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Molecular Study of Urinary Tract Infection Bacteria and their Relationship to the Present of *Oxalobacter formigenes* in Stool of Kidney Stone Patients

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Abstract

Urinary Tract Infection is widespread in Al-Basrah province, it is important to detect the causative agent for suitable treatment and which factors may affect on the disease. Fifty urine samples were collected from patients with urinary stone disease from Al-Basrah General Hospital. Bacterial growth was in 48 samples. Axenic culture appeared 82% compared with mixed or no growth. Out of all bacterial isolates (55), gram – positive was 71% versus to gram – negative. *16SrDNA* gene sequencing identified 10 *Staphylococcus haemolyticus* , 7 *Escherechia coli*, 5 *Staphylococcus epidermidis* and 3 *Enterococcus faecalis*. While *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Streptococcus agalactiae*, *Staphylococcus hominis* and *Bacillus cereus* were 2 for each. Furthermore, *Bacillus subtilis*, *Enterococcus raffinosus*, *Staphylococcus lugdunesis*, *Staphylococcus warneri*, *Streptococcus parasanguinis*, *Corynebacterium amycolatum*, *Corynebacterium tuberculostearicum*, *Corynebacterium coyleae* and *Corynebacterium aurimucosum* were 1 for each. Four of these isolates were recorded as new global strains in GenBank. The bacterial species were more frequented in patients without surgical operation (88.6%), antibiotic utilization (77.3%), kidney and oxalate stone (72.7%) for each. Most bacterial isolates had a high sensitivity to imipenem (86.4%), then followed by amikacin and ciprofloxacin (54.5%, for each), but low and no sensitivity to ceftriaxone (97.7%), ampiclox and methicillin (100%, for each). High rate of multi - drug resistance was noticed among all isolates. This is a first study confirms a relationship between the presence of some UTI bacteria and the absence of *Oxalobacter formigenes* in the gut.

Key words: Molecular; 16SrDNA; Sequencing; *Oxalobacter*; Kidney; *Corynebacterium*; *aurimucosum*.

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1. Introduction

Urinary tract infection (UTI) is a common infection experienced by people after respiratory and digestive infections, resulting the most common problem of both community and hospital acquired infections for patients admitted to hospitals [1]. Each year, about 150 million people are diagnosed with UTIs [2]. Generally, UTIs are clinical classified according to the site of infection e.g. In kidney is called pyelonephritis, in the bladder is cystitis and in urethra is arthritis [3]. Approximately 85% of UTIs cases were caused by gram negative bacteria including *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Pseudomonas aeruginosa*, whilst only 15% were by gram positive as *Enterococcus* sp, *Staphylococcus* sp and *Streptococcus* sp. [4,5,6] .

16S rDNA gene sequencing has emerged as more precise and rapid method to identify numerous bacteria providing successfully implemented in clinical laboratories [7,8]. Many bacterial genera and species have been reclassified and renamed by *16S rDNA* sequences . Also, the phylogenetic relationships among bacteria have been determined by this gene [9].

Urinary stone is one of the medical factors increasing the risk of complicating urinary tract infections [10]. The stone can cause partial or complete obstruction to the flow of urine and permitting the bacterial growth in the urinary tract leading to infection [11]. Also, the sharp edges of some urinary stones such as uric acid and calcium – oxalate damaging the epithelial layer of the urinary tract and encourage the bacterial growth [12].

In past studies, urinary tract problems, especially UTIs are quite common after surgery when the bacteria have low virulent of the skin, but become serious when entry through urosurgical operations [13].

Antibiotic sensitivity of bacteria is usually variable among species and strains, thus, the resistance to antibiotics making it difficult to treat in some infectious diseases [11]. The widespread and uncontrolled use of broad - spectrum antibiotics led to the emergence of multi-drug resistant bacteria [14].

Briefly, the present study was designed to identify the bacteria from urinary stone patients, precisely end unprejudiced, using *16SrRNA* gene sequencing. Moreover, which agents and antibiotics are affects on their frequency. Furthermore, is the presence of *Oxalobacter formigenes* affect on the frequency of some UTI bacteria?.

2. Materials and Methods

2.1 Sample Collection

Fifty midstream urine samples were collected from urolithiasis patients between 20-60 years old from the Urological Lithotripsy Unit of Al Basrah General Hospital in Iraq during October to December in 2014. The urine samples were accumulated by sterilized containers (Dollphi, Syria) after necessary precautions [15]. Samples were shipped to the lab within one hour.

2.2 Urine Culture

After vortex a urine sample, 0.1 ml was inoculated separately onto Blood agar (LAB, UK) and MacConkey agar (LAB, UK) plates and incubated aerobically at 37 ºC for 24 h. The grown colonies were calculated (CFU) per ml and followed by gram stain to initial identification [16]. All grown colonies from primary cultures were streaked onto Nutrient agar (Salucea, Netherlands) plates for the next assays.

2.3 DNA Extraction from Bacteria

Pure bacterial isolates were transported to1. 5 ml eppendroff tube. Phosphate buffer saline (PBS) was added for washing by vortex for 10 sec. , centrifuged for 5 minutes at 13,000 rpm and discarded the supernatant, this process (add, vortex and centrifuge) was repeated three times [17].

Extraction of genomic DNA was achieved by Exi prep ™ plus Bacteria Genomic DNA Kit (BIONEER, Korea) by Automated Nucleic Acid Extraction System (BIONEER, Korea), then DNA was detected by gel of 1% agarose containing 0.5 µl ethidium bromide and electrophoresed at 60 volts for 1.5 hours.

2.4 PCR for 16S Ribosomal DNA

Primers 27-forward 5'-AGAGTTTGATCCTGGC-3' and 1492-reverse 5'-GGTTACCTTGTTACGACTT-3' [18] were applied to amplify *16SrDNA* genes in eppendroff tube (50 µl) mixture (BIONEER, Korea) consisting of 11 µl Mastermix, 10 pmol primer (1.5 µl) for each bacterium sample, 1.5 µl DNA template and 34.5 µl nuclease free water. PCR program was 92 ºC for 2 min, 35 cycles of 94 ºC denaturation for 30 sec., 51.8 ºC annealing for 45 sec. and 72 ºC extension for 1.5 mim., finally, 72 ºC for 5 min. The bands of 1500 bp were observed by adding 5 µl of PCR product in 2% agarose gel with 0.5 µl ethidium bromide and electrophoresed with 5 µl of 1 kB DNA ladder (BIONEER, Korea) then photographed by Samsung camera.

