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*Full Length Research Paper*

# Mutations in β-lactamases detected in multidrug resistant gram negative bacteria isolated from community acquired urinary tract infections in Assiut, Egypt

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**The aim of this study was to characterize the beta lactamases genes of bacteria isolated from urinary tract infection (UTI) in Assiut, Egypt. Results revealed that one hundred fifty nine [31.8%] out from 500 urine samples were culture-positive. *Escherichia coli* was the most common UTI pathogen [61%] followed by *Klebsiella pneumoniae* [23.3%], *Proteus mirabilis* [8.2%] and *Pseudomonas aeruginosa* [7.5%]. Sensitivity of isolates to ampicillin was [15%], amoxicillin/clavulanic acid [43.5%], ceftriaxone [24%], imipenem [95.6%], amikacin [75%], ciprofloxacin [21.4%] and trimethoprim /sulfamethoxazole [37%]. Confirmatory phenotypic detection of extended-spectrum β-lactamases [ESBLs] by ESBL E-test method resulted in [42.7%] isolates were ESBLs producers. Genotypic characterization of ESBLs genes in phenotypically positive isolates resulted in [91.2%] were ESBL producers. The presence of CTX-M type ESBL was [75%] followed by TEM [37%], OXA [24%] and SHV [21%]. Sequencing of ESBLs genes showed that CTX-M-15, OXA [1,116], TEM-1 and SHV [1, 11,111,115] as new ESBL types. Multiple sequence alignment of sequenced genes showed mutation in L31R in SHV-11[Novel SHV-115], E29Q in SHV-1[Novel SHV-111], and P65R in TEM-1 and I97M in OXA-1 [Novel OXA-116]. This study is one from first studies in Egypt that highlights the presence of multiple mutations in ESBLs.**

**Key words:** Uropathogens, extended-spectrum β-lactamases (ESBLs), mutation, Egypt.

## INTRODUCTION

UTIs are ranked among the most common infectious diseases found in either the community or healthcare setting (Nicolle, 2005). UTIs have been described by the Egyptians as "sending forth heat from the bladder" since ancient times with the first documented description in the Ebers Papyrus 1550 BC (Al-Achi, 2008). Many studies reported that *Escherichia coli* and *Klebsiella pneumoniae* represented the most common pathogens that caused UTIs in various regions of the world,(Gupta et al., 2011) while *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter*, *Enterococcus* species and *Staphylococcus species* represented the minority of the

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detected uropathogens (Thomson et al., 1994).

Emergence of antibiotic resistance in uropathogens increased sharply over the world. It varies according to geographical regions and is directly proportional to the excessive use and misuse of antibiotics (Gupta et al., 2001). Certain microorganisms produce defensive enzymes as ESBLs, which own hydrolytic activity enabling them to attack β-lactam ring of penicillins (Paterson and Bonomo, 2005) β-lactamases possess an active site serine, and generally inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam (Livermore, 1995).

TEM-1, SHV-1 and TEM-2 enzymes have limited hydrolytic activity, while mutations in these enzyme result in extended-spectrum phenotype [ESBLs] manifested in serious structural alterations within the active site of the protein which potentiate the β-lactamase activity towards the third-generation cephalosporins (Stürenburg and Mack, 2003). Other types of ESBLs including CTXM, OXA, BES, CME, VEB, PER, SFO and GES, which characterized by potent hydrolytic activity, have been emerged which reflecting the abundance of β-lactamase genes that are available in the bacterial gene pool (Ambler et al., 1991; Livermore, 1995; Philippon et al., 2002; Poirel et al., 2002; Stürenburg and Mack, 2003).

In many studies, a remarkable increase in the ESBL rate was reported from all regions of the world (Eisner et al., 2006; Gupta, 2007; Hosoglu et al., 2007).

## MATERIALS AND METHODS

### Collection of urine samples

A total of 500 clinical samples were collected from Al Azhar university hospital, Assiut, during the period of 1 January 2014 to 1 July 2014, urine samples were collected in a sterile container according to the methods described by Cheesbrough from patients previously clinically diagnosed with UTIs (Cheesbrough, 2006).

### Isolation and purification of uropathogens

Urine samples were centrifuged at 3.000 r.p.m for 5 min and the sediment was streaked on cysteine lactose electrolyte deficient agar [Oxoid, UK] for isolation of different uropathogens.

