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Assessment role of phagocytic neutrophil cells among different Wagner's grades of diabetic foot ulcers infections

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Abstract

Background: Foot ulcers complications in diabetes mellitus (DM) patients are one of the significant medical problems and an economic burden. The aim of this study is to assessment role of phagocytic neutrophil cells and its relationship with the incidence of diabetic foot ulcers (DFU) infection of diabetic patients. A total of 60 venipuncture blood samples collected from diabetes mellitus, diabetic foot ulcer patients and healthy persons as control group. 20 swabs from the DFU patients were collected and processed for culture and susceptibility test after the ulcers classified according to Wagner's grades system. Phagocytic cells activity test was performed to determine the efficiency of phagocytic neutrophil cells in diabetic patients.

Results: Gram positive bacteria were the most prevalent in the DFU patients 57.1% with statistical significant relationship between the type of bacteria and grades of Wagner's classification followed by Gram negative bacteria in high grades of ulcers. Wagner's ulcers grade 1 and 2 were the most prevalence in DFU patients 30%. There was a weak negative correlation between the efficiency of phagocytic neutrophil cells activity and grade ulcers classified ($r = -0.323$). Amikacin and ciprofloxacin were the most effective antibiotics against 90.5% and 81% of the bacterial isolates respectively, whereas ampicillin, cefepime and cefadroxil were less effective antibiotics against the bacterial isolates.

Conclusion: When the grade of ulcer increased, the bacterial resistance to antibiotics increased, and this was emphasis the correlation with prevalent of Gram negative bacteria in the high grade of

ulcers with high resistance of antibiotics. In contrast, the grade of ulcer increased, the efficiency of phagocytic neutrophil cells decreased.

Key words: Phagocytic cells, Bacterial infection, Wagner's grades classification, Diabetic foot ulcers.

Background

Diabetes mellitus (DM) is one of the main global health problems. It's defined as a metabolic disorder resulting from a defect in insulin secretion, insulin action, or both, which leads to chronic hyperglycemia [1]. Several polymorphonuclear leukocyte cells (PMNLs) affected by hyperglycemia, neutrophil's functions impair occur in diabetic subjects including impaired migration, phagocytosis, intracellular killing and chemotaxis, which may be due to decreased PMNLs membrane fluidity [2]. There is clinical evidence pointing to the higher prevalence of infectious diseases among individuals with DM [3]. Foot ulcers remain one of the most distressing complications of a diabetic patient and it's one of the most significant and devastating complications of diabetes [4]. Wagner's ulcer grades classification is one of the most widely used and universally accepted grading systems for diabetic foot ulcers (DFU), consisting of six simplistic wound grades used to assess ulcer depth as following: grade-0; high risk foot and no ulceration, grade-1; superficial ulceration, grade-2; deep ulcer - cellulitis, grade-3; osteomyelitis with ulceration or abscess, grade-4; gangrenous patches and grade-5; gangrene of entire foot [5].

Phagocytic cells activity is affected by blood sugar levels, and the infection of lesions grades of DFU also affected by phagocytic activity of neutrophil cells. This study aimed to assessment role of phagocytic neutrophil cells and its role to fight the bacterial infection of diabetic foot ulcers classified according to Wagner's grades system, as well as to determine the antibiogram patterns of bacterial isolated from diabetic foot ulcers.

Results

Diabetic foot ulcers classification

As an assessment of grades of DFU as described by Wagner's classification system at the time of study period, the results showed that grade I and II seen in 6(30%), grade III 5(25%), grade IV 1(5%) and grade V 2(10%), table (1).

Table (1): Distribution of diabetic foot ulcers according to Wagner's classification

Ulcer grade	DFU	
	No.	%
Grade I	6	30.0
Grade II	6	30.0
Grade III	5	25.0
Grade IV	1	5.0
Grade V	2	10.0
Total	20	100.0

Distribution of bacterial isolated

Twenty one bacterial species were isolated from DFU according to the conventional methods for identification of bacteria. Gram positive bacteria isolates were *S. aureus* 3(14%), *Staphylococcus epidermidis* (*S. epidermidis*) 3(14%), *Streptococci species* 3(14%), *Staphylococcus saprophyticus* (*S. saprophyticus*) 2(10%) and methicillin resistance *Staphylococcus aureus* (MRSA) 1(5%). Other isolates of Gram negative were *Proteus mirabilis* (*P. mirabilis*) 2(9%), *Morganella morganii* (*M. morganii*) 2(9%), *Pseudomonas aeruginosa* (*P. aeruginosa*) 1(5%), *Serratia species* 1(5%), *Escherichia coli* (*Esch. coli*) 1(5%), *Klebsiella species* 1(5%) and *Citrobacter freundii* (*C. freundii*) 1(5%).

Bacterial species of DFU associated with Wagner's grades system

As shown in table (2), the superficial ulcer grades infections were caused by Gram positive bacteria, while Gram-negative bacteria showed predominant in deep ulcer grades infections with statistically significant association (P-value 0.057). The distribution of different bacterial species isolated from DFU cases according to Wagner's grades classification given in figure (1).