2.5 Sequencing and Identification of 16S rDNA Gene

The PCR 16SrDNA gene was purified with 30% polyethylene glycol (PEG -8000) to get rid of any unreacted mixture of PCR [19], then sequenced by automated DNA sequencer according to Macrogen Company conditions. The alignment for each bacteria was identified by "BLAST" from the website http://blast.ncbi.nlm.nih.gov [20], and all sequences were compared by "CLUSTAL Omega" http://www.ebi.ac.uk /Tools /msa /clustalo/.

2.6 Phylogenetic Tree

The *16SrDNA* sequences data for identified bacterial isolates were aligned for the concatenated of 524 bp , then collected together in the box of the "MAFFT" (Multiple Alignment using Fast Fourier Transform) program , then the tree visualized by "forester" software [21].

2.7 Antibiotic Susceptibility

Antibiotic susceptibility patterns for bacterial isolates were performed by the disc diffusion method onto Muller-

Hinton (LAB, UK) plates according to the guidelines of Clinical Laboratory Standards Institute [22] using the following antibiotics disc: Ampiclox (APX) 30 µg, Co-trimoxazole (SXT) 25 µg, Gentamicin (CN) 10 µg, Imipenem (IMP) 10 µg, Methicilin (ME) 10 µg and Nitrofurantoin (F) 300 µg (Bioanalyse, Turkey), and Amikacin (AK) 30 µg, Ceftriaxone (CTR) 30 µg, Ciprofloxacin (CIP) 30 µg and Tetracycline (TE) 30 µg (Titan Biotech., India).

2.8 Statistical Analysis

Statistical analysis was performed with the Chi - square test by Statistical Package for Social Sciences (SPSS).

3. Results

3.1 Growth and Gram's Staining of Bacteria

Bacterial growth appeared in 48 (96%) of samples (50) with a higher significant difference ($P< 0.01$). Axenic bacterial culture appeared in 41 (82%) of samples comparing with mixed or no growth at $P< 0.01$. Gram–positive bacteria showed 39 (71%) while gram - negative bacteria was 16 (29%) at $P< 0.01$ as in Table (1).

3.2 Identification by 16Sr DNA Gene Sequencing

16SrDNA gene from 55 bacterial isolates were observed on agarose gel at the suitable size (1500 bp) in comparison with the DNA ladder as in Figure (1). Only 44 were identified by *16SrDNA* gene sequencing and compared with their type strains. However, 18 different species were obtained. The frequency of *Staphylococcus haemolyticus* was 10 (22.72%), followed by *E. coli* 7 (15.9%), *Staphylococcus epidermidis* 5 (11.4%) and *Enterococcus faecalis* 3 (6.8%). *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Streptococcus agalactiae*, *Staphylococcus hominis* and *Bacillus cereus* were 2 (4.54%) for each. *Bacillus subtilis*, *Enterococcus raffinosus*, *Staphylococcus lugdunesis*, *Staphylococcus warneri*, *Streptococcus parasanguinis*, *Corynebacterium amycolatum*, *Corynebacterium aurimucosum*, *Corynebacterium coyleae* and *Corynebacterium tuberculostearicum* were 1 (2.27%) for each.

3.3 Phylogenetic Tree of Bacterial Species

The phylogenetic tree (Figure 2) shows the distribution and phylogenetic relationships among the studied bacterial species (only 15) and their identical type strains. Four bacterial strains showed closely related (0.002 , 0.017 , 0.005 and 0.007 for branch value) whilst all others were identical (0).

3.4 Identification of New Global Bacterial Strains

Four bacterial isolates were identified as new strains showing differences with their type strains in some numbers and placements of the bases. Thus, the new bacterial strains were published the GenBank as *Escherichia coli* strain IRQBAS14 "LNB67523" (isolate No.9) which closely related (99%) with *E.coli* strain "ATCC 25922" for its Transversion point mutation (T instead of G) at the position 1149 bp (Figure 3)

,*Corynebacterium tuberculostearicum* strain IRQBAS15 "LNB67524" (isolate No.33) which closely related (99%) with *Corynebacterium tuberculostearicum* strain "Medalle X" for its nine point mutations(TCTGCGCA and Ginstead of CGGCTAGT and C) at positions 952, 959, 960, 961, 962, 969, 970, 971 and 972 bp respectively (Figure 4), *Corynebacterium coyleae* strain IRQBAS16 "LNB67525" (isolate No.41) which closely related (99%) with *Corynebacterium coyleae* strain "V17-2011302" for its two point mutations of Transition (C instead of G) and Transversion (T instead of G) at the positions 58 and 59 bp respectively (Figure 5) and *Corynebacterium aurimucosum* strain IRQBAS17 "LNB67526" (isolate No.46) which closely related (99%) with *Corynebacterium aurimucosum* strain "H2456" for its four point mutations of Transversion (C ,C, G and G

Table 1: Growth and gram's staining of bacteria isolated from urine samples in patients with urinary stones.