### Morphological and Biochemical characterization of isolated bacteria

Purified isolates were examined macroscopically and microscopically. Catalase and oxidase tests performed for all isolates. API 20 E and API 20 NE [Biomerieux, France] were used for confirmatory identification of purified isolates from *Enterobacteriaceae,* and *Pseudomonas* spp. (Butler et al., 1975; Peladan and Monteil, 1988).

### Antimicrobial susceptibility testing

The antimicrobial susceptibility test was performed using disks [ampicillin 10 µg, amoxicillin/clavulanate 20/10 µg, ceftriaxone 30 µg, imipenem 10 µg, amikacin 30 µg, ciprofloxacin 5 µg and trimethoprim/ sulfamethoxazole 1.25/ 23.75 µg] diffusion test were performed using the routine discs diffusion procedure described by (Bauer et al., 1966) using Muller-Hinton agar [Oxoid Limited, Hampshire, England] according to the recommendations of Clinical Laboratory Standards Institute [CSLI]. *E. coli* [ATCC 25922] was used as control strain.

The assay was conducted in duplicate for each organism evaluated. The zone size around each antimicrobial disc was interpreted as susceptible, intermediate or resistant according to interpretative criteria recommended by CLSI (2013). Isolates with inhibition zone ≤ 25 mm to ceftriaxone 30 µg [Oxoid, UK] was considered as ESBLs producers (CLSI, 2013).

The ESBL-E-Test strips [AB biodisc, Solna, Sweden] ceftazidime/ceftazidime + clavulanic acid [TZ/TZL] and cefotaxime/cefotaxime + Clavulanic acid [CT/CTL] were used as per the manufacturer's instructions. An isolate was ESBL positive when the minimum inhibitory concentration [MIC] ratio was ≥ 8 and negative when the MIC ratio was < 8 (Mortensen et al., 2005; CLSI 2013).

### Genetical analysis

DNA was purified by using WIZARD Genomic DNA Purification Kit (Promega, Germany, catalog No. A1125), following instructions as directed by the manufacturer.

PCR was conducted for detection of specific genes according to the resistance phenotype using forward and reverse primers for the following genes *bla*-TEM, *bla*-SHV, *bla*-CTX-M, and *bla*-OXA (Oliver et al., 2002; Pagani et al., 2003). PCR reactions was carried out in 50 µl reactions with 2µl forward and reverse primers, 2 µl template DNA, and 10 ml of 5Xof Hot Master Mix [Solis BioDyne - Tartu Estonia]. Thermal cycling consisted of different conditions for amplification of each gene (Table 1).

### Agarose gel electrophoresis

The PCR products were visualized using agarose [1%] gel electrophoresis [Biometra-Agarose gel mini, Germany] (Brook, 2005). amplicons sizes were calculated by a comparison with 100 bp to 3kb molecular weight DNA ladder [Solis BioDyne -Tartu Estonia]. PCR products were purified using the Agencourt XP Ampure Beads [Beckam Coulter, USA]. The quality of the final products were assessed using a Bioanalyzer 2100 [Agilent Technologies, USA] and after quantification with a Qubit [Invitrogen, USA].

### Sequencing of PCR products

β-lactamases were identified by sequencing the purified PCR amplicons using the dideoxynucleotide chain termination method with fluorescent cycle sequencing using dye-labelled terminators [BigDye Terminator version3.1cycle sequencing kit; Applied Biosystems, Grand Island, NY, USA] on an ABI prism 3730 automated DNA sequencer (Sanger et al., 1977).

### Sequence assembly, analysis and alignment

The sequences obtained of ESBLs was assembled by [DNA Baser Sequence Assembler v4.32, 2015; Heracle BioSoft, http://www.DnaBaser.com] and compared with published sequences from the same genomic region available in GenBank [BLAST] (McGinnis and Madden, 2004). The multiple sequence alignment was performed by the online software Clustal Omega

