Original Article

Comparison Between Urinary Tract Infection Bacteriuria in Diabetic and Non-Diabetic Patients Identified by VITEK 2 and 16S rRNA Sequencing Against Some Biological Factors

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ABSTRACT

Objective: To investigate the prevalence and characterization of bacterial species associated with urinary tract infections in both diabetic and non-diabetic patients.

Study Design: Comparative study

Place and Duration of Study: This study was conducted at the College of Science, University of Basrah, Iraq from 1st November 2022 to 31st January 2023.

Methods: One hundred and one midstream urine samples from UTI outpatients (61 diabetic, 39 non-diabetic) were collected. Samples cultured on MacConkey, Blood and Nutrient Agar (Accumix, India) at 37°C for 24h. Macroscopic urinalysis with 10 chemical tests (glucose, protein, pH, bilirubin, blood, ketone, leukocyte, nitrite, specific gravity and urobilinogen) done on all samples. Pure colonies obtained by subculturing, maintained on Nutrient Agar slants and Broth. Gram stain used for preliminary isolate classification.

Results: Forty-eight (60.7%) were from diabetic patients compared to 31 (39.2%) from non-diabetics ($P \leq 0.05$). Gram-positive bacteria were the most prevalent in diabetics (58.3%) versus Gram-negative (54.8%) in non-diabetics.16S rRNA sequences in both groups showed Escherichia coli being the most common followed by K. pneumoniae, E. fergusonii , S. hominis, E. Hormaechei, R. Ornithinolytica and S. aureus. While in diabetes were only B. safensis, S. saprophyticus, K. rhizophila, M. vitulinus, S. epidermidis, L. bacterium, C. amalonaticus, M.luteus, P. Gergoviae and P. fragi. Whereas in non-diabetes C. aurimucosum, B. velezensis, E. cloacae, C. Erwinia and E. bugandensis. Importantly, Enterobacter bugandensis was isolated from the urinary tract infection as the first time. VITEK showed only 26.1% of species identifications. Multiple alignment of 16S rRNA showed allelic differences between diabetic and non-diabetic bacteria. Sugar lysis tests showed Gram positive isolates from diabetics had 93 reactions vs. 43 in non-diabetics ($P \leq 0.05$), with no difference in Gram negative species.

Conclusion: The diabetic case influences on the types of bacterial species presents, genetic nucleotide mutations and bacterial enzymes activity either for gram positive or gram negative bacteria

Key Words: Urinary tract infection, Diabetes, VITEK 2, 16S rRNA

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INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease causing persistent hyperglycemia due to insulin defects. It involves genetic, environmental and epigenetic factors.

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DM increases susceptibility to urinary tract infections (UTIs) because of immune dysfunction, hyperglycemia, and bladder neuropathy.[1] Urinary tract infection bacteria such as Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis and Enterococcus faecalis grow well in glucose-rich urine enhancing colonization, biofilm formation and antibiotic resistance.[2] Urinary tract infection diagnosis shifted to molecular methods like VITEK 2 which uses metabolic activity cards for fast bacterial identification and susceptibility testing.[3] Bacterial metabolic profiles involving sugar fermentation are important in diabetes.[4] High glucose in diabetic urine boosts bacterial enzyme activity increasing virulence.[5] *16S rRNA* gene sequencing is a key identification method when phenotypic methods fail.[6] This study investigates genetic and enzymatic differences of bacteria from

diabetic and non-diabetic UTI patients, focusing on urine sugar effects.

METHODS

One hundred and one midstream urine samples from UTI outpatients (61 diabetic, 39 non-diabetic) were collected from 1st November 2022 to 31st January 2023. Samples cultured on MacConkey, Blood and Nutrient Agar (Accumix, India) at 37°C for 24h. Macroscopic urinalysis with 10 chemical tests (glucose, protein, pH, bilirubin, blood, ketone, leukocyte, nitrite, specific gravity and urobilinogen) done on all samples. Pure colonies obtained by subculturing, maintained on Nutrient Agar slants and Broth. Gram stain used for preliminary isolate classification.

DNA extraction: Bacterial isolates were grown in 5 mL Nutrient Broth (Accumix, India) at 37 °C for 24 h. Genomic DNA was extracted using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan), eluted in 100 µL buffer, and stored at –20 °C. DNA integrity was checked via 1.5% agarose gel electrophoresis with ethidium bromide under UV light.

