ROLE OF AEROBIC BACTERIA ON GANGRENE AMONG SAMPLE OF DIABETES MILLITUS IRAQI PATIENTS

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ABSTRACT

This study was aimed to identify the types of aerobic bacteria in individuals with gangrene. Among 106 swabs from the site of gangrene in diabetic individuals, the percentage of females was 37 (34.99%), and a male was 69 (65.1%). The highest percentage at age group 46-55 year was in male (33.33%) and 24.32% in females, while the lowest percentage at 15-25 year in female was (5.41%) while in male was zero. The mean age was 54 years among males and females. Out of 106 swabs, 76 different types of bacteria were isolated. The Gram-positive bacteria were dominant 42 (55.3%), the most common pathogen was *Staphylococcus aureus* (57.1%), and the lowest was *Micrococcus* (4.9%). The Gram-negative bacteria was 34 (44.7%), with the highest percentage was *Pseudomonas* (52.9%) and the lowest percentage was *Proteus* (3%). Kirby-Bauer Disk Diffusion test for antibiotic sensitivity represented those Gram-negative bacteria had a high rate of resistance to vancomycin, clindamycin, and ceftazidime (94.12%) in the same proportion, while the lowest resistance was to Imipenem and Colistin (52.94%), (14.71%) respectively. Gram positive bacteria showed a high resistance to Ceftazidime (100%) and low resistance to Gentamicin and Imipenem (47.62%) then Colistin (30.95%).

Keywords: gas gangrene, foot ulcer, amputation, colistin and blood supply.

مجلة العلوم الزراعية العراقية- 2024:55(4):1270-1279 **عبدالمجيد والربيعي**

دور البكتريا الهوائية في الغرغرينا بين عينة من مرضى السكري العراقيين

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المستخلص

تهدف هذه الدراسة هو تحديد انواع البكتريا الهواية لدى مرضى السكري. بين 106 مسحة من موقع الغرغرينا في مرضى السكري، كانت نسبة الإناث 37 (34.99٪)، و69 مسحة للذكور (65.1٪). وكانت أعلى نسبة في الفئة العمرية 46-55 سنة للذكور (33.33٪) و 24.32٪ للإناث ، بينما كانت أقل نسبة عند الإناث 15-25 سنة (5.41٪) بينما كانت صفراً عند الذكور. كان متوسط العمر 54 سنة بين الذكور والإناث. عزلت 76 نوع مختلف من البكتيريا من أصل 106 مسحة. كانت البكتيريا موجبة الجرام سائدة 42 (55.3٪)، وكانت أكثر مسببات الأمراض شيوعًا هي *Staphylococcus aureus* (57.1٪) ، وأقل نسبة كانت *Micrococcus* (4.9٪). كانت البكتيريا سالبة الجرام 34 (44.7٪) ، وكانت أعلى نسبة بكتريا *Pseudomonas* (52,9٪) وأقل نسبة كانت *Proteus* (3٪). أظهر اختبار Kirby-Bauer Disk Diffusion لحساسية المضادات الحيوية أن البكتيريا سالبة الجرام لديها معدل مقاومة مرتفع لفانكومايسين ، كليندامايسين ، سيفتازيديم (94.12٪) بنفس النسبة، بينما كانت أقل مقاومة للإيميبينيم والكوليستين (52.94٪)، (14.71٪) على التوالي. أظهرت البكتيريا موجبة الجرام مقاومة عالية للسيفتازيديم (100٪) ومقاومة منخفضة للجنتاميسين والإيميبينيم (47.62٪) ثم الكوليستين (30.95٪).

كلمات مفتاحية: الغرغرينا الغازية، وقرحة القدم، والبتر، والكوليستين وإمدادات الدم.

