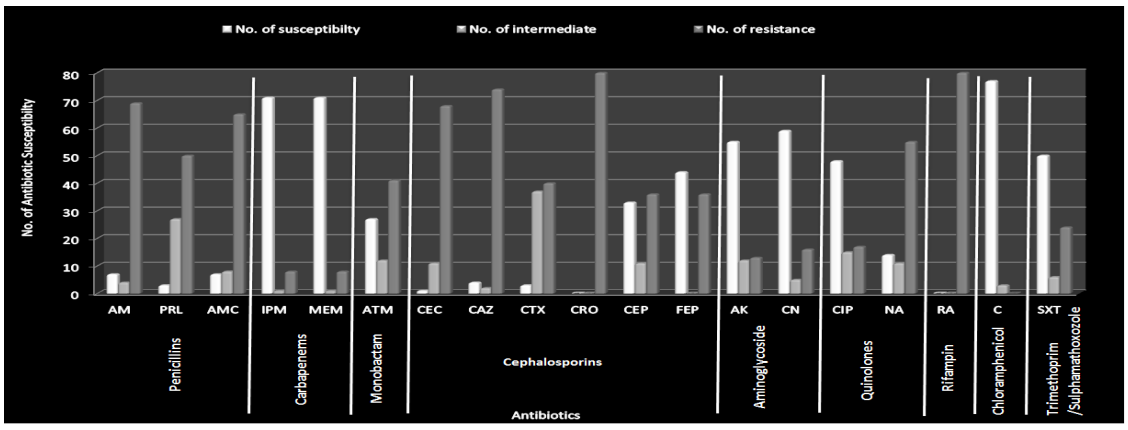

Fig. 3. Number of susceptible (S), intermediate (I) and resistance (R) *Klebsiella pneumoniae* isolates against different antibiotic groups.

Nineteen isolates out of 36 (52.8%) harbored TEM geneand 26 isolates (72.2%) harbored SHV gene, while nine isolates out of the 36 ESBL producing *K. pneumoniae* isolates (25%) harbored both genes.

TABLE 3. Detection of multi-drug resistant *K. pneumoniae* contain ESBL enzymes.

Antibiotic susceptibility	Penicillins			Carbapenems		Monob-actam	Cephalosporins						Aminoglyco-side		Quinolones		Rifampin	Chloram-phenicol	Trimethoprim /Sulphamathoxozole
	AM	PRL	AMC	IPM	MEM	ATM	CEC	CAZ	CTX	CRO	CEP	FEP	AK	CN	CIP	NA	RA	C	SXT
No. of susceptible isolates	1	0.0	0.0	27	27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	15	18	6	5	0.0	35	8
% of susceptibility	2.8	0.0	0.0	75.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	41.7	50	16.7	13.9	0.0	97.2	22.2
No. of intermediate isolates	0.0	1	3	1	1	1	0.0	0.0	0.0	0.0	0.0	0.0	9	3	14	12	0.0	1	6
% of intermediate	0.0	2.8	8.3	2.8	2.8	2.8	0.0	0.0	0.0	0.0	0.0	0.0	25	8.3	38.9	33.3	0.0	2.8	16.7
No. of resistant isolates	35	35	33	8	8	35	36	36	36	36	36	36	12	15	16	19	36	0.0	22
% of resistance	97.2	97.2	91.7	22.2	22.2	97.2	100	100	100	100	100	100	33.3	41.7	44.4	52.8	100	0.0	61.1

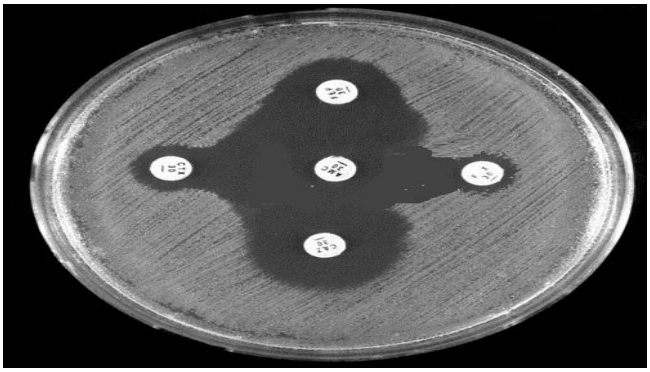

Fig. 4. Double-disk synergy test for detection of *K. pneumoniae* producing ESBL enzyme.