**Table 1.** Universal primers and PCR conditions for β-lactamases.

| Gene | Primers | Oligonucleotide sequence [5` to 3`] | PCR conditions | Reference | Expected size [bp] |
|---|---|---|---|---|---|
| *bla*TEM | TEM-**F**<br>TEM-**R** | 5`- ATGAGTATTCAACATTTCCG- 3`<br>5`- CTGACAGTTACCAATGCTTA- 3` | 1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 43°C , 1 min at 72°C; 1 cycle of 10 min at 72°C | Oliver et al. (2002) | 867 |
| *bla*SHV | SHV-**F**<br>SHV-**R** | 5`- GGTTATGCGTTATATTCGCC- 3`<br>5`- TTAGCGTTGCCAGTGCTC- 3` | 1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1min at 48°C, 1min at 72°C; 1 cycle of 10 min at 72°C | Oliver et al. (2002) | 867 |
| *bla*OXA | OXA-**F**<br>OXA-**R** | 5`- ACACAATACATATCAACTTCGC- 3`<br>5`- AGTGTGTTTAGAATGGTGATC- 3` | 1 cycle of 5 min at 96°C; 35 cycles of 1 min at 96°C, 1 min at 46°C, 2 min at 72°C; 1 cycle of 10 min at 72°C | Oliver et al. (2002) | 885 |
| *bla*CTX-M | CTX-M-**F**<br>CTX-M-**R** | 5`- ATGTGCAGYACCAGTAARGT- 3`<br>5`- TGGGTRAARTARGTSACCAGA- 3` | 1 cycle of 7 min at 94°C ; 35 cycles of 50 s at 94°C , 40 s at 50°C, 1 min at 72°C; 1 cycle of 5 min at 72°C | Pagani et al., (2003) | 593 |

**Table 2.** Total antimicrobial susceptibility pattern of UTI isolates results.

| Isolate | | *E. coli* | | | *K. pneumoniae* | | | *Proteus mirabilis* | | | *Pseudomonas aeruginosa* | | | *Total* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S | I | R | S | I | R | S | I | R | S | I | R | S | I | R |
| Amp | N | 22 | 6 | 69 | 0 | 1 | 36 | 2 | 1 | 10 | 0 | 0 | 12 | 24 | 8 | 127 |
| | % | 23 | 6 | 71 | 0 | 2.7 | 97.3 | 15.3 | 7.7 | 77 | 0 | 0 | 100 | 15 | 5 | 80 |
| CRO | N | 29 | 7 | 61 | 9 | 0 | 28 | 5 | 3 | 5 | 0 | 1 | 11 | 39 | 11 | 109 |
| | % | 30 | 7.2 | 62.8 | 24.4 | 0 | 75.6 | 35.5 | 23 | 38.5 | 0 | 8.3 | 91.7 | 24 | 7 | 76 |
| AMC | N | 51 | 25 | 21 | 12 | 12 | 13 | 6 | 2 | 5 | 0 | 0 | 12 | 69 | 39 | 51 |
| | % | 52.5 | 25.8 | 21.7 | 32.4 | 32.4 | 35.2 | 46 | 15.5 | 38.5 | 0 | 0 | 100 | 43.5 | 24.5 | 32 |
| IMP | N | 95 | 1 | 1 | 37 | 0 | 0 | 13 | 0 | 0 | 7 | 0 | 5 | 152 | 1 | 6 |
| | % | 98 | 1 | 1 | 100 | 0 | 0 | 100 | 0 | 0 | 58.3 | 0 | 41.7 | 95.6 | 0.6 | 3.8 |
| CIP | N | 18 | 40 | 39 | 3 | 13 | 21 | 3 | 7 | 3 | 10 | 1 | 1 | 34 | 61 | 64 |
| | % | 18.5 | 41.3 | 40.2 | 8 | 35 | 57 | 23 | 54 | 23 | 91.7 | 0 | 8.3 | 21.4 | 38.4 | 40.2 |
| AK | N | 75 | 11 | 11 | 23 | 2 | 12 | 10 | 1 | 2 | 11 | 0 | 1 | 119 | 14 | 26 |
| | % | 77 | 11.5 | 11.5 | 62.2 | 5.4 | 32.4 | 77 | 7.7 | 15.3 | 92 | 0 | 8 | 75 | 9 | 16 |
| SXT | N | 41 | 5 | 51 | 13 | 1 | 23 | 4 | 1 | 8 | 1 | 0 | 11 | 59 | 7 | 93 |
| | % | 42.3 | 5.1 | 52.6 | 35.1 | 2.7 | 62.2 | 30.8 | 7.7 | 61.5 | 8.3 | 0 | 93.7 | 37 | 4.5 | 58.5 |

N=number, %=percentage, S=sensitive, I=intermediate, R=resistant, amp=ampicillin, CRO=ceftriaxone, AMC=amoxicillin-clavulanic acid, Imp = imipenem, CIP=ciprofloxacin, AK=amikacin, SXT=sulfamethoxazole-trimethoprim.