Culturing n (%)		Gram's staining n (%)	
Axenic	41 (82)*	Gram-positive	39 (71)*
Mixed	7 (14)		
No growth	2 (4)	Gram-negative	16 (29)
Total	50 (100)	Total	55 (100)

* : P< 0.01

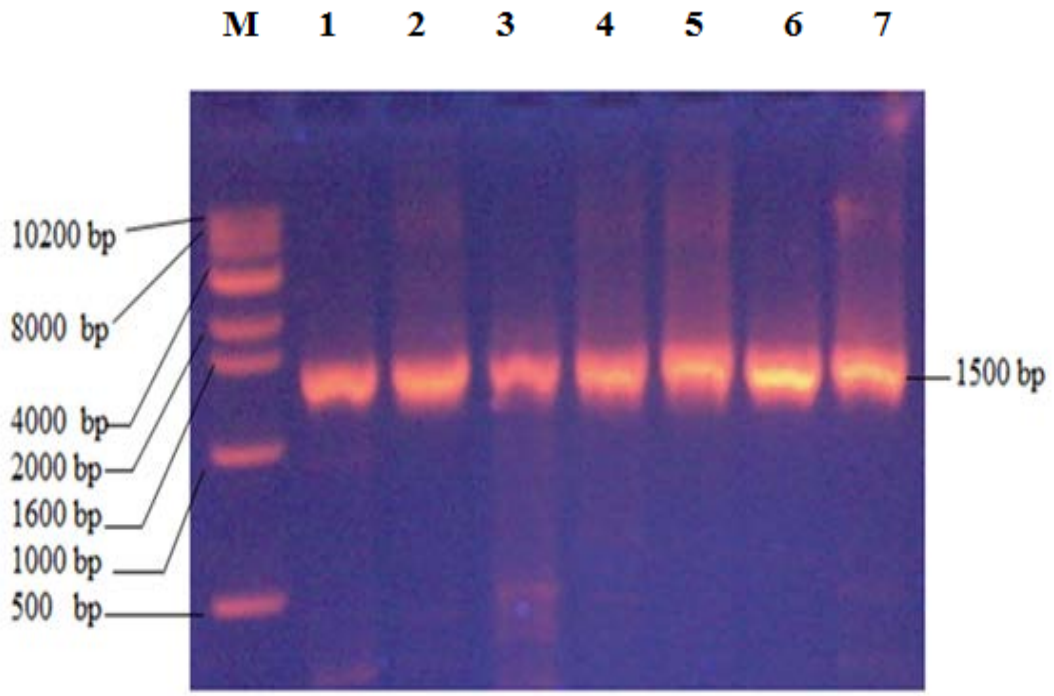

Figure 1: Gel electrophoresis of PCR products (*16Sr DNA* gene) for bacterial isolates. Lane M: (1kb DNA ladder). Lane 1-7: *16SrDNA* bands for bacterial isolates.

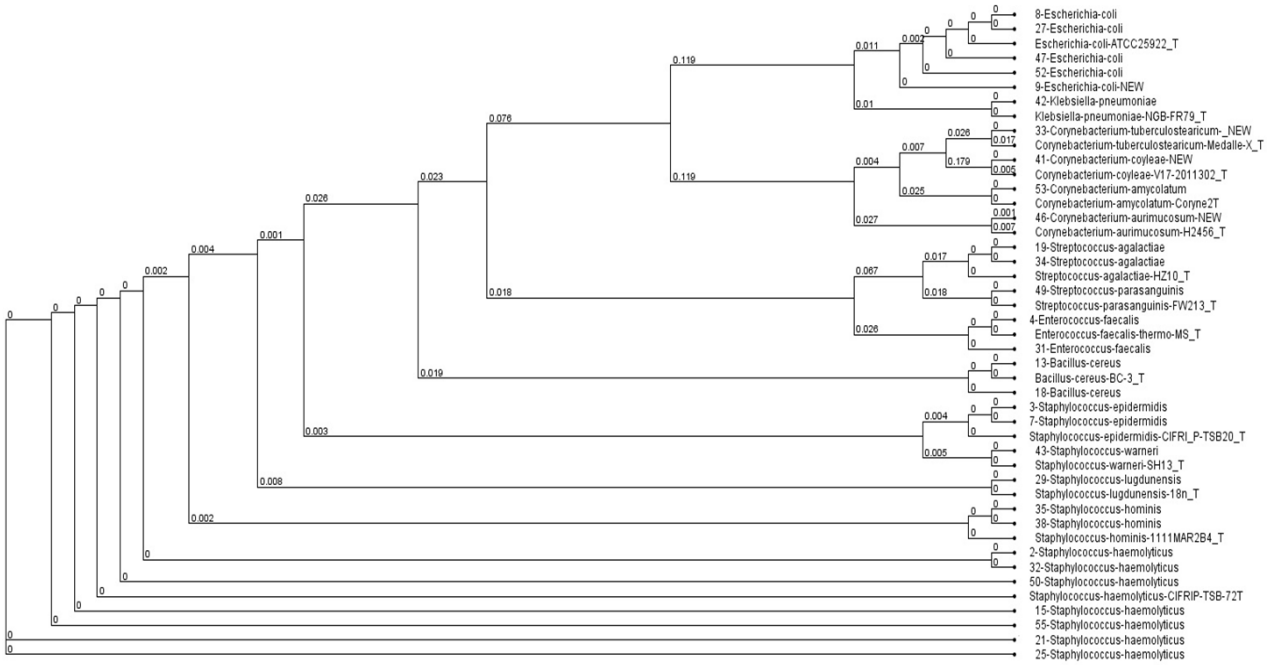

Figure 2: Phylogenetic tree showing the relationships of bacterial species (n= 15) isolated from urine samples in patients with urinary stones and type strains (T) by alignments of *16SrDNA* gene sequences, then produced by Multiple Alignment using Fast Fourier Transform program (MAFFT) and visualized using "forester"software. Bootstrap = 1000.This tree was constructed from concatenated sequences of 524 bp.

