Isolating Bacterial Strains from Diabetic Foot Patients and Testing Their Antibiotic Sensitivity

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Abstract

Background: Diabetic foot ulcers can be contaminated with a diverse range of microorganisms, including several germs that are resistant to multiple drugs. **Objective:** An experiment was conducted to isolate and diagnose different types of bacteria that infect diabetic foot patients. **Methodology:** Samples were taken from 90 diabetic patients in four hospitals in Babil, Karbala, and Diwaniyah governorates. 125 bacterial isolates were obtained from diabetic foot patients. The number of isolates was 46 Gram-positive isolates and 79 Gram-negative isolates. **Results:** After screening and collecting the isolates that were similar in appearance, the examination process was carried out using the Vitec 2 device. The results indicated that the gram-positive isolate *Kocuria rosea* had the lowest proportion of bacterial isolates, with 6.4%, while the gram-negative strain *Pseudomonas aeruginosa* had the highest percentage of isolates, 26.4%. **Conclusion:** Gram-positive bacteria were all responsive to tetracycline and gentamycin; gram-negative bacteria were sensitive to amikacin and gentamycin. [*Bangladesh Journal of Infectious Diseases, December 2024;11(2):138-145*]

Keywords: Diabetic foot; *Pseudomonas aeruginosa; Kocuria rosea*; antibiotics

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Introduction

Diabetic mellitus is characterized by being an endocrine disorder that results in a loss of the ability to regulate the level of glucose in the blood, which leads to many complications such as neurological complications, vascular disorders, neuropathy, as well as nephropathy and retinopathy[1].

Global statistics indicate that the number of people living with diabetes currently amounts to about 170 million people, and this number is expected to double by the year 2030 in another statistic, it was found that 15% of people with this disease are exposed to diabetic foot ulcers, which are located in the area under the heel of the foot and are characterized by being a source of many pathological disorders that include inflammation of

the nails, tissues, muscles, joints, tendons, and bone marrow, and may develop, infections invade deeper tissues, causing some immune disorders, in addition, 84% of those infected with these ulcers end up having their legs amputated[2].

Different types of microorganisms participate in causing infection in diabetic foot ulcers, and bacteria stand at the forefront of them through their possession of various virulence factors, firstly, and their resistance to antibiotics, secondly, acute infections are almost limited to a single type of spherical Gram-positive bacteria, while chronic infections are characterized by the presence of 3-5 bacterial types, while coagulation enzyme-negative *Staphylococcus* bacteria and *Corynebacterium* species colonize soft tissues, in addition, foot ulcers may even allow the presence of some anaerobic bacteria and fungi[3]. The diversity of microbiota in foot ulcers is affected by several factors, including the depth of the wound, variable humoral immunity, and geographical location[4].

Diabetic foot ulcer infections are characterized by being poly microbial and a mixture of aerobic and anaerobic organisms the common aerobic bacteria are represented by the genera Staphylococcus and Streptococcus, and species of the Enterobacteriaceae family are among the common anaerobic bacteria Bacteroides and Peptostreptococcus[5] .

Several bacterial species have the ability to cause diseases in humans and other organisms due to specific chemical-based or genetic characteristics that act as pathogenic factors these factors, collectively known as virulence factors, enable the bacterial cells to harm the host's body, examples of virulence factors include the production of toxins and various mechanisms[6].

Pseudomonas aeruginosa is a type of bacteria that is found in both natural environments and sterile healthcare environments, it is able to survive food shortages and other environmental stresses, *Pseudomonas* is classified as the fifth most common contaminant that causes infections in US hospitals, according to data collected between 1968 and 1998 by the CDC[7].

Staphylococcus aureus is also one of the most pathogenic bacterial species that is transmitted through contaminated food, and causes a number of common diseases around the world as a result of eating food contaminated with these bacteria or containing toxins produced from it[8]. Antibiotic overuse has made the evolution of resistant bacteria problem in different place world[9].

In this regard, it can be concluded that the two primary issues dealing with medical institutions and treatment facilities worldwide today are the rapid spread of infection in hospitals and health facilities worldwide and antibiotic resistance bacteria refer to the new strains and varieties of antibiotic-resistant pathogenic bacteria[10].