[EMBL-EBI, Hinxton, UK] (Sievers et al., 2011). A mutation was considered evident if it resulted in a unique amino acid change when compared with available NCBI sequences TEM-1 [NG_041152],OXA-1 [NG_041621], NG_039554 [SHV-1] and CTXM-15 [NG_037755] [http://www.ncbi.nlm.nih.gov].

### Statistical analysis

Data were analyzed using the Statistical Package for the Social Sciences software version 20 [SPSS Inc., Chicago, IL, USA]. A P value of <0.005 for a whole family of tests was considered statistically significant.

## RESULTS

Out of the collected samples 31.8% [159/500] were culture positive. *E. coli* was the most predominant pathogen 61% [97/159]. Other uropathogens were *K. pneumoniae* 23.3% [37/159], *P. aeruginosa* 7.5% [12/159] and *P. mirabilis* 8.2% [13/159].

The bacteria species showed varying susceptibility patterns to seven of the antimicrobial agents (Table 2).

### Performance of ESBL phenotypic detection tests

All isolates were tested for the production of the ESBLs. Preliminary screening of reduced susceptibility to ceftriaxone resulted in 75.5% [120/159], were ESBLs producers out of them *E.* coli 70% [68/97], *K. pneumoniae* 81% [30/37], *P. mirabilis* 77% [10/13], *P.*

