A NEW VARIANT OF *bla*SHV HAS BEEN REVEALED IN *KLEBSIELLA PNEUMONIA* IN SULAIMANI/IRAQ

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Abstract. In recent years, a large number of multidrug-resistant *Klebsiella pneumonia* has been revealed bearing different variants of *bla*SHV. In the current study, antibiotic sensitivity test and the rate and molecular characterization of three common beta-lactam resistance genes, *bla*SHV, *bla*OXA, and *bla*CTX were investigated in *K. pneumonia* isolated from layer chickens in Sulaimani city/Iraq. Furthermore, the molecular characterization of *bla*SHV was studied. The highest rate of resistance was to ceftazidime (92.9%) and cefotaxime (82.3%), while the lowest rate was found against meropenem (4.7) and imipenem (8.2%). Among 85 isolates of *K. pneumonia,* 33 isolates (33.8%) had CTX gene which is the highest rate among the three resistance genes. The second most common resistance gene was SHV gene, 22.3%, and OXA gene showed the lowest rate 11.7%. After PCR amplification and sequencing, it was revealed that a novel variant of *bla*SHV, SHV-Suly213 was recovered from *K. pneumonia* and it exists in the studied area. The new variant contains a hybrid mutation which is composed of a mixture of nucleotide sequence of previously known *bla*SHV, SHV-1 and SHV-213. Phylogenetic tree shows that the novel SHV is more closely related to SHV-213; therefore, it was named SHV-Suly213. The bacteria were resistant to ceftriaxone and cefotaxime, but meropenem was still active against the bacteria. In conclusion, the current study discovered a new variant of *bla*SHV, SHV-Suly213. The resistance gene compromised of mixed sequences (hybrid) of both SHV-1 and SHV-2113 that contain three nucleotide mutations in comparison to them. The bacteria are resistant to many beta-lactam antibiotics such as ceftriaxone and cefotaxime, but not meropenem.

Keywords: *bacteria, beta-lactamase resistance genes, antibiotic sensitivity, blaSHV, novel mutation*

Introduction

Beta-lactam antibiotics are counted as a first line of antibiotics against gram negative bacteria, but recently broad-spectrum resistances have developed against them due to beta-lactamase resistance genes. In the last decades, diverse types of beta-lactamases have been spotted, and they are different in their response to different classes of beta lactam antibiotics; therefore, new classification is required for the new types of beta lactamases (Ambler, 1980; Liakopoulos et al., 2016).

The *bla*SHV is one member of beta-lactamase resistance genes and it is mostly found in *Klebsiella pneumonia* and sometimes in *Escherichia coli* (*E. coli*). There are various types of SHV beta-lactamases, and they are different in their responses to beta-lactam antibiotics due to amino acid mutations and replacement in their structures. SHV1 is a beta-lactamase enzyme that was discovered in *E. coli* for the first time in 1972 (Pitton, 1972) and it has been found recently that it is mostly distributed in *K. pneumonia* and other members of Enterobacteria (Rahim et al., 2020; Shaikh et al., 2014). SHV1 is resistant against different types of Penicillin antibiotics including ampicillin and pipercillins (Livermore, 1995; Matthew et al., 1979).

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It is believed that the ancestor of SHV1 is more likely expressed from chromosomal gene that is isolated from *Klebsiella pneumonia* in feces of neonate (Hæggman et al., 1997) and its then diffused to bacterial plasmid and distributed among Enterobacteriaseae (Barthélémy et al., 1988; Shaikh et al., 2014).