Previous studies have shown that the most frequent bacterial pathogens that cause diabetic foot ulcers and cause tissue damage are aerobic bacteria, *Proteus* species, *Escherichia coli*, *Enterococcus* species, *Streptococcus* species, *Staphylococcus aureus, Pseudomonas* species[11]. *Staphylococcus aureus* it was found that bacteria and some types of Gram-positive aerobic bacteria are the main cause of foot ulcer infections, and they have been isolated in 60% of infection cases[12]. Pseudomonas aeruginosa isolates have been repeated in patients Burns, fibrosis of the bladder, and damaged tissues accompanying foot ulcers. It is one of the most important microbes that cause clinical problems resulting from its high resistance to antibiotics, which results in sepsis or amputation of the affected organ[13]. Either one type of microbe is monomicrobial, or several types are combined in it. Polymicrobial ulcer infection, and the difficulty of healing an ulcer increases when it is infected with bacteria that are multi-resistant to antibiotics, which poses a clear challenge for doctors and surgeons in dealing with them, and the reason may be due to the frequent use and incorrect handling of these antibiotics[14].

Different types of antibiotics have been used to inhibit microorganisms present in diabetic foot ulcers, and fluoroquinolones are one of the most important of these antibiotics because it has a broad inhibitory spectrum against clinically important pathogens responsible for many infections common diseases such as urinary tract infections, intestinal infections, respiratory tract infections, and diseases sexually transmitted infections, as well as skin and soft tissue infections[15]. Levofloxacin belongs to the third generation of antibiotics fluoroquinolones are characterized by their broad-spectrum antibacterial activity against bacteria, as their inhibitory effect extends both Gram-positive and Gram-negative bacteria[16]. Therefore, the study aimed to isolate and diagnose the types and numbers of bacteria isolated from diabetic foot ulcers and study of the effectiveness of the antibiotic in inhibiting bacteria under study.

Methodology

Study Settings and Population: Samples were collected between February 2024 to April 2024 from Imam Al-Sadiq Hospital (Turkish) and from Al-Diwaniyah General Hospital and Al-Husseini Hospital, Karbala, from people who were subjected to swab media swabs were taken and placed in clean and sterile refrigerated containers during transportation to the laboratories of the Musayyib Technical Institute, Department of Medical Laboratories. The sample collection period was for a period of Two months, during which (90) samples were obtained. Any adult diabetic with an ulcer on their foot. Using two sterile swabs dipped in sterile glucose broth, samples were obtained from the deepest section of the ulcer.

Preparation of the Culture Media Used: The culture media used for isolating and diagnosing bacteria, which is a general medium for all types of bacteria, were prepared according to the instructions of the supplying companies. They were sterilized in an autoclave at a temperature of 121^0 C for 15 minutes under a pressure of approximately 1.5 kg / cm2 and according to the following:

Solid Agar Medium Nutrient agar: Prepare the medium according to the manufacturer's instructions by dissolving (28g) of medium in a one liter distilled and sterilized water by using autoclave at a temperature of 121^0 C and a pressure of 15 pounds / inch 2 for 15 minutes, and after sterilization it was cooled. The medium was reduced to (45-50 m) and distributed in Petri dishes. This medium was used for the purpose of bacteria development[17].

MacConkey Media: This medium was prepared in accordance with the provided instructions of the supplying company, by dissolving 51.5 gm in a liter of distilled water and sterilized by autoclave. This medium was used to isolate dye-negative bacteria Gram and diagnosed in terms of its ability to ferment the sugar lactose.

Liquid Nutrient Medium: The liquid nutrient medium was prepared in accordance with the provided instructions of the medium manufacturer. A weight of 11 grams was taken from the medium, dissolved in a liter of distilled water, and distributed in laboratory tubes after sterilizing it in an autoclave at a temperature of 121 degrees Celsius. This medium was used for the purpose of activating bacterial isolates[17].

Mannitol Salt Agar Medium: This medium was prepared in accordance with the provided instructions of the supplying company, by dissolving 111.1 g in a liter of distilled water, and sterilized by autoclave. After completing the sterilization process, it was cooled to a degree (45-50) C This medium was used as a rich medium to isolate and bacterial diagnosis *Staphylococcus* species[17].

Transplant Samples: All samples that were collected firmly rotating the swab[18].

Short term storage of isolates: Bacterial isolates were inoculated into Nutrient agar medium using the plotting method and then incubated in plates for a whole day at 37^0 C, then stored at a temperature of 4^0 C[17].

