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Molecular Characterization of SHV-2a ESBL from Clinical Isolate Of*klebsiellapneumoniae* SLA55 in Baghdad, Iraq.

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Abstract: SHV-producing *K. pneumoniae* is mainly related to the development of plasmid-mediated extended-spectrum β-lactamases and more often involved with different infections, particularly among hospitalized patients. Fifty-nine isolates of *K. pneumoniae* collected from various hospitals from Baghdad city, Iraq. The MICs towards 17 antimicrobial agents were measured using the Vitek-2 system. A combination disc method was used for phenotypic detection of ESBL-producing isolates. All ESBLs producers were subjected to PCR-based technique to detect the existence bla_{SHV} gene using specific primers, followed by sequencing of entire genes in selected bla_{SHV}-containing isolates. The bla_{SHV-2a} gene was cloned to the pTriEx vector using the In-Fusion process, expressed in *E. coli* BL21-pLysS cells and confirmed with Coomassie Blue stain. 54 (91.53%) isolates were ESBLs producers and 48 (81.36 %) of isolates possessed the bla_{SHV} gene using the PCR-based method; the sequencing of the entire bla_{SHV} gene of selected isolates, confirmed the existence of this gene in the amplified-PCR products. The success of cloning was determined by double digestion and sequencing of the cloned bla_{SHV-2a}. The expressed SHV-2 protein was detected after 1-hour induction by IPTGand was stable after four hours induction. To the best of our knowledge, this is the first report of the molecular characterization of plasmid-borne-bla_{SHV-2a}-containing *K. pneumoniae* clinical isolate from Baghdad, Iraq, that provides evidence of the rapid spread of these enzymes among pathogens in health settings.

Keywords: *Klebsiellapneumoniae*, ESBLs, bla_{SHV-2a}, Cloning, Gene Expression.

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1. Introduction

The emergence and spread of strains resistant to multiple antimicrobial agents and documented major nosocomial outbreaks are of particular concern [1]. *K. pneumoniae* is an opportunistic pathogen that induces a wide range of infections, including pneumonia, urinary tract infection, bacteremia, and meningitis [2]. Moreover,it has many antibiotic resistance mechanisms, and the most common of which is the development of beta-lactamase production [3]. The SHV enzymes have appeared in Enterobacteriaceae in the last decade of the twentieth century, causing healthcare infections and are now present in isolates for various epidemiological settings in humans, animals as well environment [4]. The first reported SHV-1 β-lactamase had a narrow spectrum of activity. Point mutations at gene sites that affect the active site of the enzyme resulted in a family of derivatives of SHV-1. Derivatives of SHV-1 either have an extended spectrum of activity, capable of inactivating third-generation cephalosporins, or β-lactamase-inhibitors resistance [5]. The first extended-spectrum SHV-2 enzyme was described in 1983 in clinical isolates of *K. pneumoniae* and *Serratiamarcescens*[6], developing the spectrum of activity to involve the extended-spectrum cephalosporins due to amino acid changes that altered the configuration around the active site of the enzyme in which Glycine at position 238 in SHV-1 is replaced by serine in SHV-2 [7]. Currently, SHV β-lactamases encompass a large number of allelic variants including ESBL, non-ESBL, and several not classified variants. SHV-ESBLs are usually encoded by self-transmissible plasmids that frequently carrying the resistance genes to other drug classes, as consequence, their clinical significance came, furthermore their global spread in most members of Enterobacteriaceae. [8].

In Iraq, there are many studies related to ESBLs- producing Gram-negative bacteria [9–11], but to the best of our knowledge, there is a few reports dealt with the molecular characterization of SHV-ESBL producers in local isolates of *K. pneumoniae*. This present study aimed to investigate the molecular characteristics of the SHV ESBL-encoding gene from clinical isolates of *Klebsiellapneumoniae*.