Materials and methods

Sample collection and bacterial isolation

Eighty-five samples were taken and analyzed from layer chicken viscera from local slaughter houses delivered form different poultry farms of Sulaimani city in November 2019. The samples were cultured on differential media (MacConkey agar, and Eosin methylene blue) (Accumedia LAB, Neogene Culture Media, Heywood, UK), and then incubated at 37 °C for 16 h. The isolates of *Klebsiella* were further identified and confirmed through biochemical tests, such as IMViC test (Harley and Prescott, 2002) and the final confirmation was done by amplification of 16S-23S ITS (130 bp) gene using a set of primer specific to *K. pneumonia.*

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for the isolates by disc diffusion test as defined by the Clinical Laboratory Standard Institute (CLSI) guidelines for the following beta lactam antibiotics, amoxicillin (AX 25 μg), amoxicillin-clavulanic acid (AMC 30 μg), oxacillin (OX 1 μg), meropenem (MEM 10 μg), ceftriaxone (CRO 30 μg) and cefotaxime (CTX 30 μg), Imipenem (IMP10 μg), Cefixime (FX 5 μg), ceftazidime (CAZ 30 μg) (Bioanalyse, Ankara, Turkey).

Molecular identification of beta-lactamase genes, SHV, CTX, and OXA

Crude DNA of bacteria was extracted by boiling fresh colony of bacteria in 100 ul of distilled water for 15 min. *bla*SHV (800 bp), *bla*OXA (610 bp), and *bla*CTX (550 bp) genes were PCR amplified using specific primers according to the procedure and protocol described by (Ahmed et al., 2007, 2009). The PCR DNA product was then visualized under blue light, cut and gel purified after resolving on 1.5% DNA agarose gel using SmartDoc 2.0 Imaging System (Accuris, NJ, USA).

Sequence analysis

*bla*SHV gene from ten bacterial isolates were sequenced. The isolates were chosen from different poultry farms and we avoided using many repetition of the same poultry farm. The amplicons of different isolates of *Kleibsella* were sequenced through Sanger sequencing on ABI-3730XL capillary machine (Macrogen Inc., South Korea). One of the gene sequences was deposited in to GenBank National Center for Biotechnology Information (NCBI) through Bankit (Benson et al., 2015) with the accession number of OK376493.

NCBI BLASTn search tool (http://www.ncbi.nlm.nih.gov/) was used to blast the sequences and find the diversity of the gene. The multi-sequence alignment was carried out by ClustalW multi alignment tool. A phylogenic tree was built for the sequences and retrieved sequences of NCBI by the neighbor-joining (NJ) program (Phylogeny.fr) (Dereeper et al., 2010).

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Results

Isolation of klebsiella and antibiotic sensitivity testing

Eighty-five isolates of *K. pneumonia* were taken from viscera of layer chicken after slaughtering originated from the same local farm. The samples were taken by bacteriological swabs and streaked out on MacCkonkey and Eosin methylene blue agar. Then the bacteria were further identified by catalase, oxidase and common test for Enterobacteriaseae bacteria, IMViC (Indole test, Methyl red test, Voges-Proskauer test, and Citrate utilization test). The final confirmation was done by amplification of 16S-23S ITS gene using a set of primer specific to *K. pneumonia* (*Fig. 2*).

K. pneumonia isolates were analyzed to determine their antibiotic resistance pattern against nine types of beta-lactam antimicrobials. The highest rate of resistance was to ceftazidime (92.9%) and cefotaxime (82.3%), while the lowest rate was found against meropenem (4.7%) and imepenem (8.2%) (*Fig. 1A*).

The isolates that harbored SHV gene containing new mutations were resistant to oxacillin, amoxicillin, cefotaxime, and ceftriaxone, but they were sensitive to amoxicillin-clavulanic acid, cefixime, imipenem and meropenem.

