Bacterial Infections and Inflammatory Markers in Diabetic Foot Ulcers: Assessing the Roles of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Vascular Endothelial Growth Factor, and Interleukin-6

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Abstract

Background: Diabetic foot ulcers (DFUs) are severe complications of diabetes, leading to osteomyelitis, gangrene, and limb amputation. These outcomes increase mortality risk and bacterial resistance in surviving patients. The selection of the most effective antibiotics for DFUs remains a growing challenge. **Objectives:** The study aimed to identify risk factors, bacterial pathogens, and drug resistance in DFU patients, and explore the correlation between interleukin-6 (IL-6), vascular endothelial growth factor (VEGF) levels, and DFUs. **Materials and Methods:** Sixty isolates of bacteria were isolated aerobically from 100 samples of DFU (30 isolates from *Pseudomonas aeruginosa* and 30 isolates from *Staphylococcus aureus*). All isolates were identified by biochemical tests, then confirmation diagnosis was done by using VITEK-2. Determination multiple drug resistance isolates by susceptibility tests, then estimation of the activity of silver nanoparticles (AgNPs) as antibacterial with different concentrations were done. VEGF and IL-6 levels were determined by using Enzyme-linked immunosorbent assay (ELISA) technique. **Results:** The study revealed that many bacteria-resistant antibiotics, but AgNPs significantly inhibit multi-drug resistant bacteria, suggesting their potential as an alternative treatment. VEGF and IL-6 levels were significantly higher (P-value < 0.001) in DFU patients, with IL-6 negatively correlating with VEGF. **Conclusion:** Diabetic foot infections, caused by various bacteria, require understanding of antibiotic sensitivity for effective treatment. AgNPs showed strong antimicrobial effects against common bacteria, suggesting potential as an alternative treatment. The study also found elevated VEGF and IL-6 levels in DFU patients, with IL-6 potentially inhibiting VEGF, thus impairing healing.

Keywords: AgNPs, DFU, IL-6, MDR, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, VEGF

Introduction

Diabetes mellitus, which can present diverse symptoms depending on the severity of blood glucose levels, is a global health concern. Between 1980 and 2014, the number of affected individuals increased fourfold from 108 million to 422 million, highlighting the need for effective diabetes management strategies.[1,2]

Foot ulcers represent a major diabetes-related issue, affecting around a quarter of people living with this disease at some point in their lives. Those with diabetes face an amputation likelihood that is 15–30 times greater, and in fact, before 85% of lower limb removals in these individuals, an ulcer typically forms.[3]

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Scientific research into the microbiology of diabetic foot ulcers (DFUs) demonstrates them to be generally polymicrobial, with the specific pathogens differing based on individual and geographic factors. A range of bacteria including *Streptococcus, Staphylococcus* spp., *Enterococcus* spp., *Escherichia coli, Proteus mirabilis*, and

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Klebsiella pneumoniae are regularly found. However, in nations with limited resources, *Pseudomonas aeruginosa* tends to be the primary culprit due to its aggressive traits such as biofilm production.[4-10]

Staphylococcus aureus often implicates in the acute phase of diabetic foot infections (DFIs).[11] Antibiotic resistance among microorganisms isolated from diabetic foot patients is a growing issue, with DFIs associated with multi-antibiotic-resistant methicillin-resistant *S. aureus* (MRSA).[12-15] The prevalence of MRSA and *P. aeruginosa* in foot ulcer patients has increased, often leading to amputations.[16-18]

Vascular endothelial growth factor (VEGF) plays a central role in physiological and pathological angiogenesis, including tumor growth, diabetic retinopathy, macular degeneration, ischemia, inflammation, and preeclampsia.[19] VEGF supports neurogenesis, angiogenesis, and microcirculatory angiogenesis related to peripheral arterial disease caused by atherosclerosis.[20,21]

In this research, we obtained pathogenic bacteria from DFUs and examined their response to antibiotic treatments. The antibacterial potency of silver nanoparticles (AgNPs) against these harmful bacterial strains was scrutinized, and both their minimum concentration for inhibition and bactericidal action were determined. Additionally, we assessed the levels of interleukin-6 (IL-6) and VEGF in the serum, offering a thorough understanding of both the obstacles and potential strategies to handle diabetes and its associated complications.

