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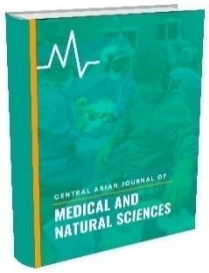

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Phenotypic and Molecular Detection of *Escherichia Coli* in Patients with Diabetic Foot Infections in Basrah, Iraq

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Abstract: Diabetic foot ulcers (DFU) are one of the most common complications for those with poorly controlled diabetes mellitus. chronic infection, hypoxia, hyperglycemia. The depth of the wound, the severity of the infection, and the degree of vascular insufficiency are used to categorize DFUs. This classification system aids in the development of DFU A greater number of individuals are at risk from enterobacteriaceae since they are also challenging to treat and more common. (E. coli): cefepime, ceftazidime, or cefotaxime. To detect the phenotypic characteristics of E. coli, MacConkey with crystal violet agar and (EMB agar were used. Targeting the 16S rDNA oligonucleotide16S rDNA primers was used for the PCR analysis of isolated E. coli. E. coli accounted for about 36.84% of the total isolates, in the study E.coli is the most common species among the bacteria that's causing of DFI from enterobacteriaceae. It was strongly associated with the type of infection and wounds grade. E. coli was present in all isolates and at 100%, and that a molecular weight of 585.

Key words: Diabetic foot ulcers, E. coli, 16S rDNA.

Introduction:

Diabetes mellitus (DM) refers to a group of chronic metabolic disorders characterized by persistent hyperglycemia (Goyal *et al.,* 2022). According to the International Diabetes Federation, 784 million people worldwide will have diabetes by 2045 (Atlas, 2021). (DM) can appear in a variety of ways, but the most common are as follows: are diabetes mellitus types 1 (T1DM) and 2 (T2DM). (Ibrahim,2018). Diabetes foot infection (DFI), a multi-microbial infection of the soft tissues and bones in the lower extremities of diabetes patients, several factors predispose diabetic patients to developing a DFI (Pitocco *et al.,* 2019), including an infection, ulceration, neuropathy, vasculopathy, immunopathy, peripheral vascular illnesses, foot biomechanics, and destruction of the deep tissues linked to neurological abnormalities in the lower extremities (Aruoah, 2021). An ulcer is any open, slowly healing sore on the skin's or mucous membrane's surface that is also accompanied by tissue

death, necrosis, and disintegration. Diabetic foot ulcer (DFU), venous, and pressure ulcers are three primary ulcer types that are regularly seen in clinics. (Leong and Gouliouris, 2021). (DFUs), which are localized sores to the surface and/or underlying tissue of the foot, can occur in people with diabetes mellitus (Mavrogenis *et al.*, 2018).If these individuals have foot problems, they are more likely to need an amputation. In half of instances, DFUs impact the plantar surface, while in the other half, they affect other areas of the foot. The primary dangers of DFU are neuropathy (PAD), motor neuropathy-related foot abnormalities, minor foot injuries, infection, and osteomyelitis. (Mavrogenis *et al.*, 2018). Moderate and severe infections include those caused by facultative anaerobic Gram-negative microorganisms. The pathogens often have synergistic relationships such as *E. coli* (Tacconelli *et al.*, 2018; Gupta *et al.*, 2019). *E. coli* is considered the most important of the Enterobacteriaceae family of bacteria. Some strains of *E. coli* are opportunistic (Gharajalar and Sofiani, 2017; Batt, 2019; Braz *et al.*, 2020). *E. coli* are a genetically diverse group of bacteria that form part of the normal intestinal microflora of humans and animals. The *E. coli* strains are capable of causing a wide range of diseases. A rod-shaped, Gram-negative, non-spore-forming bacteria that moves around using its flagella (Santos *et al.*, 2020; Neema, 2022). *E. coli* colonies often have a smooth surface and a convex shape. On Eosin Methylene Blue (EMB) agar, their colonies appear to have a colorful "sheen," but on MacConkey agar, they look flat, dry, and pink in hue. By using sorbitol MacConkey, Most strains have the ability to produce the enzyme glucuronidase. The best conditions for development are 36–37 °C and a pH in the growth range of 4.4-6.9 (Naser, 2015; Wanger *et al.*, 2017; Riedel *et al.*, 2019; Sujatha *et al.*, 2020). The majority of strains of bacteria provide positive results for the Indole test while having negative results for the oxidase, urease, and nitrite tests (Wagner, 2017; Patel, 2019). Phylogenic allies diverge from commensal and enteropathogenic strains. Therefore, *E. coli* can be categorized genetically and clinically into three major clusters: commensal, pathogenic, and extra-intestinal pathogenic *E. coli* (El-Baz *et al.*, 2022). *E. coli* isolates that cause skin and soft tissue infections: characterization (SSTIs) One of the most common pathogenic Gram-negative bacteria found in these ulcers (Lienard *et al.*, 2021). New strains of *E. coli* develop through genetic processes such as mutation genes and horizontal gene transfer that account for about (18%) of their genome. Bacterial strains *E.coli* And when used for laboratory purposes, some of them appear to harm the host (Abdulla, 2020). The genome of bacteria contains between two (5500-4000) genes, but the total number of different genes among all bacterial isolates may exceed (16000) one gene. The reason for the great diversity in the genes of bacterial isolates like *E. coli* is due to the process of horizontal gene transfer (Cass *et al.*, 2016; Bonham *et al.*, 2017).