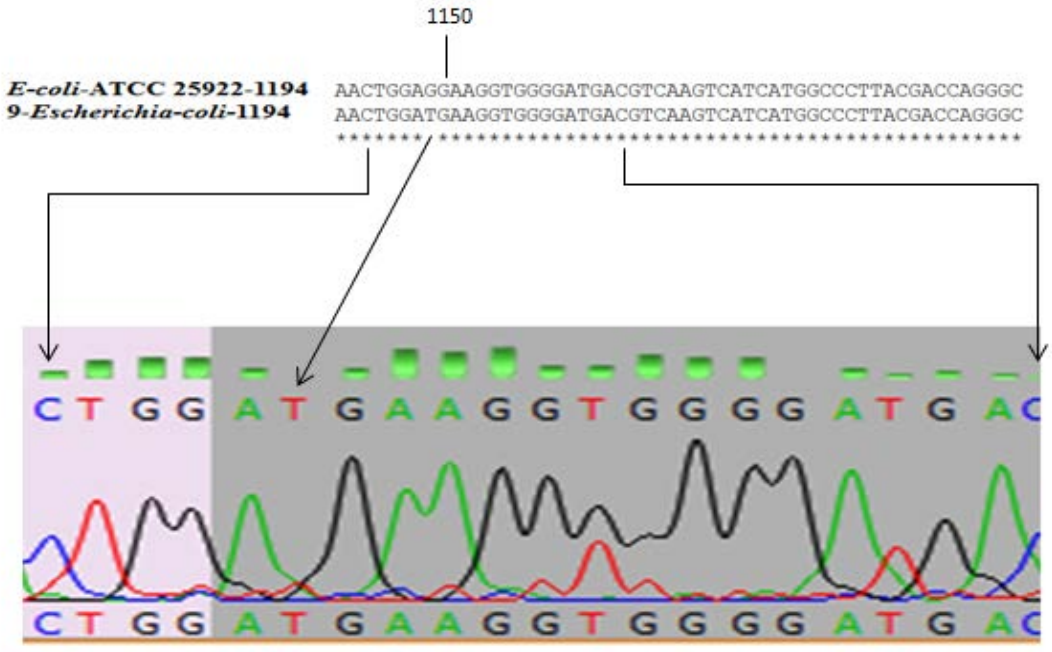

Figure 3: Comparison of 16S rDNA nucleotide sequences (1194 bp) for the isolate *E. coli* (No.9) from present study and type strain ATCC 25922. A gene or point mutation type Transversion (T instead G) at the position 1149 bp.

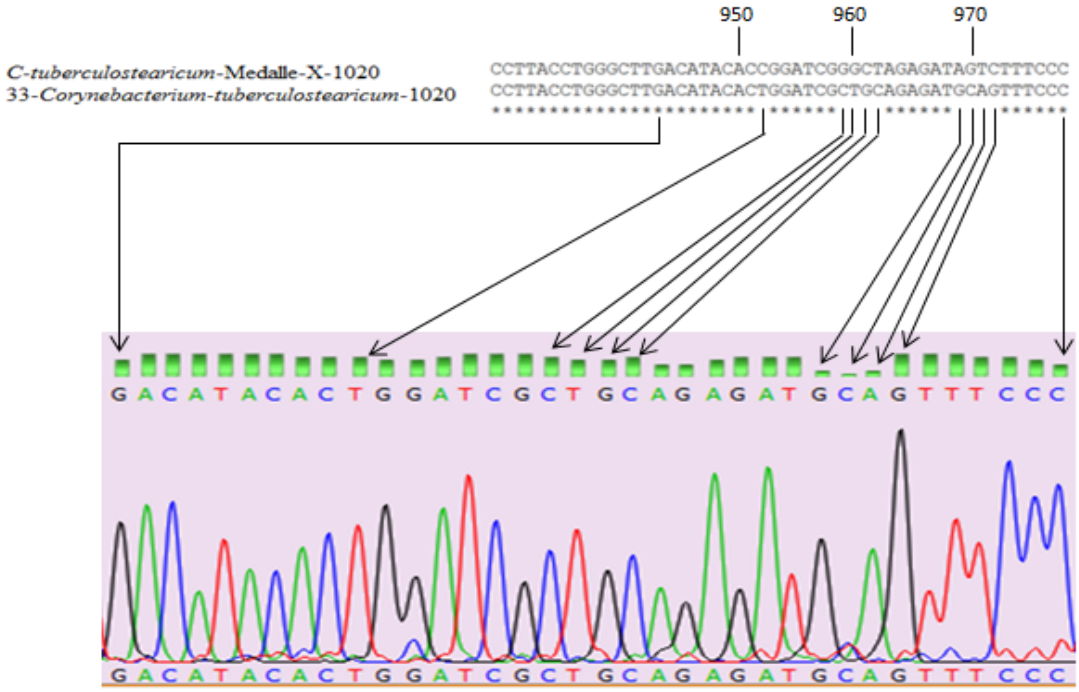

Figure 4: Comparison of 16S rDNA nucleotide sequences (1020 bp) for the isolate *Corynebacterium tuberclustearicum* (No.33) from present study and type strain Medalle X. A nine point mutation types Transversion and Transition TCTGCGCA and G instead of CGGCTAGT and C at positions 952,959,960,961,962,969,970,971 and 972 respectively.

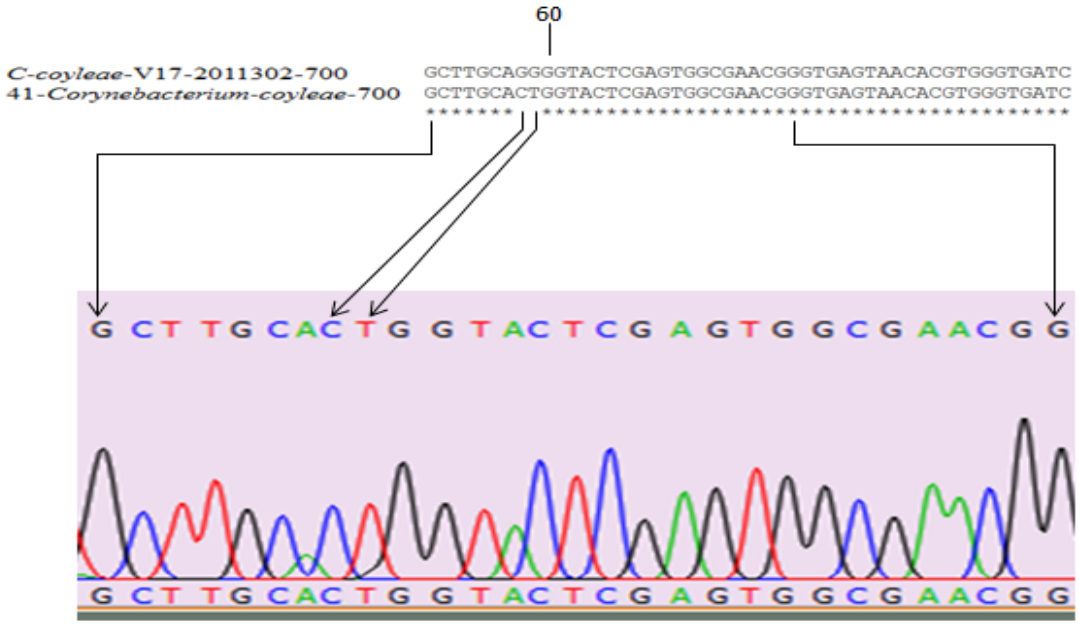

Figure 5: Comparison of 16S rDNA nucleotide sequences (700 bp) for the isolate *Corynebacterium coyleae* (No.41) from present study and type strain V17-2011302. Two gene or point mutations type Transition (C instead G) and Transversion (T instead G) respectively at positions 58 and 59 bp.

instead of A,G,C and T)at positions 42 , 43 , 52 and 53 respectively , moreover , a frame shift mutation (insertion T between T and G) at the position between 44 and 45 (Figure 6).

