Molecular detection of *bla SHV* gene in clinical and foodborne *Klebsiella pneumoniae* isolates

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Abstract

The study involved 200 samples. One hundred clinical specimens were obtained from patients in Baghdad hospitals and streaked on MacConkey agar. One hundred food samples were collected randomly from different local markets in Baghdad. Bacterial isolation from the collected food samples was done by pour-plate method using MacConkey agar.

Two groups of bacteria appeared lactose fermenter and non-lactose fermenter. Using both microscopic and macroscopic characteristics on the selective and differential media, the isolated lactose fermenting bacteria were identified as *Klebsiella* and *Escherichia coli.* Then vitek system was adopted to confirm the identification to *Klebsiella pneumoniae* as the prevalent species in the collected samples. The results revealed that 32 bacterial isolates are *K.pneumoniae* (18 clinical and 14 food origin).

Thereafter the isolates were tested for Extended Spectrum B-Lactamase (ESBL) production using a phenotypic method and the results revealed the presence of ESBL producers among clinical and food-origin isolates of *K. pneumoniae*. The results obtained by this study revealed that *bla SHV* gene is dominant in the Iraqi isolates of *K. pneumoniae* that were isolated from clinical and food samples. The results obtained by PCR technique for *bla SHV* gene (435 bp).

Key words: SHV, Klebsiella, Clinical, ESBL, food

INTRODUCTION

The emergence and dissemination of resistant bacteria to antimicrobial agents is considered a challenging and threat to global public health as antibiotic resistance results in higher medical costs, longer hospital stays, and higher mortality rates [1]. β-lactam antibiotics are among the most commonly prescribed antibiotics due to their minimal side effects and broad antibacterial spectrum. However, various mechanisms are responsible for resistance to β-lactam compounds, such as the production of degrading enzymes, alteration of the drug target (modification of penicillin-binding proteins), decreased membrane permeability, and drug efflux pump [2].

Hospital acquired infections, known as nosocomial infections, are the major cause of death and, making clinicians' jobs difficult [3]. Extended-spectrum -lactamases (ESBLs) have played a major role in the clinical area in recent decades in the wide-ranging and complicated world of B-lactamases. Their significance stems from the fact that they greatly broaden the range of earlier B-lactamases, allowing them to hydrolyze all penicillins, cephalosporins (excluding cephamycins), and aztreonam. Furthermore, most ESBL genes are located on plasmids that confer antibiotic resistance to many types of antibiotics and are easily transferred between and among bacterial pathogens [4].

Materials and Methods

Bacterial Isolation

The collected clinical samples were cultured on selective and differential media to isolate *Klebsiella pneumoniae*. One hundred clinical samples all were inoculated on MacConkey agar, and incubated at 37˚C for 24 hours. Later the grown colonies were further investigated.

Bacterial Identification

Bacterial isolates were identified to the genus level using both microscopic and macroscopic characteristic on selective and differential media, according to MacFaddin [5]. Vitek system confirmed the identification to the species level.

Antimicrobial Susceptibility Testing

The disc diffusion method was used to test the isolates for their antibiotic susceptibilities in accordance with CLSI standards [6]. The subsequent antibiotics were utilized: cefotaxime (30µg), ceftazidime (30µg), amoxicillin-clavulanate (20/10 µg).The source of all the antibiotic discs was Hi-media / India.

Double Disc Synergy Test (DDST)

For the DDST, three antibiotics were used: cefotaxime (30 m µg), ceftazidime (30 µg), and amoxicillin-clavulanate (20/10 µg). A disc containing amoxicillin and clavulanic acid was positioned in the middle, spaced 1.5 cm apart. A putative ESBL positive organism was indicated by the development of the zone of inhibition towards the Clavulanate disc at 37°C after 24 hours of incubation [7].

Molecular Detection
DNA Extraction

DNA extracted using Genomic DNA mini kit, (Geneaid, Thailand) protocol for gram negative.