Amplification of 16S rDNA: The 16S rDNA of 42 isolates was PCR-amplified using universal primers[7]: forward (27F) 5′-AGAGTTTGATCCTGGCTCAG-3′, reverse (1492R) 5′-GGTTACCTTGTTACGACTT-3′. PCR (50 µl) contained 25 µl GoTaq Master Mix, 2 µl DNA, 2 µl forward primer, 2 µl reverse primer, and 19 µl nuclease-free water. Program: 95 °C 5 min; 35 cycles of 95 °C 30s, 55 °C 30s, 72 °C 1min; final extension 72 °C 5 min. Amplification confirmed by 1.5% agarose gel electrophoresis in 1× TBE buffer; 1 µl PCR product loaded; 100 bp ladder used. Electrophoresis at 70 V for 1h; bands visualized under UV and photographed. Products stored at −20 °C until sequencing.

Bacterial isolates (22) were identified using Basic Local Alignment Search Tool (BLAST) and National Center for Biotechnology Information (NCBI) as in [8]. After proofreading, nucleotide sequences were submitted to BLAST which compared them with GenBank type strains to identify bacterial species.

he phylogenetic tree was constructed using MAFFT (Multiple Alignment Program for Nucleotide Sequences) at http://mafft.cbrc.jp/alignment/server/ after concatenating nucleotide sequences. Sequences were merged using Clustal Omega.[8]

VITEK 2 System: 42 isolates as Gram-positive and Gram-negative were identified by VITEK 2 (BioMérieux, France) and some bacterial susceptibility profile were done such asthe biochemical profiles associated with bacterial capacity to ferment the substrates with a focus on glucose, mannitol and lactose, as well as identification of the enzymes.

RESULTS

There were 61 (60.3%) diabetic patients and 40 (39.6%) non-diabetic patients. Out of 79 bacterial isolates, 48 (60.7%) obtained from diabetes was higher than 31 (39.2%) from non-diabetes ($P \leq 0.05$). The isolates were also divided into Gram negative 20 (41.6%) and Gram positive 28(58.3%) versus 17 (54.8%) and 14 (45.1%) respectively (Table 1). The 16S rRNA gene of 42 bacterial isolates was shown as a single band for each isolate on agarose gel electrophoresis at a position 1500 bp in comparison with a standard molecular DNA ladder (Fig. 1).

Twenty two different bacterial species were identified from 42 alignments. However, the bacterial species were 8 of Escherichia coli, 4 for both Klebsiella pneumoniae and Escherichia fergusonii, 3 of Staphylococcus hominis, 2 for both Bacillus safensis, Corynebacterium aurimucosum, Enterobacter hormaechei, Staphylococcus aureus and Raoultella ornithinolytica, and the other remaining species were only one for Pseudomonas fragi, Enterobacter cloacae, Pluralibacter gergoviae, Enterobacter bugandensis, Citrobacter amalonaticus, Kocuria rhizophila, Mammaliicoccus vitulinus, Candidatus Erwinia, Staphylococcus saprophyticus, Staphylococcus epidermidis, Bacillusvelezensis, Micrococcus luteus and lachnospiraceae bacterium. Each isolate sequence was aligned with its type strain in NCBI.

The distribution of bacterial species isolated from diabetic patients was analyzed to determine the prevalence of each bacterial type. Escherichia coli was the most prevalent accounting for 3 (6.2%) of the total isolates. This was followed by Klebsiella pneumoniae, Escherichia fergusonii and Bacillus safensis with a frequency of 2 (4.1%), while only 1(2%) for Staphylococcus hominis, Enterobacter hormaechei, Raoultella ornithinolytica, Kocuria rhizophila, Mammaliicoccus vitulinus, Staphylococcus saprophyticus, Lachnospiraceae bacterium, Staphylococcus epidermidis, Citrobacter amalonaticus, Micrococcus luteus, Pluralibacter gergoviae , Pseudomonas fragi and Staphylococcus aureus (Table 2). The bacterial species isolated from non-diabetic individuals were examined to determine their relative occurrence. The results showed that Escherichia coli was the most frequently detected accounting 5 (16.1%) of the total isolates. This was followed by 2 (6.4%) for Klebsiella pneumoniae, Staphylococcus hominis, Escherichia fergusonii and Corynebacterium aurimucosum, with 1(3.2%) for other species like Enterobacter hormaechei, Raoultella ornithinolytica, Bacillus velezensis, Enterobacter cloacae, Staphylococcus aureus, Candidatus Erwinia and Enterobacter bugandensis (Fig. 2).