2. Results

2.1 Antimicrobial Susceptibility and ESβLs production

The finding of the MIC values showed the resistance rates towards antimicrobials was 97.91% to Cefazolin, 83.33% to Ceftazidime, Ceftriaxone, and Azteronam, and 81.25% to

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Ampicillin/Sulbactam and Cefepime, on the other hand, the lowest rates of resistance of *K. pneumoniae* isolates were recorded for Ciprofloxacin and Etrapenem which had 2.08% (Table 1).

Table 1. The MICs of *bla*$_{SHV}$-containing*Klebsiellapneumoniae* isolates

Antimicrobial agents	**Resistance percentage**	**Intermediate percentage**	**Sensitive percentage**
	*S (%)	**I (%)	***R (%)
Ampicillin/sulbactam	6(12.5)	3(6.25)	39(81.25)
Pipracillin/Tazobactam	41(85.42)	4(8.33)	3(6.25)
Cefazolin	1(2.08)	0(0)	47(97.92)
Ceftazdime	8(16.67)	0(0)	40(83.33)
Ceftriaxone	8(16.67)	0(0)	40(83.33)
Cefepime	10(20.83)	0(0)	38(79.17)
Aztreonam	8(16.67)	0(0)	40(83.33)
Ertapenem	47(97.92)	0(0)	1(2.08)
Imipenem	48(100)	0(0)	0(0)
Meropenem	44(91.67)	0(0)	4(8.33)
Amikaicin	48(100)	0(0)	0(0)
Gentamicin	18(37.5)	0(0)	30(62.5)
Tobramycin	23(47.92)	6(12.5)	19(39.58)
Ciprofloxacin	33(68.75)	14(29.17)	1(2.08)
Levofloxacin	45(93.75)	0(0)	3(6.25)
Tigecycline	48(100)	0(0)	0(0)
Trimethoprim/sulfamethoxazole	18(37.5)	0(0)	30(62.5)

***S: Susceptibile, **I: Intermediate, ***R: Resistant**

Out of the 59-studied *K. pneumoniae* isolates 54 (91%) isolates of *K. pneumoniae* showed positive results for production of ESBLs, while 5(8%) isolates were phenotypically non-ESBLs producers.

2.2 The occurrence of SHV encoding genes

Forty-eight (81.36%) isolates possessed the *bla*$_{SHV}$gene using the PCR-based method with specific primers for amplifying partial and entire coding sequence, followed by the sequencing of the entire coding sequence of the selected isolates (21 isolates), of which 17 isolates possessed broad-spectrum SHV coding genes (2 *bla*$_{SHV-1}$ and 15 *bla*$_{SHV-11}$), while the rest four isolates belonged to the type of *bla*$_{SHV-2a}$ESBLs.

2.3 Expression of the *bla*$_{SHV}$-2a using the In-Fusion expression system

The strategy for the cloning and expression of *bla*$_{SHV-2a}$gene is shown in Figure 1.