Materials and methods:

Sample collections:

A total of 114 wounds, ulcers, and amputation swab samples were collected from patients of different ages suffering from diabetic foot infections. The samples were collected from diabetic foot patient's forefoot, midfoot, and hindfoot from November 2021 to May 2022 in Basrah Al-Sader Teaching Hospital. Wounds, ulcers, and amputations samples were collected from patients admitted to the three main hospitals in Basra and popular clinics. Patients' wound swabs from diabetic foot ulcers were taken from the center of the wound, washed with normal salt (NS) at 0.9%, and then deeply dipped using a cross-wound zigzag approach. (Cooper, 1996). Transport media swabs were streaked on

MacConkey agar, and growth on the plates was seen after a 24-hour incubation at 37°C using eosin methylene blue (EMB) plates.

Isolation and Identification:

Each sample was grown by streaking it on MacConkey and EMB agar plates, which were then kept at 37 °C for 24 hours. We looked at the colony's morphology and characteristics. Gram stain was used to identify the colonies, and pure colonies were removed and re-cultured in nutrient agar plates for further experiments as well as in nutrient agar slants as stocks. (Roberts and Greenwood, 2008). Conventional biochemical identification of *E. coli* isolates the samples were subjected to biochemical tests, which include the indole and citrate tests, the methyl red test, and Voges-Proskauer's test. It was positive for the indole and methyl red tests, negative for Voges- VITEK-2 Compact, a cutting-edge colorimetric method for bacterial identification and antibiotic susceptibility testing, was used to confirm the identity and antibiotic sensitivity test results for 42 isolates of *E. coli.* Isolates were shown to have a minimum inhibitory concentration (MIC).

Genetic work:

The bacterial *16S* rDNA gene was amplified by Polymerase Chain Reaction (PCR) using specific primers (Salama,2017), Table (1) The specific primer Sequence for 16SrDNA

Table (1) Primers used for amplification of *16S* DNA

Genes	Primer sequence (5′-3′)	Length (pb)	Amplification products (bp)	Reference (Tonu *et al.*, 2011)
ECO-f	GACCTCGGTTTAGTTCACAGA	21	585	
ECO-r	CACACGCTGACGCTGACCA	18	585	

Table (2) Primers used for amplification of *16S* DNA

Table (2)Reagents and their volumes used in PCR amplification of *16S*r DNA

No	Reagents;	volume
1	Genomic DNA	1 µl
2	Forward primer	1 µl
3	Reverse primer	1 µl
4	Master Mix	12.5 µl
5	Nuslease –free water	9,5 µl
6	Total volumes	25 µl