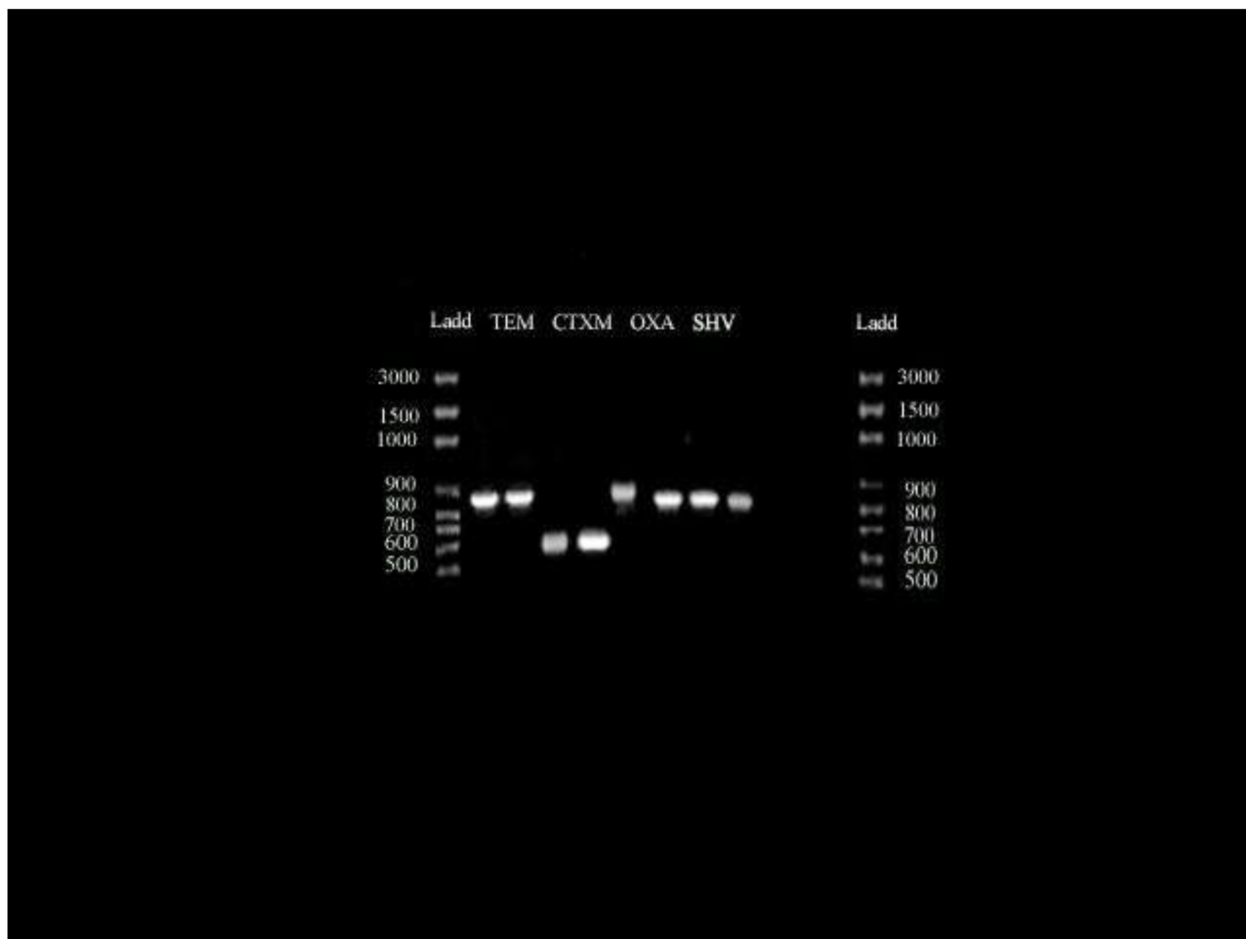


**Figure 1.** Agarose gel electrophoresis of PCR amplified β-lactamases genes. Lane lad: ladder with size range from 100bp to 3000bp. Lane OXA: OXA gene appear at 585bp. Lane CTX-M: CTX-M gene appear at 593bp. Lane TEM: TEM gene appear at 867bp. Lane SHV: SHV gene appear at 885 bp.

*aeruginosa* 100% [12/12]. Confirmatory test by ESBL-E-Test resulted in 42.8% [68/159], were ESBLs producers out of them *E.* coli 35% [34/97], *K. pneumoniae* 62% [23/37], *P. mirabilis* 38.5% [5/13] and *P. aeruginosa* 50% [6/12].

## Genotypic detection of β-lactamases producers by PCR

Amplification of *bla*-TEM, *bla*-SHV, *bla*-OXA and *bla*-CTX-M genes by PCR using universal primers for 68 isolates of phenotypic ESBL producers (Figure 1).

TEM gene detected with 37% [25/68], SHV 21% [14/68], OXA 24% [16/68] and CTX-M 75% [51/68] which is most predominant gene detected. TEM gene were detected in *E. coli* 41% [14/34], *K. pneumoniae* 26% [6/23], *Proteus mirabilis* 40% [2/5] and highly percentage detected in *P. aeruginosa* 50% [3/6]. SHV gene was detected in *E. coli* 12% [4/34], *K. pneumoniae* 26% [6/23], *P. aeruginosa 33*% [2/6] and highly percentage detected in *P. mirabilis* 40% [2/5]. OXA gene were detected in *K. pneumoniae* 22% [5/23], *P. aeruginosa* 17% [1/6], *P. mirabilis* 20% [1/5] and highly percentage detected in *E. coli* 26% [9/34]. CTX-M gene was detected in *K. pneumoniae* 74% [17/23], *P. aeruginosa 67*% [4/6], *P. mirabilis* 20% [1/5] and highly percentage detected in *E. coli* 85% [29/34] (Table 3).

## Detected types of β-lactamases by sequencing

Alignment of sequenced β -lactamases genes with BLAST resulted in, CTX-15 ESBL type, the only detected among CTX-M gene 100% [51/68]. OXA-1 type present with 93.5% and OXA-116 present with 6.5% among OXA type ESBL gene. TEM-1 β -lactamase the only detected among TEM gene. SHV-1 β-lactamase present with 57% [8/14], SHV-11 -lactamase type present with 29% [4/14], SHV-111 ESBL type present with 7% [1/14] and SHV-115 ESBL type present with 7%[1/14] among SHV ESBL gene.