Received:22/5/2022, Accepted:7/8/2022

INTRODUCTION

The gangrene: Defining it as a condition resulting from infection, inflammation, or complications of contaminated wounds and chronic diseases such as diabetes, resulting in tissue damage. This term is used to characterize the condition of an organ or tissue deterioration or death due to a lack of blood supply or a serious bacterial infection. Gangrene can infect any portion of the body, although the extremities are the most afflicted (1). Diabetic foot ulcers often suffer from infection with Gram-positive organisms such *as Staphylococcus aureus* (10), Gram-negative like *Pseudomonas aeruginosa* and others, in addition to resistant anaerobes. The microorganism that colonizes the superficial wound can provide a suitable place for the invasion that causes infection, and this is what these studies indicated (12). "Gangrene includes many types: Dry gangrene, Wet or moist gangrene, Gas gangrene and Fournier's gangrene" (32). There are several causes of gangrene, both direct and indirect. Direct causes of gangrene include mechanical, physical, chemical, biochemical, and microbial damage to the tissues, as well as indirect alterations in the tissue owing to a lack of vital components (nutrition, utilization of energy and immunity) (29). Gas-gangrene is a disease caused by anaerobic bacteria, most often *Clostridium perfringens* (11). Poor prognosis leads to amputation of the affected organ and sometimes death. Muscle tissue necrosis is one of the most common symptoms of gas gangrene, in which the muscle skin turns red to black then necrosis occurs, the mortality rate among patients is around (20%). The amputation rate remains high (25-88%) (2,3). "Effects of Gangrene: septicemia, delirium, skin discoloration, pain, swelling, foul-smelling discharge and flu-like symptoms" (1). Routine bacteriological procedures can be used to detect aerobic bacteria and drug sensitivity testing (30). The main aim of this study is to find the relation between aerobic bacterial infection with Gangrene in patients with diabetes, so we try tofind the most prevalent types of aerobic bacteria and their effect on disease and causing Gangrene. The steps for the work are as follows: by (Ali and Sultana) (I) Isolation and regular diagnosis of aerobic bacteria from diabetic patients in Baghdad hospitals from various Gangrene infections. (II) Examination of the drug sensitivity of isolated aerobic bacteria using the usual methods of several groups of antibiotics, especially penicillin's, cephalosporin, and others.

MATERIALS AND METHODS

Collection of samples

All samples including wound swabs of Gangrenous patient were 140 swabs collected from patients admitted to surgical unite in medical city Hospital, Al-kindy educational hospital, Baghdad hospital laboratories, and educational laboratories/medical city from gangrene foot ulcer during the period from 20 November 2019 to 25 December 2020 by using a sterile swab with transport medium from each patient. The information concerning the patients' name, age, the onset of the disease etc. were recorded. Gangrene samples were cultured immediately after collection for Diagnosis. According to COVID-19 situation, many samples were discarded which contaminated due to long time preservation in a bad electricity cooling condition. The net number was One hundred and six midstream gangrene samples were collected from patients (diabetic foot ulcer and other area than foot from gangrene among sample of diabetes mellitus Iraqi patients) suffering from symptoms referred to as gangrene.

Cultural characteristics

Different culture media, such as Mannitol salt, Blood-agar, MacConkey, and cetrimide, were used to inoculate the isolates; The cultured samples were incubated aerobically for 24 hours at 37°C, Bacterial colonies were identified phenotypically and biochemically, through their growth on plates, microscopic examination and the use of routine biochemical tests where no vitek system or more complex methods were used.

Antibiotic susceptibility testing for aerobic bacteria: Kirby-Bauer (37) single disk diffusion method was used to test the susceptibility of 76 isolates to different antimicrobial agents (Amoxicillin, Piperacillin, Clindamycin, Vancomycin, Azithromycin, Gentamicin, Ciprofloxacin, Colistin, Ceftazidime and Imipenem). Mueller-Hinton-plates were prepared depending on the

produce company HI media / India. Inoculums were prepared, by transferring 3-5 colonies into a tube of 5ml of normal saline NaCl 0.85%, mixed well and compared with McFarland tube to obtains growth with 1.5×10^8 CFU/ml, and adaptation to turbidity of McFarland 0.5, suspensions were used within 30 minutes of preparation. The diameter of the inhibition zone was measured and compared to the chart provided by Clinical and Laboratory Standard Institute (18).