3.5 The Frequency of Urine Bacterial Species from Urolithiasis Patients According to Some Factors

Broadly speaking, the bacterial species (n=44) were more frequented in patients without surgical operation (88.6%), antibiotic utilization (77.3%), kidney and oxalate stone (72.7%, for each) with high significance at P< 0.01, while they were 59.1% and 52.3% in the males and age group (40-60 years) respectively, simply without significant conflicts. *S. haemolyticus* was frequented (100%) in patients without surgical operation, also it was more frequented in patients with antibiotic use (90%), males and kidney stone (70%, for each) at P< 0.01, and oxalate stone at P< 0.05. *E. coli* was frequented (100%) in patients with kidney stone, antibiotic use (85.7%), male patients, age group (40-60 years), oxalate stone and without surgical operation (71.4%) at P< 0.01. *S. epidermidis* was frequented in patients with antibiotic use (80%) at P< 0.01, and in males, age group (20-39 years), kidney, oxalate stone and without surgical operation (60%, for each) at P< 0.05. *E. faecalis* was frequented in patients with kidney, oxalate stone and without surgical operation (100%, for each), while it was more frequented in males, age group (40-60 years) and antibiotic use (66.7%, for each) at P< 0.01. However, the frequency was100% for all the following: *P. aeruginosa* in patients with kidney, oxalate stone; *K. pneumonia* in male patients, age group (20-39 years), oxalate stone, antibiotic use and without surgical operation (100%, for each); *S. agalactiae* in patients with age group (40-60 years), oxalate stone and without surgical operation; *S. hominis* in patients with age group (40-60 years), antibiotic usage and without surgical operation; *B. cereus* in patients oxalate stones and without surgical operation; *B. subtilis* and *E. raffinosus* in male patients, age group (40-60 years), Kidney, oxalate stone, antibiotic usage and without surgical operation; *S. lugdunensis* in male, age.

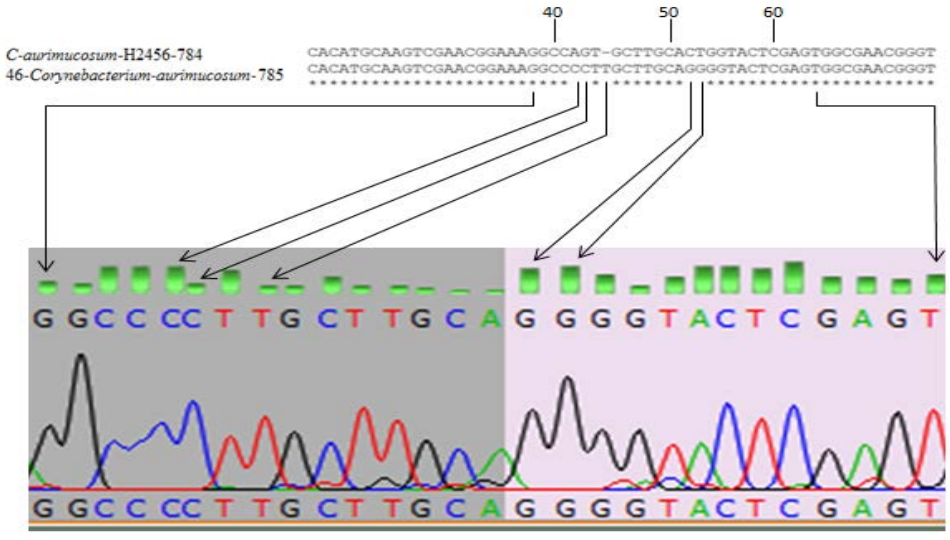

Figure 6: Comparison of 16S rDNA nucleotide sequences (784 bp) for the isolate *Corynebacterium aurimucosum* (No.46) from present study and type strain H2456. Four genes or point mutation types Transversion C, C, G and G instead of A, G, C and T at positions 42,43,52 and 53 bp respectively. A frame shift mutation insertion T between position of 44 and 45 bp.

group (40-60 years), ureter, non-oxalate stone, antibiotic usage and without surgical operation; *S. warneri* in male, age group (20-39 years), ureter, oxalate stone, antibiotic usage and without surgical operation; *S. parasanguinis* and *C. amycolutum* in female patients, age group (20-39 years), kidney, oxalate stone, without antibiotic use and without surgical operation; *C. tuberculostearicum* in male, age group (20-39 years), kidney, non- oxalate stone, antibiotic usage and without surgical operation , and finally *C. aurimucosum* in female, age group (20-39 years), kidney, non-oxalate stone, antibiotic usage and without surgical operation as in Table (2).