Medium term storage of isolates: Bacterial isolates were inoculated into Nutrient Broth medium using sterile tubes they were then incubated the tubes were stored for a whole day at 37^0 C, then stored at a temperature of 4°C for the duration of storage at this time. The method lasts for three weeks and is then renewed after this period ends[17].

Long-term storage of isolates: Bacterial isolates were stored for a long period of up to more than three months in Nutrient medium broth, which contains 15.0% glycerol. The tubes were sterilized and then cooled and transported one colony per tube, then the tubes were incubated for 6 hours at 37^0 C and then stored at -20^0 C[17].

Phenotypic examinations of bacterial colonies: After obtaining the bacterial colonies, they were classified according to the morphology of the colonies, the color of the colony, and the nature of the colony. Thus, the numbers of isolates were listed according to the mentioned characteristics and also The initial diagnosis of the isolates was made based on bacterial growth on the diagnostic media used in the research (MacConkey for negative bacteria and Mannitol for positive bacteria).

Isolation and diagnosis of the studied bacteria: Gram stain was used to differentiate between negative and positive bacteria and to determine the appearance of the bacteria under the microscope. A microscopic examination of the isolates was conducted by taking a bacterial swab, staining it with gram stain, and then examining it under a microscope to observe its interaction with the stain.

It is scientifically known that the color of bacteria found in the Gram stain depends on the type of chemical composition of the bacterial cell wall[19].

Diagnosing chemical characteristics with the Vitek2 Compact System: The diagnostic process was carried out using the Vitek2 device for the obtained isolates, which included 64 tests for positive bacteria as well as for negative bacteria[20].

Testing the sensitivity of isolated and identified bacteria to antibiotics: The work was done using Muller-Hinton agar medium poured into a Petri dish and using a bacterial suspension with a concentration of 1.5×10^8 CFU/ml. The suspension bacteria were distributed homogeneously on the top of the solid medium using a cotton swab, and then antibiotic discs were applied to the surface of the cultured medium after it dried at a rate 5 tablets per plate. The Petri dishes were placed in the incubator and kept at a temperature of 37°C for a period of 20-24 hours, then the diameters of the no-growth halos were measured and compared with reference tables, and the results were expressed as resistant (R), sensitive (S) or moderately sensitive (I). The discs were all from Bio-analyse company. Antibiotic tablets prescribed for treatment in the local market were used: tetracycline (TE, 30 mcg), amoxicillin with clavulanate (AUG, 30 mcg), amikacin (AMK, 30 mcg), ceftriaxone (CRO, 30 mcg), ampicillin (AM, 10 mcg), gentamycin (GEN, 10 mcg) and azithromycin (AZM, 15 mcg).

Statistical Analysis: The results were analyzed statistically to compare the averages statistically and at the level of probability ($p \leq 0.05$). The results were analyzed using the Statistical program SPSS version 16 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp).

Ethical Clearance: Ethical clearance was obtained from Imam Al-Sadiq Hospital and from Al-Diwaniyah General Hospital and Al-Husseini Hospital, Karbala. Written informed consent was obtained from the patients or from their legal guardian. Patients confidentiality were strictly maintained. No name, address or contact details of the patients were divulged.

Results

Clinical characteristics of the study participants: Ninety (90) study participants were included in the present study. The number of males were 58 (64.44%) and 32 (35.56%) were female. The average age of the study participants was 57.0±5.0, and majority were between the ages of 50 and 75. Just 38.89% of the research participants came from rural areas; the bulk (61.11%) were citizens of city centres. Type I diabetes affected most of the individuals (Table 1).

Table 1: Clinical Characteristics of The Study Participants

Characteristics	Frequency	Percent
Gender		
Male	58	**64.44**
Female	32	35.56
Age Group		
50 to 59	51	56.67
60 to 69	23	25.56
70 to 75	**16**	17.78
Residence		
City Centers	55	61.11
Rural Areas	35	38.89
Type of Diabetes		
Type I	82	91.11
Type II	**8**	8.89

Diagnosis of the Selected Isolates: The chosen isolates were cultured, and as Table 2 shows, bacterial colonies appeared after a 24-hour incubation period at 37 °C on the MacConkey and Mannitol medium. Concerning the microscopic features, the bacterial isolates emerged 48 hours after incubation following a gram staining.

