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Molecular study of some pathogenic bacteria associated with the diabetes patients

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Abstract---The study aimed to isolation and identification of aerobic bacteria from diabetic foot ulcers in diabetic patients and investigation the resistance of bacteria to antibiotic agents phenotypically and genotypically. The present study included 50 specimens collected from patients suffering from diabetic foot ulcer for both sexes with an age ranged between (20 -60) years. The results showed that 11 (22%) % isolates were belonged to *P. aeruginosa*, 8 (16%) isolates were *K. pneumonia*, followed by *Acintobacter baumanii* with 8(16%) isolates, *Proteus mirabilis* 6(12%), *E.coli* 5(10%), *S. aereus* 11(22%), and *S. epidermidis* 4(10%). The dominance bacterial isolates, were *P. aeruginosa* 11(22%), *K.pneumonia* 8(16%), and *A. baumanii* 8(16%) that isolated and diagnosed from clinical specimens of diabetic foot ulcer and detect the antibacterial resistance phenotypically and genotypically.

Keywords---molecular study, pathogenic bacteria, diabetes, patients, Iraq.

Introduction

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerve (1). Foot infections are common and serious problems in persons with diabetes. Diabetic foot infections (DFIs) typically begin in a wound, most often a neuropathic ulceration. While all wounds are colonized with microorganisms. Infections are then classified into mild (superficial and limited in size and depth), moderate (deeper or more extensive), or severe (accompanied by systemic signs or metabolic perturbations) (2). The principal

contributory factors that might result in foot ulcer development are peripheral neuropathy, peripheral vascular disease, poor glycemic control, foot deformity, past foot ulcer history, previous amputation, visual impairment, diabetic nephropathy (especially patients on dialysis), and cigarette smoking (3). Antibiotic therapy should be based on bacterial culture results and the antibiotic's toxicity capability. Uncontrolled or poorly controlled diabetes will reduce the effectiveness of immune cells fighting against bacteria where even a small cut may lead to an open sore called ulcer. In patients with diabetes there may be impaired microvascular circulation to the region of diabetic foot which limits the access of phagocytic cells resulting in bacterial infection (4). Bacterial resistance to antimicrobial agents is a serious problem worldwide with regard to treatment of infectious diseases. Understanding of the molecular basis of how resistance genes are acquired and transmitted may contribute to the creation of new antimicrobial strategies. The spread of antibiotic resistance is usually associated with either the clonal spread of an epidemic strain or through independent acquisition of the resistance genes on plasmids, transposons or integrons (5). Naturally occurring gene expression elements called "integrons" have been described as vehicles for the acquisition of resistance genes carried by mobile elements. Integrons are one of the mobile genetic elements which can carry genes of resistance to different antibiotics, which contain integrase gene (5). The aim of the present study to perform a molecular analysis of multidrug resistance bacteria causing DF ulcer and to determine their antibiotic resistance pattern as well as comparing these recent findings with those reported in our previous study.

Materials and Methods

The study included 100 specimens (swab specimens) collected from patients suffering from diabetic foot ulcer who attending to Alsader medical city, ALNajaf/ Iraq , during the period extended from September 2021 to December 2022 for both sexes with an age ranged between (20 -70) years.as in figure 1. A 100 pus samples swab specimens from diabetic foot infection ulcer then transported by sterile transport swabs to the department of bacteriology laboratory and inoculated using direct method of inoculation on culture of selective media namely MacConkey, Blood, Mannitol and Cetrimide agar as figure (1), then inoculated at 37°C for 18-24 hours (6).

Bacterial Preparation

The collected specimens were inoculated on three types from culture media which included blood agar, mannitol salt agar and MacConkey agar. The plates were incubated at 37°C for 24 hours then a single pure isolated colony was transferred to trypticase soya agar for the preservation and to carry out other biochemical tests that confirmed the identification of isolates (7). Suspension was prepared according to the manufacturer's recommendations of bioMérieux by transferring sufficient number of colonies from overnight pure culture by swab and suspending the microorganism in 3.0 ml of sterile saline in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity was adjusted to equivalent to a McFarland No. 0.5 using a turbidity meter called DensiChek. The same suspension was used in identification with VITEK2 compact system.