Table(3) Program used in PCR amplification

Steps	Temperture	Tim	No of Cycle
Initial denaturation	94.C	4 min	1
denaturation	94.C	90sec	30
Annealing	62.0	90sec	30
Extenation	72.0	2min	30
Final extanation	72.0	7min	1

Antibiotic sensitivity:

Table (4) Antibiotics used in the present study (Zone Diameter Interpretive Standard Chart and their Concentrations) Sabir *et al.* (2014)

Susceptible (S)	Intermediate (I)	Resistant (R)	Concentration µcg/disc	Disc symbol	Drugs
≥26	22-25	≤21	5	CIP	Ciprofloxacin
≥15	13-14	≤12	30	Tob	Tobramycin
≥17	15-16	≤14	30	AK	Amikacin
≥21	17-20	≤16	5	LEV	Levofloxacin
≥21	18-20	≤17	110	PIT	Piperacillin-tazobactam
≥15	12-14	≤11	30	TE	Tetracycline
≥14	11-13	≤10	30	DX	Doxycycline
≥18	15-17	≤14	30	FOX	cefoxitin
≥21	18-20	≤17	30	CAZ	Ceftazidime
≥25	19-24	≤18	30	FEP	Cefepime
≥23	20-22	≤19	10	IMI	Imipenem
≥23	20-22	≤19	10	MRP	Meropenem
≥18	13-17	≤13	30	C	Chloramphenicol

Results:

Sample Collection

A total of 114 samples were collected from patients with diabetic foot ulcers. the *E. coli* accounted for about 36.84% of the total isolates, while about 23.68% were mixed with *E. coli* and Klebsiella pneumonia, and about 33.33% were *E. coli* and other types.

Morphological Examination

Identification of *E. coli*

Initial characterization of bacterial isolates was done using cultural morphology. The results of the culture indicated pink-dry colonies on MacConkey agar, with bile salt precipitating around the colonies concerning *E. coli*. While the *E. coli* cultured on EMB selective media revealed a green metallic sheen, Figure (1) illustrates

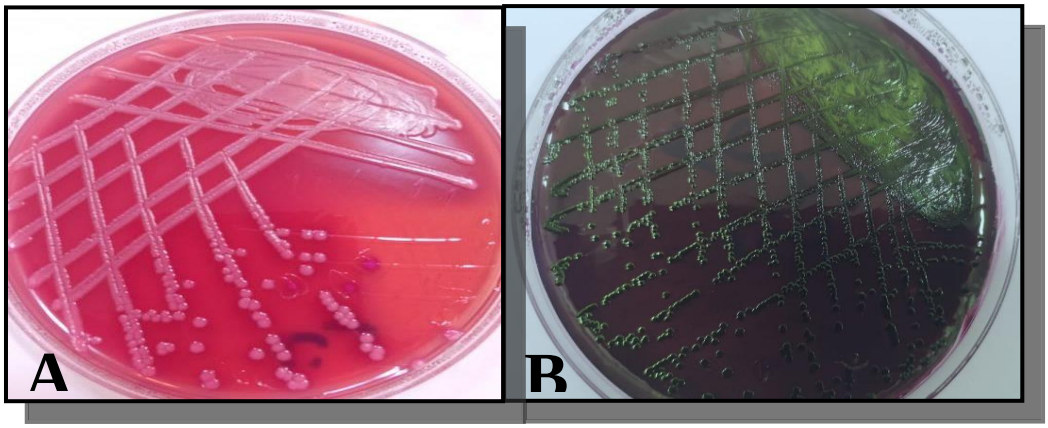

Figure (1) A. *E. coli* on MacConkey agar culture media; B. *E. coli* on Eosin methylene blue agar.