Table 3 compares the identification results from the VITEK system and 16S rRNA gene sequencing. The

differences between the two techniques indicate a possibility of misidentification when using VITEK is approximately 73.9% to be identical with 16S rRNA in only 26.1% with no significant difference at ($P \leq 0.05$).

Twelve Gram-positive species were identified, including multiple isolates of Staphylococcus hominis, Bacillus safensis, S. aureus and Corynebacterium aurimucosum while others like Kocuriarhizophila, M. vitulinus, Candidatus Erwinia, S. saprophyticus, S. epidermidis, B. velezensis, M. Luteus Lachnospiraceae bacterium appeared once. Gram-positive isolates showed 93 positive vs. 193 negative biochemical reactions in diabetics and 43 vs. 113 in non-diabetics ($P \leq 0.05$) (Table 4).

Nine Gram-negative species were identified: multiple isolates of E. fergusonii, K. pneumoniae, E. hormaechei and R. ornithinolytica, single isolates of C. amalonaticus, E. bugandensis, P. gergoviae, E. cloacae and P. fragi. Biochemical profiles (17 isolates) showed 98 positive vs. 91 negative reactions in diabetics and 94 vs. 74 in non-diabetics, with no significant difference (Table 5).

Table No.1: Gram-positive and Gram-negative isolates in diabetics and non-diabetics cases

Urinary Tract Infection Samples	Bacte-rial Isolates	Diabetes		Non-diabetes	
		Gr +ve	Gr-ve	Gr+ve	Gr-ve
101	79	28 (58.3%)	20 (41.6%)	14 (45.1%)	17 (54.8%)
Total		48 (60.7%)*		31 (39.3%)	

*P≤0.05

Table No.2: The distribution of bacterial isolates between diabetics and non-diabetic cases

Bacteria species	No. (%)	Diabetics	Non-diabetics
Escherichia coli	8 (10%)	3 (6.2%)	5(16.1%)
Klebsiella pneumoniae	4 (5%)	2 (4.1%)	2 (6.4%)
Staphylococcus hominis	3 (3.7%)	1 (2%)	2 (6.4%)
Escherichia fergusonii	4 (4%)	2 (4.1%)	2 (6.4%)
Enterobacter hormaechei	2 (2.5%)	1 (2%)	1 (3.2%)
Raoultella ornithinolytica	2 (2.5%)	1 (2%)	1 (3.2%)
Staphylococcus aureus	2 (2.5%)	1 (2%)	1 (3.2%)
Bacillus safensis	2 (2.5%)	2 (4.1%)	-
Kocuria rhizophila	1 (1.2%)	1 (2%)	-
Mammaliicoccus vitulinus	1 (1.2%)	1 (2%)	-
Staphylococcus saprophyticus	1 (1.2%)	1 (2%)	-
Lachnospiraceae bacterium	1 (1.2%)	1 (2%)	-
Staphylococcus epidermidis	1 (1.2%)	1 (2%)	-
*Gr+ve cocci	25 (31.6%)	19 (39.5%)	6 (19.3%)
Citrobacter amalonaticus	1 (1.2%)	1 (2%)	-
Micrococcus luteus	1 (1.2%)	1 (2%)	-
Pluralibacter gergoviae	1 (1.2%)	1 (2%)	-
Pseudomonas fragi	1 (1.2%)	1 (2%)	-
Corynebacterium aurimucosum	2 (2.5%)	-	2 (4.6%)
Bacillus velezensis	1 (1.25)	-	1 (3.2%)
Enterobactercloacae	1 (1.2%)	-	1 (3.2%)
*Gr-ve rode	11 (13.9%)	7 (14.5%)	4 (12.9%)
Candidatus Erwinia	1 (1.2%)	-	1 (3.2%)
Enterobacter bugandensis	1 (1.2%)	-	1 (3.2%)
*Gr+ve rode	1 (1.26%)	-	1 (3.2%)
Total	79	48 (60.7%)*	31 (39.2%)

*P≤0.05

Table No. 3: Evaluation of bacterial identification by 16Sr RNA gene sequencing versus VITEK system

