



Research article

Detection and identification of the extended- spectrum β -lactamases (*bla* SHV and *bla* CTX –M) *Klebsiella pneumonia* by PCR technique

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(Received 11/7/2017, Accepted 19/10/2017)

Abstract

Klebsiella pneumonia was the most important nosocomial infections pathogen. It was causing several morbidity and mortality in sick animals and human. Its identification and detection performed by usage of conventional cultural characters, biochemical tests and Polymerase chain reaction technique (PCR). One-hundred clinical samples were divided into (50) samples of sheep suffering from pneumonia and (50) samples of human with UTI, collected from different regions of Al-Diwaniyah city. Thirty-four (68%) sheep samples were positive for *K. pneumonia* identification, while 38 (76%) urine samples of a human with UTI cases gave *K. pneumoniae* isolates. The results show only (72) isolates were identification by PCR technique. Thirty isolates of human samples (78.9%) were positive for detection of *bla* CTX-M ESBLs, while its detection did not determine in sheep. Sixty-three isolates from total isolates were positive to detection of *bla* SHV, these positive isolates divided into 53 isolate for a human and 28 sheep. Molecular characterization of ESBL provide information about the prevalence of ESBL producing *K. pneumoniae* in Al-Diwaniyah. The aim of this study was determination of CTX-M and SHV genes presence in extended-spectrum β -lactamase (ESBL) producing *Klebsiella pneumoniae*.

Keywords: ESBL, *Klebsiella pneumonia*, CTX-M gene, SHV gene, Polymerase change reaction, Drug resistance

Introduction

One of the most common nosocomial bacteria is *Klebsiella pneumonia*. It was causing several morbidity and mortality in a sick animals and human. Chromosomally encoded SHV-1 β -lactamase presenting the most *K. pneumoniae* isolates (1). Since 1983, Enterobacteriaceae, especially in *Klebsiella* spp have plasmid-encoded extended-spectrum β -lactamases (ESBLs) derived from the CTX and SHV families (2, 3). Scarcely, we can report *K. pneumoniae* producing two or more ESBLs, but commonly they include - SHV and/or CTX -derived enzymes (4, 5). In 1983, *Klebsiella pneumoniae* with ESBLs were first reported in Germany (6). The most common mechanism of antibiotic resistance to beta

lactam antibiotics was synthesis of beta lactamase. Aerobic Gram positive, Gram negative and anaerobes bacteria can produce these enzymes (7). The name of these enzymes called extended- spectrum β -lactamases (ESBLs) Because of their broad spectrum of activity, especially against the oxyimino-cephalosporins (8). The mutations of CTX and SHV genes were responsible for producing these enzymes in family Enterobacteriaceae especially *Klebsiella pneumonia* (9). *Klebsiella* species were difficult to eradicate due to rapidly become the most common ESBL producing organism. (9)The genus *Klebsiella* in particular *Klebsiella pneumoniae*, frequently cause



human infections the accounts for a significant proportion of urinary tract infections, pneumonia, septicemias, and soft tissue infections (10). ESBLs enzymes have capacity to hydrolysis of penicillins, broadspectrum cephalosporins monobactams, and generally derived from CTX and SHV type enzymes (11). The aims of study were identification of *Klebsiella pneumonia* by using specific primer of PCR and detection extended- spectrum β -lactamases (ESBLs) of *bla* SHV and *bla* CTX -M

Materials and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 374

Sample collection:

Fifty urine samples were collected from human suffering from urinary infection in Al-Diwaniyah teaching hospital and fifty nasal swap samples were collected from sheep suffering from respiratory infection. All samples were cultured on MacConkey's and blood agar plates and incubated for 24 hours at 37°C according to standard procedure (12). The isolates were activated by inoculated on Chromagar Orientation and incubated at 37°C for overnight, Identification of isolates based on morphology of colonies and by biochemical tests according to MacFaddin (13).