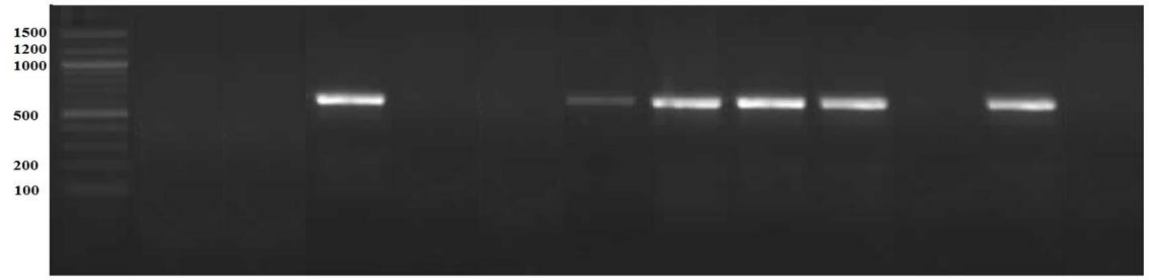

Fig. 5. Agarose gel (1.5%) electrophoresis of amplified 478bp DNA fragment TEM gene of *K. pneumoniae.*

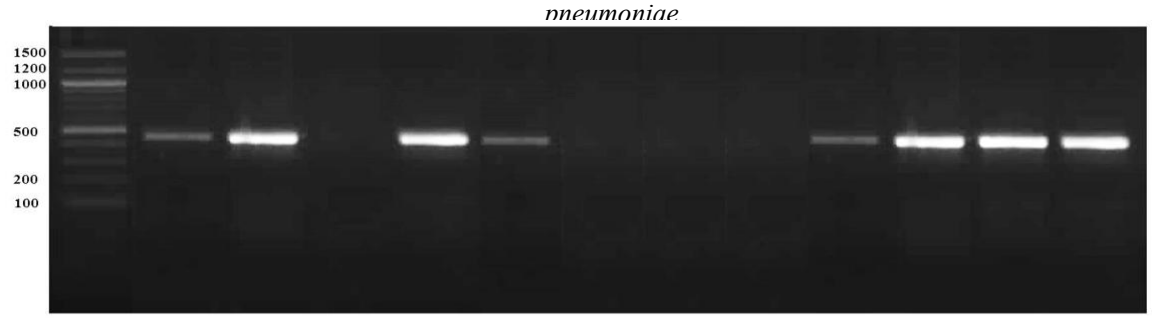

Fig. 6. Agarose gel (1.5%) electrophoresis of amplified 606bp DNA fragment SHV gene of *K. pneumoniae.*

Nucleic acid sequencealignment of bla_{TEM} and bla_{SHV} genes for the Egyptian *K. pneumoniae* isolates was done on gene bank database on the World Wide Web and revealed the presence of some mutations. Table 4 illustrated three types of mutations in bla_{TEM} including deletion, substitution and insertion, while in case of bla_{SHV} gene there were two types of mutations including deletion and substitution (Table 5).

Effect of Glycyrrhizic acid nanoparticles on ESBL producing *Klebsiella pneumoniae,* different concentrations of Glycyrrhizic acid nano-particle were tested for their antibacterial activity on three ESBL producing *K. pneumoniae* isolates, one contains TEM gene, one contains SHV gene and one contains both genes. Nano Glycyrrhizic acid was scanned by scanning electron microscope (Fig. 7).

The Table 6 shows that the minimal bactericidal concentration (MBC) for the bacterial isolates which contain both TEM or SHV genes and that containing both genes was 10µg/ml Nano Glycyrrhizic acid. The minimal inhibitory concentration (MIC) for the isolate that contains TEM gene was 20µg/ml and that for the isolate contains SHV gene was 40µg/ml while that for the isolate containing both genes was 30µg/ml.

TABLE 4. Type of mutations detected in a complex region of TEM gene of Egyptian isolates of *K. pneumoniae* compared to the same region of *K. pneumoniae* included in the GenBank database.