Materials and Methods

Solution and media

Media	Origin
Mueller-Hinton media, Mueller-Hinton agar, Nutrient agar, Nutrient broth, Ames transport, Blood agar base, Brain heart agar	Himedia, Mumbai, India
AgNPs (25 nm)	Zhengzhou Dongyao Nano Materials Co., Ltd., Zhengzhou City, Henan Province, China
Antibiotic disks	Bioanalyse, Turkey

Sample collection, isolation, and characterization of microbial species

During the period from September 2022 to January 2023, a total of 100 DFU and blood specimens were collected from Hillah Teaching Hospital, Iraq, and transported to a laboratory for examination. The patients ages were ranging from 41 to 70 years and they comprised 40 male patients and 60 female patients. Every specimen was planted on nutrient, MacConkey's, and blood agar dishes and then cultivated at 37°C spanning 24–48 h. Biochemical evaluations were used to identify the separated strains, and their identification was then validated utilizing a Vitek-2 compact apparatus (Biomérieux, France).[22]

Assessment of antibiotic susceptibility and multiple antibiotic resistance

Bacterial strains that were isolated, such as *P. aeruginosa* and *S. aureus*, underwent antibiotic resistance assessment against 13 and 18 antibiotic discs correspondingly. The technique of Kirby-Bauer disk diffusion was used for antibiotic resistance determination on Mueller-Hinton agar dishes (Carl Roth, Germany). Every identified bacterial strain was then re-dissolved to reach a standard cloudiness [equivalent to 1.5×10^8 units forming colonies per milliliter (cfu/mL)]. This was followed by analyses for antibiotic sensitivity and mid-level sensitivity, aligned with the guidelines provided by the Clinical Laboratory and Standards Institute (CLSI).[22]

Analysis of antibacterial activity of silver nanoparticles employing agar disk diffusion method

We evaluated the antimicrobial capacity of AgNPs against human disease-causing bacteria, which included *P. aeruginosa* and *S. aureus*, following the guidelines set by the CLSI.[22] A disk diffusion test was conducted to examine the bacterial resistance to antibiotics and AgNPs. The AgNPs were prepared in a set of three at concentrations varying between 31.25 µg/mL and 500 µg/mL in sterile deionized water. Initially, the samples were incubated at 4°C for a quarter of an hour, succeeded by overnight incubation at 37°C. After this incubation period, an inhibition zone around the well indicated a positive test result, the diameter of which was determined using a digital vernier caliper.[23-25]

Determination of vascular endothelial growth factor and interleukin-6 levels

The levels of IL-6 and human VEGF A were determined using ELISA technique kits were provided form Shanghai YL Biotech (China), respectively, the test assay was done according to manufacturer instructions.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to the document number 4348 dated September 4, 2022.

Results

Isolation of pathogenic bacteria from clinical specimens

Out of the 100 samples analyzed, 24% comprised *P. aeruginosa* isolates, 24% consisted of *S. aureus* isolates, 12% contained isolates of various bacteria, and 40% yielded negative culture results [Figure 1]. Bacterial strains were procured and characterized from DFU patients from Hilla Teaching Hospital, Iraq. The isolates underwent verification through a series of biochemical examinations

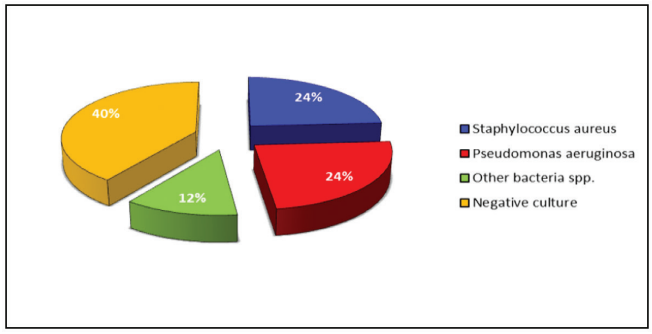

Figure 1: Isolation rate of bacterial species

Table 1: The probability of identifying gram-negative and gram-positive bacterial isolates from various sources using the Vitek-2 compact system