DNA extraction

Fresh bacterial genomic DNA of *K. pneumonia* was extracted from 1ml nutrient broth samples in 1.5ml microcentrifuge tubes by using (Presto TM mini g DNA Bacteria Kit, Geneaid .USA) , the extract gDNA was checked by nanodrop spectrophotometer and store in -20°C at refrigerator until usage.

Detection of *K. pneumonia*, SHV and CTX genes by PCR reaction

PCR reaction was used to detect 16S rRNA as identification of *K. pneumonia*, SHV and CTX genes in the multidrug resistance bacterial strain. PCR was performed by use, 5 μ l of the template DNA, 12 PCR water Bioneer (South Korea). Amplification was carried out in thermocycler (Eppendorf mastercycler ®) (bioneer-south korea). Agarose gel electrophoresis (1.5%) of PCR products was carried out using mM Tris-Borate- EDTA(TBE) buffer at 70V for 2hour, and the DNA bands were stained with ethidium bromid (sinaclon iran) 100bp DNA ladder was used to confirm the size specific. To detect 16S rRNA as diagnosis of *K. pneumonia*, CTX and SHV of , PCR reactions performed in a total volume of 25 μ l containing 2 μ l of the DNA sample, 1 μ m of each primers, 2 μ m Magnesium chloride (MgCl₂), 5 mL of 10 _ PCR buffer AMS, 200 μ m dNTPs, and 1 unit of Taq DNA polymerase (CinnaGen Co., Tehran, Iran). The PCR assay was performed at 95°C for 5 minutes and then for 35 cycles of 94°C for 30 second , 60°C for 40 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes, with a final hold at 4°C in a thermal cycler (Thermo cycler; Eppendorf, Germany). For SHV and CTX amplifications, conditions for thermal cycling remained the same except for the annealing temperature (57°C). The primer sequences for 16S rRNA as diagnosis of *K. pneumonia* CTX and SHV are shown in Table (1). Agarose gel 1.5% was used to run the amplified products and staining with ethidium bromide (3 μ l) in a darkness. The electrode buffer used was Tris-borate- EDTA (TBE), which consists of Tris-base 10.8 g 89 mM, boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 mL of 0.5 M EDTA (pH 8.0) (all components were combined in sufficient H₂O and stirred to dissolve). A 100-bp ladder molecular weight marker (Roche, New Jersey, USA) was used to measure the molecular weight of the amplified products. Aliquots (10 μ l) of PCR products were applied to the gel. A constant voltage of 84 V for 1 hour used for product separation. The images of ethidium bromide stained DNA bands were digitized using an UVItec (UVItec, Paisley, UK).



Table (1): The primer sequences for 16S rRNA as diagnosis of *K. pneumonia* CTX and SHV

Primer	Sequence		Amplification
KP-16S rRNA, strain K-18	F	GGAAGTACACACGGTCCAG	770 bp
	R	CCAGGTAAGGTTCTTCGCGT	
<i>bla</i> _{SHV}	F	ATGCGTTATATTCGCCTGTG	753 bp
	R	TGCTTTGTTATTCGGCCAA	
<i>bla</i> _{CTX-M}	F	CGCTTTCGCATGTGCAG	550bp
	R	ACCGCGATATCGTTGGT	

Results

Isolation and identification:

Cultural characters of *K. pneumonia* growth on MacConkey and Orientation media showed in table (1). Pink, mucoid, lactose fermented colonies were considered to be *Klebsiella* spp on MacConkey agar while on orientation medium, colonies is metallic blue color, large, rounded Figure (1).

