Genotypic detection of GES and VEB extended spectrum β-lactamase among aerobic Gram-negative bacteria isolated from diabetic foot ulcers

Najah Mahdi Lukey¹, Fatima Moeen Abbas²
¹The Iraqi Ministry of Interior, Department of Medical Services, Iraq.
²Department of Biology, College of Sciences for Women, University of Babylon, Iraq.
najahn983@gmail.com

Abstract
Objective: Gram-negative bacteria with Extended-Spectrum β-Lactamase (ESBL) genes are of concern due to their susceptibility to multi-drug resistance. The goal of this research is to investigate the gene coding of resistance of ESBLs encoded by the VEB gene and GES gene to modern β-lactams. Method: seventy wound swabs were taken from diabetic foot ulcer patients in period from October (2019) to February (2020). The collected samples were cultured on different media agar to identify by morphological, biochemical tests and Vitek 2 system. To determine VEB gene and GES gene using the methodology of Polymerase chain reaction (PCR) on the isolated bacteria. Result: a total of 50 Gram negative bacteria. The distribution of VEB gene was Proteus ssp. 7, Morganella morganii.ssp. 2, Klebsiella oxytoeca 1, Acinetobacter baumannii 2. While the GES gene distribution was Escherichia coli 1, Proteus ssp 1, Morganella morganii.ssp 4, Acinetobacter baumannii 1. Conclusion: The VEB gene and GES gene plays an important role in the resistance to new β-lactams of ESBL-producing isolates.

Keywords: Gram-negative bacteria, diabetic foot ulcer, ESBL, GES and VEB gene, PCR.
1. Introduction

Diabetes mellitus (DM) is a chronic disease that affects a large number of people worldwide and is a major public health concern. The prevalence of diabetes was estimated to be 2.8% in 2000 and 4.4% in 2030, for all age groups worldwide. It is estimated that the total number of people with diabetes will grow from 171 million in 2000 to 366 million in 2030 (Sarah Wild, 2004). There are several complications associated with diabetes mellitus, including peripheral neuropathy and Peripheral vascular disease development, which contribute to an increased risk of developing foot ulcers (Lipsky et al., 2004). Many opportunistic pathogenic microorganisms may be responsible for the occurrence of diabetic foot ulcers, depending on the availability of appropriate conditions for the growth of these bacteria, such as the aerobic Gram-negative bacteria, which is one of the types that cause diabetic foot injury (Pitout et al., 2005). The bacteria have developed many mechanisms to avoid the drug effect such as extending spectrum beta-lactamase (ESBL) which is a strain of microorganisms that produce beta-lactamases. β-Lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis, which result in ineffective compounds (Pitout et al., 2005). These microorganisms contain genes or encode special genes that descript the secretion of enzymes that work to break down the benzene ring present as a countermeasure. Antimicrobial resistance can even increase further by the acquisition of genetic elements, for example, plasmids carrying SHV-, TEM-, and extended-spectrum β-lactamases (ESBLs) or by PER-, VEB-, GES-, BEL-, and PME-type ESBLs (Maurya et al., 2015).

The Guiana-Extended Spectrum (GES-1) ESBL, first discovered in Klebsiella pneumoniae isolated from a patient in Guyana, has been gradually isolated from Enterobacteriaceae and other bacteria from various geographical areas (Garza-Ramos et al., 2015). Some molecular studies showed that both OXA-10 and GES-1 genes were found in Pseudomonas aeruginosa multidrug-resistant strains isolated from nosocomial infections and in a report on the distribution of the prevalence of ESBLs among burn patients was about 56% and 20% respectively (Tawfik et al., 2012). The GES gene consider A class enzyme, and are not to be related to any other plasmid-mediated β-lactamases. The enzymes show resistance to penicillin and show less activity against aztreonam and imipenem, it also hydrolyzes carbapenems (Snyder, 2001). The GES gene is difficult to identify in the lab due to nuclear nomenclature and paucity. There are different types of GES which have different hydrolysis properties have been designed by identical names (Snyder, 2001). Class A group of lactamases may be classified into several classes, and the VEB group is one of the smaller subgroups of Class A lactamases with only 12 confirmed variants. The VEB enzymes tend to be found frequently in non fermenter organisms such as Pseudomonas aeruginosa and Acinetobacter as well as in other Enterobacteriaceae spp. and their prevalence is growing (Akinci and Vahaboglu, 2010).
This study aimed to determine the frequency of aerobic Gram-negative bacteria associated with diabetic foot ulcer patients, detect their susceptibility profiles and investigation the presence of GES and VEB ESBLs using conventional Polymerase Chain Reaction (PCR) technique.