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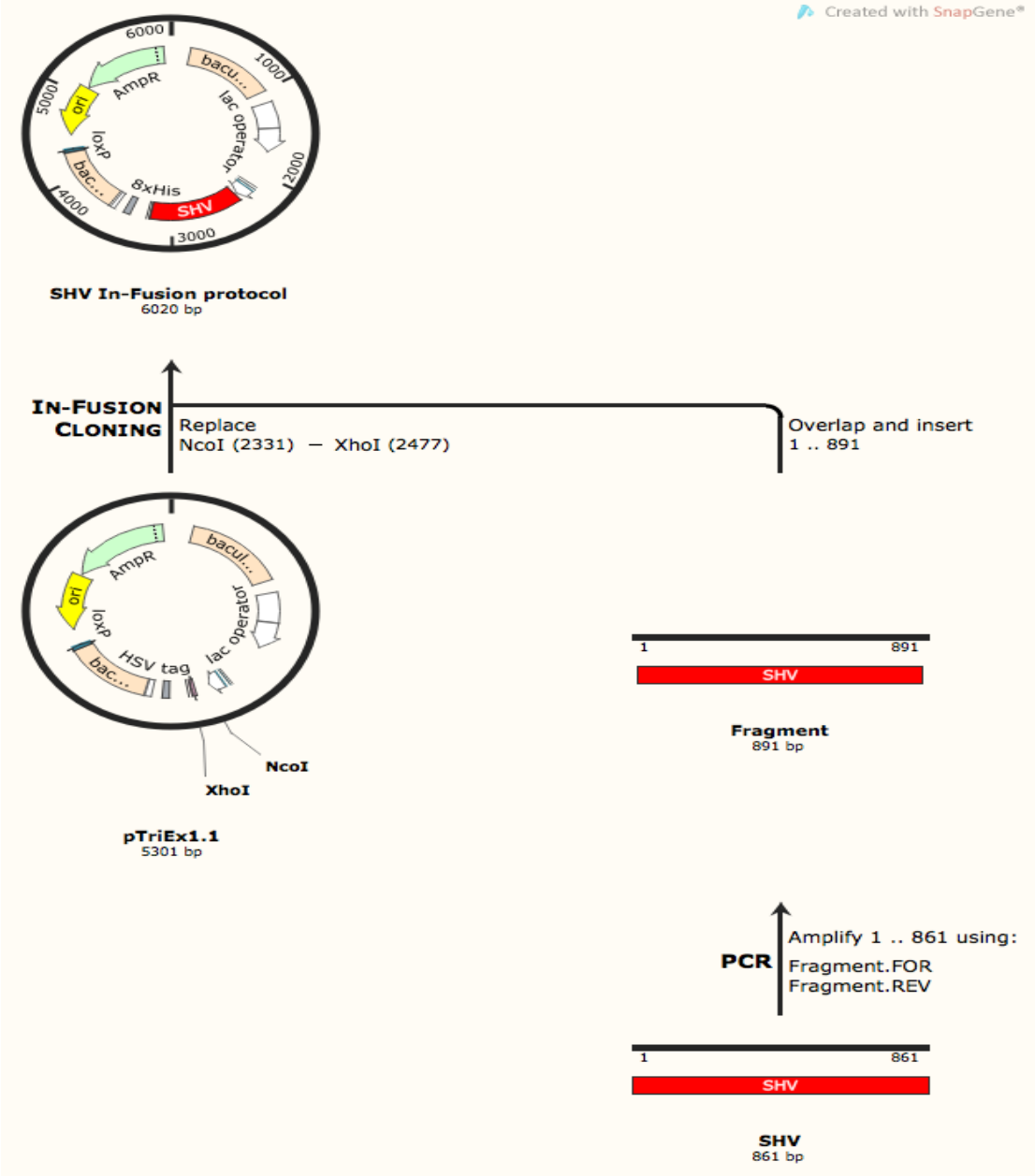

Figure1. Design steps for cloning process using In-Fusion protocol (SnapGene).

The entire *bla*$_{SHV-2a}$gene was cloned into the pTriEx to obtain a recombinant vector by insertion of the *bla*$_{SHV-2a}$gene between the *NcoI* and *XhoI* sites. The *bla*$_{SHV-2a}$-pTriEx was transformed into chemical-competent Stellar^{TM}*E .coli* cells. 15 colonies were selected randomly for colony PCR using TriExUp and TriExDOWN primers. After electrophoresis of agarose gel, amplification products were evaluated. The results showed a single band in clone 15 of1216 bp (Figure 2).