Table (2): Distribution of bacterial isolated according to Wagner's grades of ulcers

Bacterial groups	Wagner's ulcer grades					P-value
	Grade I	Grade II	Grade III	Grade IV	Grade V	
Gram positive bacteria	9.6%	33.5%	9.6%	4.8%	-	0.057*
Gram negative bacteria	14.4%	-	14.4%	4.8%	9.6%	

*Statistical significant at P-value 0.05

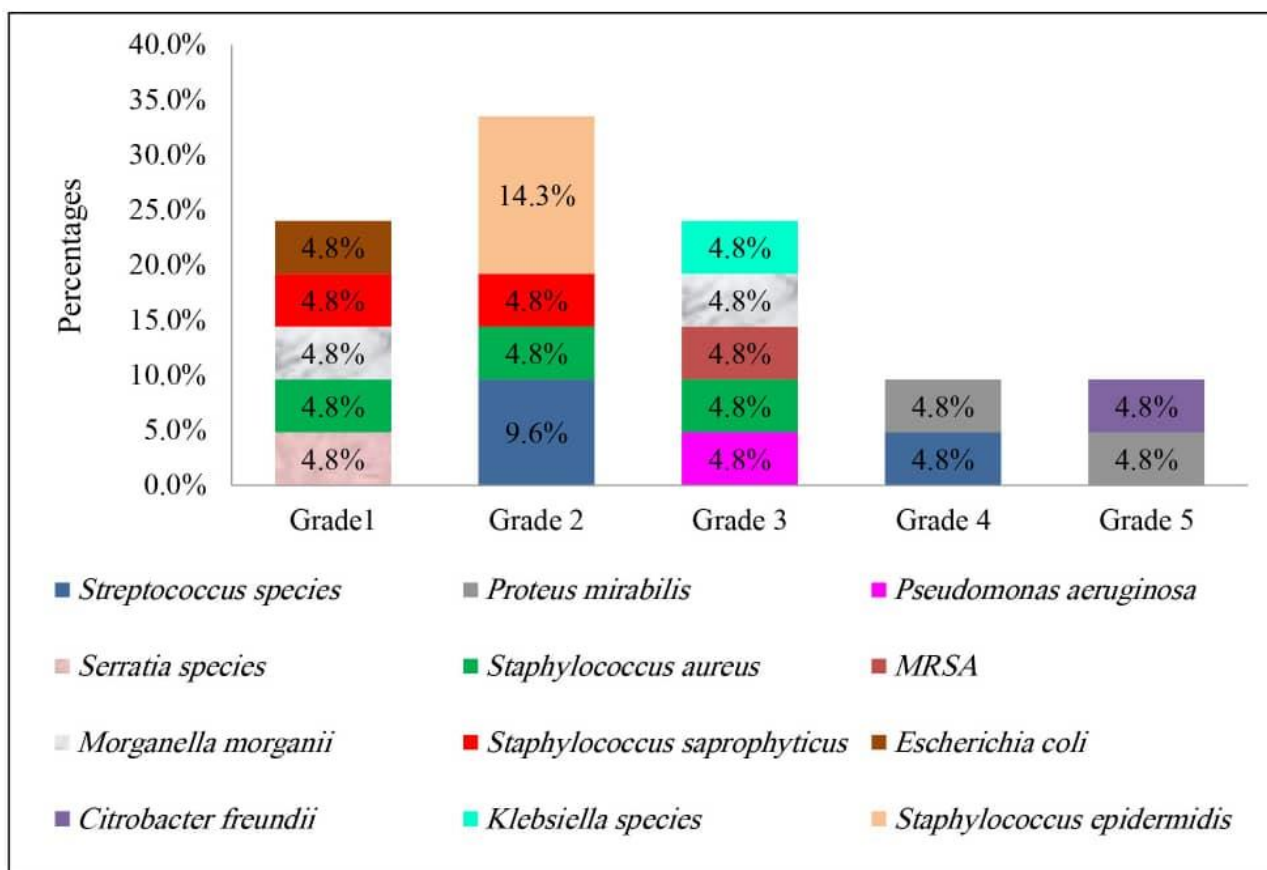


Figure (1): Bacterial species of DFU according to Wagner's ulcers grades classification

Antibiotics resistance patterns of bacterial isolates

In this study, *Staphylococci species* were resistant for ampicillin 100% and high sensitive to ciprofloxacin, amikacin, trimethoprim and cefaclor 100%. MRSA showed high resistant to cefixime, ampicillin, cefadroxil and trimethoprim antibiotics 100% and high sensitive for vancomycin, amikacin, ciprofloxacin, amoxyclav and cefepime 100%. *Streptococci species* were resistant for cefepime, cefixime, ampicillin, cefadroxil and cefaclor 100%, and sensitive for ciprofloxacin and amikacin 100%.

Gram negative bacteria showed high resistance to ampicillin and cefixime 88.8% followed by cefadroxil, cefaclor, cefepime 77.8% and trimethoprim 55.6%. Most of the isolates were susceptible to amikacin 77.8% and ciprofloxacin 55.6%, table (3).

Table (3): Antibiotics sensitive, intermediate sensitive and resistance patterns of bacterial isolates

Patterns	Antibiotics discs									
	CIP	AMC	CPM	AK	CFM	AMP	CF	TR	CFR	VA
S No.(%)	17(81.0)	12(61.9)	10(47.6)	19(90.4)	2(9.5)	0(0.0)	11(52.4)	11(52.4)	9(42.9)	10(83.3)
I No.(%)	0(0.0)	1(4.8)	1(4.8)	1(4.8)	0(0.0)	1(4.8)	0(0.0)	4(19.0)	1(4.8)	0(0.0)
R No.(%)	4(19.0)	8(33.3)	10(47.6)	1(4.8)	19(90.5)	20(95.2)	10(47.6)	6(28.6)	11(52.4)	2(16.7)

Antibiotics resistance patterns associated with Wagner's ulcers grades

There was an increased resistance of bacterial isolates with increased grades of Wagner's classification as shown in figure (2). According to Pearson correlation test there was statistically significant correlation (P-value = 0.05 ; r = 0.369).