Microscopic Examination

smear obtained from pure colonies stained with a Gram stain and examined under a microscope. The bacteria cells appear as short, red, and not spore-forming rods. Figure (2) illustrates

Biochemical characterization of the isolates

Results of the biochemical tests performed on *E.* coli revealed positive findings for the enzymes catalase, indole, and methyl red but negative results for the enzymes oxidase, Voges-Proskauer, Simmons citrate, and the TSI test, which revealed A/A with gas but no H_2S.

Confirmatory test:

Vitek®2 System test for identification of bacteria

Test results on the Vitek®2 System revealed that 42 of the isolates

Antibiotic sensitivity tests *E.coli*

in Table 4. The results show that *E. coli* showed their sensitivity to antibiotics, as they showed the highest resistance to the antibiotic CAZ (95.24%) and the lowest sensitivity to the two antibiotics, C (7.14%) and AK (7.14%).

Distribution of bacterial growth according to age and sex patients.

The existence or non-existence of bacterial growth was categorized according to age groups, and compared with sex, there was a significant association between age and sex among growth groups. Males were significantly more prevalent among the older age groups of 23 (46.0%), while females were significantly more prevalent among the younger age groups of 30 (46.0%). Either in the presence or absence of bacterial growth, there was no significant statistical association. In total, there was a significant statistical association between age and sex. Males were found to be more common in the 61-70 age range, while females were more common in the 40-50 age range. In Table 5.

Culture			Sex		Total	Sig.
			Male	Female		
Growth	Age group	40 to 50 years	11	27	38	0.043*
			22.9%	45.8%	35.5%	
		51 to 60 years	15	15	30	
			31.3%	25.4%	28.0%	
		61 to 70	22	17	39	
			45.8%	28.8%	36.4%	
	Total		48	59	107	
			100.0%	100.0%	100.0%	
No growth	Age group	40 to 50 years	1	3	4	0.190*
			50.0%	60.0%	57.1%	
		51 to 60 years	0	2	2	
			0.0%	40.0%	28.6%	
		61 to 70	1	0	1	
			50.0%	0.0%	14.3%	
	Total		2	5	7	
			100.0%	100.0%	100.0%	
Total	Age group	40 to 50 years	12	30	42	0.024**
			24.0%	46.9%	36.8%	

		51 to 60 years	15	17	32	
			30.0%	26.6%	28.1%	
		61 to 70	23	17	40	
			46.0%	26.6%	35.1%	
	Total		50	64	114	
			100.0%	100.0%	100.0%	

Relationship between type of infection and wound grade

The diabetic foot has been divided into four grades, depending on the severity of ulceration. In **Figure (5)** wound grade was significantly and statistically associated with infection type. Moderate infections were associated with grade 1 wounds (81.1)%while severe infections were associated with grade 2 wounds (75.3%) and grade 3 wounds (19.5%). According to the University of Texas classification