Bacterial isolates	Gram stain	Probability%
P. aeruginosa	G ⁻ve (n = 30)	94–99
S. aureas	G +ve (n = 30)	94–99
Total	60	

Table 2: Biochemical tests for identification of *P. aeruginosa* isolates and *S. aureas*

Test	*P. aeruginosa*	*Staph. aureas*
	Result	Result
Gram stain	–	+
Oxidase test	+	–
Catalase test	+	+
Pigments production	+	–
Hemolysis (β-hemolysis)	–	+
Indole test	–	–
Methyl-red	–	+
Voges-proskauer	–	+
Simmon's citrate	+	+
Urease production	–	+
H_2S production	–	–
Lipase	+	+
Phosphatase	+	+
Nitrate reduction	+	+
Hyaluronidase	–	+
DNase	+	+

and were subsequently corroborated using the Vitek-2 compact system (Biomérieux) [Table 1 and 2].

Antibiotic sensitivity of *Staphylococcus aureus* and *Pseudomonas aeruginosa* as multi-drug resistance

Compliance with antibiotic susceptibility testing rules and cutoff points was ensured by using the regulations of the European Committee on Antimicrobial Susceptibility Testing, the CLSI, and the Food and Drug Administration of the United States. The antibiotic susceptibility of bacterial isolates was examined, employing thirteen antibiotics for susceptibility evaluation of *P. aeruginosa* isolates and eighteen antibiotics for susceptibility evaluation of *S. aureus* isolates. Following this, the identification of multi-drug-resistant (MDR) bacteria was conducted, as demonstrated in Tables 3 and 4.

Table 3: Antibiotic resistance patterns of *Pseudomonas aeruginosa* isolates

Pattern type	Percent %
XDR	63.3
MDR	20
Not resistance	16.7

MDR, multiple drug resistance; XDR, extensively drug-resistant

Table 4: Antibiotic resistance patterns of *Staphylococcus aureus* isolates

Pattern type	Percent %
XDR	20
MDR	43.3
Not resistance	36.7

MDR, multiple drug resistance; XDR, extensively drug-resistant

Antibacterial efficacy of silver nanoparticles

AgNPs demonstrated potent antibacterial activity against multi-drug-resistant *P. aeruginosa* and *S. aureus* isolates. Compared to various antibiotics, AgNPs showed a clear increase in the inhibition zone diameter with rising nanoparticle concentration. The largest inhibition zone against the test organisms was found at a concentration of 500 µg/mL, indicating high sensitivity across all isolates. AgNPs also exhibited significant inhibition against *S. aureus*, even surpassing the selected antibiotics. Figures 2 and 4 suggest that all AgNP concentrations affect all MDR isolates, even at the minimum concentration tested [Figures 3 and 5].

Interleukin-6 and human vascular endothelial cell growth factor A

The concentrations of VEGF and IL-6 were significantly higher in the patient cohort than in the control group [Table 5]. This was true across all age and gender groups [Table 6]. The data also showed a trend of VEGF regulation in patients, with levels decreasing with age but remaining higher than in healthy individuals. Figure 7 shows a correlation between VEGF and IL-6.

Discussion

To substantiate the initial identification outcomes for strains obtained from diverse patients, the Vitek-2 approach was implemented, resulting in a probability range of 94%–99%, as depicted in Table 1. The identification of these isolates depends on the microscopic characteristics. Bacteria appeared as gram-negative and gram-positive, with morphological characteristics such as colony size, shape, color, natural pigment, clearness,