RESULTS AND DISCUSSION

Incidence of gangrene

The incidence of Gangrene according to age group and gender: The enrolled cases in this study were (106 cases); the percentage of female was 37 (34.99%), while the percentage of male was 69 (65.1%). The distribution of gangrene infections according to age group of patients was investigate in this study and grouped into six groups as shown in the figure (1). The highest percentage of infected Gangrene patient was 33.33% in male and 24.32% in female at age groups 46-55 year respectively, while the lowest percentage of infected gangrene was at age group 15-25 year in which the infected female percentage was 5.41% while in male was zero. The mean age was 54 year among male and female. A study by (23) showed that the percentage of females was (58.6%) "with an average age of 69.6 (±7.16) years old while for men's was 70.4 (±7.26) years old and the women's aged 80 years old and overs had 6 times higher chance for developing diabetic foot than those aged between sixteen and sixty nine years old." (31) Found that the prevalence in male was more than that in female with a male to female ratio of 1.18:1. It may be explained as a result that the male showed less care to their foot than female. Also, the variation in infection gangrene may be explained as a result of the low socio-economic status, chronic diseases and other observed risk factors for gangrene beside that it could be explained to the presence of unique structure in gram-negative and gram positive bacteria which help attachment to the cells, that allowed multiplication, diffusion and tissue invasion, resulting in invasive infection and as a complicated cases.

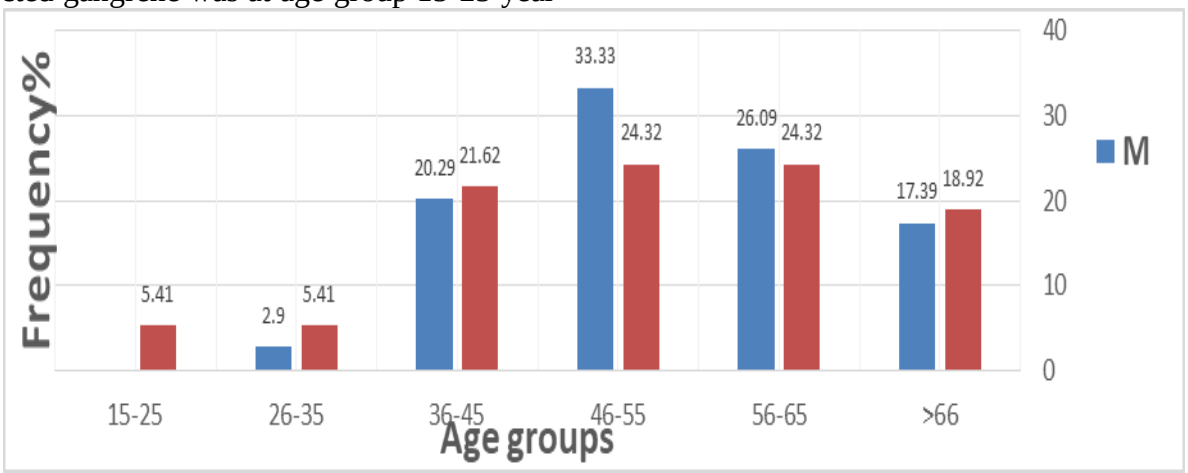


Figure 1. Distribution of the Gangrene infections among a sample of diabetes mellitus patients according to the Gender and age group

M: Male, F: Female

Distribution the gangrene infection Incidence in patients according to the infection period of patients.

The distribution of gangrene infection according to infected days of diabetic foot and other from gangrene among sample of diabetes mellitus patients was investigated in this study and grouped into three categories group as shown in the table (1).

The distribution of gangrene infections according to the date of collection.