2. Materials and Methods

Collection of Samples and Microbiological analysis

Seventy wound swabs were collected from diabetic foot ulcer patients from Al-Kindi Hospital and private specialized clinic in Baghdad, from October (2019) to February (2020). The ages of the patients were ranged from 30 - 80 years from both sexes. The specimens were taken by swab the wound by gently rotating sterile calcium alginate or rayon swab between your fingers. Swab the wound from margin to margin in a 10-point zigzag fashion. Use enough pressure to express fluid from within the wound tissue. All samples were labeled and transferred to microbiology laboratory / Al-Kindi Hospital. The wound samples were cultured on MacConkey’s agar and Blood agar, then incubated overnight at 37˚C under aerobic conditions. The cultured that have growth were selected and cultured on slant broth for further isolation and biochemical identification. Species identification was done according to morphological, biochemical tests as described by (Mahon, et al., 2018), then confirmed by VITK 2 system.

Antimicrobial Susceptibility Testing

All bacterial isolates were subjected to susceptibility testing using Kirby –Bauer disk diffusion method on plates of Mueller Hinton agar (Bauer et al., 1966). The following disks were tested: amikacin (30 µg), ceftiraxone (30µg), cefotaxime (30µg), ceftazidime (30µg), ciprofloxacin (5µg), imipenem (10 µg), meropenem (10 µg), amoxicillin clavulanic acid (30 µg), piperacillin (100 µg) and azithromycin (15 µg). The diameter of the growth inhibition zones on plates around the disks was measured and compared with Clinical and Laboratory Standard Institute (CLSI,2017) guidelines.

Molecular Detection of GES and VEB β- Lactamase Genes

The DNA of bacterial isolates was extracted using kit (promega/USA). Conventional PCR technique was used to determine GES and VEB genes using primers detailed in (Table-1). PCR thermocycling conditions were as follows: initial denaturation at 95˚C for 2 min,35 cycles of denaturation at 95˚C for 30 sec, annealing at 50˚C for 1 min for GES gene and 55˚C for 1 min for VEB gene, extension at 72˚C for 2 min an final extension at 72˚C for 10 min.

Table 1: Primer sequences used for ESBLs genes amplification.

| Primer | Sequence (5’-3’) | Amplicon | References |
|--------|-----------------|----------|------------|


3. Results and Discussion

A total of 70 samples were collected from Ulcer Foot Diabetic patients admitted to Al-Kindi Hospital and private specialized clinic in Baghdad province, from October (2019) to February (2020). The male patients were higher risk of infection than female, with 42 (60%) of male and 28 (40%) of female (Table -2).

Table (2) Distribution of patients according to the type of gender.

| Gender | No. | %  |
|--------|-----|----|
| Male   | 42  | 60 |
| Female | 28  | 40 |
| Total  | 70  | 100% |

The result identifies differences between genders in relation to the risk of diabetic foot. Patients with DM from both genders may differ the way to handle the disease and the way they adhere to the care necessary to keep the disease under control. Men in particular, are more careless about their feet (Pscherer et al., 2012), resulting in a higher proportion of amputations among them. On the other hand, due to the challenge of modifying habits, especially the implementation of an eating plan and daily physical exercise, women have greater difficulty retaining glycemic and lipid regulation (Schroeder et al., 2014).

The study shows that 30-40 years’ patients form 5 (7.14%) of total patients. While 41-50 years were 15 (21.4%), and 51-60 years were form 33 (47.1%) which is the higher infection rate among the total ages of samples. The elders of ages 61-70 were 12 (17.1%), however, the ages 71-80 were 5 (7.1%) of the total sample collected (Table -3).

The higher rate of infection is among patients from age 51-60 years old, due to low physical activity, shows association with the presence of general complications, especially with retinopathy and nephropathy (Santos et al., 2015).

Table (3) Distribution of Gram-negative bacterial infection in relation to age groups.

| Age group | No. | %     |
|-----------|-----|-------|
| 30-40     | 5   | 7.14% |
| 41-50     | 15  | 21.42%|
| 51-60     | 33  | 47.14%|
| 61-70     | 12  | 17.14%|
| 71-80     | 5   | 7.14% |
The major patients that the sample were collected were Type II diabetic 65 (92.8%), while the 5 (7.14%) of the samples were Type I (Table-4).