PCR Amplification of ESBL coding genes

The primers used to detect ESBL coding gene blaSHV in *K. pneumoniae* were designated in this study: bla SHV-F: ´5 -TTCGCCTGTGTATTATCTCC-´3, bla SHV – R: ´5 -GCAAAAAGGCAGTCAATCC-´3 , the product size was 435 base pair.

These primers were provided in a lyophilized form. They were dissolved in sterile deionized distilled water to give a final concentration of100 pmol/µL. Primer stock and working portion were stored in the deep freezer until used in PCR amplification.

The extracted DNA, primers and PCR premix, were thawed at 4^{o}C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes. PCR mixture was set up in a total volume of 25µL included 12.5µL of PCR premix, 1.5µL of each primer and 4µL of template DNA have been used. The rest volume was completed with sterile de-ionized distilled water, then vortexed. Negative control contained all material except template DNA, so instead that distilled water was added. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermo-cycler PCR instrument where DNA was amplified as the program: one cycle of Initial denaturation at 95°C for 1 minute, then 35 cycle of: (Denaturation at 95°C for 30 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 30 seconds) and one cycle of final extension at 72°C for 1 minute.

Determination of PCR Product Specificity

Agarose gel electrophoresis was adopted to detect the specificity of PCR products. Agarose gel was prepared in 1.5 % concentration for the determination of the PCR specificity by dissolving 1.5 g of agarose powder in 100 ml of 1X TBE buffer, and boiling, then the agarose was cooled to 50-60^{o}C, 5 µL of red safe dye was added with mixing, agarose was poured out into the jar, then cooled to 20°C. When agarose gel was poured, several wells were carefully made with a comb at one side of the gel about 5-10 mm away from the end of gel; after final solidification, the comb was removed. The jar was put in the electrophoresis tank. Six microliters of the 100 bp DNA ladder were placed in the first left well or the middle one of the agarose electrophoresis gel, then 10 µL of each PCR product were put in the rest wells of the agarose gel. Thereafter the electrophoresis tank closed with its special lid, and electric current was matched (75 volt for 1h) [8]. The red safe stained bands in gel were visualized using Gel documentation system.

RESULTS AND DISCUSSION

***Klebsiella pneumoniae* isolation and identification**

Two hundred samples of clinical and food origin were cultured on selective and differential culture media to isolate *Klebsiella pneumoniae*. The results revealed the isolation of 120 bacterial isolates from clinical and food samples. Two types of colonies appeared on MacConkey agar; 81 isolate of lactose fermenters (67.5%) and 39 isolate of non-lactose fermenters (32.5%).

Figure 1 shows the initial isolation results clarifying the percentage of lactose fermenters and lactose non-fermenters on MacConkey agar.

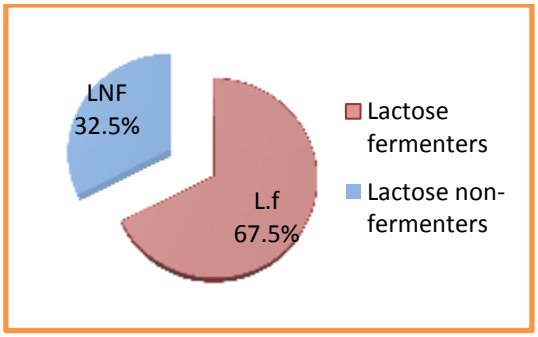

Figure 1 Percentage of lactose fermenters and non-lactose fermenters as a result of initial isolation

MacConkey agar is selective for Gram negative Enterobacteriaceae and differential between lactose fermenter which appear as pink colonies and non -lactose fermenter which appeared as pale colonies [9]. The lactose fermenters were of two principal types of colonies; small pink flattened colonies which were subjected to another culture step on eosin methylene blue agar to confirm the identification and the results revealed the appearance of colonies with green metallic sheen (Figure 2).