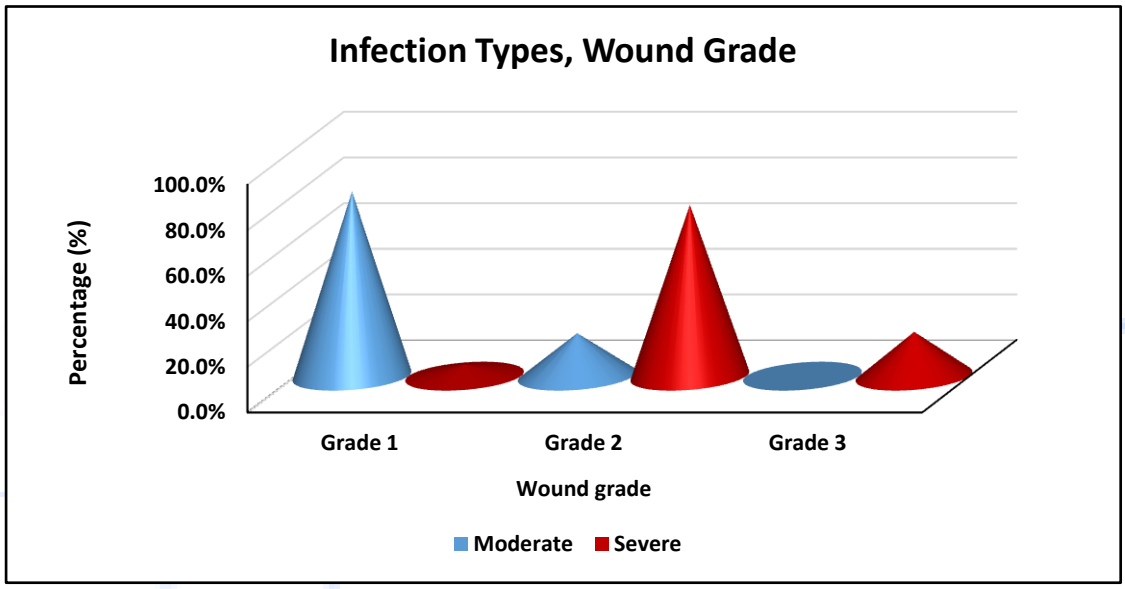

Figure (5) relationship between type of infection and Wound grad

Relationship between *E. coli* and type of infection

According to Table (14), there is a significant statistical association between the presence of *E. coli* and type of infection. *E. coli* is associated with the severe infection 20 (25.97%) and the moderate infection 22 (59.5%).

Table (6) *E. coli,* and type of infection

		Infection type		Total	Sig.*
		Moderate	Severe		
E. coli	Presen	22	20	42	0.0001
	t	59.5%	25.97%	36.0%	
	Absent	15	58	73	
		40.5%	75.3%	64.0%	
Total		37	77	114	
		100%	100%	100%	

Genetic profiling of bacterial species *E. coli* isolates ribosomal *16srDNA:*

By comparison with a conventional molecular DNA ladder, (2000 bp) a single band of *16S* rDNA was identified at (585 bp) figures (6) .(The DNA extracted underwent PCR for *16S* rDNA amplification.