Position	Type of mutation			
(Query)	Deletion	Substitution		Insertion
		Inversion	Transversion	
436	-T			
437	-A			
438		A→G		
442				+C
443				+C
452			A→C	

TABLE 5. Type of mutations detected in a complex region of SHV gene of Egyptian isolates of *K. pneumoniae* compared to the same region of *K. pneumoniae* included in the GenBank database.

Position	Type of mutation		
(Query)	Deletion	Substitution	
		Inversion	Transversion
100			T→G
152		T→C	
497		G→A	
524		T→C	
568	-C		
571			G→C
576			A→T
578			A→C

TABLE 6. Antibacterial activity of different concentrations of Glycyrrhizic acid nano particle on ESBL producing *K. pneumoniae* isolates.

Concentrations of Glycyrrhizic acid nanoparticle (µg/ml)	Measuring on spectrophotometer at 600nm (optical density)		
	ESBL producing *K. pneumoniae* have TEM gene	ESBL producing *K. pneumoniae* have SHV gene	ESBL producing *K. pneumoniae* have both TEM, SHV genes
10	0.057 (MBC)	0.077 (MBC)	0.069 (MBC)
20	0.073 (MIC)	0.086	0.072
30	0.200	0.088	0.082 (MIC)
40	0.084	0.092 (MIC)	0.099
50	0.118	0.357	0.083
60	0.096	0.104	0.081
70	0.107	0.113	0.090
80	0.570	0.111	0.105
90	0.167	0.160	0.296
100	0.108	0.435	0.256

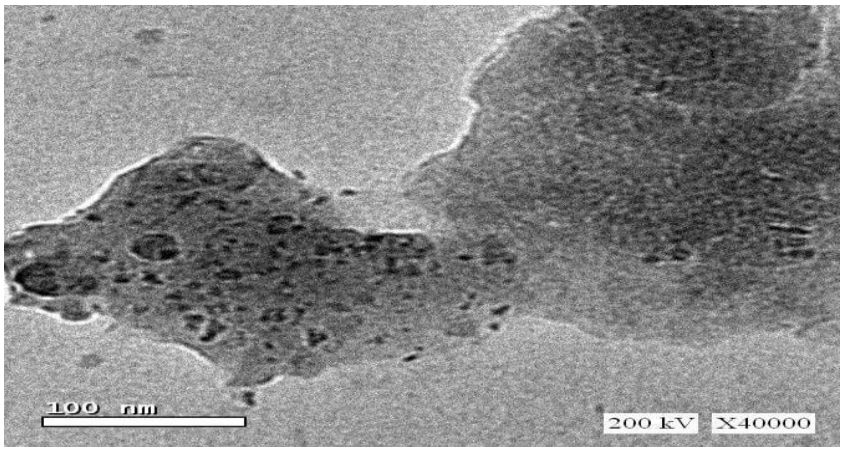

Fig. 7. Scanning electron microscope image of the nano Glycyrrhizic acid, a spherical in shape.

Discussion

The present study reveals that *K. pneumoniae* was detected in 53.3% of the total collected samples. Seventy one percent of them were found to be multi-drug resistant strains. Conversely to this result, Archana & Harsh (2011) illustrated that11.8% confirmed *K. pneumoniae* isolates were further tested for antimicrobial drug sensitivity and almost fifty percent of them were found to be multidrug resistant. A possible subtle difference with 'non-Egyptian' isolates of *K. pneumonia* that patients neglect the time and right dose of treatment and take medication without consulting the doctor until bacterial genes develop resistance.

In the present study, *Klebsiella pneumoniae* isolates was predominant in males (62%) than in females (38%). *K. pneumoniae* infection seen in patients aged 20-60. Conversely, to this result Shashidhar Vishwanath et al. (2013) illustrated that the high percentage of infection in patients admitted to Iran hospital were females with a percentage (45.45%) than males (31.57%), *Klebsiella pneumoniae* infection seen in persons aged 45-60. This may be due to the Egyptian Traditions is that females are used to stay at home so they are protected from infection while males are responsible for working and may have bad habits like smoking.