3.6 Antibiotic Susceptibility Pattern of Bacteria

Antibiotic susceptibility test for urine bacterial isolates (44) showed highly sensitive to imipenem (86.4%) with significant difference at P< 0.01, followed by amikacin and ciprofloxacin (54.5%, for each), while low sensitivity to gentamicin (43.2%) and tetracycline (25%). On the other hand, they were a high rate of resistance against ampiclox and methicillin (100%, for each), ceftriaxone (97.7%), nitrofurantoin and Co-trimaxazole (86.4%, for each) at P< 0.01 for each, as in (Table 3). *S. haemolyticus* (10) appeared sensitive to imipenem (90%) with high significant differences (P< 0.01), followed by ciprofloxacin (60%) at P< 0.05. *E. coli* (7) showed significant resistant to all antibiotics with the exception of imipenem, amikacin, gentamicin and ciprofloxacin at P< 0.05. *S. epidermidis* (5) was sensitive to imipenem (100%) followed by amikacin (60%) at P< 0.05. *E. faecalis* (3) was sensitive to imipenem, amikacin, gentamicin and ciprofloxacin (66.7% for each) at P< 0.01. *P. aeruginosa* (2) was sensitive only to imipenem (100%). *K. pneumoniae* (2) was sensitive to imipenem (100%). *S. hominis* (2) was sensitive to imipenem (100%). *S. agalactiae* (2) was sensitive to imipenem, gentamicin, ciprofloxacin and tetracycline (100% for each). *B.cereus* (2) was sensitive to imipenem IMP: Imipenem, AK: Amikacin, CN: Gentamicin, CIP: Ciprofloxacin, SXT: Co-trimoxazole, TE: Tetracycline F: Nitrofurantoin, CTR: Ceftriaxone, APX: Ampiclox, ME: Methicillin**.** and amikacin (100% for each).

The other bacterial species were sensitive (100%) to different antibiotics, since, the single isolate of all the following species including:

B. subtilis was sensitive to imipenem, amikacin, gentamicin, ciprofloxacin and ceftriaxone; *E. raffinosus* to imipenem and Co-trimaxazole; *S. lugdunensis* to imipenem and gentamicin; *S. warneri* to imipenem, ciprofloxacin and Co-trimaxazole; *S. parasunguinis* to imipenem, ciprofloxacin and nitrofurantoin; *C. amycolutum* to imipenem, ciprofloxacin and tetracycline; *C. aurimucosum* to imipenem, amikacin, gentamicin, ciprofloxacin and tetracycline, and finally *C. coyleae* to imipenem, amikacin, gentamicin and ciprofloxacin. Exclusively, *C. tuberculostearicum* (1) showed resistant to all antibiotics.

3.7 The Relationship Between the Frequency of Urine Bacterial Species and Oxalobacter formigenes from Stool

Generally, the urine bacterial species (44) were more frequented (75%) in oxalate urinary stone patients associated with the absence of *O. formigenes* with the significant differences at P< 0.01, while they were 58.3% in non-oxalate stone patients with the presence of *O. formigenes* isolating from the same patients of a previous study by Jasim and Abed Al-Abbas (23), but without significant differences (Table 4).

Table 2: The Frequency of Bacterial Species from Urine Samples of Urolithiasis Patients and Some Factors

Bacterial Species		**Sex n (%)**		**Age n (%)**		**Site of Stone n (%)**			**Type of Stone n (%)**		**Antibiotic usage n (%)**		**Surgical operation n (%)**	
	n	Male	Female	20-39 year	40-60 year	Kidney	Ureter	Bladder	Oxalate	Non-oxalate	Yes	No	Yes	No
Staphylococcus haemolyticus	10	7(70)*	3(30)	5(50)	5(50)	7(70)*	3(30)	0(0)	6(60)**	4(40)	9(90)*	1(10)	0(0)	10(100)
Escherichia coli	7	5(71.4)*	2(28.6)	2(28.6)	5(71.4)*	7(100)	0(0)	0(0)	5(71.4)*	2(28.6)	6(85.7)*	1(14.3)	2(28.6)	5(71.4)*
Staphylococcus epidermidis	5	3(60)**	2(40)	3(60)**	2(40)	3(60)**	2(40)	0(0)	3(60)**	2(40)	4(80)*	1(20)	2(40)	3(60)**
Enterococcus Faecalis	3	2(66.7)*	1(33.3)	1(33.3)	2(66.7)*	3(100)	0(0)	0(0)	3(100)	0(0)	2(66.7)*	1(33.3)	0(0)	3(100)
Pseudomonas aeruginosa	2	1(50)	1(50)	1(50)	1(50)	2(100)	0(0)	0(0)	2(100)	0(0)	1(50)	1(50)	1(50)	1(50)
Klebsiella pneumonia	2	0(0)	2(100)	2(100)	0(0)	1(50)	1(50)	0(0)	2(100)	0(0)	2(100)	0(0)	0(0)	2(100)
Streptococcus. agalactiae	2	1(50)	1(50)	0(0)	2(100)	0(0)	1(50)	1(50)	2(100)	0(0)	1(50)	1(50)	0(0)	2(100)
Staphylococcus hominis	2	1(50)	1(50)	0(0)	2(100)	1(50)	1(50)	0(0)	1(50)	1(50)	2(100)	0(0)	0(0)	2(100)
Bacillus cereus	2	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	0(0)	2(100)	0(0)	1(50)	1(50)	0(0)	2(100)
Bacillus subtilis	1	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)

Enterococcus raffinosus	1	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)
Staphylococcus lugdunensis	1	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	1(100)
Staphylococcus warneri	1	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)
Streptococcus parasanguinis	1	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)
Corynebacterium amycolutum	1	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)
Corynebacterium tuberculostearicum	1	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	1(100)
Corynebacterium coyleae	1	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	0(0)	1(100)
Corynebacterium aurimucosum	1	0(0)	1(100)	1(100)	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	0(0)	1(100)	0(0)	1(100)
Total n (%)	44	26(59.1)	18(40.9)	21(47.7)	23(52.3)	32(72.7)*	11(25)	1(2.3)	32(72.7)*	12(27.3)	34(77.3)*	10(22.7)	5(11.4)	39(88.6)*

* P< 0.01,** P< 0.05

Table 3: Antibiotic Susceptibility Patterns of Urine Bacterial Species from Patients with Urinary Stones