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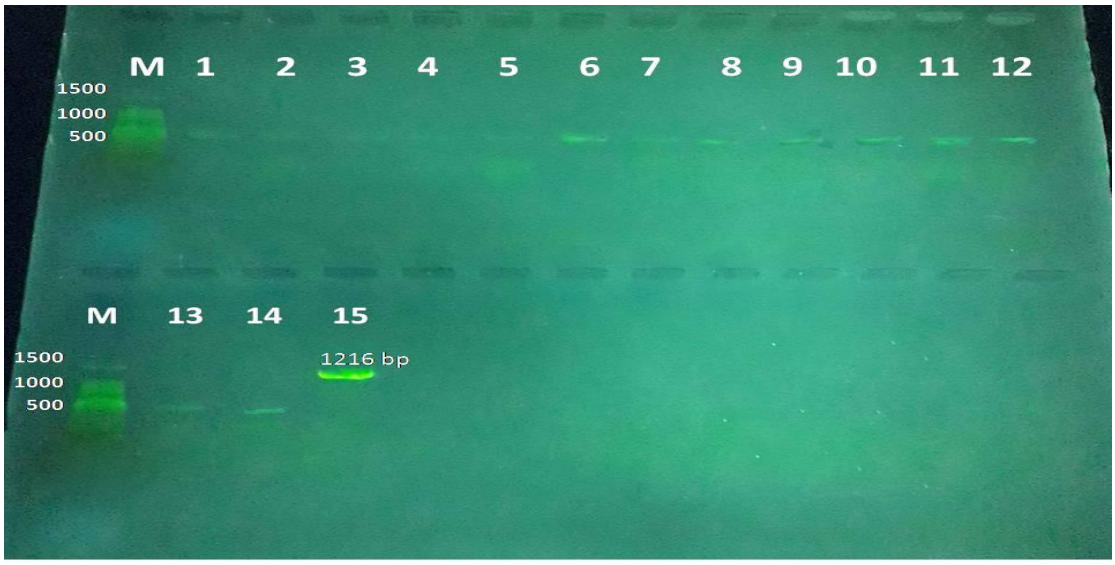

Figure 2. The *bla*$_{SHV-2a}$ gene screened by colony PCR. 15 colonies were screened for the presence of *the bla*$_{SHV-2a}$ gene. Lane 1: Marker (1500 bp) DNA ladder, Lanes 2-16: colonies numbered from 1- 15.

The *bla*$_{SHV-2a}$clone 15 was confirmed by double digestion with *NcoI* and *XhoI* restriction enzymes. The double-digestion agarose gel revealed a single 5.3 kbp band corresponding to the pTriEx vector and the 891 bp band equal to the *bla*$_{SHV-2a}$gene (Figure 3).

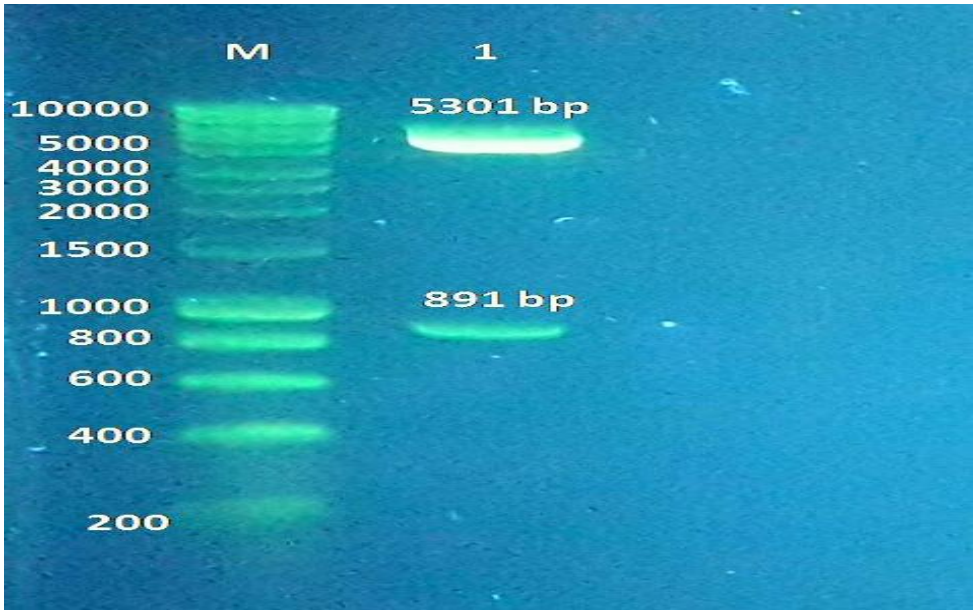

Figure 3. Gel electrophoresis of double digest *bla*$_{SHV-2a}$-pTriEx. Lane M: Marker (10000 bp) DNA ladder, Lanes: 1 pTriEx1.1-*bla*$_{SHV-2a}$transformants double digested with *Nco*I and *Xho*l, pTriEx1.1 (5301bp) and *bla*$_{SHV-2a}$(891bp), stained with Red Safe dye and visualized on a UV transilluminator.