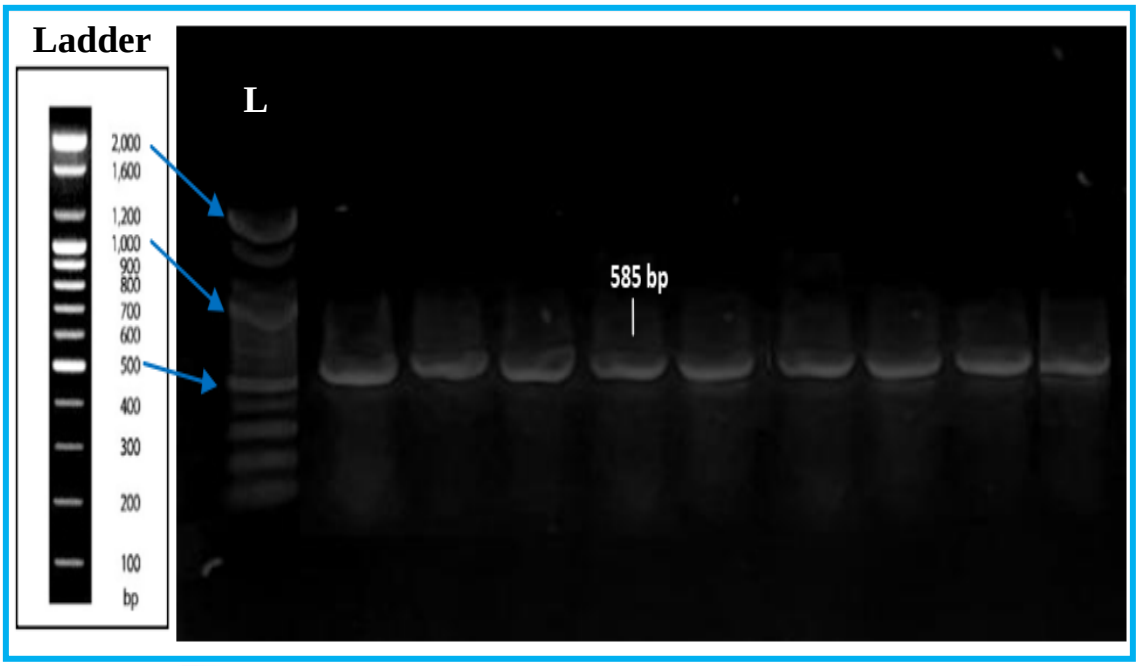

Figure (6) PCR-amplified *16S* rDNA products are visible in the patterns of agarose electrophoresis. Lane1: (2000 bp DNA ladder), Lane: (no. 2-10) *E.coli* isolates' *16S* rDNA band. using 1% agarose gel, Temperatures, 62ºC,70V, and 45 min.

Conclusion

The results of *E. coli* (36.8%) This is in perfect agreement with the previous study. Ibrahim (2018), the rate of *E. coli* was 36.6%. and agrees with the study (Qadir *et al*., 2020), *E. coli* reached 32.0%. while dis agree with the (Abdul Ameer *et al.*, 2021) their study. reported *E. coli* (26%). Frequency of *Klebsiella pneumoniae*: 23.68% this results agreed with the previous studies, respectively, by Ibrahim (2018) (23.3%) and Albadri (2021). They reported the rate of *Klebsiella pneumoniae* was 24% (Habeeb *et al.*, 2021)., 23.2% were reached. *E. coli* and *Klebsiella pneumoniae* mixed whereas they were isolated from one another in other research (Qadir *et al.*, 2020; Habeeb *et al.*, 2021). Additionally, other studies by King *et al.* (2006) and Ibrahim (2018) mixed infections of foot contained anaerobic bacteria like *Clostridium spp*. as well as aerobic Gram-negative like *E. coli*, *Klebsiella spp*., In addition to vascular diseases, failure to wear clean socks and appropriate medical shoes, and foot deformities, other contributing factors to the predominance of bacteria include dry skin, ischemia because of the occurrence of ulcers, a lack of health awareness, a lack of interest in personal hygiene and the environment of the affected person, as well as a lack of control over blood sugar measurement. The isolates of *E. coli* were diagnosed based on the differential medium, MacConkey agar. Which contains crystal violet dye and yellow salts that inhibit the growth of gram-positive bacteria. Identical outcomes with Albadri (2021) To ensure purity, they were grown on the selective medium EMB. The isolates appeared green with a metallic sheen, which was bright. Identical outcomes were found in Naser *et al.* (2015) and Antony *et al.* (2016). as a result of the fermentation of lactose sugar and the appearance of the acidic substance in the media, which encourages the colonies to absorb the dye from the media and give it this bright appearance. Using the disc diffusion technique as per the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2016), The sensitivity of the bacterial isolates to 13 different antibiotics was tested, and the bacteria were variable in their resistance to these tested antibiotics, as all the isolates were multi-antibiotic-resistant bacteria.