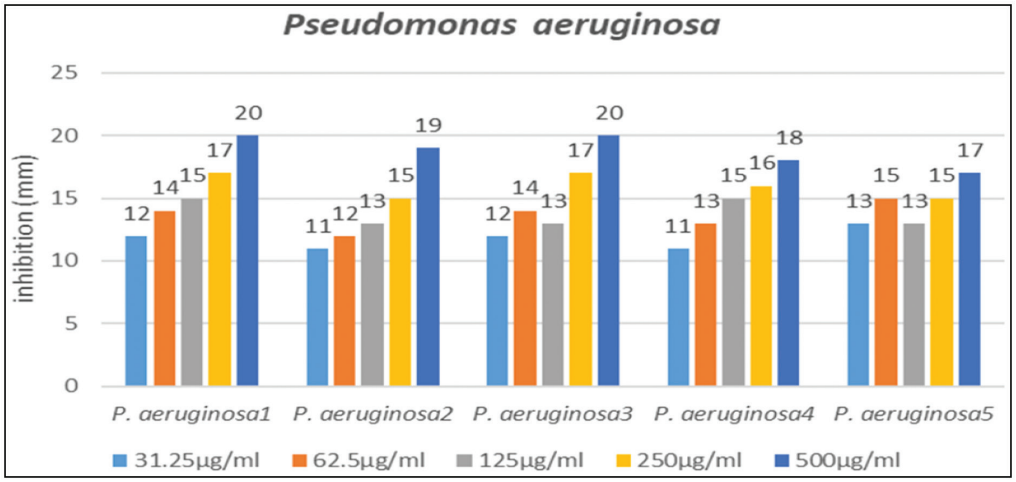

Figure 2: Antibacterial activity of AgNPs against 5 isolates of MDR *P. aeurogenosa*

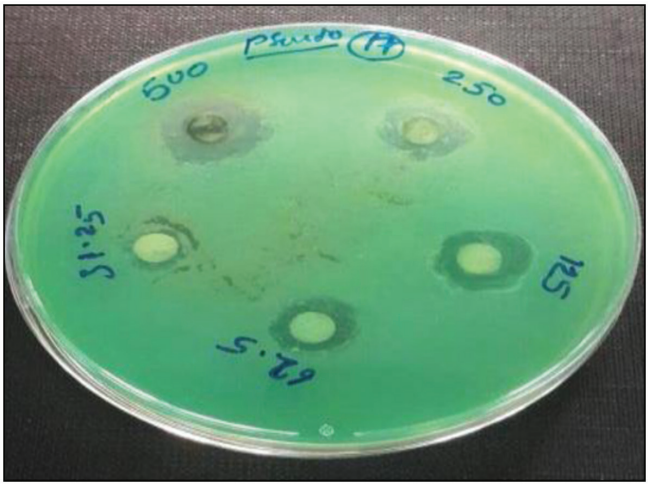

Figure 3: Antibacterial activity of AgNPs on MDR *P. aeurogenosa*

elevation, texture, lactose fermentation on Mac Conkey agar, and the formation of hemolysis on blood agar being essential for their identification,[22] as shown in Table 2.

Results from Table 3 indicated that entirely isolates (20%) were resistant to at least three groups of antibiotics; therefore, these isolates were classified as (MDR), (63.3%) of which 30 isolates were marked as extensively drug-resistant (XDR) and five isolates (16.7%) of *P. aeruginosa* isolates were not resistant to all existing agents in all the classes of antibiotics tested. Antibiotic resistance may be caused by the coordinated action of multi-drug, efflux pumps thru antibiotic resistance genes then decreased penetrability of bacterial cell covers. Apart from self-resistance, *P. aeruginosa* can easily acquire resistance through chromosomal mutations in genes encoded by chromosomes or through gene transfer for determinants of resistance.[26]

From Table 4 showed indicated that entirely isolates (43.3%) were resistant to at least three groups of antibiotics, therefore these isolates were classified as (MDR), (20%) of which 30 isolates was marked as XDR and 11 isolates (36.7%) of *S. aureus* isolates were not resistant to all existing agents in all the classes of antibiotics tested. Prior research has indicated that infections caused by *S. aureus* are more prevalent among patients who have previously received antibiotic treatment compared to those who have not been exposed to antibiotics.[27] *Staphylococcus aureus* is responsible for soft tissue and bone infections, with a significant proportion located in the lower extremities. This bacterium is capable of invading osteoblasts, fibroblasts, and endothelial cells, and exhibits considerable resistance to antibiotics and antibiotic therapy.[28]