Table 2: Gram Stain Tests for Bacterial Isolates

Medium type	Gram stain	N(%)
MacConkey agar	Negative	79(63.2%)
Mannitol agar	Positive	46(36.8%)

Table 3: Number and Percentage of Diagnosed Bacterial Isolates

Samples of Bacterial Isolates	Gram stain	Frequency	Percent
S. epidermidis	+	17	13.6
S. lugdunensis	+	11	8.8
S. gallinarum	+	10	8
K. rosea	+	8	6.4
P. aeruginosa	-	33	26.4
P. putida	-	18	14.4
E. coli	-	15	12
A. baumannii complex	-	13	10.4

Table 4: Testing the Sensitivity of Bacterial Isolates to Antibiotics

Bacterial isolates	Antibiotics						
	TE	AUG	AMK	CRO	AM	GEN	AZM
S. epidermidis	S	R	R	R	S	S	R
S. lugdunensis	S	R	I	R	R	S	R
S. gallinarum	S	R	R	R	S	S	R
K. rosea	S	R	S	R	I	S	R
P. aeruginosa	R	I	S	S	R	S	I
P. putida	R	R	S	I	R	S	R
E. coli	I	R	S	S	R	S	R
A. baumannii complex	R	R	S	I	R	S	R

There exist Gram-positive and Gram-negative bacterial isolates. Ninety (90) individuals with diabetic foot ulcers produced one hundred and twenty-five (125) bacterial isolates, 46 isolates (36.2%) of these were a Gram positive and 79 isolates (63.2%) were a Gram negative (Table 2).

After examination isolations with the Vitek 2 device, we find different types of bacteria most common of these identified were *Pseudomonas aeruginosa* 26.4% (33/125), followed by *Pseudomonas putida* 14.4% (18/125), and *Staphylococcus epidermidis* 13.6% (17/125) followed by *Escherichia coli* 12% (15/125). Other isolates included *Acinetobacter baumannii* complex (10.4%), *Staphylococcus lugdunensis* (8.8%), *Staphylococcus gallinarum* (8.0%), *Kocuria rosea* (6.4%) (Table 3).

Testing the Sensitivity of Bacterial Isolates to Antibiotics: The sensitivity of Gram-negative and Gram-positive bacteria isolates to a number of antibiotics was tested, including amoxicillin with clavulanate, tetracycline, amikacin, ceftriaxone, ampicillin, gentamycin and azithromycin.

The data presented in table 4 indicated that all Gram-positive bacterial isolates exhibited complete resistance (100%) to the medications amoxicillin with clavulanate (AUG), Ceftriaxone (CRO), and azithromycin (AZM). All Gram-positive bacterial isolates exhibited 100% susceptibility to tetracycline (TE) and gentamycin (GEN). While other drugs shown varying degrees of resistance, sensitivity, and moderate sensitivity towards bacterial isolates. Table 4 indicated that all gram-negative bacterial isolates exhibited complete resistance (100%) to the antibiotic ampicillin (AM). Additionally, it was observed that these isolates were resistant to both azithromycin (AZM) and amoxicillin with clavulanate (AUG), with the exception of the *Pseudomonas aeruginosa* isolate,

which displayed moderate sensitivity (I) to both antibiotics. All Gram-negative isolates exhibit 100% susceptibility to both amikacin (AMK) and gentamycin (GEN) medicines.

Discussion

The most common problem that can happen with diabetes is wounds on the feet. If the patients don't treat diabetic foot wounds, they can get infected and lead to other problems like gangrene, osteomyelitis, and even amputation[3]. Surgery and antibiotics are the two main ways to treat this problem[21]. The purpose of this study was to find out which pathogenic bacteria caused the most illnesses at the study sites. Like other studies done in other countries Shah *et al.*[22] found that most of the people who participated were men. These results may be attributed to the higher likelihood of men engaging in outdoor activities, which might result in accidents and the development of ulcers. Likewise, a majority of individuals with Diabetic foot ulcers fell between the age range of 50 to 75, consistent with research conducted in other nations[23].