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The transformant plasmid DNA with the predicted size insert was sequenced at Macrogen with Sanger sequencing using TriExUP and TriExDOWEN primers. To confirm each insert right sequence, snapgen software alignments were carried out. The *bla*$_{SHV-2a}$gene reading frame was in-frame with the six histidine-tagged downstream sequence on the C-terminal end of the vector.

2.4 Expression of the *bla*$_{SHV-2a}$ gene

For the expression of the construct, pTriEx-*bla*$_{SHV-2a}$was transformed in host cells BL21 (DE3) pLysSand induced by IPTG. Induced cell lysate samples showed appropriate 32.25 kDaprotein bands for the SDS-PAGE gel SHV His-tag protein.

The results in Figure 4 showed that the expression of the SHV-2aprotein began after one hour of induction and that the protein was stable after four hours induction according to the coomassie staining.

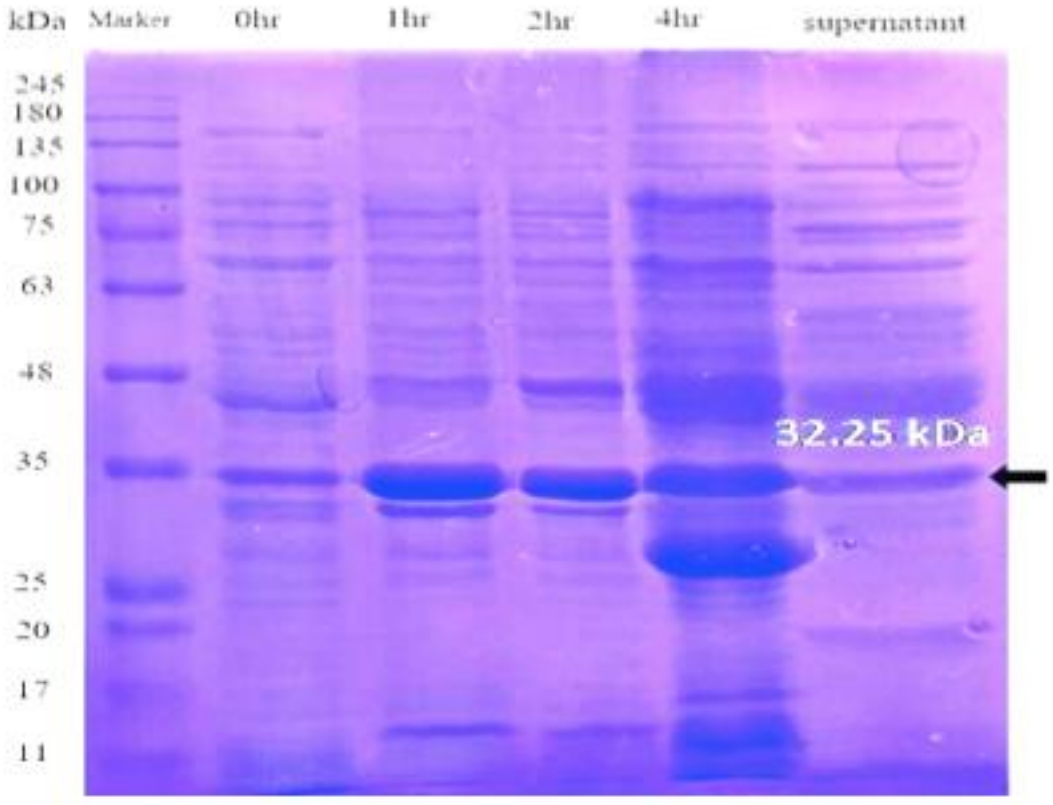

Figure 4. Expression of SHV protein in BL21 (DE3) pLysS competent cells. The SHV-2a protein was converted as outlined in materials and methods with vector pTriEX and triggered with IPTG when the O.D.600 reached 0.3. Lane 1: Marker; Lane 2: total (uninduced) lysate; Lane 3: complete lysate (induced after 1 hour); Lane 4: complete lysate (induced after 2 hours);