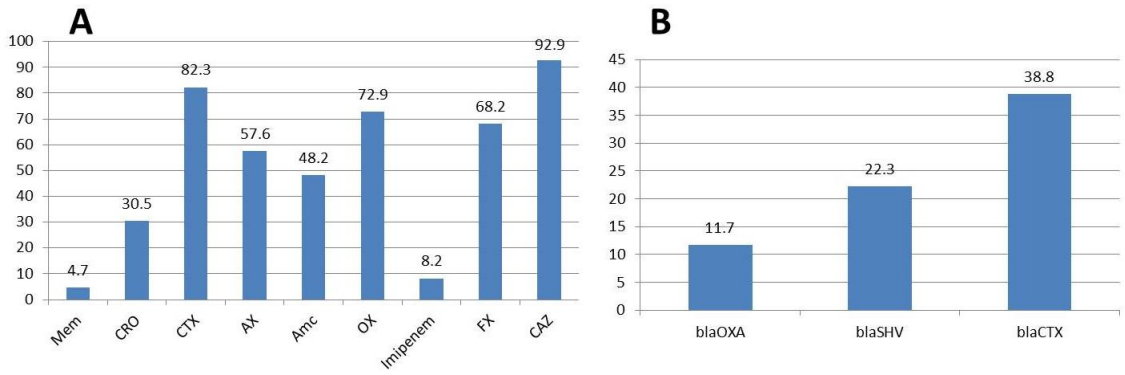

Figure 1. *Antibiotic resistance pattern and the rate of beta-lactam resistance genes in K. pneumonia isolates in different samples of layer chickens. (A) The percentage of resistant K. pneumonia isolates against nine beta-lactam antibiotics. (B) The rate of different beta-lactam resistance genes in K. pneumonia isolates*

Investigation of resistance gene, blaSHV, blaOXA and blaCTX

To investigate the variants of *bla*SHV, *bla*OXA and *bla*CTX, specific primers were used to amplify each of the resistance genes using conventional PCR techniques (*Fig. 2B*). Among 85 isolates of *K. pneumonia*, 33 isolates (33.8%) had CTX gene which is the highest rate among the three resistance genes. The second most common resistance gene was SHV gene which was found in 19 isolates (22.3%) and OXA gene showed the lowest rate which was discovered in 10 samples (11.7%) (*Fig. 1B*).

Ten of SHV genes were sequenced using Sanger sequencing. The sequences of the resulted genes were blasted and aligned to the available sequences on the website, NCBI. The results showed that the sequence of all studied *bla*SHV contain three nucleotide mutations in comparison to two previously found *bla*SHV, SHV1 and SHV-213. During investigation the *bla*SHV gene via sequencing and phylogenetic tree, a novel allele was discovered (*Figs. 3* and *4*) and named as *bla*SHV-Suly213 and the sequence was deposited into NCBI with accession number (OK376493).

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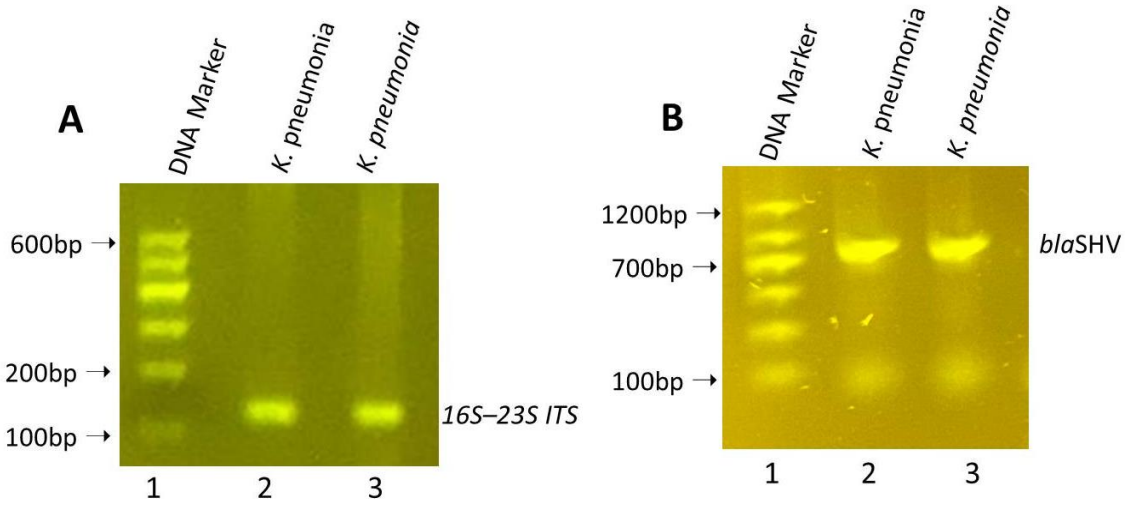