In this particular study, certain samples of *P. aeruginosa* and *S. aureus* were classified as multi-drug resistant due to their evolved resistance to at least a single agent in three or more antimicrobial categories, suggesting a wide-ranging antibiotic resistance. The emergence of multi-drug resistance in bacteria could be a result of the accumulation of resistance R plasmids or gene transposons, each encoding for a specific agent, or the activity of multi-drug efflux pumps capable of expelling various drugs.[15,16]

The exact process of AgNPs' antibacterial effect remains somewhat elusive, although numerous theories have been proposed. AgNPs are capable of gradually releasing silver ions, which might be the primary means of microbial eradication. Because of their affinity for sulfur-containing proteins, these ions can bind to the cell wall and cytoplasmic membrane, thus escalating membrane permeability and potentially leading to bacterial envelope disruption. Inside the bacterial cell, free silver ions can inactivate respiratory enzymes, disrupt adenosine triphosphate production, and foster the generation of reactive oxygen species, which could inflict cell membrane damage and DNA changes. The interplay between silver ions and components within the DNA could hinder replication and cellular reproduction, or even result in microbial eradication. Silver ions might also obstruct

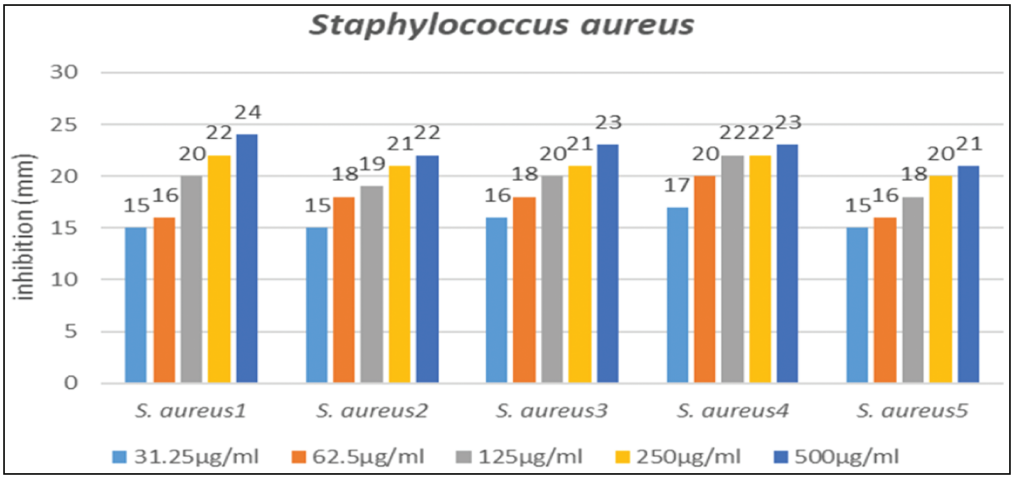

Figure 4: Antibacterial activity of AgNPs against 5 isolates of MDR *S. aureus*

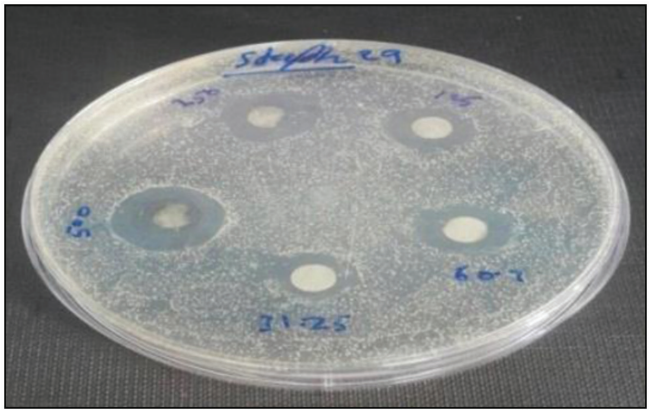

Figure 5: Antibacterial activity of AgNPs on MDR *S. aureus*

protein production by denaturing cytoplasmic ribosomes. Furthermore, the research underscored the efficacy of AgNPs against antibiotic-resistant strains, even at reduced concentrations, underlining their potential role in controlling multi-drug resistant infections.[29-31]