Table (4) Distribution of patients according to the type of diabetes.

| Type of Diabetes | No. | %   |
|------------------|-----|-----|
| Type 1           | 5   | 7.14%|
| Type 2           | 65  | 92.85%|
| Total            | 70  | 100 %|

Type 2 DM (formerly known as non-insulin dependent DM) is the most common type of DM characterized by hyperglycemia, insulin resistance, and relative insulin deficiency. Most patients with diabetes mellitus have either type 1 diabetes (which is immune-mediated or idiopathic). Type 2 DM is the most common type of DM results from interaction between genetic, environmental and behavioral risk factors (Sarah Wild, 2004).

The total 70 sample were positive bacterial growth. The number of bacteria isolated were 78 strain, most of it 50 (64.1%) were Gram negative bacteria, while the remaining 28 (35.8%) were Gram positive bacteria.

Table (5) Distribution of Isolate According to Positive and Negative Culture.

| No. of culture | Gram negative culture | Gram positive culture |
|----------------|-----------------------|-----------------------|
| 78             | 50                    | 28                    |
| 100 %          | 64.1 %                | 35.8 %                |

In the present study the Gram negative bacteria are more prevalence in the foot ulcer patients than gram positive bacteria. Due to morphological structure such as the thickness of cell wall membrane with the peptidoglycan layer and presence or absence of the outer lipid membrane. Any modification of Gram-negative bacteria in the outer membrane, such as modification of hydrophobic properties or mutations in porins and other factors, may create resistance. This substantial layer lacks Gram-positive bacteria, which makes Gram-negative bacteria more resistant to antibiotics than Gram-positive bacteria (Miller, 2016). The result shows that (64.1 %) of the total sample were Gram negative bacteria of the diabetic foot ulcer patients, this agreement with Mohammed in Al-Hilla with (61.7 %) Gram negative bacteria (Al-Allak et al.,2019). While in
Basrah they reported with (23.3 %), in Sulaimani was (29.1%) (Muhsin, et al., 2018). A study in Saudi Arabia shows (83.1 %) (Al Ayed et al., 2018).

Table (6): Prevalence of Gram-negative bacteria recovered from patients with diabetic foot ulcer.

| Gram negative culture aerobes |
|------------------------------|
| Type of Bacteria             | No. | %     |
| Escherichia coli             | 15  | 30 %  |
| Proteus mirabilis            | 9   | 18 %  |
| Proteus penneri             | 1   | 2 %   |
| Proteus hauseri             | 1   | 2 %   |
| Morganella morganii.ssp.    | 7   | 14 %  |
| Klebsiella pneumoniae       | 4   | 8 %   |
| Klebsiella oxytoca          | 2   | 4 %   |
| Pseudomonas aeruginosa      | 5   | 10 %  |
| Enterobacter cloacae complex| 3   | 6 %   |
| Acinetobacter baumannii     | 2   | 4 %   |
| Acinetobacter lwoffii       | 1   | 2 %   |
| Total                       | 50  | 100 % |

The result shows that the highest bacteria isolated was *Escherichia coli* (30 %) in diabetic foot ulcer. The study corresponds with other result in Al Hillah (14.8 %), in Basrah (36.6 %), in Sulaimani (33.3 %). In other countries like Saudi Arabia the result was (6%), and in Iran was (20.5 %). The *Escherichia coli* was the predominant pathogen due to the virulence factors that include outer membrane protease, cytotoxic necrotizing factor 1, drug resistance, and hemolysin (Petkovšek et al., 2009 ).The second most common bacterium isolated was *Proteus mirabilis*, and it consider one of the most common agent infection in diabetic foot ulcer (Perim et al., 2015). The most significant is that it demonstrates a strong tolerance to many antimicrobial agents that cause diabetic foot ulcers worse and postpone care. On a genetic basis, the virulence of *P. mirabilis* is defined as chromosomally incorporated or extra chromosomally imported. *P. mirabilis* virulence factors are wide-ranging, elastic fimbria ,flagella swarming motility and toxin production such as hemolysin and extracellular enzymes such as protease and urease all function together to improve pathogenesis (Armbruster and Mobley, 2012).