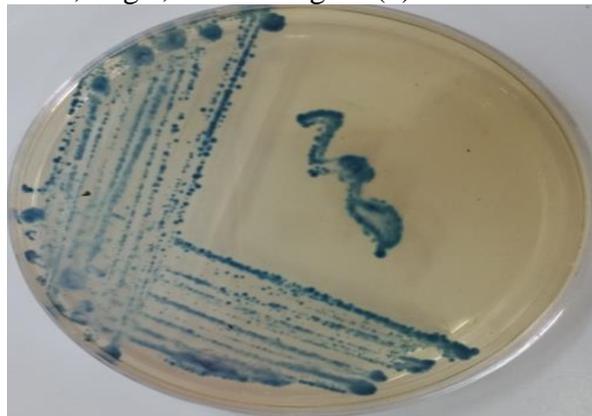


Figure (1): Metallic blue, rounded and large colonies of *Klebsiella pneumonia* on Orientation chrome agar

These isolates were positive citrate utilization and catalase while negative for H₂S production and oxidase reaction. (12). Thirty four (68%) samples from 50 nasal swap collected from sheep suffering from

respiratory sings were positive for *K. pneumoniae*, while 38 (76) urine samples of human with UTI cases gave *K. pneumoniae* isolates table (2).

Identification of *Klebsiella pneumonia* -16S rRNA, strain K-1

Seventy-two *K. pneumonia* isolates tested by PCR technique by usage of specific primer sequences with product sizes of 770bp. (Figure 2).

Detection of *bla* SHV

A Sixty three isolates from total 72 isolates were positive to Detection of *bla* SHV, these positive isolates divided to 53 isolate (%) for human and 28 (%) sheep. The *bla* SHV gene detection were performed by PCR by usage specific primer sequences with product sizes of 753bp. (Table 4) (Figure 3).

Detection of *bla* CTX-M ESBLs

Out of total 38 isolates, 30 isolate of human samples (78.9%) were positive for gene determination to be CTX-M ESBLs. PCR performed by usage of specific primer sequences with product sizes of 550bp, while not determined this gen in sheep. Table (4) Figure (4).

Table (1): cultural characters of *Klebsiella Pneumonia* on selective media

Bacterial isolates	EMB	Triple Sugar Iron(TSI) Slant/butt	MacConkey agar	Orientation chrome agar
<i>Klebsiella Pneumonia</i>	purple	y/y, gas, no H ₂ S	pink colonies	metallic blue color



Table (2): identification of *Klebsiella Pneumonia* by biochemical test.

Bacteria	Biochemical test														
	Cat.	Oxi.	Ind	MR.	VP.	Urs.	Cit.	H ₂ S	Moti.	Gel.	Nit.	Glu	Lac	Suc	Man
<i>K. pneumonia</i>	+	-	-	-	+	+	+	-	-	-	+	+	+	+	+

Y=yellow, H₂S=sulfur hydrate production, EMB=Eosin Methylene Blue.

Table (3): *K. pneumoniae* number and their isolation percentages of from sheep and human samples.

Type of sample	<i>Klebsiella pneumoniae</i>		Total
	Positive	Negative	
Nasal swap of sheep	34 (68%)	16 (32%)	50 (100%)
UTI human	38 (76%)	12 (24%)	50 (100%)
Total	72 (72%)	28 (28%)	100 (100%)