Bacterial Species		**Antibiotic sensitivity n (%)**									
	n	IMP	AK	CN	CIP	SXT	TE	F	CTR	APX	ME
Staphylococcus haemolyticus	10	9 (90)	4 (40)	3 (30)	6 (60)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
Escherichia coli	7	4(57.1)	4(57.1)	3(42.6)	3(42.6)	1(14.3)	0 (0)	1(14.3)	0 (0)	0 (0)	0 (0)
Staphylococcus epidermidis	5	5 (100)	3 (60)	1 (20)	1 (20)	1 (20)	4 (40)	1 (20)	0 (0)	0 (0)	0 (0)
Enterococcus Faecalis	3	2(66.7)	2(66.7)	2(66.7)	2(66.7)	1(33.3)	1(33.3)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus hominis	2	2 (100)	1 (50)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)
Streptococcus agalactiae	2	2 (100)	1 (50)	2 (100)	2 (100)	0 (0)	2 (100)	1 (50)	0 (0)	0 (0)	0 (0)
Pseudomonas aeruginosa	2	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Klebsiella pneumonia	2	2 (100)	1 (50)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bacillus cereus	2	2 (100)	2 (100)	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bacillus subtilis	2	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Enterococcus raffinosus	1	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus lugdunensis	1	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Staphylococcus warneri	1	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptococcus parasanguinis	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
Corynebacterium amycolatum	1	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Corynebacterium aurimucosum	1	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Corynebacterium coyleae	1	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Corynebacterium tuberclustearicum	1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total n (%)	44	38* (86.4)	24 (54.5)	19 (43.2)	24 (54.5)	6 (13.6)	11 (25)	6 (13.6)	1 (2.3)	0(0)	0 (0)

*P< 0.01

The frequencies of *S. haemolyticus*, *S. epidermidis*, *K. pneumoniae*, *S. agalactiae*, *E. raffinosus*, *S. warneri*, *C. coyleae* (100% for each), *E. coli* (80%) and *E. faecalis* (66.7%) were the predominant species in patients with oxalate stone have not *O. formigenes* ($P< 0.01$). On the other hand, the frequencies of *E. coli*, *S. hominis* and *C. aurimucosum* were 100% for each, in non-oxalate stone patients with the presence of *O. formigenes*, while *S. lugdunensis* and *C. tuberculostericum* were 100% for each, in non- oxalate stone patients with the negative of *O. formigenes*.

4. Discussion

Axenic culture was appeared in 82% of samples compared with mixed or no growth, this result in agreement with Hassan [24] founding 90% of the samples gave axenic cultures and the remaining 10% gave mixed and no growth.

The axenic means pure colonies from the first culture. Relatively, most urinary tract infections are caused by a single pathogen, but sometimes can be caused by a two types of the pathogens, especially in patients with immunocompromised. Also, the present study appeared that a large proportion of bacteria were gram positive isolates comparing to gram negative, as in (Table 1). This may be due to the widespread using of antibiotics leading to a shift in the etiological agent of disease. However, this result is inconsistent with previous studies [25,26,27].

Out of all 55 bacterial isolates, only 44 were identified by *16SrDNA* and provided 18 different species, while the remaining 11 isolates were unidentified genetically as a result of their failed by sequencing .

The results revealed that *S. haemolyticus* was the most common bacteria with 10 isolates (22.27%), followed by *E. coli* 7 isolates (15.9%) then *S. epidermidis* 5 isolates (11.4%) and *E. faecalis* 3 isolates (6.8%) as Table (2). Relatively, urinary tract infections occur when bacterial flora from the gastrointestinal tract and the skin can be clinging to the opening of the urethra and then colonization, once these bacteria gain access to the bladder they may multiply and moving up through ureters to the kidneys, this is called ascending pathway [15]. *S. haemolyticus* is a part of the commensal bacteria in the skin, mucusal surfaces, urethra and periurethra of human, showing the increasing importance as a cause of acquired-hospital infections, especially, when isolated from urine as a cause of UTIs [28,29,30]. Some of its virulence factors play an important role in its pathogenicity, including hemolysin, cytolysin, biofilm formation which increases antibiotic resistance and often leads to persistent infections [31,32,33,34]. Nevertheless, the urinary stone contributes significantly to increase the adhesion of bacteria, thus increase its ability to colonize the urinary tract and then the ability to infect [35]. On the other hand, *E. coli* is normally resides in the colon, it is the most common bacterium causing UTIs in patients with urinary stone disease in both community acquired and nosocomial infections [1,27,36]. *E. coli* have many virulence factors which enable to colonize the urethra and spread up the urinary tract to the bladder including ″P-fimbria‟ to bind urinary tract epithelial cells, other factors known to contribute to the virulence are the production of hemolysins, colicins and cytotoxic necrotizing factor [37]. *S. epidermidis* has been isolated from urine as a cause of UTIs in patients with catheterization and urinary stone disease [27,38]. However, this bacterium can cause infection in immunocompromised patients and is particularly adept to form

biofilm on surgical implants and indwelling catheters [35]. Although, the patients did not have any indwelling foreign devices, but the urinary stones providing a surface for the bacteria to form a biofilm, thus, leading to urinary tract infection [35]. *Enterococcus faecalis* is an opportunistic pathogen and is the causative agent for urinary tract infection in patients with urolithiasis [27,39] and it has many virulence factors playing an important role in pathogenicity such as aggregation substance, surface adhesions (ESP) and hemolysin [40,41].

Table 4: The Relationship between the Frequency of Urine Bacterial Species and *Oxalobacter formigenes*

Bacterial species		***Oxalobacter formigenes* (*oxc* gene)**			
		Oxalate stone patients n=32		**Non- oxalate stone patients n=12**	
	n	**Yes n (%)**	**No n (%)**	**Yes n (%)**	**No n (%)**
Staphylococcus haemolyticus	10	0(0)	6(100)	2(50)	2(50)
Escherichia coli	7	1(20)	4(80)*	2(100)	0(0)
Staphylococcus epidermidis	5	0(0)	3(100)	1(50)	1(50)
Enterococcus faecalis	3	1(33.3)	2(66.7)*	-	-
Pseudomonas aeruginosa	2	1(50)	1(50)	-	-
Klebsiella pneumonia	2	0(0)	2(100)	-	-
Streptococcus agalactiae	2	0(0)	2(100)	-	-
Staphylococcus hominis	2	1(100)	0(0)	1(100)	0(0)
Bacillus cereus	2	1(50)	1(50)	-	-
Bacillus subtilis	1	1(100)	0(0)	-	-
Enterococcus raffinosus	1	0(0)	1(100)	-	-
Staphylococcus lugdunensis	1	-	-	0(0)	1(100)
Staphylococcus warneri	1	0(0)	1(100)	-	-
Streptococcus parasanguinis	1	1(100)	0(0)	-	-
Corynebacterium amycolutum	1	1(100	0(0)	-	-
Corynebacterium tuberculostearicum	1	-	-	0(0)	1(100)
Corynebacterium coyleae	1	0(0)	1(100)	-	-
Corynebacterium aurimucosum	1	-	-	1(100)	0(0)
Total n (%)	44	8(25)	24(75)**	7(58.3)	5(41.7)

*: Previous study (Jasim and Abd Al-Abbas., 2016)

** : P< 0.01

- : Not present

The previous studies have reported that *E. coli* is the most common species in UTI, but the present study

detected a wide variation in the frequency of all the bacterial species under the study. It is thought that the widespread and uncontrolled use of antibiotics in our country following the type of pathogen and their resistance to antibiotics changing with over time.