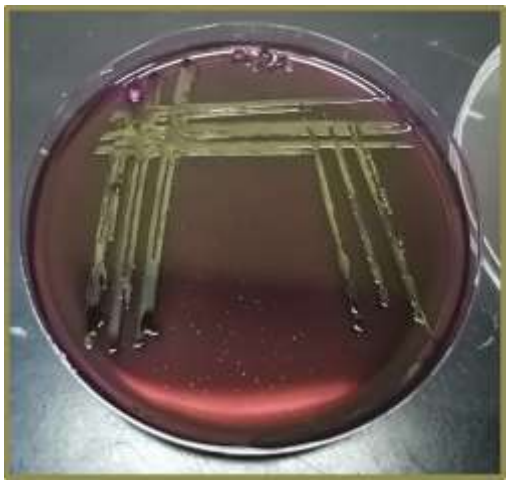

Figure 2 *Escherichia coli* isolate on EMB agar after 24h of incubation at 37°C

Large mucoid convex pink colonies which were suspected to be *Klebsiella* subjected to further identification steps such as Chromogenic media (Figures 3, 4).

Figure 3 *Klebsiella pneumoniae* isolate on MacConkey agar after 24h of incubation at 37°C

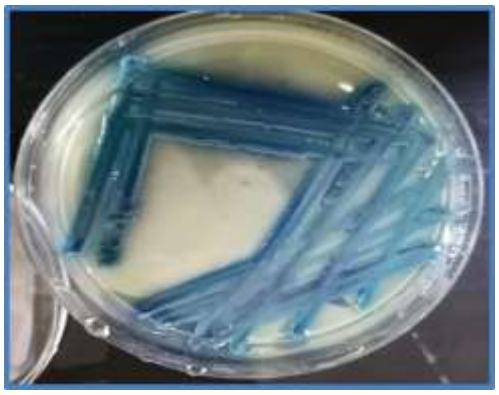

Figure 4 *Klebsiella pneumoniae* isolate on chromogenic agar after 24h of incubation at 37°C

Out of 120 bacterial isolates isolated from clinical and food sources; thirty two *K. pneumoniae* isolates, eighteen isolates from clinical sources and fourteen isolates from food sources. The isolated bacteria were identified depending on biochemical tests, morphological and cultural characteristics and the identification was confirmed by vitek system as *K. pneumoniae*.

While other lactose fermenters lack such characteristics were further investigated on Eosin methylene blue agar. The results revealed 30 isolate of *Escherichia coli* (49.38%) and other lactose fermenters neither *Klebsiella* nor *E. coli* were 19 isolates (23.45%) (Figure 5).

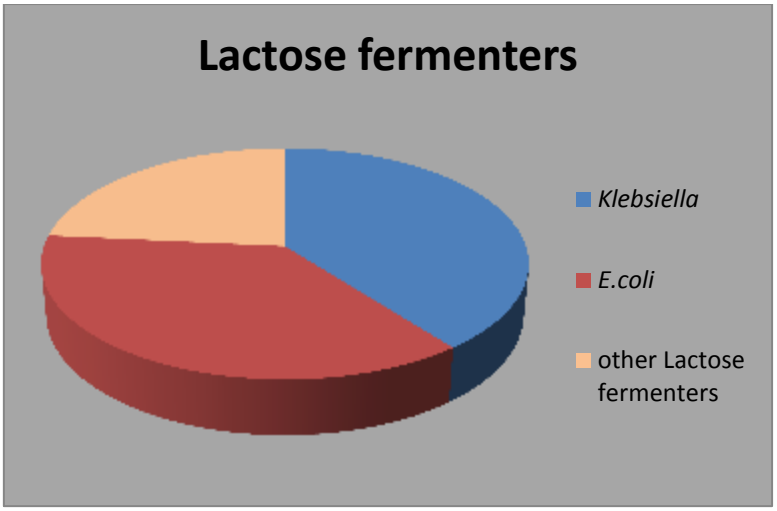

Figure 5: Percentage of the two type of isolates *E. coli* and *Klebsiella*.