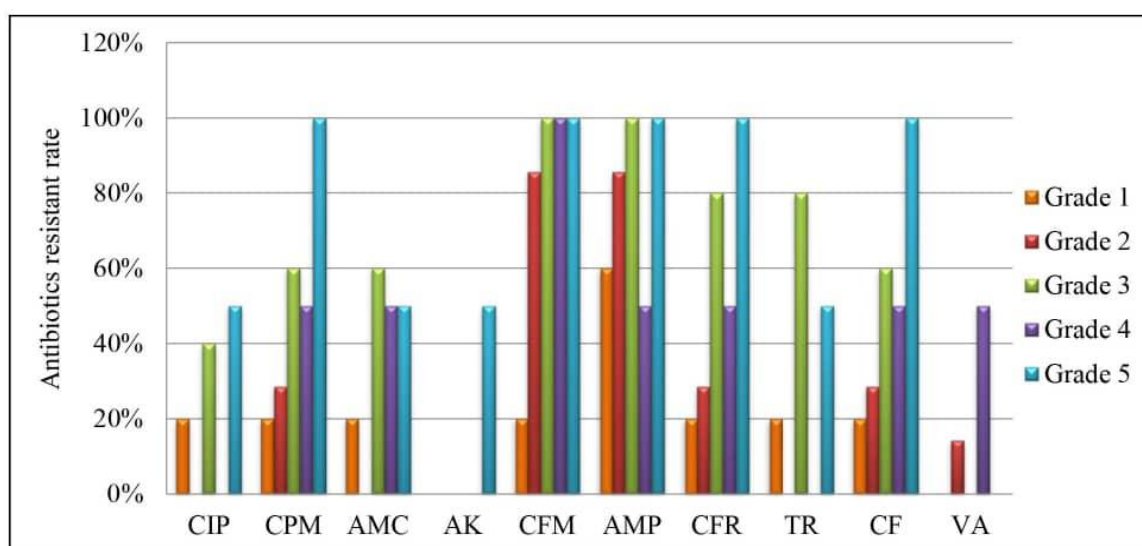


Figure (2): Antibiotics resistance patterns of bacterial isolated in different Wagner's grades

Phagocytic neutrophil cells activity test

According to phagocytic cells activity test, DM and DFU patients had no high efficient phagocytic neutrophil activity, 1 and 0 respectively as given in table (4).

Table (4): Phagocytic neutrophil cells activity among studied groups

Group	Phagocytic neutrophil cells activity			
	Low	Medium	High	Total
Control	0	10	10	20
DM	2	17	1	20
DFU	6	14	0	20
Total	8	41	11	60

The mean of phagocytosis was completely different between the three groups studied (control, DM and DFU patients) using LSD with high significant difference statistics as shown in table (5).

Table (5): The mean of phagocytic neutrophil cells activity among studied groups

Group	Mean %		
	Control (71.35%)	DM (52.70%)	DFU (43.05%)
Control	-	0.000*	0.000*
DM	0.000*	-	0.001*
DFU	0.000*	0.001*	-

*Statistical significant at P-value < 0.05

In this study we correlated the degree of wounds with phagocytic efficiency to understand the development of the ulcer grade and role of neutrophil phagocytic cells. Statistically, there was low insignificant correlation between these two variables as given in table (6) and figure (3).

Table (6): Correlation between phagocytic neutrophil cells activity and Wagner's grades of ulcer

Variable	No.	P-value	Pearson correlation (r)
Grades of ulcers	20	0.164	-0.323
Phagocytosis	20		