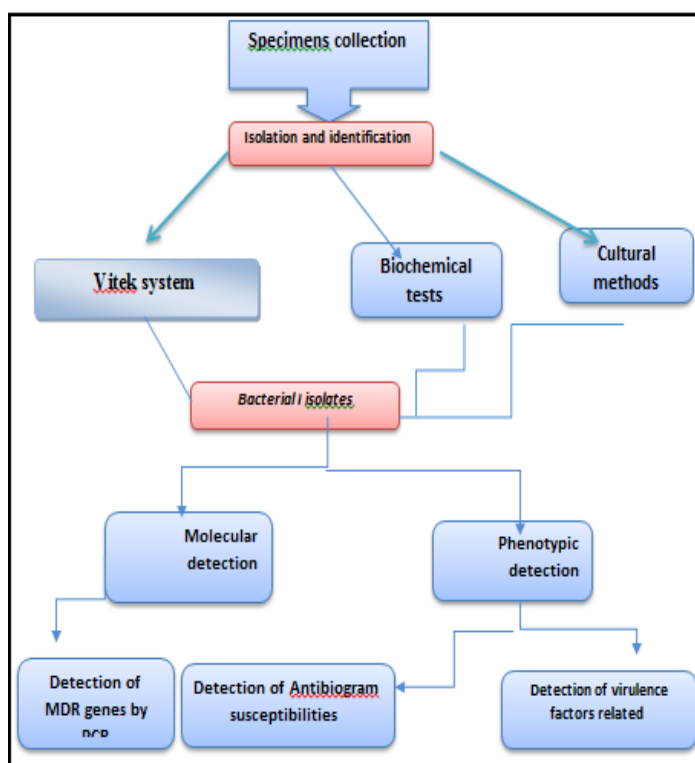


Figure 1: Scheme of the current study

Morphological and Cultural Characteristics

A single colony was taken from each primary positive culture on blood agar, MacConkey agar, mannitol salt agar and repeat growth for gain pure culture and then it was identified depending on its morphological and cultural characteristics (blood hemolysis, lactose fermentation, mannitol fermentation, colony shape, size, colour, borders, and texture) and then it was examined under the microscope after making smear from pure colony on clean slid and stain with Gram's stain for observation arrangement and reaction bacteria with stain (8).

Vitek2 Identification

Gram positive (GP) and Gram negative (GN) identification card has been used for identification Gram positive and Gram negative bacteria. The bacterial suspension was adjusted to McFarland standard of 0.5 in 2.5ml of a 0.45% sodium chloride solution with a Vitek2 instrument (bioMérieux, France). The time was always less than 30 min. The GP, GN identification card is a fully closed system to which no reagents have to be added. The card was put on the cassette designed for use with the Vitek2 system, placed in the instrument, automatically filled in a vacuum chamber, sealed, incubated at 35.5°C, and automatically subjected to colorimetric measurement (with a new reading head) every 15 min for a maximum incubation period of 8 hours. Data were analyzed using Vitek2 databases, which allow organism identification in a kinetic mode beginning 180 min after the start of incubation (9).

Antibiotic Susceptibility (Test Disk Diffusion Method)

It was performed according to Clinical Laboratory Standard Institute (10):

1. With a sterile wire loop, the tips of 4-5 isolated colonies of the organism to test tube containing 5 ml of sterile normal saline in a cell density equivalent to turbidity of McFarland tube No. (0.5) which approximately equal to bacterial cells density of 1.5×10^8 cells/ml
2. A sterile cotton swab was dipped into the standardized bacterial suspension. The excess fluid was removed by rotating the swab firmly against the inside of the tube above fluid level. The swab was then streaked onto the dried surface of a Muller-Hinton plate in 2 different planes to obtain an even distribution of the inoculums.
3. The plate lids were replaced and the inoculated plates were allowed to remain on a flat and level surface undisturbed for 3-5 min to allow absorption of excess moisture.
4. With the sterile forceps, the selected discs were placed on the inoculated plate and pressed gently into the agar. Within 15 min the inoculated plates were incubated at 37 °C for 18 -24 hr in an inverted position.
5. After incubation, the diameters of the complete inhibition zone were noted and measured using reflected light and a ruler. The end point, measured to the nearest millimeter, was taken as the area showing no visible growth.
6. The results interpreted according to (CLSI, 2018), the critical diameters and to the leaflet of antibiotics manufactures.

Extraction of Genomic DNA

DNA of bacterial isolates was prepared by boiling method. Briefly, colonies were suspended in 100 microliters of sterile distilled water and boiled at 100°C in the water bath for 15 minutes then rapidly cooled at -20°C for one hour, then centrifugation and the supernatant were preserved for the used in the amplification-processes (11).