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Lane 5: complete lysate (induced after 4 hours); Lane 6: complete lysate (supernatant.). The black arrow indicates the SHV-2a protein that has been expressed.

3. Discussion

Through the Antibiogram profile, it is clear that most isolates were highly resistant to extended-spectrum cephalosporin and Aztreonam, this can give at first glance that these isolates can be the ESBLs producers. This consequence was confirmed using the combination disc method to the determination of ESBL production, where most isolates (91%) were ESBLs producers.

ESBLs-producing *Klebsiellapneumoniae* was increasingly recognized in hospital settings throughout the world including regional and neighboring countries of Iraq [12–14].

In Iraq, there is a lack of information about the infection caused by ESBLs-producing bacterial, especially, before 2003 because embargo applied on Iraq which affected negatively all scientific fields. After that, many studies related to phenotypic detection of bacterial ESBLs production began, particularly at the beginning of the last decade [15–17]. These reports indicated that infections caused by ESBL-producing bacteria increased dramatically [18].

The SHV types of enzymes are mostly found in *Klebsiella*species (especially *K. pneumoniae*) most often housed by a plasmid. The existence of bla$_{SHV}$on plasmids has contributed to the worldwide spread of genes for which bla$_{SHV}$can now be found in many Enterobacteriaceae species [19]. SHV beta-lactamase has few variants other than TEM and CTX-M. So far, 178 variants of SHV based on amino acid sequence composition were identified (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/SHV). SHV-2 was an enzyme that originated from a point mutation in SHV-1, which resulted in the substitution of glycine by serine at the 238 positions [20].

Globally, several studies reported the occurrence and prevalence of SHV-2a-containing K. pneumonia [21–24]. In Iraq, many studies reported the possessing of *K. pneumoniae* to SHV beta-lactamase [17,18,25]. On the other hand, to our knowledge, there are no reports about investigating the characteristics of SHV variants at a molecular level. The spread of ESBLs producers among the clinically important Gram-negative bacteria is one of Iraq rising problems in particular. As a result of that, this problem becomes further complicated by the lack of studies identifying the resistance mechanisms; so an in-depth molecular study of one selected isolate of four SHV-2a-possessing *K. pneumoniae* was conducted.During cloning the *bla*$_{\text{SHV-2a}}$gene was characterized; furthermore, the expression of this gene was shown. In addition to some basic

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research purposes, the functionally active form is essential for the study of the *bla*$_{SHV-2a}$gene, such as activity and structure. Several new cloning systems for in-vitro DNA fragment were introduced due to the rapid developments of molecular biology techniques, such as uracil-specific cloning g of excision reagents [26]. The In-Fusion differs from other conventional cloning methods by being able to enter any section of DNA that has a 15 bp identity at its end and without point mutations. Furthermore, the In-Fusion system arecapable of cloning multiple DNA fragments with nearly the same efficiency as a single fragment in one single reaction with the correct coding sequence [27]. Cloning and expression of SHV-2a protein were demonstrated in this study as a histidine-tagged fusion protein in *E. coli*. The pTri-*bla*$_{SHV-2a}$recombinant plasmid was transformed in*E.coli* BL21 (DE3) pLysS for protein expression. Optimum protein expression time was calculated by analyzing the protein expression profile of the SDS-PAGE gel. The expression of the SHV-2a protein started after 1-hour induction and increased on the base on the band thickness observed as a function of time. The protein displayed even after 4 hours of induction (Figure 4).