**Table 3.** Frequency distribution of β -lactamases among urinary isolates.

| *Gene* | Occurrence | | | | Total |
|---|---|---|---|---|---|
| | *E. coli* | *K. pneumonia* | *Proteus mirabilis* | *Pseudomonas aeruginosa* | |
| ZERO | 1 | 3 | 2 | 0 | 6 |
| TEM | 0 | 0 | 1 | 0 | 1 |
| SHV | 0 | 1 | 0 | 0 | 1 |
| CTX-M | 9 | 6 | 0 | 0 | 15 |
| OXA | 2 | 0 | 0 | 0 | 2 |
| CTX-M +TEM | 1 | 4 | 0 | 1 | 6 |
| CTX-M+SHV | 2 | 1 | 0 | 0 | 3 |
| TEM+SHV | 0 | 0 | 1 | 1 | 2 |
| CTX-M+OXA | 6 | 3 | 0 | 2 | 11 |
| OXA+TEM | 2 | 0 | 0 | 0 | 2 |
| OXA+SHV | 0 | 2 | 0 | 1 | 3 |
| CTX-M +TEM+SHV | 2 | 0 | 0 | 0 | 2 |
| CTX-M+OXA+TEM | 9 | 0 | 0 | 1 | 10 |
| CTX-M+OXA+SHV | 0 | 1 | 1 | 0 | 2 |
| CTX-M +TEM +OXA+ SHV | 0 | 2 | 0 | 0 | 2 |
| Total | 34 | 23 | 5 | 6 | 68 |

**Table 4.** Mutations detected in sequenced β-lactamases genes.

| Gene | TEM-1 | SHV-1 | SHV-11 | OXA-1 |
|---|---|---|---|---|
| **Microorganism** | *E. coli* | *E. coli* | *K. pneumoniae* | *K. pneumoniae* |
| **Strain** | NORAN2014 | esam1980 | MR1982 | RAWAN2015 |
| **Mutation** | P65R | E29Q | L31R | I97M |
| **Novel ESBL** | ……….. | SHV-111 | SHV-115 | OXA-116 |
| **Other ESBLs detected** | CTX-M, OXA | CTX-M | CTX-M, OXA | CTX-M,SHV |
| **Accession number** | KR632744 | KR780480 | KR780481 | KR780478 |

## Mutations in sequenced β-lactamases

Multiple sequence alignment of ESBL genes resulted in detection of multiple mutations and development of novel ESBL types (Table 4).

## DISCUSSION

In our results, the leading pathogen causing UTI was *E. coli* [61%] followed by *K. pneumonia* [23.3%], *P. mirabilis* [8.2%] and *P. aeruginosa* [7.5%] which nearly similar to that reported by Ibrahim et al. (2014) in Egypt. *E. coli* was the most common pathogen causing UTI in the world this in agreement with our study (Gupta et al., 2011). Blindly treatment of UTI leads to increase the resistance rate of these pathogens to antibiotics especially β–lactam antibiotics due to excessive and misuse of these antibiotics. Imipenem was the most effective antibiotic against UTI and activity more than 95% because of less used due to economic considerations.

In this study production of ESBLs varies from type of isolate to another. *P. aeruginosa* was the most powerful ESBLs producers [100%], followed by *E. coli* 97%, *K. pneumonia*e 82.6% and finally *P. mirabilis* 82%. In our study sequenced CTX-M showed that most CTX-M genes were CTX-M-15 as that detected in Egypt and middle east area (Amin et al., 2005; Thabit et al., 2011).

In our study, multiple mutations detected among β-lactamases; in SHV-11 gene there is mutation in position 31, amino acid Arginine instead of amino acid Leucine [L31R], results in novel SHV-115 *K. pneumoniae* strain MR1982 ESBL with accession number [KR780481]. L31R mutation in *Klebsiella* was the first detected in middle east and the second detected in world after Mendonça et al. (2009) in Portugal from *Klebsiella* results in novel SHV 61. TEM-1 gene show mutation in position 65, amino acid Arginine instead of amino acid Proline [P65R] results in *E. coli* strain NORAN2014 β-lactamase TEM-1with accession number [KR632744.1]. In SHV-1 gene there is mutation in position 29, amino acid Glutamine instead of amino acid Glutamate [E29Q]

results in novel SHV-111 ESBL, *E. coli* strain esam1980 with accession number [KR780480.1]. In OXA-1 gene there is mutation in position 97, amino acid Methionine instead of amino acid Isoleucine [I97M] results in novel OXA-116, *K. pneumoniae* strain RAWAN2015 ESBL with accession number [KR780478]. [http://www.ncbi.nlm.nih.gov].

## Conclusion

Frequent consumption and misuse of antibiotics lead to mutations and the emergence of new genes more aggressive and more resistant to antibiotics, which leads to increased mortality and which calls for the search for new antibiotics and open new horizons in how to address these pathogens.

## Conflict of Interests

The authors have not declared any conflict of interests.

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