Figure 2. *PCR amplification of 16S-23S ITS and blaSHV gene of K. pneumonia. (A) PCR confirmation of K. pneumonia using specific primer to amplify16S-23S ITS gene. (B) PCR amplification of beta-lactamase resistance genes, blaSHV are shown in lane 2 and 3 that recovered from nineteen isolates of Kleibsella*

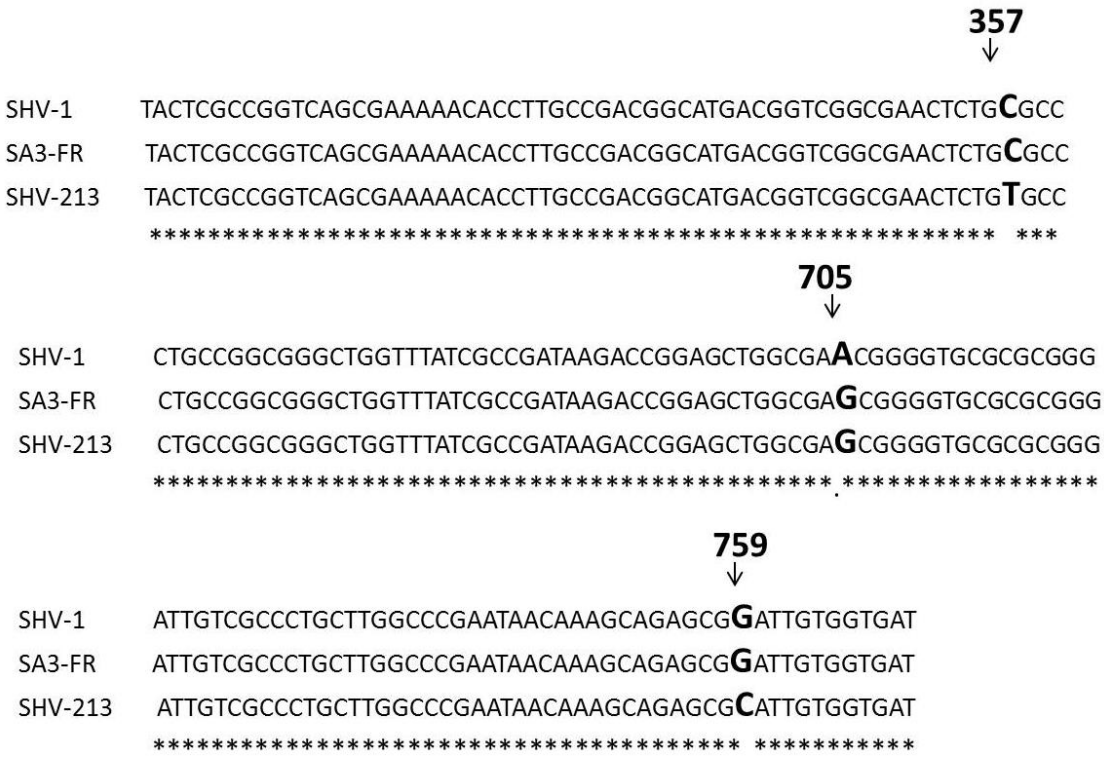

Figure 3. *Multiple sequence alignment of blaSHV gene recovered from K. pneumonia. SHV gene in K. pneumonia isolated from layer chicken in Sulaimaniyah in comparison with published SHV-1 and SHV-213 from GenBank*

In comparison to SHV-213, the gene of the current study contains two mutations which are located at the nucleotide number 357 starting from the starting codon and at position number 759 from the starting codon where guanine replaced by cytosine. In

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comparison to SHV1, it contains a mutation at locations number 705 where guanine replaced by adenine (*Fig. 3*). The diversity of the gene was compared to different related sequences available online. 11 gene sequences of SHV enzyme were taken and put into a phylogenetic tree to find the diversity of the gene found in this study (*Fig. 4*).