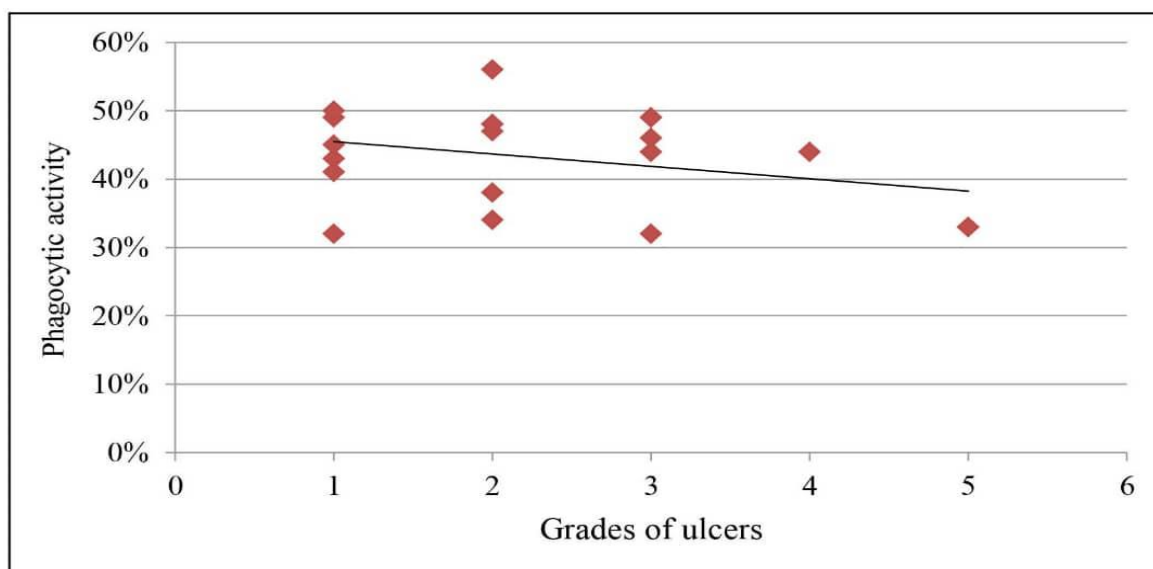


Figure (3): The correlation between phagocytic neutrophil cells activity and Wagner's grades of ulcers

Discussion

Identifying the risk factors of DFU might help to develop better prevention strategies in diabetic patients. In this study we attempted to find if there is a relationship between the phagocytic neutrophil cells activity and the Wagner's grades of the diabetic ulcers. Wagner's grade 1 and 2 ulcers classification were the most common prevalence 30% in ulcers of diabetic patients, and these results were compatible with other studies conducted in different countries [13-15], but disagree with a previous study reported that Wagner's grade 2 and 3 ulcers were the most common prevalence [16]. Grade 4 was the lowest prevalence in this study, and this contrast with other studies revealed that the grade 4 was the most common prevalence in DFU [17, 18]. Other studies showed the grade 4 was commonly infected with mixed variety of bacteria including extended spectrum beta-lactamase (ESBL) *Klebsiella species* and MRSA [17], also, highly multiple bacterial infection were more incidence in DFU grade 4 [19]. Our study results found that multiple bacterial infections was associated with grade 2 and 4 of Wagner's ulcers classification, while other study revealed that non-significant difference of the mean number of isolates and diabetic deep tissue infection of different grades [20].

In this study, Gram positive bacteria showed an increased prevalence 57.1% in diabetic ulcers with similar results of different studies 77% [21], 35% [22] and 51.1% [5]. The most common bacterial isolates from diabetic ulcers in this study were *Staphylococci species* 40.8% compatible to other findings showed that *Staphylococci species* accounted 43.37% [23], whereas Gram negative

bacteria were the most frequently pathogenic agents reported in other studies 51% [24], 56.1% [25], 61% [26], 67% [27] and 51.2% [28].

Interestingly, our study revealed high rate of *S. aureus* 14.3%, *S. epidermidis* 14.3%, *S. saprophyticus* 9.5% and MRSA 4.8%. These results were compatible with the findings of other studies showed that *S. aureus* 19.4%, *S. epidermidis* 18.4% and other *Staphylococci species* 3.6% [29], Other study showed the most commonly isolated *S. aureus* 30%, followed by *S. saprophyticus* 19% and *S. epidermidis* 10% [30]. Another study showed that *S. aureus* 25%, *S. saprophyticus* 15% and *S. epidermidis* 7% were the most isolated from DFU [14].

Streptococci species were the second most commonly isolated pathogens in our study 14.3%, this is nearly with other studies isolated *Streptococci species* 17% [31], 16% [32] and 15.6% [33]. Poor control of blood glucose was associated with a high relative abundant colonization by *Staphylococci species* and *Streptococci species* [34]. So, the most cases of DFU who have mixed infection of *Staphylococci species* and *Streptococci species* were poor glycemic control patients in our study.

P. mirabilis and *M. morganii* accounted 9.5% of predominant Gram negative bacteria in DFU in this study. High prevalence of *Proteus species* was reported in different studies [22, 30, 35], while *M. morgani* showed isolated low prevalence 3.16% [36]. *P. aeruginosa* accounted 4.8% with resistance for the most antibiotics used in this study. Similar results found that *Pseudomonas species* were the most common isolated from DFU [5, 31, 32, 37-39]. *P. aeruginosa* may cause an invasive form, sometimes in diabetics which fails to respond to topical antibiotic therapy [40]. Additionally, significant delay in wound healing cause of *P. aeruginosa* biofilms inhibit neutrophil movement [41]. So the coordinated control of the production of virulence and antibiotic resistance factors and the ability to adapt to various environmental changes is a likely and important reason that *P. aeruginosa* is a successful and common pathogen [42].

The prevalence of *Klebsiella species* and *Esch. coli* was 4.8% in this study, this agreed with other study results 4% [14], and disagreed with other study recorded a high rates of *Klebsiella species* 12.6% and *Esch. coli* 17.9% isolated from DFU [36]. Also, *C. freundii* and *Serratia species* isolates accounted 4.8% in this study, and is comparable with other results 5% [22], 3.85% [5], 4.3% [43] for *C. freundii* and 2.3% for *Serratia species* [44]. Different geographical area could have contributed to different types of bacterial isolated [35].

The results of numerous studies conducted on the bacterial profiles of DFU showed a varied and often contradictory findings [39]. The reason for this difference in findings could be attributed to the difference in the causative agents, geographic variations or the severity of the infections [32].