The current study indicated that non ESBL producing *K. pneumoniae* isolates were found to be highly susceptible to Carbapenems (imipenem 88.75% and meropenem 88.75), chloramphenicol 96.25%, Aminoglycosides (amikacin 68.75% and gentamycin 73.75%), Trimethoprim/ Sulphamathoxazole 62.5% and Quinolones (ciprofloxacin 60%). On the other hand, ESBL producing *K. pneumoniae* isolates were found to be highly susceptible to Carbapenems (imipenem 75.0% and meropenem 75.0), chloramphenicol 91.7% and aminoglycosides (amikacin 41.7% and gentamycin 50.0%).

In previous studies, *K. pneumoniae* strains were found to be highly susceptible to aminoglycosides and quinolones. On the other hand over 60% strains were resistant to chloramphenicol, tetracycline and cephalosporins (ceftizoxime and cefotaxime). Cephalosporins were the drug of choice in combination with aminoglycosides to treat *Klebsiella* infection (Archana & Harsh, 2011). Carbapenems are stable in the presence of hydrolytic effects of ESBLs, which may explain the consistent finding that >98% of ESBL-producing organisms retain susceptibility to either imipenem or meropenem (Babini & Livermore, 2000; Goossens, 2001 and Winokur et al., 2001).

The present study showed that 36 isolates of *K. pneumoniae* out of eighty bacterial isolates were found to be ESBL producing *K. pneumoniae* (sputum n=26 and blood n=10). They also showed a positive result for double disk synergy test for the production of extended spectrum β-lactamases. These ESBL multi-drug resistant *K. pneumoniae* were found to be highly resistance to Penicillins group (Ampicillin 97.2%, Piperacillin 97.2% and Amoxicillin/Clavulanic acid 91.7%), Monobactam group (Aztreonam 97.2%), Cephalosporins group (2nd generation 100%, 3rd generation 100% and 4th generation

100%), Rifampin 100%, Quinolones group (Ciprofloxacin 44.4% and Nalidixic acid 52.8%) and Trimethoprim /Sulphamathoxozole 61.1%. Positive ESBLs are predominantly responsible for drug resistance to β-lactam antibiotics (Liu et al., 2014 and Lahlaoui et al., 2014). The chromosomally encoded β- lactamases could be responsible for this intrinsic resistance (Sahly et al., 2004). Overall resistance was high on account of the production of extended spectrum β-lactamases (ESBLs) by the *K. pneumoniae*.

ESBLs are grouped into four classes A, B, C and D enzymes. Temoneira (TEM) and sulfhydryl variable (SHV) are class A ESBLs (Shahid et al., 2011). The most common ESBLs observed in the isolated *K. pneumoniae* plasmids are encoding Temoniera (TEM) and Sulfhydryl variable (SVH), which are active against Cephalosporins (Vuotto et al., 2014) so in the present study, PCR was performed to determine the presence of TEM and SHV genes as antibiotic resistance factors of *K. pneumoniae*.

Plasmids encoding Temoniera (TEM) and Sulfhydryl variable (SVH) ESBLs are the most common to be found in isolated *K. pneumoniae,* which are active against cephaloporins. The plasmids that encode the ESBL genes also have been found to carry genes that express resistance for drugs other than beta-lactams, such as aminoglycosides (Vuotto et al., 2014). So in this study the detection TEM and SHV genes was used as a mark of multi-drug resistant in *K. pneumoniae*.

The results indicated that 52.8% contained the TEM gene and 72.2% contained the SHV gene, while 25% of the multi-drug resistant isolated has both genes (TEM and SHV). Multidrug-resistant (MDR) and extended-spectrum β-lactamase producing *Klebsiella pneumoniae* pose serious antibiotic management problem as resistance genes are easily transferred from one organism to another (Lim et al., 2009).

Fouzia & Damle (2015) made a study on the amplification of bla_{TEM} and bla_{SHV} and she found that seventy percent of isolates showed a presence of TEM gene, while 50% isolates showed a presence of SHV gene and 20% isolates had both bla_{TEM} and bla_{SHV} genes.

In a study by Amita Jain & Rajesh Mondal using the same set of primers, they found a presence of bla_{TEM} gene in ESBL producing *Klebsiella* sp. as more common (48.4 %) than bla_{SHV} (20.3%) gene. while (26.5%) isolates presented both TEM and SHV genes (Jain & Mondal, 2008).