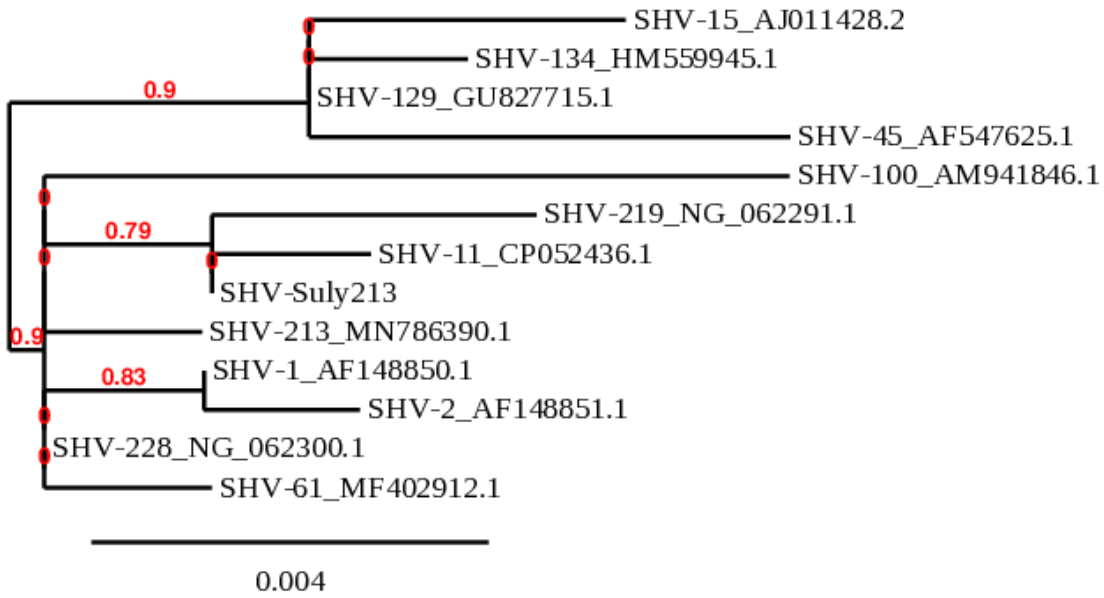

Figure 4. *Phylogenic analysis of SHV-Suly213. Phylogenetic tree was built to the sequence of SHV-Suly213 in comparison to very closely related sequences and 11 available sequences retrieved from NCBI Blast*

Discussion

Antibiotics are strong agents still used to treat bacterial infections in human and animals and beta-lactam antibiotic is known as frontline protection antimicrobials against bacterial infection (Bradford, 2001). On the other hand, bacterial resistance to common antibiotics causes difficulties in treatment and high morbidity and mortality in animal and poultry (Grover et al., 2013). Local and wild birds bearing resistant bacteria may spread these bacteria into the environment and to human (Peirano et al., 2011; Alaa et al., 2020). Therefore, antibiotic resistance phenotype of *K. pneumonia* to nine common beta-lactam antibiotics was tested including amoxicillin-clavulanic acid, amoxicillin, oxacillin, ceftriaxone, meropenem, ceftazidim, and cefotaxime. The highest rate of resistance was to ceftazidime and cefotaxime, while the lowest rate was found against meropenem and imepenem. Therefore, imipenem and merpenem remains as the most active antibiotic against *K. pneumonia*, but the bacteria was resistant to most antibiotics at an alarming level. The distribution of resistant *K. pneumonia* to beta-lactam antibiotics is similarly highly distributed in different countries. The result of our studies agrees with the study in Egypt where highly resistant bacteria is recorded against beta-lactam antibiotics (Abdel-Rhman SH, 2020) and similar result was also recorded in Turkey (Aktas et al., 2002).