Estimation of DNA Concentration

The concentration and purity of DNA can be determined by reading the optical density of a sample at 260 and 280 nm in spectrophotometry, 5 µL of each DNA sample were added to 995 µL. of distilled water and mixed well. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. For pure double-stranded DNA, 1 OD₂₆₀ = 50 µg/ mL (12). The ratio between the readings at 260 and 280 nm (OD₂₆₀/OD₂₈₀) provides an estimate of the purity of the nucleic acid. Pure preparation of DNA has an OD₂₆₀/OD₂₈₀ value of 1.8, the concentration of DNA was calculated by the formula: DNA concentration (µg/ml) = O.D 260nm × 50 × dilution factor

Results and Discussion

This study was conducted on 50 specimens from wounds of diabetic foot of suspected patients during the period from November 2021 to December in the AL-Sader hospital in Al-Najaf City, Iraq, DFU = 50 (50%), and Control = 50 (50%).

According to the results of the study the male patients outnumbered the female patients by a margin of 42 % to 58 % for DFU compared with control as illustrated in figure (2).

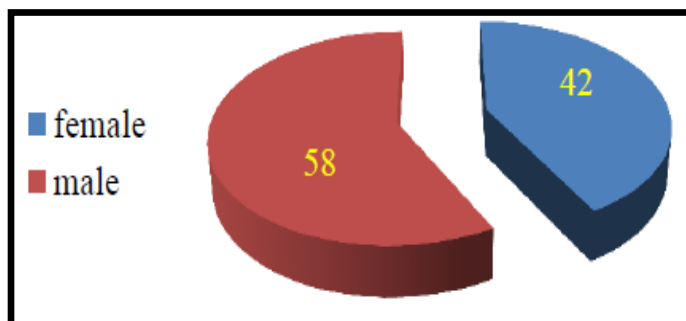


Figure 2: Distribution of patients with Foot ulcer according to sex

Increased male to female frequency similar with the result of (Shekhar, *et al.*, 2014) they illustrated that male recorded 72.2% (Bansal, *et al.*, 2008), observed that male more frequency than female (78.64% vs 21.36%) and dissimilar with the result of (13) they found The majority of diabetic foot patients were female (58.6 %) (14). Male predominance in DFU could be linked to factors such as gender-related differences in life styles and professional roles that require the feet to tolerate more pressure as a result of work, Increased level of outdoor work and poor compliance to foot care practices. The wounds healing in females more better than in male, this may be due to differences in hormones and explain that increase estrogen receptor in female increase healing wound which act as endogenous enhancers of healing process while in male increased the level of androgen was considered harmful for wound healing since androgenic species decrease repair of dermis (15, 16).

Isolation of bacteria species associated with DFU

In this study a total of (50) clinical specimens was collected from patients suffering from diabetic foot ulcer patients. 38(92%) specimens gave positive growth bacteria and the other 8(10%) specimens were mix growth of bacteria and other no growth as show in figure (3).

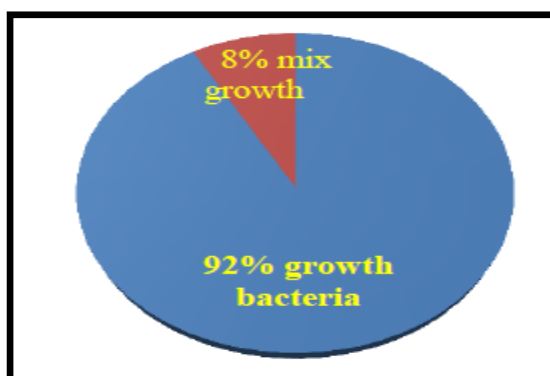


Figure 3: Percentages of bacterial isolation from diabetic foot ulcer patients.

The final identification was performed with the automated vitek-2 compact system using GP,GN-ID cards which contained 64 biochemical tests and one negative control .Exactly 38 isolates performed identification and confirmed by vitek-2 system by using five kits (for GN-ID cards and GP-ID cards). The finding show that a total number of 50 swabs were 38(92%) show positive culture of bacterial growth versus 12(8%) show negative results for culturing. The result of negative growth may be as a result of the other causative agents of infection, anaerobic bacteria, fungi ,and viruses (17), or may be as a result of either the wounds were not infected at the time of the study or the antibiotics prescribed were effective (18, 19). Culture investigation depending on morphological and biochemical test shows that Gram negative bacteria reveals a high rate 38(76%) which include *P. aeruginosa* that show a high percentage 11(22%) , then *K. pneumonia* with 8(16%) followed by *Acintobacteria baumannii* with 8(16%) , *Proteus mirabilis* 6(12%), and *E. coli* with 5(10%), as in figure (4-4) whereas G +ve bacteria recorded 11(22%) which include *S. aureus* was the most isolated bacteria in this study with percentage 7(20%) followed by *S. epidermidis* 4(10%) as in figure (4).