The present study revealed that sequencing alignment of bla_{TEM} and bla_{SHV} genes showed some mutations in both genes such as deletion and substitution in both genes and insertion in TEM gene only. The mutation presented in both genes may be due to the excess or improper use of antibiotics in Egypt.

Glycyrrhizic acid interferes with arylamine N-acetyltransferase activity in bacteria, thus showing antibacterial effects against *Klebsiella* spp. (Tanaka et al., 2001 and Krausse et al., 2004).

Glycyrrhizic acid should be considered, one of the proposed chemopreventive drugs, that could inhibit arylamine N-acetyltransferase (NAT) activity in *Klebsiella pneumoniae*. The NAT activity in *K. pneumoniae* was inhibited by Glycyrrhizic acid in a dose-dependent manner (Hsueh-Hsia et al., 1997).

Conclusion

The present study showed that nano Glycyrrhizic acid was found to be highly effective on MDR *K. pneumoniae*. The minimal inhibitory concentration (MIC) for the isolate that contains TEM gene was 20% and that for the isolate contains SHV gene was 40% while that for the isolate containing both genes was 30%.

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الكشف المعملي لتأثير جزيئات النانو لحمض الجليسريزك ضد بكتيريا الكليبسيلة الرئوية المنتجه لانزيم البيتا لاكتام الممتد المفعول

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تعد سلالات الكليبسيلة الرئوية المنتجة لإنزيم البيتا لاكتام الممتد المفعول من السلالات المقاومة لكثير من مجموعات المضادات الحيوية وذلك لإحتوائهم على جينات مسئولة عن تعدد المقاومة للمضادات الحيوية. الهدف الرئيسى من البحث عزل وتعريف سلالات الكليبسيلة الرئوية المتعددة المقاومة للمضادات الحيوية من عزلات سريرية مختلفة مع تعريف وتحديد التتابع النيوكليوتيدى للجينين TEM وSHV المسئولين عن هذه المقاومة كما تهدف الرسالة لتحديد تأثير جزيئات النانو لحمض الجليسيريزيك فى معالجة هذه العزلات المتعددة المقاومة للمضادات الحيوية وخاصة التى تحتوى على انزيم البيتا لاكتام الممتد المفعول. تم تجميع 150 عزلة من مصادر مختلفة وتم تعريف 80 عزلة منهم كليبسيلة الرئوية بنسبة 53.3% . وبدراسة اختبار المضادات الحيوية على 80 عزلة كليبسيلة الرئوية بطريقة الأنتشار القرصى وتحديد وجود انزيم البيتا لاكتام ممتد المفعول بطريقة انتشار الأقراص الثنائى وجد ان عدد 57/80 عزلة بنسبة 71% متعددة المقاومة للمضادات الحيوية وعدد 36/57 عزلة بنسبة 63% تحتوى على هذا الأنزيم. ثم تم تحديد وجود جينين TEM وSHV بهذه العزلات 36/57 باستخدام تفاعل البلمرة المتسلسل. اوضحت النتيجة ان 52.8% تحتوى TEM جين, 72.2% تحتوى SHV جين بينما 25% كانت تحتوى جينين. تم عمل التتابع النيوكليوتيدى لهذين الجينين ومقارنتهم ببنك الجينات فوجد أنه هناك بعض الإختلاف ما بين اختفاء واحلال وتبديل فى تتابع الجينين. وقد اظهر الكلورمفينكول تأثير جيد على العزلات التى بها انزيم البيتا لاكتام الممتد المفعول والمتعدد المقاومة للعديد من المضادات الحيوية بنسبة 97.2%. تم دراسة تأثير الجسيمات النانوية لحمض الجليسريزيك على الكليبسيلة الرئوية التى تحتوى انزيم البيتا لاكتام الممتد المفعول باستخدام تركيزات مختلفة. وكشف النشاط المضاد للبكتيريا من جزيئات النانو لحمض الجليسريزيك 10 ميكروجرام/ مل هو اقل تركيز قاتل للبكتريا الكليبسيلة الرئوية التى تحتوى انزيم البيتا لاكتام الممتد المفعول.