Resistance genes were found in most of the isolates in high rates. Among 85 isolates of *K. pneumonia,* 33 isolates (33.8%) had CTX gene which is the highest rate among

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three resistance genes. The second most common resistance gene was SHV gene which was found in 19 isolates (22.3%) and the lowest rate was OXA gene which was discovered in 10 samples (11.7%) (*Fig. 1B*). This indicates that bacteria harboring resistance gene are endemic, and they are circulating in the hosts of the study area. The high rate of beta-lactamase genes in *K. pneumonia* is not recorded only in this studied area, but also in other countries such as in Brazil (Ferreira et al., 2019). The isolates harboring SHV genes containing new mutations were highly resistant to oxacillin, amoxicillin, cefotaxime, and ceftriaxone, but they were sensitive to amoxicillin-clavulanic acid. Meropenem and imipenem are still the most active antibiotics against *K. pneumonia* and the bacteria were completely sensitive to the latter antibiotic.

Finally, the interesting result of this study, other than antibacterial resistance pattern of *K. pneumonia* is finding the novel *bla*SHV gene variant which is different from all previously discovered variants of *bla*SHV. The bacteria containing new SHV gene variants recovered from ten isolates of *K. pneumonia* harboring SHV genes. The bacteria were isolated from the viscera of layer chicken after slaughtering in local slaughterhouses. The novel allele was named *bla*SHV-Suly213 because it is more closely related to *bla*SHV213 variant (Aung et al., 2021) (*Fig. 3*). The new variants contain three nucleotide mutations in comparison to both SHV-1 (CP052436.1) and SHV-213 (*Fig. 2*). In comparison to SHV-213, the new gene contains two mutations which are located at the nucleotide number 357 starting from the starting codon sequence and at position number 759 from the starting codon sequence where guanine is replaced by cytosine. In comparison to SHV1, it contains one mutation at locations number 705 where guanine is replaced by adenine (*Fig. 2*). The diversity of the gene was compared to different related sequences available online. 11 different gene sequences of SHV enzyme were taken and put into a phylogenetic tree to find the diversity of the gene found in this study and it was revealed that the novel allele is more closely related to SHV-213 and SHV-1 (*Fig. 3*).

Interestingly, all three mutations are silent mutations. The nucleotide number 357 thymine was changed to cytosine (TGT/TGC) and both codons translate to the same amino acid, cysteine. The second mutation at the position number 705 where adenine is replaced by guanine (GAA/GAG) and both codons generate the same amino acid, glutamate. The third mutation, cytosine is replaced by guanine at position number 759 (CGC/CGG) and both codons make arginine. Nucleotides have been mutated but the amino acid sequence and open reading frame remained intact. Therefore, the mutations may not be advantageous for the bacteria, but the strategy of the bacteria to have these mutations and the reason of changing these nucleotides remains unclear.

Conclusion

In summary, the current study analyzed the molecular study of antibiotic resistance of clinical isolates, *K. pneumonia* originated from layer chicken in Sulaimani city. The data discovered a new variant of *bla*SHV, SHV-Suly213. The resistance gene compromised mixed sequences (hybrid) of both SHV-1 and SHV-213 that contain three nucleotide mutations. The bacteria are resistant to ceftriaxone, and cefotaxime, but not meropenem. These findings show the genetic diversity of beta lactamase gene, *bla*SHV and generation of new variants of SHV genes in bacteria generated through periodic mutations.

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