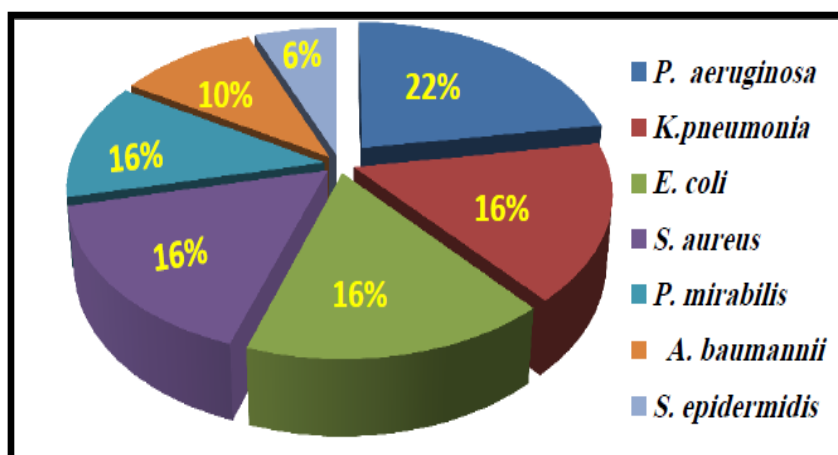


Figure 4: The percentage of bacteria isolated from clinical specimens.

Molecular Identification of Antimicrobial Drug Resistance

blaOXA gene

The result showed that the *blaOXA* gene (928 bp) resistance gene was detected in 3/5 (60%) *A. baumannii*, 3/5 (60%) *P. aeruginosa*, and 4/5(80%) *K. pneumonia* as show in figure (5).

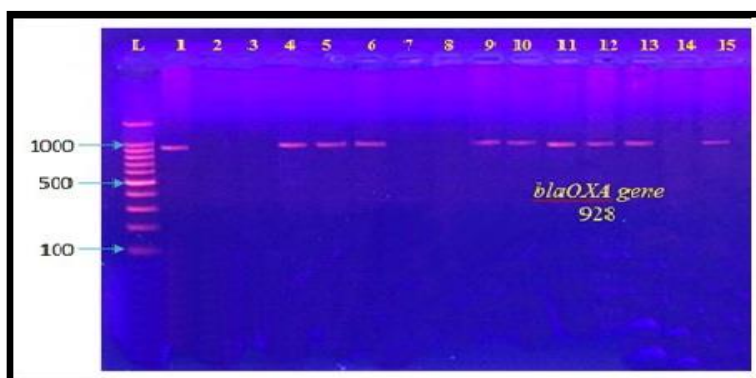


Figure 5: PCR amplification products of bacteria isolates that amplified with *blaOXA* gene primers with product 928 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 4, 5, 6, 9,10, 11, 12,13, 15) show positive results with the *blaOXA* gene.

Carbapenemases are the main mechanism by which resistance to carbapenems occurs and they belong to three of the four β -lactamase classes A, B and D. Class D carbapenemases are the OXA- β -lactamases, further subdivided into various sub-groups mainly *blaOXA*-23, *blaOXA*-24/40, *blaOXA*-58, *blaOXA*-48, *blaOXA*-51 and *blaOXA*-143. These OXA-type β -lactamases occur widely in *P. aeruginosa* with the most abundant being *blaOXA*-51, which is chromosomally encoded hence intrinsic to these species but it may confer resistance to carbapenems when its expression is up-regulated by genetic re-organization (20). Class B carbapenemases are also known as the metallo- β -lactamases (MBLs), they are mostly encoded by integron borne mobile gene cassettes and hence, they are transferable amongst various bacteria via horizontal gene transfer mechanisms notably conjugation. Class A carbapenemases include the *K. pneumoniae* carbapenemase (KPC) family that can be plasmid encoded or chromosomal (21).