VEGF and IL-6 are two important factors to consider in this patient group. The significantly higher concentrations of both factors in patients, regardless of age and gender, indicate their potential involvement in the disease process. VEGF levels were upregulated in patients, especially in middle-aged individuals, which could be attributed to the body's increased gene encoding for VEGF at this stage of life.[32,33]

However, as patients aged, VEGF levels decreased, though they remained higher than those of healthy individuals [Figure 6]. This might be due to declining angiogenesis processes and endothelial function in old age, or a response to increased metabolic pathways and hypoxia.[34,35]

IL-6 levels, on the other hand, were consistently higher in patients, reflecting its role in infection and inflammation. In fact, high levels of IL-6 have been associated with hyperglycemia in type 2 diabetes mellitus patients, and are considered an early diagnostic marker for predicting the incidence of DFU.[36-38]

Furthermore, the negative correlation between IL-6 and VEGF suggests that IL-6-driven inflammation could be suppressing VEGF production or activity. This could lead to impaired angiogenesis and tissue repair, exacerbating the severity of DFUs.[39] An imbalance between pro-inflammatory IL-6 and pro-angiogenic VEGF may also contribute to persistent, non-healing ulcers.[40]

Conclusion

Diabetic foot infections, caused by both Gram-positive and Gram-negative bacteria, require understanding of antibiotic sensitivity patterns for effective treatment. AgNPs showed strong inhibition and antimicrobial activity against *P. aeruginosa* and *S. aureus*, suggesting their potential as alternative antibacterial agents, mitigating antibiotic resistance risks. The study also found elevated levels of VEGF and IL-6 in DFU patients across all age and gender groups, with VEGF upregulation prominent in middle-aged patients. This could be due to increased stimulation of VEGF-encoding genes during middle age. Notably, a negative correlation between IL-6 and VEGF suggests that IL-6 driven inflammation may hinder VEGF production or activity, impairing angiogenesis and tissue repair, and worsening DFU severity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Table 5: Results of VEGF and IL-6 in patients and control group

Groups parameters	Patient	Control	P
	Mean ±S.D		
Vascular endothelial growth factor (**pg**/m**L**)	163.59±5.6	81.86±3.5	<0.001
IL-6 (pg/m**L**)	19.76±0.45	11.35±0.36	<0.001

Table 6: Levels of VEGF and IL-6 in patients and control group according to gender and age

Groups Parameters	Age (year)	Male		Female		$LSD_{(0.05)}$
		Patient	Control	Patient	Control	
		Mean ± SD				
Vascular endothelial growth factor (pg/mL)	41–50	230.83±9.8	101.59±1.6	216.66±9.4	100.68±10.3	14.761
	51–60	148.75±5.7	101.48±5.7	163.19±7.1	97.81±9.7	
	61–70	111.29±4.9	48.53±7.8	110.81±1.6	41.08±8.4	
IL-6 (pg/mL)	41–50	15.95±1.7	9.97±0.8	18.20±2.6	9.60±2.2	2.196
	51–60	20.34±2.3	11.95±1.1	22.30±1.7	12.50±3.7	
	61–70	20.75±2.1	12.10±0.5	21.02±3.3	11.96±1.2	

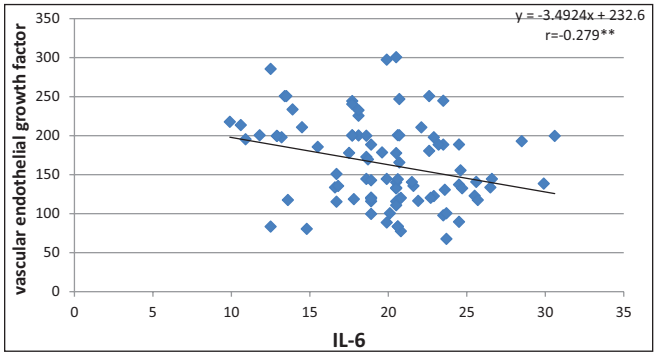

Figure 6: Correlation coefficient between IL-6 and vascular endothelial growth factor of patients

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