16sr RNA	VITEK	Identical
Corynebacterium aurimucosum	Mammaliicoccus vitulinus	-
Escherichiacoli	Escherichia coli	+
Staphylococcus aureus	Mammaliicoccus vitulinus	-
Staphylococcus hominis	Mammaliicoccus vitulinus	-
Kocuriarhizophila	Mammaliicoccus vitulinus	-
Escherichia fergusonii	Escherichia coli	-
Escherichia coli	Pseudomonas fluorescens	-
Bacillus velezensis	Klebsiella pneumoniae	-
Enterobacter cloacae	Escherichia coli	-
Bacillus safensis	Unidentified Organism	-
Staphylococcus aureus	Staphylococcus haemolyticus	-
Staphylococcus vitulinus	Staphylococcus vitulinus	+
Staphylococcus hominis	Staphylococcus hominis	+
Escherichia fergusonii	Escherichia coli	-
Escherichia coli	Escherichia coli	+
Klebsiella pneumoniae	Klebsiella pneumoniae	+
Candidatus Erwinia	Staphylococcus haemolyticus	-
Klebsiella pneumoniae	Enterobacter aerogenes	-
Escherichia coli	Pasteurella testudinis	-
Enterobacter hormaechei	Pantoea spp.	-

Enterobacter bugandensis	Enterobacter cloacae complex	-
Enterobacter hormaechei	Enterobacter cloacae	-
Escherichia coli	Escherichia coli	+
Raoultella ornithinolytica	Raoultella planticola	-
Raoultella ornithinolytica	Raoultella planticola	-
Escherichia coli	Enterobacter aerogenes	-
Escherichia coli	Escherichia coli	+
Klebsiella pneumoniae	Klebsiella pneumoniae	+
Corynebacterium aurimucosum	Mammaliicoccus vitulinus	-
Escherichia coli	Escherichia coli	+
Staphylococcus saprophyticus	Staphylococcus saprophyticus	+
Lachnospiraceae bacterium	Leuconostoc mesenteroides	-
Staphylococcus epidermidis	Staphylococcus haemolyticus	-
Citrobacter amalonaticus	Unidentified Organism	-
Micrococcus luteus	Kocuria kristinae	-
Bacillus safensis	Escherichia coli	-
Staphylococcus hominis	Mammaliicoccus vitulinus	-
Pluralibacter gergoviae	Unidentified Organism	-
Pseudomonas fragi	Pseudomonas fluorescens	-
Escherichia fergusonii	Klebsiella pneumoniae	-
Klebsiella pneumoniae	Klebsiella pneumoniae	+
Escherichia fergusonii	Rhizobium radiobacter	-
Total	42 (100%)*	31 (26.1%)

*P≤0.05

Table No.4: Biochemical tests for different Gr+ve bacterial species in diabetic and non-diabetic cases

Type of bacteria (No. 17)	Bio-chemical test	Diabetics (No. 11)		Non-diabetics (No. 6)	
		+	-	+	-
Staphylococcus aureus (No. 2)	AMY	1	10	0	6
	PIPLC	1	10	0	6
Staphylococcus hominis (No. 3)	dXYL	4	7	3	3
	ADH1	10	1	5	1
Bacillus safensis (No. 2)	BGAL	2	9	0	6
	AGLU	5	6	3	3
Bacillus velezensis (No. 1)	APPA	1	10	0	6
	CDEX	0	11	0	6
Corynebacterium aurimucosum (No. 2)	AMAN	1	10	0	6
	BGURr	0	11	0	6
Kocuria rhizophila (No. 1)	AGAL	0	11	0	6
	AlaA	2	9	1	5
Mammaliicoccus vitulinus (No.1)	dSOR	2	9	0	6
	URE	1	10	1	5
Staphylococcus saprophyticus (No,1)	POLYB	0	11	0	6
	dGAL	1	10	0	6
Staphylococcus epidermidis(no:1)	dRIB	7	4	3	3
	dMAL	3	8	2	4
Micrococcus luteus (No. 1)	NC6.5	9	2	4	2
	dMAN	7	4	4	2
	dMNE	6	5	4	2
Lachnospiraceae bacterium (No. 1)	MBdG	5	6	4	2
	dRAF	3	8	0	6
Candidatus Erwinia (No. 1)**	SAL	3	8	0	6
	SAC	10	1	5	1
	dTRE	9	2	4	2
Total	26	93*	193	43*	113

*P≤0.05

**Candidatus Erwinia was recorded as Gr+ve

Table No.5: Biochemical tests for different Gram-negative bacterial species in diabetic and non-diabetic cases