***blaGES* gene**

The result showed the *blaGES* resistance genes(692bp) was detected in 5/5 (100%) *A. baumannii* ,2/5(40%) *P. aeruginosa* , and 3/5(60%) *K. pneumoniae* as show in figure (6). This study aimed to investigate the prevalence of *blaGES* genes in β -lactam-resistant *P. aeruginosa*, which the increased prevalence of β -lactamase is one of the main factors in resistance to β -lactams in *P. aeruginosa*. Twenty-six GES variations have been identified, with some of being classed as carbapenemases *bla* (22). *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* have all been found to be hosted by GES-5. Only GES-3 and GES-4 have been found in Japan prior to the epidemic described in this study (in *K. pneumoniae*). The MDRP for the original instance was either obtained locally or imported from another country. We will be able to monitor the likely spread of GES-5 in the surrounding region because this is the first report of GES-5 discovery in Japan. To establish adequate antibiotic therapy and evaluate and monitor the emergence of drug-resistant *P. aeruginosa*, molecular identification and monitoring of resistance genes is critical (23).

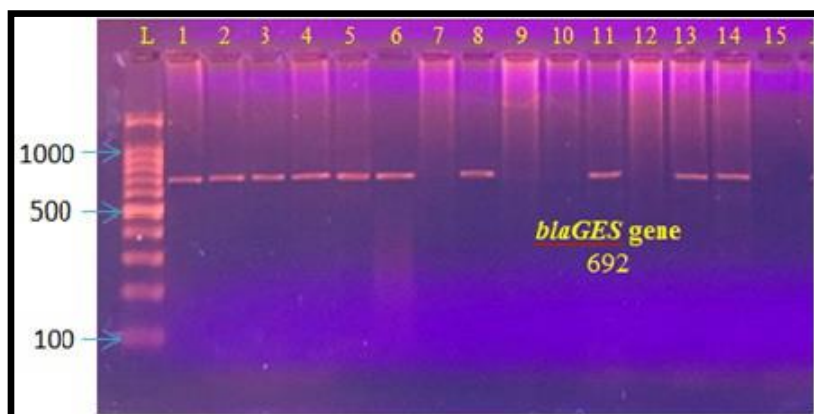


Figure 6: PCR amplification products of bacteria isolates that amplified with *blaGES* gene primers with product 692 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (1, 2, 3, 4, 5, 6, 8, 11, 13, 14) show positive results with the *blaGES* gene.

***blaTEM* genes**

The result showed the *blaTEM* resistance genes (766 bp) was detected in 2/5 (40%) *A. baumannii*, 3/5 (60%) *P. aeruginosa*, and 5/5 (100%) *K. pneumoniae*. The *blaTEM* genes are found on a family of similar β -lactamase plasmids, with the Asian, African, and almost indistinguishable Rio and Toronto plasmids (called due on their epidemiological origin) being the most often characterized. Other forms of β -lactamase-producing plasmids, such as those found in Nimes, New Zealand, Australia, and Johannesburg, have also been discovered in gonococci. The Asian plasmid is thought to be the primordial plasmid from which all other plasmids developed by deletions and/or insertions. As a result, these plasmids that produce β -lactamases may be classified as deletion derivatives of the Asian plasmid (Africa, Rio/Toronto, and Johannesburg) or insertion derivatives of the Asian (New Zealand) or African (Nimes) plasmids (24).

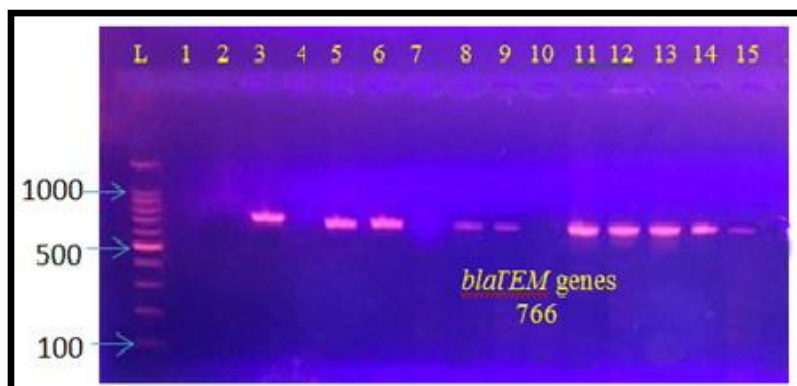


Figure 7: PCR amplification products of bacteria isolates that amplified with *blaTEM* gene primers with product 766 bp. Lane (L), DNA molecular size marker (100-bp ladder), Lanes (3, 5, 6, 8, 9, 11, 13, 14, 15) show positive results with the *blaTEM* gene.

Conclusions

The dominance bacterial isolates, were *P. aeruginosa* 11(22%), *K.pneumonia* 8(16%), and *A. baumannii* 8(16%) that isolated and diagnosed from clinical specimens of diabetic foot ulcer and detect the antibacterial resistance phenotypically and genotypically.

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