Phylogenetic tree is a diagram refer to evolutionary relationships among different species based on similarities and differences in their genetic characteristics [42]. The phylogenetic tree was designed according to the bacterial isolates which showed 99% or 100% similarity with the type strains (Figure 2), giving four bacterial isolates as new strains, each have 1% difference with its type strain. A mutation is a permanent alteration in the sequence of the nitrogen base of the DNA that is generally may change the end product of the specific gene [43]. Mutations lead to change the genes that are very important in the bacterial evolution. Mutations may occur by exposuring the bacteria to certain environmental factors such as radiation and chemical mutagens [44]. Also, overuse of broad spectrum antibiotics leads to a mutation and emergence a new strain [14,45]. Furthermore, the transfer of bacteria from the original living environment to another may cause mutation [46]. Since, *Escherichia coli* is colonize the gastrointestinal tract and transmitted from the anus to the urinary tract by ascending route [15]. *Corynebacterium tuberculostearicum, C. coyleae* and *C. aurimucosum* are members of non-diphtheria corynebacteria causing the major components of the commensal bacteria on human skin, urethra and mucous surfaces. Although, these bacteria are commonly isolated from clinical samples [47] and originally thought to be essentially contaminants, but recently have been reported as pathogens, especially in patient with immunocompromised and indwelling medical device [48]. However, these bacteria isolated from urine samples as causative agents of urinary tract infections [49,50,51]. No previous studies have reported the relationships between these bacteria and urinary stones, but believed that the stone contributed to adhere the bacteria then invasive urinary tract .

Although the majority of bacterial species have low virulent on the skin, but they become with high virulence when enter through the surgical operations of the urinary tract and hence to increase their ability to infect [13]. Most patients with urinary stones had been prescribed antibiotics, but the indiscriminate use of antibiotics and lack of quality control on some sources of antibiotics lead to the emergence of multi-drug resistant by bacterial species, thus, increasing the ability of infection [52]. The complex structure of the kidneys and slowly flow of urine in some parts in addition to the presence of oxalate stones with sharp edges damaging the epithelium of the urinary tract and encourage the bacterial growth [12]. Moreover, oxalate stones often established in the urinary tract providing a surface for bacteria to form a biofilm [35]. These factors contribute significantly to increase the ability of bacteria to colonize and invade the urinary tract and thus increases their ability to infect.

Table (3) showed that imipinem was 100% active against all bacterial species with the exception of *S. haemolyticus, E. faecalis, E. coli* and *C. tuberculostericum* (90%, 66.7%, 57.1% and 0% respectively). No previous study about the sensitivity of *S. haemolyticus* to imipenem in the urine because it rarely isolated as a cause of UTI. Some studies have reported that *E. faecalis* and *E.coli* were 100% and 98% (respectively) sensitive to imipenem [6,53,54,55]. Nevertheless, the present study showed imipenem is moderate activity against *E. faecalis* and *E. coli*. During the antibiotic therapy, the exposure of bacterial pathogens to high concentration of antibiotics for long periods creates severe selection pressure leading to emergence the resistance [56]. However, the multi resistance of *C. tuberculostericum* to all antibiotics, may be due to the

occurrence of nine mutations in different locations of its genome changing some genes that responsible on antibiotic receptors, in addition, its structural properties of the cell wall having fatty acid profile comprised tuberculostearic acid can play an important role in antibiotics resistance [57]. All bacterial isolates appeared high resistance to ampiclox and methicillin (100%, for each) which is due to the acquisition of *mec A* gene by plasmid transferring [58,59]. On the other hand, many bacterial species appeared resistance rate ranging from 4 to 8 antibiotics, while *Pseudomonas aeruginosa* was resistant to all antibiotics with the exception to imipenem, since the multidrug resistance in the bacterium could be mediated by many mechanisms including enzyme production, multidrug efflux systems, external membrane protein (Porin) loss and target mutations [60].

The absence of *O. formigenes* from the intestinal microflora leads to increase absorption of oxalate by the kidneys and thus to hyperoxaluria and eventually to oxalate urinary stone formation [61]. Hence, the oxalate stone covered with sharp projections that damage the epithelium of the urinary tract and encouraging the bacterial growth by forming the nudus and thereby increase their ability to infect [12]. The present study showed that *S. haemolyticus* and *S. epidermidis* were frequented (100%, for each) in oxalate stone patients with the absence of *O. formigenes*, while *E. coli* and *E. faecalis* were 80% and 66.7% respectively with statistical significance differences. These results supported the absence of *O. formigenes* from the gut microflora can indirectly increase the ability of bacterial species to colonize and invade the urinary tract . This explanation is suitable for other bacterial species in the present study (*K. pneumoniae, S. agalactiae, E. raffinosus, S. warneri and C. coyleae*) but because their small number, the result is considered not precise.

5. Conclusion

The most common bacterial species associated with urolithiasis in Basrah province were *Staphylococcus haemolyticus, Escherichia coli* then *Staphylococcus epidermidis* and *Enterococcus faecalis*. However, the precise identification discovered four bacterial isolates were recorded as new global forms. Although, the high rate of multi-drug resistance among all urinary isolates, but imipenem is a very effective one. Significantly, there was a relationship between the presence of O. *formigenes* in the gastrointestinal tract and the absence of other bacterial species in the urine patients having urinary stone.

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Competing interests

The authors did not have any conflict of interest

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