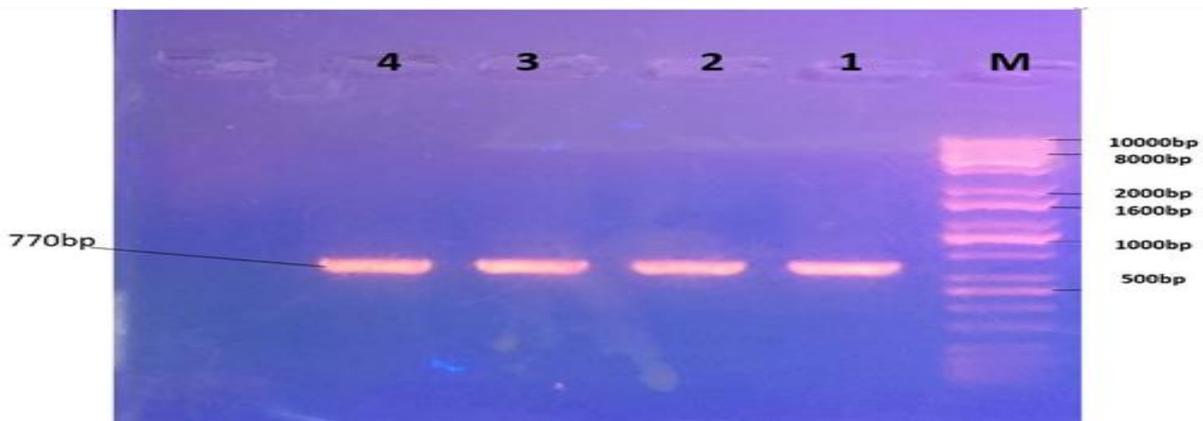


Figure (2) Agarose gel electrophoresis image that show the PCR product of *Klebsiella pneumoniae* partial 16S rRNA gene

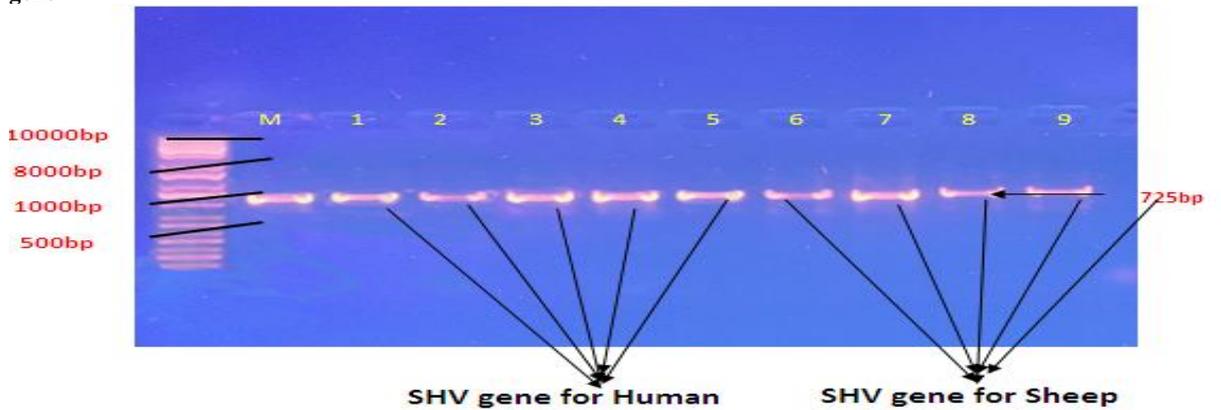


Figure (3): Agarose gel electrophoresis image that show the PCR product analysis of antibiotic resistance gene *bla* (SHV) of *Kleb. Pneumonia*.

Table (4): The genes distribution with the numbers of the total isolation

No. of isolated <i>K. pneumonia</i>		Genes		
		KP-16S rRNA, strain K-18	<i>bla</i> _{SHV}	<i>bla</i> _{CTX-M}
Sheep	34	34 (100%)	28 (82%)	0 (0%)
human	38	38 (100%)	35 (92%)	30 (78.9%)
Total	72	72 (100%)	65 (90%)	30 (41.6%)