The distribution of gangrene infections according to the date of collection was investigated in this study and the patients according to their date were grouped into three categories group as shown in the table (1). The highest percentage of infected gangrene was 60 patients (42.9 %) represented in group A, while the least percentage of infected gangrene was 37 patients (26.4 %) represented in group B. The differences and variation among A and B groups was very high even group A cases

collected within 1 month and group B within 2 months, this may be due to decrease in hospital admissions during emergency of COVID-19 pandemic. Sample collection should be among winter and summer to make a comparison which also not included in this study due to the same reason. A study by (24), showed, a seasonal variation among diabetic foot ulcer patients and the Majority incidence of ulceration in winter (64.93%) and (35.04%) presented in summer categories group as shown in the table (2), the incidence of diabetic foot ulceration and amputations might be reduced by education and clustering of medical services.

Table 1. Spread of gangrene infection Incidence in patients according to the infection period of patients.

period/days or week	Frequency	Percentage %
1 st trimester (1-3 days)	30	21.4%
2 nd trimester (3-7 days)	53	37.9%
3 rd trimester (1-2 weeks)	57	40.7%
Total	140	100%

Table 2. Distribution of Incidence of gangrene infections According to the date of collection sample from patients

Name	Date	Frequency	Percentage%
Group A	20 November to 25 December 2019	60	42.9%
Group B	28 December 2019 to 10 February 2020	37	26.4%
Group C	11 October to 25 December 2020	43	30.7%
Total	7 months	140	100%

Isolation and identification of aerobic bacteria : All bacterial isolates were described using Bergeys-Manual of Systematic Bacteriology (26) and additional characteristics given by others (9). Cultural, morphological and biochemical characteristics for each bacterial genus, revealed the appearance of many kinds of bacteria, includes (G+ve and G-ve) bacteria within 76 samples of diabetic foot ulcer swabs.

Cultural and morphological characteristics

Primary identification of bacterial isolates was done after being incubated aerobically on different kind of agar plates at 37 Cº for 24-48 hrs. The medium used as an enrichment medium for most bacterial isolates was blood agar. Thus, bacterial colonies appeared clearly on this medium. This allows studying their shapes, colors, texture, hemolysis pattern (β, α, γ) by observing the regions surrounding the colonies, and other morphological characters. The enrichment Blood agar medium is used to differentiate *Klebsiella* from other bacterial species that is similar with growth on MacConkey agar but heamolytic blood like *Serratia* spp. (22). *S. aureus* grew and showed smooth, translucent, creamy, yellow pigmented colonies on mannitol salt agar and ferment mannitol while *S. epidermidis* grew on mannitol salt agar but did not ferment mannitol. *Proteus* spp. (12) was identified by the swarming phenomenon on blood agar in which the bacterial growth covered all the surface of the agar with a moldy fish smell and the bacteria on the MacConkey agar appeared pale because the genus did not ferment lactose. *Pseudomonas* spp. has non-lactose fermenting colonies, clear, convex colonies with a metallic sheen and dark green pigmentation on MacConkey medium. *Acinetobacter* spp. (10) were diagnosed by its growth on MacConkey agar, appeared pale as small and non-lactose fermenting colonies, but on blood agar colonies appeared opaque creamy and non-heamolytic. *Klebsiella* spp. colonies were lactose fermenting and gave pink color; round regular edge was produced on MacConkey's medium (26).

Microscopic examination

Gram stain allows distinguishing for type of (G+ve) and (G-ve) microorganisms. Under the microscope, their color was observed in response to gram stain, shapes (rods, oval, cocci, and others) and their arrangement. *Staphylococcus* spp. was positive for gram stain and appeared as grape-like clusters, non-moving, no formation of spores. *Streptococcus* spp. was (G+ve) cocci, arranged in long or short chains and may be found as single cocci, bacteria. Other bacteria were related to Enterobacteriaceae such as *Proteus* spp., *Pseudomonas* spp. gram negative rods and

cocci, *Acinetobacter* spp. gram negative cocco-bacilli, *E. coli* gram negative cocci. *Klebsiella* spp. were found to be (G-ve) Non-moving, tiny straight bacilli grouped, singly or in Dual under a Compound Light Micro-scope, as described by (Chang et al.) (20).