Type of bacteria (No. 17)	Biochemical test	Diabetics (No. 11)		Non-diabetics (No. 6)	
		+	-	+	-
Escherichia fergusonii (No. 4)	ADO	5	4	5	3
	IARL	1	8	0	8
Klebsiella pneumoniae(No. 4)	dCEL	7	2	5	3
	BGAL	6	3	8	0
Citrobacter amalonaticus (No. 1)	H2S	0	9	0	8
	AGLU	0	9	0	8
Enterobacter hormaechei (No.2)	BGLU	7	2	3	5
	dMAL	6	3	8	0
	dMAN	7	2	8	0
Enterobacter bugandensis (No.1)	dMNE	8	1	8	0
	BXYL	5	4	4	4
Raoultella ornithinolytica (No.2)	LIP	1	8	0	8
	PLE	5	4	5	3
	URE	2	7	1	7
	dSOR	6	3	8	0
Pluralibacter gergoviae (No. 1)	SAC	7	2	5	3
	dTAG	3	6	1	7
	dTRE	7	2	8	0
Enterobacter cloacae (No. 1)	MNT	3	6	4	4
Pseudomonas fragi (No. 1)	ILATK	6	3	6	2
	AGAL	6	3	7	1
Total	21	98	91	94	74

*P≤0.05

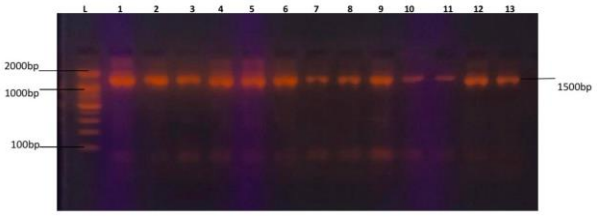

Figure No. 1: A model of agarose gel electrophoresis (1.57%) patterns showing PCR amplified products of 16Sr RNA Lane L: 100 bp DNA ladder, lanes 1-13: 16 Sr RNA bands of bacterial isolates

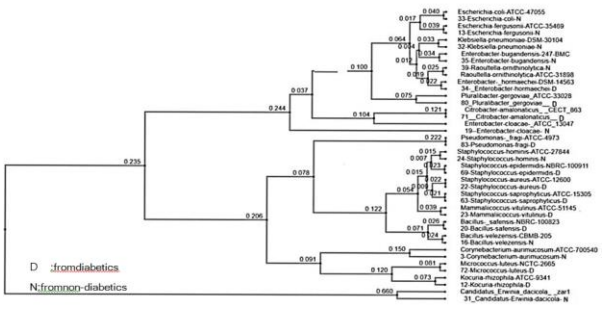

Figure No. 2: Rooted neighbour joining phylogenetic tree constructed sequences derived from an alignment of 16S rRNA sequences 21 different bacterial species from UTI patients (with different concatenation for each species) including diabetic and non-diseased cases isolates with their reference strain

DISCUSSION

In the present study, 79 (78%) bacterial isolates were obtained. This rate may be influenced by factors such as low bacterial concentration, poor storage or prior antibiotic use that inhibited growth. In addition, not all UTIs are caused by bacteria; viral or fungal infections may also occur. From the isolates, 43 predominant strains were selected for further testing. Gram-positive bacteria were more common in diabetic patients (58.3%) than in non-diabetics (45.1%), while Gram-negative were higher in non-diabetics (54.8%). This agrees with previous studies.[9,10] The overall isolation rate was significantly higher in diabetic patients (60.7%) compared to non-diabetics (39.2%) ($P \leq 0.05$), which supports the findings of Utku[11] suggesting that high glucose levels in diabetics may promote bacterial growth. However, one study contradicted this, reporting higher UTI prevalence in non-diabetics.[12]

This study showed that 43 isolates were selected for molecular identification using the conserved 16S rRNA gene, considered the gold standard for bacterial taxonomy. 21 species were confirmed, while 1 isolate with 74.11% similarity to Lachnospiraceae was excluded. A phylogenetic tree confirmed species identification by comparing sequences with GenBank references.[8] Comparison of 7 species,16S rRNA sequences revealed nucleotide differences between diabetic and non-diabetic isolates, notably E. fergusonii (13 differences), R. ornithinolytica (26 differences) and E. hormaechei (9 differences), while S. hominis and S. aureus showed minimal variation.