In our study, the diabetic patients have superficial wounds of Wagner's grades classification I and II were mostly infected with Gram positive bacteria. *Staphylococci* and *Streptococci species* were the predominant pathogens, whereas Gram negative bacteria were more frequently isolated from Wagner's wounds classification grades III and V. These findings are in agreement with various studies concluded that an increasing of Wagner's grades, the proportion of bacterial infections were increased [2, 24, 35, 44, 45]. However, the percentage of *S. aureus* decreased with increased Wagner's grades similar findings reported in other study [24]. MRSA is an increasing problem in industrialized and developing countries. It is commonly believed to be an important cause of poor outcome, increased duration of hospital stay, increased cost and mortality [17, 46]. In this study, MRSA were associated with Wagner's grade 3 ulcer classification, and other study showed that Wagner's ulcers grade 3 were commonly infected with MRSA [17].

This study showed high level resistance of bacterial isolates to ampicillin 95%, this ratio showed in other studies reported 92.4% [44], 94.9% [47, 48], and 50% [35]. This increased of resistance may be attributed to the fact that ampicillin has been widely abused and frequently implicated in self-medication [47]. Cefepime was active against 47.6% of isolates, this result agreed with other reports 41% [43], 61.9% [49], 42.4% [32], 46.7% [50].

Our results revealed that amikacin was the most effective antibiotic against 90.5% of the bacterial isolates. This result was similar to that showed 75.4% [51], 96.7% [44], 82.9% [52], 90% [26], while it's less activity showed in other studies 54.7% [51], 54.5% [54], 62.7% [45]. Also, ciprofloxacin showed greater susceptibility to the isolates 81%, this result was approved with several studies reported sensitivity of ciprofloxacin was 63.7% [55], 61.8% [50], 78.8% [33] and 77.3% [56], while other studies showed high resistant bacteria to ciprofloxacin 51.2% [57], 71.8% [54].

In this study, vancomycin showed 83.3% sensitivity of Gram positive bacteria isolated. This similar to other studies 90.1% [55], 95% [36], 98.2% [24], while other study showed low sensitivity of vancomycin 44.5% [44]. However, other studies showed 100% of Gram positive bacteria susceptible to vancomycin [5, 27, 32, 35, 50, 58].

Amoxyclav was active against bacterial isolated 61.9% in this study, and this result was in accordance with the studies showed sensitivity amoxyclav were 67.4% [27], 49% [55], 57.2% [57]. In contrast, other studies showed high resistance amoxyclav 79% [44], 81.8% [54]. Cefexime showed low sensitivity 9.5% for the isolated bacteria in our study. Similar low rate reported in other study 3.2% [59]. While the sensitivity for Gram negative bacteria to cefexime was 70.7% [5], and for Gram positive bacteria was 77.3% [33]. The sensitivity of cefaclor was 47.6% of bacterial

isolates in this study. This was compatible with the results of other study showed the second generation of cephalosporin was 50% [44]. Other studies showed that cefaclor was active 58.4% against Gram positive bacteria [54], and 92.8% against Gram negative bacteria [35].

Trimethoprim had sensitivity 52.4% of bacterial isolates in this study. It's similar with the results of other studies 50% [44], 70.3% [35], while the sensitivity to co-trimoxazole was low in other studies 16% [36], 14.3% [56]. Cefadroxil was active 52.4% against bacterial isolates. Other study indicated that first generation of cephalosporin was sensitive 41.7% [43], 16.1% [32]. Other studies showed the sensitivity of cefadroxil to Gram negative bacteria was 44.4% [50] and 11% [24].

Our study revealed that a low significant positive correlation between the antibiotics resistant and increasing of Wagner's grades classification. Such relation have previously reported that an increasing of Wagner's grades, the resistance rates to some antibiotics increased [60].

This study indicated a significant reduction in phagocytic activity in DM and DFU patients compared with control group. In addition, our baseline results confirm and extend previous reports concerning the impairment of phagocytosis that occurs in neutrophil cells from diabetic patients than non-diabetic patients [61]. Other studies showed a significant reduction in phagocytic cells activity in Type-1 DM and Type-2 DM [62, 63]. This difference may be due to that hyperglycemia impairs granulocyte functions including adherence, chemotaxis, phagocytosis and bactericidal activity [64]. So, hyperglycemia reduced response of neutrophil function and disorders of humoral immunity as one of the long-term effects of elevated mean blood glucose (MBG) or HbA1c, also the impaired micro-vascular circulation in patients with diabetic foot limits the access of phagocytes favouring development of infection [3, 26, 65].

One of the strengths of our study results find the relationship between the manner of immune response involving phagocytic cells activity and the degree of wound. The study results showed a weak inverse correlation between the ratio of phagocytic cells activity and the degree of wound in Wagner's classification (P-value 0.164 ; $r = -0.323$). However, in our knowledge, there have been no reports in the literature regarding the correlation between phagocytic cells activity and the degree of wound in diabetic foot patients to support our result or to know the reasons of this relation we need further deep studies to be clarified.

Conclusion

When the grade of ulcer increased, the bacterial resistance to antibiotics increased, and this was emphasis the correlation with prevalent of Gram negative bacteria in the high grade of ulcers with high resistance of antibiotics. In contrast, the grade of ulcer increased, the efficiency of neutrophil

phagocytic cells decreased. Further immunological and bacteriological investigations needed to control the increasing of antibiotics resistance in diabetic foot ulcers patients. We recommended when the grade of ulcer classified is high, a bacterial culture and sensitivity testing should be done to avoid complications of the increasing bacterial resistance to antibiotics.