All Gram-negative bacteria (n=50) were screened against 10 antimicrobial agents belonging to various groups showed that the isolates differed in their antibiotic resistance . Overall, all Gram-negative bacteria isolated in this study were sensitive to Meropenem form class Carbapenems (88 %), while the Imipenem were (84 %) sensitivity. The rate of sensitivity to aminoglycoside (amikacin) were (72%). The Macrolides class show sensitivity (36%) such as Azithromycin. The penicillin agents such as Piperacillin (6 %), and the β-lactamase inhibitors combination, Amoxicillin - Clavulanic acid rate was (4% ).
Table (7): Susceptibility profiles of Gram-negative bacterial isolates against various antibiotics (n = 50).

| Antibiotic          | Symbol | S    | %    | I    | %    | R    | %    |
|---------------------|--------|------|------|------|------|------|------|
| meropenem           | MEM    | 44   | 88%  | 3    | 6%   | 3    | 6%   |
| imipenem            | IMP    | 42   | 84%  | 5    | 10%  | 3    | 6%   |
| amikacin            | AK     | 36   | 72%  | 5    | 10%  | 9    | 18%  |
| ceftazidime         | CAZ    | 35   | 70%  | 7    | 14%  | 8    | 16%  |
| cefotaxime          | CTX    | 28   | 56%  | 10   | 20%  | 12   | 24%  |
| ceftriaxone         | CTR    | 27   | 54%  | 8    | 16%  | 15   | 30%  |
| ciprofloxacin       | CIP    | 30   | 60%  | 2    | 4%   | 18   | 36%  |
| azithromycin        | ATH    | 18   | 36%  | 15   | 30%  | 17   | 34%  |
| Piperacillin        | PRL    | 3    | 6%   | 8    | 16%  | 39   | 78%  |
| Amoxicillin-Clavulanic acid | AMC  | 2    | 4%   | 5    | 10%  | 43   | 86%  |

S: Sensitive   I: Intermediate   R : Resist

This study revealed that the most susceptible antibiotic was Meropenem and Imipenem followed by Amikacin, the present study was nearly from a study done in Basrah shows that most of isolated bacteria were susceptible mainly to Meropenem, ciprofloxacin, gentamicin, followed by Kanamycin and Lincomycin, then Piperacillin (Jeber and Saeed, 2013). While in Al-Hilla the result shows that Gram-negative bacteria demonstrated strong immunity to Amikacin, Imipenem, and Aztreonam (Al-Allak et al., 2019). In Iran, susceptibility results identified Meropenem antibiotic as a more effective agent against E. coli spp. (Ahadishooli et al., 2020).
The PCR results detected GES gene with 7(3.5 %), while the VEB gene 12 (6 %) (Fig-1) and (Fig-2). In the recent studies in Egypt, there was no GES gene found in the E. coli stains, the first study announced the only (3.85 %) was carrying GES gene (El-Badawy et al., 2017). Other studies identified 16.7% (6/36) and 8% (8/100) prevalence rates for VEB gene by conventional PCR technique (Udomsantisuk et al., 2011; Rezai et al., 2015). In Iran, the GES gene were (16%) and the VEB gene was (13.3 %) of the total ESBLs genes isolated (Amirkamali et al., 2017). In a similar study for the GES gene of Pseudomonas aeruginosa isolated from Nosocomial Infections in Ismailia, Egypt, the result was 12/21 (26.7%) (Shehata et al., 2015).

Figure (1): PCR amplified products from extracted. Gram negative isolates DNA amplified with primer for ESBLs gene VEB /642dp.

Lane: (M), DNA Ladder size. marker (1.500 bP ladder).
Lanes: 1, 2,3,4,5 and 6 isolates of \textit{Proteus mirabilis}.
Lane: 7 isolates of \textit{Proteus hauseri}.
Lanes: 8 and 9 isolates of \textit{Morganella morganii.ssp}.
Lane: 10 isolates of \textit{Klebsiella oxytoca}.
Lanes: 11 and 12 isolates of \textit{Acinetobacter baumannii}.
Lanes: C - It’s negative.
Lanes: C+ It’s positive.
Figure (2): PCR amplified products from extracted. Gram negative isolates DNA amplified with primer for ESBLs gene GES /827 dp.
Lane: (M), DNA Ladder size. marker (1.500 bp ladder).
Lane: 1 isolates of E. coli.
Lane: 2 isolates of Proteus penneri.
Lanes: 3,4,5, and 6 isolates of Morganella morganii.ssp.
Lane: 7 isolates of Acinetobacter baumannii.
Lanes: C - It’s negative.
Lanes: C+ It’s positive.

4. Conclusions
This study shows the best alternative treatment for ESBL producers of Gram-negative bacterial infections diabetic foot ulcer is Carbapenems (Imipenem and Meropenem). The VEB and GES genes have a significant role in the resistance to new β-lactams of ESBL-producing isolates.

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