Cells of *K. pneumoniae* appeared by Gram staining as Gram-negative bacilli. Vitek system was used to confirm the identification of the isolated bacteria to species level and to detect the antimicrobial sensitivity. Results revealed that 32 *K. pnemoniae* were isolated in this study. Most of *K. pneumoniae* isolates were multidrug resistant (MDR) (Figure 6).

Organism Quantity:
Selected Organism : Klebsiella pneumoniae

Source: Urine **Collected:**

Comments:	

Susceptibility Information	**Analysis Time:** 8.82 hours	**Status:** Final

Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
+Amoxicillin		R	Aztreonam	>= 64	R
+Ampicillin		R	Imipenem	>= 16	R
+Amoxicillin/Clavulanic Acid		R	Meropenem	>= 16	R
+Ampicillin/Sulbactam		R	Amikacin	>= 64	R
+Carbenicillin		R	Gentamicin	>= 16	R
Ticarcillin	>= 128	R	Tobramycin	>= 16	R
Ticarcillin/Clavulanic Acid	>= 128	R	Ciprofloxacin	>= 4	R
+Azlocillin		R	+Levofloxacin		R
Piperacillin	>= 128	R	+Lomefloxacin		R
Piperacillin/Tazobactam	>= 128	R	+Norfloxacin		R
+Ceflobiprole		R	+Ofloxacin		R
+Cefixime		R	+Doxycycline		R
+Cefpodoxime		R	Minocycline	>= 16	R
Ceftazidime	>= 64	R	+Trimethoprim		R
+Ceftriaxone		R	Trimethoprim/ Sulfamethoxazole	>= 320	R
Cefepime	>= 64	R			

AES Findings	
Confidence:	Consistent

Figure 6 Vitek report of MDR *Klebsiella pneumoniae*

Despite the renewed interest in K. pneumoniae species complex (KpSC) epidemiology, boosted by the increasing involvement of K. pneumoniae sensu stricto (Kp1) in human infections associated with high levels of antibiotic resistance and virulence [10], the contribution of nonclinical sources, such as food products, to the current emergence of KpSC is poorly understood. This is partly due to the lack of standardized protocols for the detection and isolation of K. pneumoniae strains in environmental, food, or animal samples.

Different selective culture media have been previously developed for Klebsiella spp. Among the most recognized is MacConkey inositol-carbenicillin agar [11].

The result of this study appeared the high prevalence of ESBL producing K. pneumoniae isolated from Iraqi patients and less frequency among the food origin isolates (Figure 7).

ESBL phenotypic detection

Figure 7 Antibiotics that were used in the detection of ESBL: Cefotaxime (CTX), Ceftazidime and Amoxicillin-clavulanate (AMC) for food-origin Klebsiella pneumoniae isolate on Muller Hinton agar after 24h of incubation at 37°C

The result of this study appeared the high prevalence of ESBL producers among *K. pneumoniae* isolated from Iraqi patients. Over the course of time, bacteria have developed resistance to different, conventionally used antibiotics. The study showed that ten isolates of *K. pneumoniae* were resistance to three or more antimicrobial classes, and it is defined as multi-drug resistance (MDR) [12].

Results of molecular study

DNA concentration and purity

After DNA extraction by Genomic DNA Mini Bacteria Kit, DNA concentration was between 76 and 91 ng/ml; whereas, purity was about 1.89 -1.99. A ratio of 1.8 -2.0 is generally accepted as "pure" for DNA. If the ratio is appreciably lower than the indicated ratio, it may specify the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm [13].