Methods

Study design and samples collection

A case-control and analytical study was conducted during a period from November 2018 to May 2019 on diabetes mellitus and diabetic foot ulcer patients in different hospitals of Mukalla city - Hadhramout / Yemen. A total of 60 volunteers were selected randomly and classified into 20 patients of DFU, 20 patients of DM and 20 healthy persons as control group. About 10ml of venipuncture blood were collected in ethylene diamine tetraacetic acid (EDTA) for phagocytic neutrophil activity test.

A sterile swabs were collected from ulcers after cleaned with sterile saline to remove accumulated drainage and transient skin flora. The swabs moistened with sterile saline if the wounds were dry. Then the swabs kept at room temperature and delivered to the microbiology laboratory for culture [6].

Classification of diabetic ulcers

The ulcers of diabetic foot patients were classified according to Wagner's grades classification system as an assessment of five grades at the time of study period.

Phagocytic neutrophil cells activity test

Phagocytic cells activity test was performed according to the methods described by [7-10] with minor modifications. In briefly, normal saline suspension of pathogenic strain *Staphylococcus aureus* (*S. aureus*) colonies was prepared and the visible turbidity was adjusted to 0.5 McFarland turbidity standard yielding an approximately 1.5×10^8 CFU/ml. About 1ml of EDTA whole blood mixed with 1ml of saline bacterial suspension, then incubated for one hour at 37°C. One drop of incubated mixture was placed on a slide of microscope to make a thin smear. The smear was lifted to dry for 3 minutes, and fixed with ethanol 96%, then placed in hematoxyline stain for 10 minutes. After water washing, the smear placed in eosin stain for 30 seconds, then washed by water and examined under light microscope with 100x oil immersion. The total number of *S. aureus* ingested within 100 neutrophil cells were counted and divided by 100 to give the percentage of phagocytic cells activity.

Isolation and identification of bacterial pathogens

All swab samples of ulcers were inoculated on the blood agar and MacConkey agar media (Oxoid/England). The culture plates were incubated under aerobic conditions at 37°C for 24 hours. After incubation, the growing of bacterial isolates were recognizable by colonies features. Identification of bacterial species was made based on reaction of Gram stain, morphology and biochemical characteristics using different available tests, catalase; coagulase; DNase; mannitol fermentation; urea; citrate; Kligler iron agar, sulphide indole motility and oxidase [11].

Antibiotic susceptibility testing

Antibiotic susceptibility testing was done using Kirby-Bauer disc diffusion method on Mueller Hinton agar (Oxoid/England) according to the standard guidelines of the Clinical Laboratory Standards Institute (CLSI) for testing ampicillin, cefepime, cefadroxil, ciprofloxacin, cefixime, cefaclor, trimethoprim, amoxyclav, methicillin, amikacin and vancomycin [12].

Statistical analysis

Data analyzed using the software of Statistical Package for Social Sciences (SPSS) version 25. The graphs presented using the software program (Excel for Windows Microsoft) version 10. Descriptive statistics (frequencies and percentages) for study variables were obtained and compared using least significant difference test (LSD) and one way ANOVA test. The association between different groups of the explanatory variables was measured and compared using Pearson Chi-square (χ^2) test. The relationship between the variables examined by the Pearson correlation (r) test. The level of significance was set at P-value less than 0.05.

Abbreviations

DM: Diabetes mellitus, DFU: Diabetic foot ulcers, PMNLs: Polymorphonuclear leukocyte cells, CFU: Colony forming unit, EDTA: Ethylene diamine tetraacetic acid, CLSI: Clinical and Laboratory Standards Institute, SPSS: Statistical Package for Social Sciences, LSD: Least significant difference test, MRSA: Methicillin resistance *Staphylococcus aureus*, S: Sensitive, I: Intermediate sensitive, R: Resistance, CIP: Ciprofloxacin, CPM: Cefepime, AMC: Amoxiclav, AK: Amikacin, CFM: Cefixime, AMP: Ampicillin, CFR: Cefadroxil, TR: Trimethoprim, CF: Cefaclor, VA: Vancomycin.

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Authors' contributions

All authors conceived, designed the experiments and wrote the manuscript. Maryam Hamed Baras analyzed the experimental results, performed the statistical analysis and interpreted all of the data. Eidha Ali Bin-Hameed reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Ethical approval for this study was obtained from Hadhramout University before commencing the study. The information were taken from the participants after they agreed to it verbally according to the informed consent with confidentiality of each participant as well as the results.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