Biochemical tests

The results of biochemical tests as shown in the Tables (3, 4), represent the characteristics of gram-positive and gram-negative bacteria as in this part by (25, 19). Biochemical tests were differential for bacterial isolates, *Staphylococcus* spp. were positive for catalase test and for coagulase test. *Pseudomonas* spp. showed oxidase and catalase positive tests, so these tests considered as differential tests for them. IMVIC test were differential for Enterobacteriaceae members; *E. coli* and *Proteus* spp gave indole and methyl-red positive result and negative for vogesproskauer and cimmon citrate tests. IMVIC test for *Klebsiella* spp. and *Enterococcus faecalis* were opposite to the result observed by *E. coli* and *Proteus* spp. Urea agar was differential test for *Proteus* spp. As they appear positive in the oxidized state in purple and clearly negative in the reduced state (4). Catalase test: In catalase-positive bacteria, there is the formation of bubbles due to the oxygen produced by the decomposition of hydrogen peroxide, and this is due to the presence of the enzyme catalase, which is produced by bacteria. This enzyme breaks down hydrogen peroxide into water and oxygen (21). Indole test: by using Kovac's reagent to detect the indole producing bacteria by forming a red ring which indicate the positive result, while non-producing indole bacteria showed a brown ring which indicate a negative result. Regarding urease test, which used to differentiate microorganisms according to their ability on hydrolyzing urea by urease enzyme urease, the positive result showed a change in the color from yellow as a negative to pink a positive due to phenol red as an indicator (33). Coagulase test is an important test used to distinguish *S. aureus* from Coagulase Negative Staphylococci (CONS), the secretion of coagulase aids in the fibrins thrombus formation that give protection to the bacteria from phagocytes and from the immune system. The Coagulase positive result represented by thrombus formation within 30-60 minutes, while a negative result represented by no coagulation formation (18) and (35). Hemolysis test used blood agar to differentiate bacteria based on their hemolytic characteristics (alpha, beta and gamma), a change in blood agar to z green that surrounds a bacterial colony refer to alpha hemolysis due to partially hemolysis. Beta hemolysis showed a clear zone around a colony due to complete breakdown of the hemoglobin of the red blood cells like that appears in *Streptococcus pyogenes* and *S. aureus*. No hemolysis was found in the area surrounding the growing of microorganisms is called gamma hemolysis, where a brown color appears in the implanted plate as a result of the growth conditions used and the presence of carbon dioxide (for absence of the enzyme hemolysin). *Enterococcus faecalis* showed gamma hemolysis (6). The results of IMVIC: Methyl red test used for detection of fermentation of glucose and production of acid. By the presence of methyl red as indicator, the color changed to red due to low pH less than 5 (5, 26). Vogas-Proskauer test positive result showed a pink-red color at the surface as a result to glucose fermentation and acetone production after using the indicator due to low pH less than 5 (5, 26). Simmon citrate test used to differentiate bacteria according to their ability to hydrolyze the citrate which is the carbon source and change the color from green to blue by the presence of the indicator Bromothymol blue. The results in biochemical tests show in figure (2).

Table 3. The biochemical tests of gram-positive bacteria

Test	*S. aureus*	*S. epidermidis*
Catalase	+	-
Oxidase	-	-
Coagulase	+	-
Mannitol fermentation	+	-

Table 4. The biochemical tests of Gram- negative bacteria

Test	*E. coli*	*Klebsiella* spp.	*Enterobacter* spp.	*Pseudomonas* spp.	*Proteus* spp.
Oxidase	-	-	-	+	-
Catalase	+	+	+	+	+
Indole	+	-	-	-	+
Methyl red	+	-	-	-	+
Voges proskauer	-	+	+	-	-
Citrate utilization	-	+	+	+	-
Urease	-	+	-	ND	+
Motility	+	-	+	+	+