4. **Materials and methods**

4.1 Identification of isolates

Fifty-nine *K. pneumoniae* isolates were obtained from some hospitals based in Baghdad, Iraqbetween 2014-2018. Isolates were collected from different specimens as follow: 39 (66.1%), 8 (13.5%), 5 (8.4%), 5 (8.4%), and 2(2.3%) isolates from blood, urine, burn, and ear respectively. Identification of isolates was carried out using Vitek-2 compact system phenotypically and was confirmed genotypically by detecting the housekeeping (*rpoB*) gene using PCR-based technique.

4.2 Susceptibility testing and determination of ESBL production

The antimicrobial susceptibility test towards 17 antimicrobials including Ampicillin/sulbactam, piperacillin/Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Aztreonam, Etrapenem, imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Tigecycline, and Trimethoprim/ sulfamethoxazole was performed by the Vitek-2 system (bioMerieux, USA) according to the manufacturer's instructions. A combination disc method was used for phenotypic detection of ESBL producers, the results were interpreted based on CLSI 2017 [28], as result of that, considered positive when the difference of ≥5 mm in the

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Received 05 March 2021; Accepted 01 April 2021.

zone of growth inhibition diameter between cefepime disks and their respective cefepime /clavulanate disk.

4.3 Genotypic detection of isolates

Bacterial genomic DNA was extracted using a genomic purification kit (Promega / USA). The RNA polymerase (*rpoB*) housekeeping gene β subunit was amplified to confirm the identifying of isolates using the primers VIC3 (5-GGCGAAATGGCWGAGAACCA-3) and VIC2 (5-GAGTCTTCGAAGTTGTAACC-3)[29]. The reaction conditions involved initial denaturation at 94 ^{0}C for 5 min followed by 35 cycles of 94 ^{0}C for 30 s, 58 ^{0}C for 50 S, and 72 ^{0}C for 1 min, and a final elongation step at 72 ^{0}C for 5 min. Negative controls which contain all mixture reagents without DNA were included for all the reactions.

4.5 Amplification of SHV encoding gene

The sequence of SHV primers Fw-5¯ATGCGTTATATTCGCCTGTG-¯3 and Rv-5¯ TTAGCGTTGCCAGTGCTCGA-¯3 were used to detect partial coding sequence of an 861bp of SHV encoding gene using genomic DNA as a template, while the primers Fw-5¯ TGTCGCTTCTTTACTCGCCT-¯3 and Rv-5¯ATATCGCCCGGCACGCCT-¯3 was designed in this study to amplify the entire *bla*$_{\text{SHV}}$gene with PCR product of a 975 bp, amplification conditions were used as previously described [30].

4.6 Sequencing of Entire bla$_{SHV}$ gene and nucleotide sequence analysis

Sequencing of the entire *bla*$_{\text{SHV}}$gene for selected isolates were performed using the chain-termination method (Sanger sequencing) with ABI Big Dye Terminator chemistry on an ABI 3730 automated sequencer (Applied Biosystems, Inc.) at Macrogen, Inc. (Seoul, South Korea). The sequencing data obtained were analyzed using the BLASTn program available on the National Center for Biotechnology Information (NCBI) website. The *bla*$_{\text{SHV}}$ gene sequences were deposited in the Genbank database under the accession numbers **MT791286 to MT791306**.

***4.7* Cloning and expression of *bla$_{SHV-2a}$*from*Klebsiellapneumoniae* isolate SLA55**

***4.7.1* Construction of*bla$_{SHV-2a}$* in the expression vector**

Initially, *bla*$_{\text{SHV-2a}}$was amplified using designed primers for the In-Fusion method from clinical isolate *Klebsiellapneumoniae* SLA55 (accession number: MT791287) Fw-5¯-AGGAGATATACCATGGCCATGCGTTATATTCGCCTGTGT-3 and Rv-5¯-AGGAGATATACCATGGCcATGCGTTATATTCGCCTGTGT-¯3. The amplification units used were: for Initial denaturation for 3min.at 95^{o}C, Denaturation 94^{o}C (40 sec), Annealing 60^{o}C