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Figures

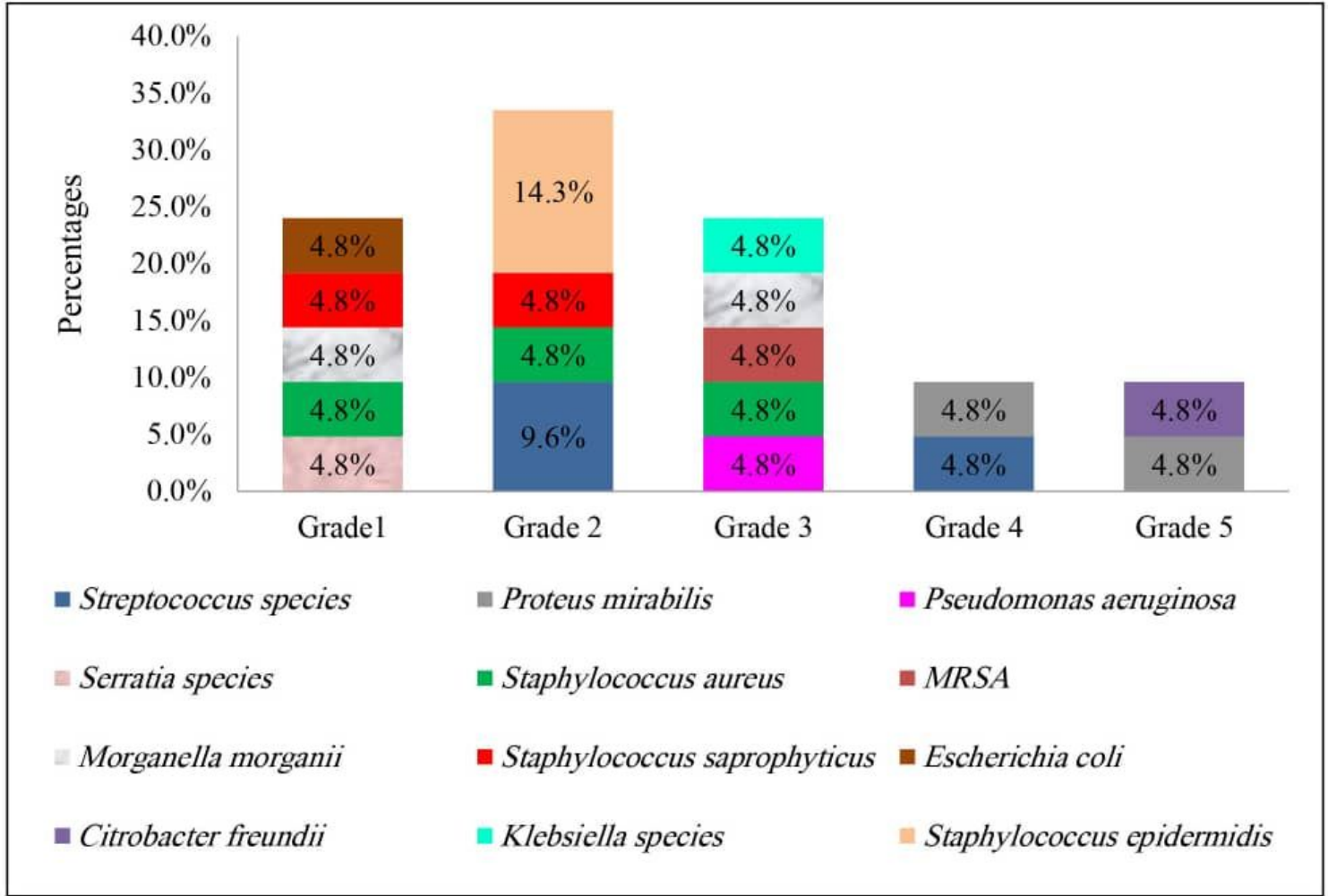


Figure 1

Bacterial species of DFU according to Wagner’s ulcers grades classification

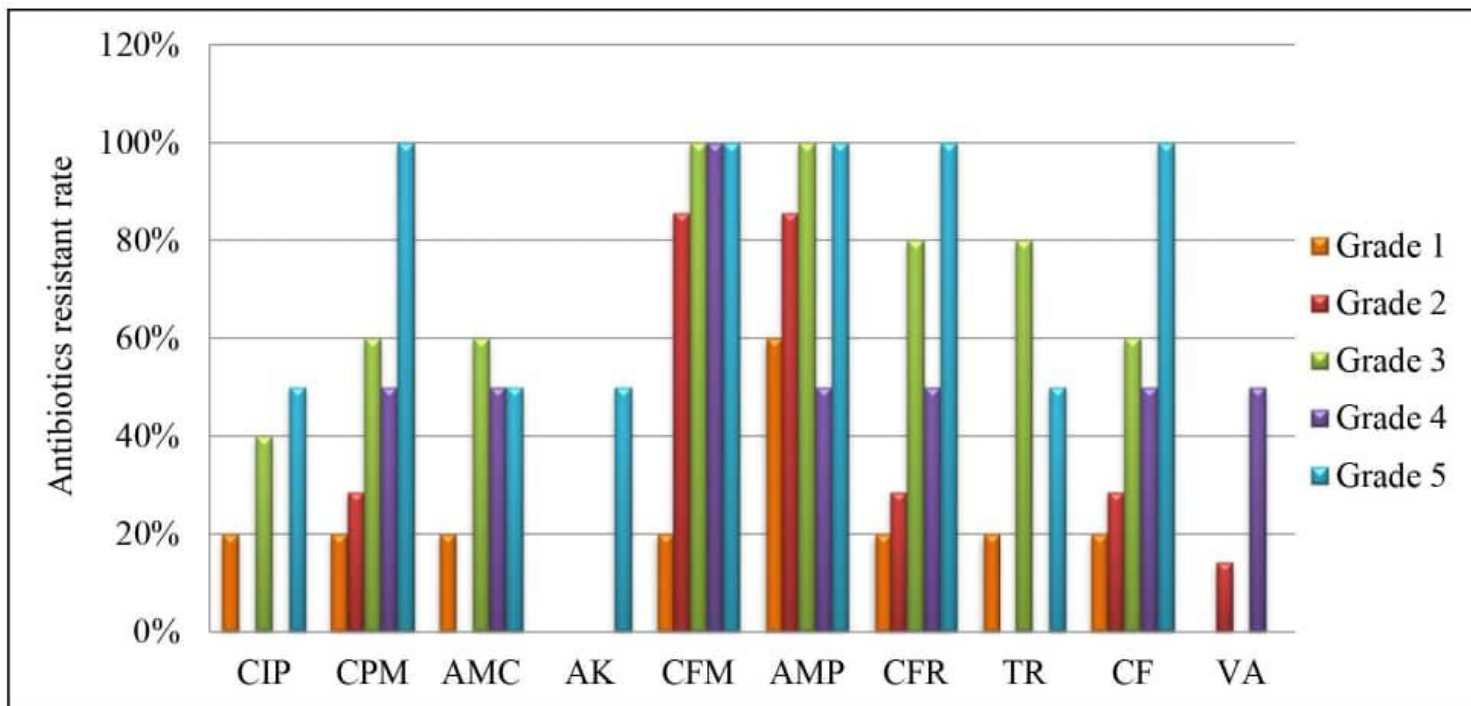