In the present study, out of 79 isolates, 42 were selected based on dominant colony morphology and sequenced using the conserved 16S rRNA gene, identifying 22 bacterial species. This gene is the gold standard for bacterial taxonomy and phylogeny.[13] Escherichia coli was the most prevalent accounting for 3 (6.2%) of the total isolates. This was followed by Klebsiella pneumoniae, Escherichia fergusonii and Bacillus safensis with a frequency of 2 (4.1%), while only 1 (2%) for Staphylococcus hominis, Enterobacter hormaechei, Raoultella ornithinolytica, Kocuria rhizophila, Mammaliicoccus vitulinus, Staphylococcus saprophyticus, Lachnospiraceae bacterium, Staphylococcus epidermidis, Citrobacter amalonaticus, Micrococcus luteus, Pluralibacter gergoviae, Pseudomonas fragi and Staphylococcus aureus (Table 2) . On the other hand, Escherichia coli was the most frequently 5(16.1%) of the total isolates in the non-diabetes followed by 2 (6.4%) for Klebsiella pneumoniae, Staphylococcus hominis, Escherichia fergusonii and Coryne bacterium aurimucosum, with 1 (3.2%) for other species like Enterobacter hormaechei, Raoultella ornithinolytica, Bacillus velezensis, Enterobacter cloacae, Staphylococcus aureus, Candidatus Erwinia and Enterobacter bugandensis. Bacterial percentage was 60.7% in diabetics and 39.2% in non-diabetics, with a significant difference ($P \leq 0.05$), supporting that high sugar in diabetic UTIs may enhance bacterial growth and diversity.[3]

The identification agreement between VITEK and 16S rRNA sequencing was limited to 26.1%, as shown in Table 3, while 16S rRNA achieved 100% accuracy with significant differences ($P \leq 0.05$). This percentage may not be entirely precise due to some sequences being too short or poorly readable. Despite this, 16S rRNA is considered one of the best techniques for bacterial identification due to its high sensitivity and specificity.[13,14] Unlike biochemical methods like VITEK, which depend on metabolic reactions that may overlap among related species or be affected by environmental factors, 16S rRNA targets conserved genetic regions, allowing more accurate classification at the species level. This is especially helpful in UTI cases, where mixed infections and antibiotic resistance often interfere with biochemical identification. In this study, 16S sequencing not only confirmed dominant culturable bacteria but also detected Enterobacter bugandensis, which would have been missed by automated biochemical methods.

12 Gram-positive species were identified, including multiple isolates of Staphylococcus hominis, Bacillus safensis, S. aureus and Corynebacterium aurimucosum, while others appeared once. Gram-positive isolates exhibited significantly more non-fermenting biochemical reactions in both diabetics (93 positive vs. 193 negative) and non-diabetics (43 positive vs. 113 negative) with $P \leq 0.05$.[15] The prevalence of non-fermenters among Gram-positive bacteria may be attributed to antibiotic use, which suppresses fermentation and metabolic activity in these less adaptable organisms, consistent with their structural vulnerability and metabolic limitations.

A total of 9 Gram-negative species were identified including multiple isolates of Escherichia fergusonii, Klebsiella pneumoniae, Enterobacter hormaechei and

Raoultella ornithinolytica and single isolates of Citrobacter amalonaticus, Enterobacter bugandensis, Pantoea gergoviae, Enterobacter cloacae and Pseudomonas fragi. Biochemical profiling of 17 isolates revealed 98 positive versus 91 negative reactions in diabetic patients and 94 versus 74 in non-diabetic patients, with no statistically significant difference. Fermenting strains were predominantly observed among Gram-negative isolates, indicating a preserved ability for sugar metabolism under stress conditions.E. coli fermentation is regulated by host immunity, microbial competition and antibiotics.[16] Unlike Gram-negatives, Gram-positives lack an outer membrane exposing teichoic acids and allowing β-lactams to inhibit peptidoglycan synthesis causing cell lysis.[17]

CONCLUSION

The diabetic case influences on the which types of bacterial species presents, genetic nucleotide mutationsand bacterial enzymes activity either for gram positive or gram negative bacteria.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Zainab S. Baqer, Munaff J. Abd Al-Abbas
Drafting or Revising Critically:	Zainab S. Baqer, Munaff J. Abd Al-Abbas
Final Approval of version:	All the above authors
Agreement to accountable for all aspects of work:	All the above authors

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