ND: not detected

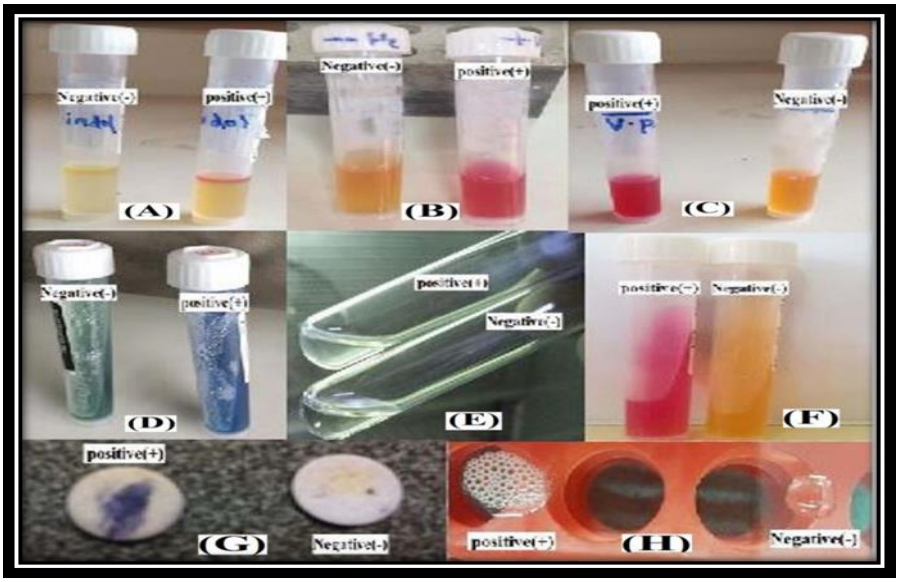


Figure 2. Biochemical tests for Identification of bacterial isolate, "a": indole-test, "b": methyl-red-test, "c": vogesproskauer-test, "d": Simmons-citrate-test, "e": Coagulase-test, "f": Urease-test, "g": oxidase-test, "h": Catalase test.

Bacterial isolation and distribution

Aerobic bacteria isolated from 106 samples were 76 isolates, this due to either no growth and discarding many samples due to contamination. Urease-producing bacteria were recorded in 55 swabs (72.3%) of the 76 patients who had been infected for ulcers. Non-urease-producing bacteria were cultured 21 swabs (27.7%) of the 76 patients. Of the 76 bacterial isolates, 63 (73.2%) showed mixed infection. While the anaerobic bacteria detected by PCR were 21 (15.09%) samples. The most prevalent organism was S. aureus, which accounted for 24 of 76 (31.6%) different organisms identified, followed by Pseudomonas then E. coli, which accounted for 18 (23.7%) and 13 (17.1%) respectively of the organisms identified (Table 5) as in the study (15).

Table 5. Identification of bacteria from diabetic foot ulcer from 76 patients.

Bacterium	Females (n = 33) No. %	Males (n = 43) No. %	Total (n = 76) No. %
Urease producing (55)			
Staphylococcus aureus	**9 (11.8%)**	**15 (19.7%)**	**24 (31.6%)**
NCP			**8 (10.5%)**
***Proteus* spp**	**1 (1.3%)**	**0 (0.0%)**	**1 (1.3%)**
***Klebsiella* spp.**	**1 (1.3%)**	**1 (1.3%)**	**2 (2.6%)**
Pseudomonas aeruginosa	**6 (7.9%)**	**12 (15.38%)**	**18 (23.7%)**
Micrococcus	**0 (0.0%)**	**2 (2.6%)**	**2 (1.5%)**
Non-urease producing (21)			
E. coli	**5 (6.6%)**	**8 (10.5%)**	**13 (17.1%)**
Enterococcus	**3 (3.9%)**	**5 (6.6%)**	**8 (10.5%)**