Figure 2

Antibiotics resistance patterns of bacterial isolated in different Wagner's grades

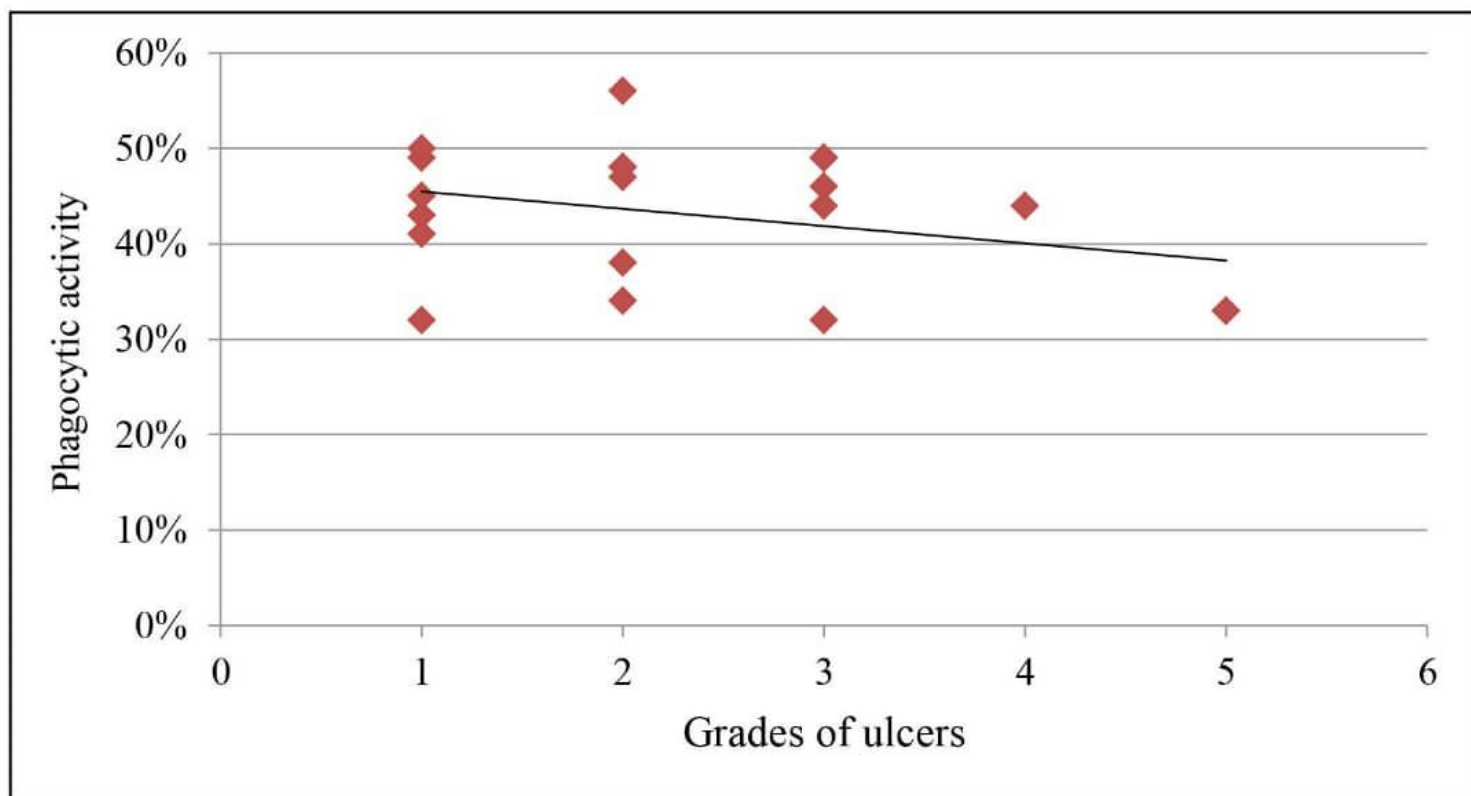


Figure 3

The correlation between phagocytic neutrophil cells activity and Wagner's grades of ulcers