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 10993 - 11006
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(10 sec) and Extension for 35 cycles at 72^{o}C (50 sec). The estimated product size (891bp) was analyzed by agarose gel electrophoresis. The vector pTriEx was doubly digested with *NcoI* and *XhoI*restriction sites and purified. The expression system pTriEx enables the expression of recombinant SHV-2aprotein at the C-terminal end with a six histidine-tagged sequence. The recombinant vector was transformed into *E. coli* competent cell as directed by the producer by heat shock (clontech). The transformed mixture was cultured on Luria Bertani agar with Ampicillins (100µg/ml). 15 randomly selected colonies were screened by colony PCR for the presence of *bla*$_{SHV-2a}$gene using TriExUp as a forward primer Fw: 5¯ GGTTATTGTGCTGTCTCATCA-¯3 and TriExDOWN as a reverse primer Rv: 5¯ TCGATCTCAGTGGTATTTGTG-¯3, to confirm that the *bla*$_{SHV-2a}$gene construct was correct and that the gene was in the frame, double digestion with *NcoI* and *XhoI* restriction enzymes was also performed.

*4.7.2 bla$_{SHV-2a}$*expression in*E .coli*

The pTri-*bla*$_{SHV}$ plasmid DNA was transformed into BL21 (DE3)-pLysS cells for expression of *bla*$_{SHV-2a}$gene. The next day only one colony was inoculated into a medium of 10 ml LB broth and incubated with chloramphenicol (34µg/ml) and 100µg/ml ampicillin in the shaker at 37^{o}C. The overnight culture was inoculated the next day in 10 ml of fresh LB broth with ampicillin (100µg/ml) and incubated in the shaker at 37°C for 3 hours. The SHV-2A protein expression was induced when the culture reached O.D. 600 of 0.3 by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). Before the addition of IPTG, 1 ml of the culture was collected and centrifuged. 1 ml of the culture was also collected at 1,2,3 and 4 hours after IPTG was added to determine the optimum induction time. The sodium dodecyl sulfThe pTri-*bla*$_{SHV}$ plasmid DNA was transformed into BL21 (DE3)-pLysS cells for expression of *bla*$_{SHV-2a}$gene. The next day only one colony was inoculated into a medium of 10 ml LB broth and incubated with chloramphenicol (34µg/ml) and 100µg/ml ampicillin in the shaker at 37^{o}C. The overnight culture was inoculated the next day in 10 ml of fresh LB broth with ampicillin (100µg/ml) and incubated in the shaker at 37°C for 3 hours. The SHV-2A protein expression was induced when the culture reached O.D. 600 of 0.3 by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). Before the addition of IPTG, 1 ml of the culture was collected and centrifuged. 1 ml of the culture was also collected at 1,2,3 and 4 hours after IPTG was added to determine the optimum induction time. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to

confirm the expression of the protein. The bacterial pellets were re-suspended in 50µlof chilled PBS 30µl of samples were combined with 10 µl of LDS (Novex) sample buffer incubated for 10 minutes at 100 °C and centrifuged for 2 min before loading into 4-12 % of SDS-PAGE (life Technologies) gel. Later samples were separated for 30 minutes at a constant voltage of 170v in a 1x MES running buffer (Life Technologies). The gel was stained with coomassie blue for analysis.

5. Conclusion

One of the most possible risk factors which probably responsible of the prevalence of ESBLs-producing organisms are antimicrobials misused in Iraq especially between low education society levels or limited scientific culture. In addition to that, the increasing numbers of migrant workers to Iraq, particularly from Indian subcontinent which considered as one of the endemic regions of infections caused by ESBLs producers, and lack of applying fines against some health institutions that are not committed to the laws. In this research, we suggested the activating of the health surveillance system, strict regulation by authorities to stop dispensing antibiotics without a medical prescription, and conducting in-depth studies on molecular mechanisms of antimicrobial resistance is necessary to address this critical issue.

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Conflicts of Interest: The authors declare no conflict of interest.

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