The study showed that *E. coli* had the highest resistance to ceftazidime 40 (95.24%). Cefepime 39(92.86%) Piperacillin-tazobactam, Levofloxacin. They have the same proportions, respectively: 32 (76.19%), cefoxitin (28 (66.66%), and imipenem (27 (64.29%). Tetracycline 26(61.04%). Tobramycin 24(57.14%). Doxycycline 23(54.76%). while the lower resistance has been Meropenem 20 (47.52%). Chloramphenicol, Amikacin 3 (7.14%). They have the same proportion, respectively: ciprofloxacin 27 (6.43%). Whereas the best drug, Chloramphenicol 38 (90.48%), is used in the treatment of diabetic foot, it is followed by Amikacin 35 (83.33%). The study recorded high resistance to the generation third, from the antibiotic CAZ- (95.24%). And these results disagreed previous study (Huda *et al*., 2022). CAZ (79%); and disagreed with previous study Qadir *et al.* (2020) CAZ (47.1%). Also, recorded study the antibiotic FEP (92.86%) The result agreed with (Huda *et al.*, 2022) study FEP (89.4%). and antibiotic LEV (76.19%) The result agreed with (Huda *et al*., 2022 study LEV (79%), This result disagree with (Huda *et al*., 2022) study FOX (89.4%), antibiotic IMP (64.29%). This result disagrees with (Qadir *et al.*, 2020) study (0%) and antibiotic TOP (57.14%). This result agrees with Albadri, 2021) study TOP (50%), While disagree with (Qadir *et al.*, 2020) study (75%). extended-spectrum beta-lactamase (ESBL) is resistant to more recent third-generation cephalosporins. 85 to 100 percent of *E. coli* isolates in 17 of the 22 European nations. (State, 2015). The overuse or improper use of antibiotics by the general public and healthcare professionals is just one of the many reasons that have contributed to the rise in antimicrobial resistance rates (Magiorakos *et al.*, 2012; Katongole *et al.*, 2019). Excessive use of antibiotics has led to the development of some resistant bacterial species (Le Page *et al.*, 2019). Males were significantly more prevalent among the older age groups of 23 (46.0%). This is consistent with the Salih and Abbas (2022) study from Thi Qar, Iraq (43.75%), and this does not agree with İçer and Durguns (2017) study. 31 (53.4%) while females were significantly more prevalent among the younger age groups of 30 (46.0%). consistent with the Icier and Durgun (2017) study. (46.6%) and is not compatible with Salih and Abbas (2022). Results of the current study indicated that diabetic patients typically develop diabetic foot ulcers (DFUs) in their fourth and fifth decades of life. This development may be caused by a variety of factors, including obesity, genetics, diet, high blood pressure, advancing age, and poor personal hygiene. These results are in agreement with the study of Misbah and Iqra (2018). According to the University of Texas classification the current study differed from other studies in the classification of wounds. The relationship between type of infection and wound grade It is a significant statistical relationship. According to the study results, there is a significant statistical association between the presence of *E. coli* and the type of infection. severe infection 20 (25.97%), and the moderate infection 22 (59.5%). The results were higher than in the previous study for diabetic foot ulcers. Giurato *et al.* (2017) found that infection is present in 10%–15% of moderate infections and less than 50% of severe infections. The emergence of this high percentage may be due to several reasons, including Lack of interest in personal hygiene, poor health care provided by hospital workers, frequent use of drugs that increase bacterial resistance, and lack of control of sugar and pressure. The sequence of the region was used for the diagnosis of isolates of *E. coli*, and at 100% and in comparison, with the DNA ladder, it was found to have a molecular weight of 585 bp. All isolates were subjected to molecular diagnostics using PCR technology based on the diagnostic 16S rDNA, which is characterized by being a stable region with little heterogeneity for a long time, and this is evidence of the efficiency of the method in diagnosis, so it can be considered an effective method. This agrees with previous studies by Maleki *et al.* (2017) (Gamal *et al.*, 2017). All isolates of *E.coli* examined achieved a successful amplification of the 16S rRNA gene at 585 bp. Since phenotypic methods are not sufficient for identifying bacterial species, determining the phenotype of these bacterial species may be difficult or impossible in microbiology laboratories (Clark *et al*., 2013; Hanan, 2022). The sequencing of the *16S* rDNA allows for a fast comparison of published sequences in microbial genome databases (Janda and Abbott, 2002; Faisal, 2022).

Conclusion:
This is a summary of the results of a study:
Results revealed that the diagnostic gene sequence for E. coli was present in all isolates and at 100%, and that a molecular weight of 585 comparison to the DNA ladder.

E. coli is associated with the severe infection and the moderate infection. Moderate infections were associated with wounds grade 1 while severe infections were associated with wounds grade 2 and wounds grade 3 wounds According to the University of Texas classification.

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