Result of ESBL coding genes detection by PCR

The results obtained by this study revealed that *bla SHV* gene is dominant in the Iraqi isolates of *K. pneumoniae* that were isolated from clinical and food samples. The results obtained by PCR technique for *bla SHV* gene (435 bp) are shown in figure (8).

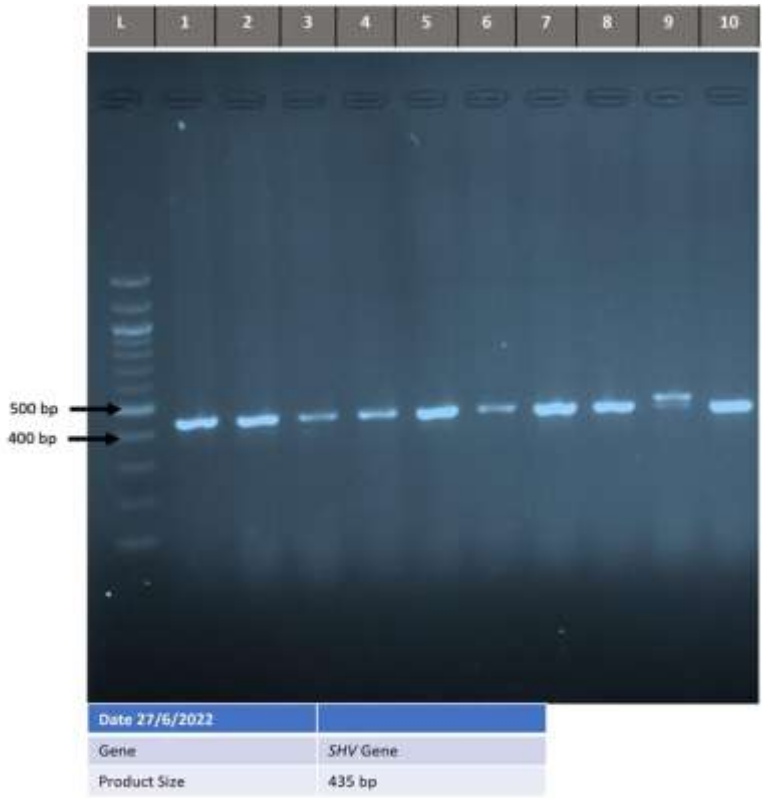

Figure 8 Agarose gel electrophoresis of PCR product of bla SHV (435bp), 1.5% agarose, red safe stain, TBE buffer, 75 volt, 1hr L: DNA ladder, lan1: K1, lan2:K4 lan3:K5, lan4:K6 , lan5:K7 , lan6:K8, lan7 :K9, lan8:K10, lan9:K11, lan10:K13.

Discussion

Detection of ESBL-producing Enterobacteriaceae has traditionally relied on phenotypic methods for detection in clinical microbiology laboratories. These methods exploit the fact that ESBLs are inhibited by traditional b-lactamase inhibitors such as clavulanate. Both CLSI and EUCAST have endorsed screening and confirmatory tests for detection of ESBL producers and guidance on use of these tests varies based on the cephalosporin breakpoints applied by the laboratory [14; 15]. Although the methods described by these standards setting organizations are similar, differences exist in the recommended organisms to test, screening and confirmatory test methods and interpretations. These ESBL methods require overnight incubation and have known limitations that affect both sensitivity (e.g. false negatives due to the co-production of an AmpC b-lactamase) and specificity (e.g. false positivity due to hyperproduction of narrower-spectrum b-lactamases combined with altered permeability) [16].