As further research published in various locations, including Nigeria, China, and India, shows the degree to which bacteria impact the healing process of diabetic foot ulcers, there was a corresponding rise in both the frequency of bacterial isolation and the diversity of bacterial species present in the ulcer[24]. In this investigation, *Pseudomonas aeruginosa* was the most common isolate, accounting for 26.4% (33/125) of the samples. In their investigations done in Ethiopia, Amogne *et al.*[25] discovered that *Klebsiella* species were the most common bacteria, accounting for 23.9% (22/92) of the samples, *Proteus* species followed behind at 18.47% (17/92). In Egypt, *Proteus mirabilis* accounts for 16.8% of the isolates[26]. Whereas in Saudi Arabia bacteria species

Pseudomonas make up 15.6%[27]. In South America, Pseudomonas species are the most prevalent isolates, representing 18.8%[28]. This demonstrates that the primary bacteria responsible for diabetic foot ulcers infections may differ in various environments.

The most prevalent gram-negative isolates in this investigation were *Pseudomonas aeruginosa* and *Pseudomonas putida*, with percentages of 26.4 and 14.4%, respectively. This which had a similar prevalence to other studies conducted in India (23.2% of isolates), Libya (17.5% of isolates) and another study in India (23.6% of isolates) meanwhile in contrast, a prior study conducted in Ethiopia found that none of the 92 cultivated samples contained any Pseudomonas species[3]. Differences in sample sizes between the several studies and unique features unique to each study site may be the causes of the observed difference.

Since the past decades, bacterial resistance to antibiotics has spread and become resistant to multiple drugs, which represents a threat to human health These bacterial strains have since become a major public concern and cause high rates of hospital infections, reaching 80-90%[29].

Bacteria that are resistant to antibiotics may be bacteria that cannot be completely inhibited or killed by the antibiotic, or the antibiotics may have worked effectively before resistance occurred, leading to the bacteria becoming resistant to the antibiotic by functionally adapting in a way that prevents the antibiotic to resisting it[30].

Antibiotics AUG, CRO, and AM are classified into a group bacterial resistance to these antibiotics can be achieved through three mechanisms: the first is by secreting beta-lactamase enzymes that hydrolyze the beta-lactamase ring present in the antibiotics, and the second is to reduce the bacteria's permeability to antibodies and thus prevent them from entering the cell, the last is depends on changing the specific target of the antigen in the cell, which is impossible for the antigen to detect binding to the intended target, and thus not killing the bacteria[31].

The antibiotics Amikacin and Gentamicin belong to the group of aminoglycosides, and bacteria resist these antibiotics through several mechanisms first is inhibition of the antigen through an enzyme that changes the structure of the antigen by transferring an effective phosphoryl, ribosyl, and acyl group (to the antigen, such as functional groups) thiol by

changing the structure of the antigen by the enzyme nucleotide transfers and the second it involves modifying the target of the antibiotic by methylation of the DNA and rRNA, and the third is reducing the permeability of the bacterial wall to antibiotics and thus preventing them from entry into the bacterial cell[32]. This work revealed that the majority of the gram positive bacteria from diabetic foot patient samples were partially or totally resistant to the antibiotics utilized in the study. Using antibiotics for too long, using antibiotics in the wrong way, bacteria being able to form biofilms, and one of the bacterial polysaccharide components stopping host antibodies from working are some of the things that could make bacterial isolates from chronic wounds resistant to antibiotics. In this work, the majority of the isolates exhibited resistance to several antibiotic classes, suggesting that diabetic foot patients are developing both gram-positive and gram-negative bacteria resistant to several medicines. The colonisation of many drug-resistant bacteria at the wound site of diabetic patients might extend the treatment period and limit the action of a wide range of antimicrobials because of the formation of self-secreting biofilms surrounding the microbial colony. This implies that a new approach is required to eradicate colonization, multi-antibiotic resistant, and potentially fatal bacteria in diabetic wounds[33].

Conclusion

Many different kinds of bacteria, including some that are resistant to numerous medications, can contaminate diabetic foot ulcers. The study found that *Pseudomonas* spp. were the most prevalent bacteria, with other gram-negative & positive bacteria also present.

Contribution to authors:
Asmaa Abd Ali ALrifai : Conception and design, or design of the research; AMMAR K. JASMAN & Asmaa Abd Ali ALrifai : the acquisition, analysis, or interpretation of data; conceptualized and designed the overall study; Azhaar Mousa Jaffar & Asmaa Abd Ali ALrifai: involved in data collection; Asmaa Abd Ali ALrifai: Drafting the manuscript or revising it critically for important intellectual content; Asmaa Abd Ali ALrifai: involved in data input and data cleaning; Asmaa Abd Ali ALrifai & Azhaar Mousa Jaffar: conducted data analysis;

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