NCP: Non coagulase positive

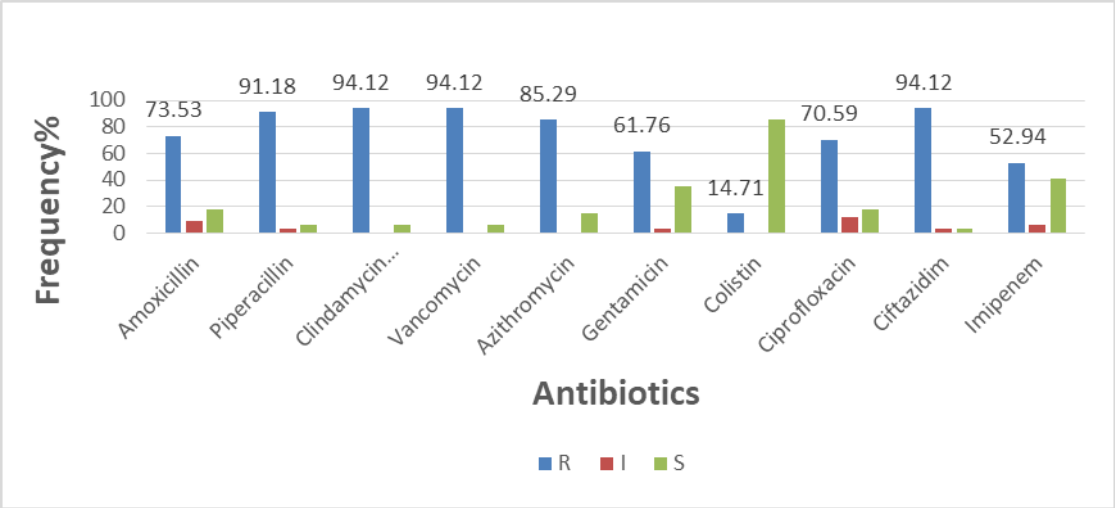
The study showed that Gram-positive bacteria were dominant among the cultured isolates, reaching (55.3%) while Gram-negative bacteria only (44.7%). Among a (G+ve) isolates is most common pathogens was *S. aureus* (57.1%), followed by *Enterococcus* (19.0%) and *Micrococcus* (4.9%). Gram-negative isolates, the percentage distribution in which Pseudomonas was (52.9%) is one of the opportunistic human pathogens (37), followed by *E. coli* (38.2%), then *Klebsiella* spp. (5.9%) and *Proteus* (2.9%). The highest percentage of *S. aureus* (57.1%) Perhaps the reason is that it is present in the skin and nasal membranes with a dreadful pathogenic potential to cause a variety of and hospital-acquired infections (8, 38). Being found in up to 80 percent of healthy people. It is one of the most common causes that affect and inflammation occurs the skin and pose a danger to the soft tissues of the body (7), while the low frequency of *Micrococcus* may be related to that these organisms are not pathogenic bacteria and consider as normal resident skin flora in different anatomic site, However, *S. aureus*, which is not generally found as a part in the resident flora, can be isolated from the nasal (20–40%) and intertriginous tissues (20%) and consider the most pathogen isolated from infected surgical wounds (38). These results in disagreement with a study by (36) who found that the spread of (G-ve) bacteria was prevalent in a diabatic foot problems (71.27 percentage); where's (G+ve) only (28.73 percentages). Between a (G-ve) cultivated isolate, but there is an agreement with the most pathogen isolation was *Pseudomonas* (24.49%), also they found that the (G+ve) isolated consist from *S. aureus* with a high percent's (66.77%) (Table 6).

Table 6. Distribution (G+ve and G-ve) bacteria among 76 isolates from patients with diabetic foot ulcer.

Gram positive	Number	Percentage %	Gram Negative	Number	Percentage %
S. aureus	**24**	**57.1%**	***Pseudomonas***	**18**	**52.9%**
Micrococcus	**2**	**4.9%**	***E. coli***	**13**	**38.2%**
NCP	**8**	**19.0%**	***Klebsiella***	**2**	**5.9%**
Enterococcus	**8**	**19.0%**	***Proteus***	**1**	**3%**
Total	**42**	**100%**	**Total**	**34**	**100%**