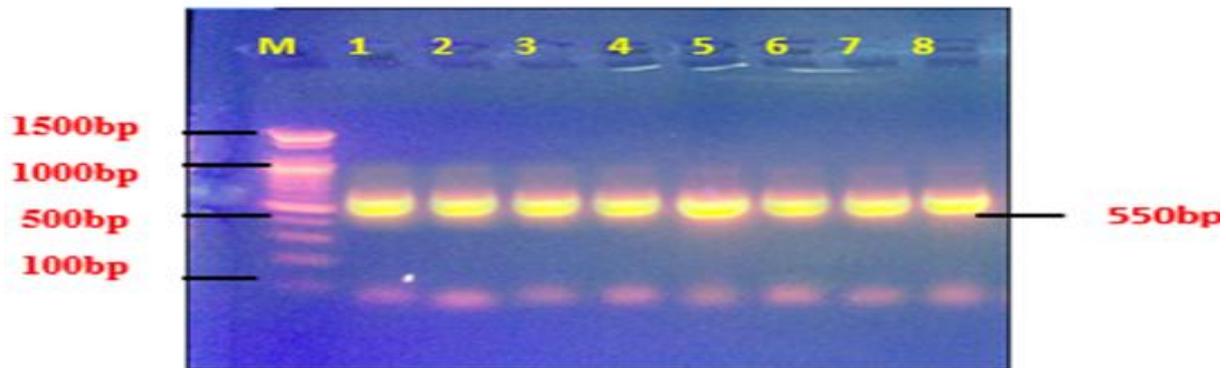


Figure (4): Agarose gel electrophoresis image that show the PCR product analysis of antibiotic resistance gene bla(ctx) of *Klebsiella pneumoniae*.

Discussion

K. pneumoniae, one of the most common bacteria of Gram-negative sepsis, usually lives the alimentary tract of human and animal (14, 15). β -lactamase can be produced by some of gram-negative bacilli such as *K. pneumoniae* and *Escherichia coli* strains. Beta-lactamases, were produced by Enterobacteriaceae, were encoded by plasmids. SHV and CTX are the most important beta-lactamases (16, 17). Drug resistance to β -lactam antibiotics was mainly result from Positive ESBLs (18, 19). The rate of detection of ESBLs in *K. pneumoniae* was calculated to be (90, 41.6) %; this was higher than the rate observed in Iran and Beijing. However, the rate of detection of CTX-M ESBLs in *K. pneumoniae* (78.9%) in human and while this gene did not determined in sheep. Similar result was seen in Beijing (84.80%), and higher than that in Iran (20). Therefore, we concluded that the production of ESBLs and CTX-M ESBLs differed regionally; this may be attributed to the increasing habit of antibiotic use, or the choice of detection method. This study was discovered the CTX-M genes in 30 (78.9%) of 38 strains of CTX-M- ESBL-producing *K. pneumoniae* in UTI case of human coexisted in the plasmid and chromosome DNA. The CTX-M ESBL genes present in both plasmids and chromosomes propagated with greater ease than those presented only in the plasmid or chromosome only; because of the CTX-M

ESBL genes coexisting in the plasmids and chromosomes could spread in the plasmid or chromosome through the function of ISEcpI (21). The coexistence of CTX-M ESBL genes in the plasmid and chromosome may be attributed to the widespread use of antibiotics in clinics, and the frequent use of antibiotic stock farming; cumulatively, that is lead to increase in drug-resistant genes in bacteria. On the other hand, other studies reported low percentages of ESBL producing organisms, (24) found that ESBL production *K. pneumoniae* were 4.9% of total *K. pneumoniae* isolated in a hospital of Japan (25) from Hyderabad, India, reported that 19.8% of Enterobacteriaceae species isolated over a period of one year were ESBL producers. Other study in Korea showed SHV is the most common ESBL (26). In India, another study reported frequency of ESBL-producing *Klebsiella* spp. was between 6% - 87%. (27, 28) *K. pneumoniae* has the progenitor of the SHV class of enzymes, SHV-1, is universally found with this bacterium. Many strains of *K. pneumoniae*, the gene encoding SHV-1, or its apparent precursor, LEN-1, resides found within a bacterial chromosome; it may be that gene of SHV-1 β -lactamase evolved as a chromosomal gene in *Klebsiella*, The SHV-1 β -lactamase is responsible for up to 20% of the plasmid-mediated ampicillin resistance in *K. pneumoniae* species (29).



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