This point is worth noting, as it potentially could lead to treatment failure, prolonged diseases, and elevated morbidity and mortality rates. The transmission of resistance is a plasmid-mediated genetic determinant, may be attributed to the development of MDR among these isolates [17]. Studies have shown a rising pattern in the incidences of *K. pneumoniae* isolates with multiple antibiotic resistance. It has also been reported that infections with multiple antibiotic resistance attributes have a negative impact on the treatment of bacterial infections, especially in elderly, children, and immune-compromised individuals [18]. The use of available antibiotics in appropriate combinations is the only promising alternative technique to treat MDR bacterial infections [19]. This increased in the β-lactam antibiotic resistance isolates among *Klebsiella* strains can be explained in most cases to the production of β-lactamase enzyme that destroyed the β-lactam ring and inactivated it and this enzyme was encoded by plasmid that easy to transfer among strains [20]. Drug

inactivation or modification like *K. pneumoniae* producing beta-lactamase destroying ampicillin (intrinsic). Reduction of drug accumulation or access to the cell example: *E. coli* with altered porins or efflux pumps that reduce the antibiotic concentration of tetracyclines or anaerobic bacteria that lack oxidative metabolism to drive uptake of aminoglycosides [21].
The SHV-type b-lactamases (so named for sulfhydryl reagent variable) originated as chromosomally encoded enzymes in *K. pneumoniae* [22].

The first ESBL described in 1985 was SHV-2 and was found in a single strain of *K. ozaenae* isolated in Germany that differed from SHV-1 by a single amino acid substitution of Gly to Ser at position 238 [23].

Similar to what is seen in TEM type ESBLs, themajority of SHV-type ESBLs also havemutations at Ambler positions 238 (Gly to Ser) and 240 (Lys to Glu) [24]. In European surveillance, SHV-type ESBLs were found in 3.1%–17.0% of clinical isolates of K. pneumoniae, depending on region [25].
The results obtained by this study revealed that *bla SHV* gene is common in the Iraqi isolates of *K. pneumoniae* in percentage (83.33% and 50%) in clinical and food origin isolates respectively.
According to the majority of studies, the three ESBL classes *SHV*, *TEM*, and *CTX-M* appear to be the most frequently occurring kinds among K. pneumoniae community isolates. The SHV (90.5%) was frequent Beta-lactamase type in the current study was. Our local isolates had a high prevalence of *SHV* type ESBL, which is consistent with several prior results from Iran [26],
followed by *blaSHV* and *blaTEM* in the Asian Pacific area. The most common gene types found in surgical site infection isolates from patients in Nigeria were *blaSHV*, *blaCTX -M*, and *blaOXA*. The most common ESBL resistance genes in Burkina Faso's Enterobacteriaceae were *blaCTX-M* (40.1%), *blaTEM* (26.2%), and *blaSHV* (5.9%). Together with the current findings, these data demonstrated that there are geographical and local variations in the frequency of different ESBL gene types [27]. There are regional differences in the distribution of ESBLs throughout the world, and some ESBLs are absent from some regions while being more common in others . In a related study conducted in Indonesia, Severin and colleagues found *BlaSHV* and *BlaCTX-M* but no *BlaTEM*, and they attributed this to variances in the epidemiological distribution of ESBLs [28].

Even though *TEM* and *SHV* variants are the most widespread ESBLs, it appears that they have been less frequent than *CTX-M* over the previous ten years [29]. According to other publications who reported the *blaSHV-1* gene occasionally in connection with *CTX* genes, in both animals and humans, we found the blaSHV-1 gene in all K. pneumoniae strains. The ESBLs SHV have also been found in wildlife and animal species [30].

Only 73% of the ESBL-producing isolates in this investigation included ESBL expressing genes, and there are two possible explanations for this. One explanation could be because phenotypic techniques, similar to those employed in this investigation, are less precise in a contrast with molecular techniques. Regarding molecular characterization and confirmation of ESBLs in *K. pneumoniae* by phenotypic analysis *E. coli* demonstrated that several of the phenotypically abnormal PCR results for the positive ESBL-producers were negative. ESBL genes were examined. It might have been linked to the creation of ESBLs [28].

Conclusions

The presence of *Klebsiella* in both food and clinical isolates and prevalence of *bla SHV* gene in *K. pneumoniae* of clinical and food samples.

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