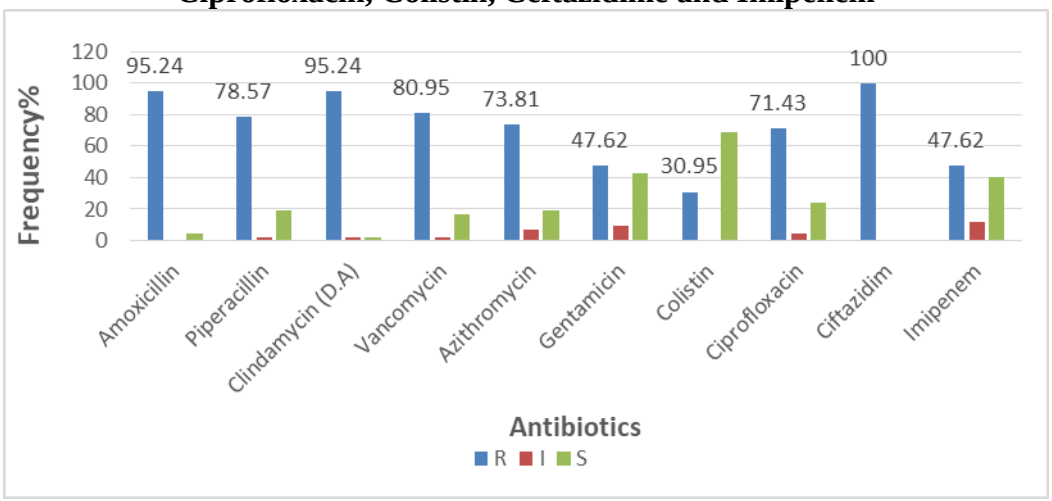
Antimicrobial susceptibility of bacterial isolates: All Gram negative isolates were resistant to ≥ 1 of the 10 antimicrobial agents tested. The most effective agents were Colistin followed by Imipenem and gentamicin: all isolates were sensitive to them. The least effective agents were Vancomycin, Ceftazidime and Clindamycin as most the isolates were resistant to them (Figure 3). High percentage of resistance (94.12%) for Vancomycin as reported by (Rampal et al.) (36), who showed that all (G-ve) are entirely resistance to treat Vancomycin, due to the outer membrane are impermeable to big glyco-peptide molecules. Also, the same percentage of resistance (94.12%) against for Clindamycin which occurrences because mutation in the gene 23S-rRNAt hat target by the drugs, the changes in flow, and Erythromycin resistant methylase gene inherently as reported by (Card RM et al.) (16). Ceftazidime resistance also showed high prevalence (94.12%) which in disagree with a study done by (FantaGashe et al.) (24) Who showed 149 (60.1%) of the total bacterial isolates. While highest sensitivity was toward Colistin (70.59%) among 34gram negative bacterial isolates, 5 (14.7%) isolates showed resistance to Colistin. Monitoring the beginning of Colistin resistance distribution. Colistin resistance developed by adaptive or mutational mechanism due to alteration in outer membrane of gram negative. Also, Colistin resistance may be mediated by plasmid distribution among human and mediated by plasmid distribution (28, 39). Regarding Gram positive isolates 42, they showed resistant to ≥ 1 of the 10 antimicrobial agents tested. The most effective agents were the same as that in gram negative bacteria which were Colistin followed by Imipenem and gentamicin: most isolates were sensitive to them. The less effective agents were Ceftazidime (100%) followed by Amoxicillin and Clindamycin (95.24%) as most the isolates were resistant to them (Figure 3), (Table 7). Vancomycin resistance was high (80.95%) which disagree with (34) who represented that the vancomycin resistance of *Staphylococcus aureus* was (26%), this either due to the

presence of no coagulase positive bacteria and micrococcus within all resistant group or may be due to Colistin antagonism effect against Vancomycin (17), Figure 4.



R:Resistant, I:Intermediate, S:Sensitive

Figure 3. Antimicrobial sensitivity test of Gram- negative isolates from foot ulcer samples Amoxicillin, Piperacillin, Clindamycin, Vancomycin, Azithromycin, Gentamicin, Ciprofloxacin, Colistin, Ceftazidime and Imipenem



R:Resistant, I:Intermediate, S:Sensitive

Figure 4. Antimicrobial sensitivity test of Gram- positive isolates from foot ulcer samples

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