Phylogenetic relationship of *Citrobacter* spp. and Other Enterobacteriaceae Isolated from Stool Samples of Healthy and Patients with Colorectal Cancer in Al-Najaf Al-Ashraf/Iraq

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Abstract

The current study aimed to detect phylogenetic relationships among enterobacteriaceae isolates. A total of 110 stool samples were collected from healthy and patients with coleractal cancer during the period 2016-2017. The results of PCR technique for amplification of *tuf* showed that 55 isolate (55%) were belong to *Enterobacteriaceae* (suspected *Citrobacter*) by appearance amplicon with molecular weight 871 bp while 45 isolate (45%) were belong to *E.coli* by appearance of amplicon with molecular wight 884 bp. Also, the results of electrophoresis of *atpD1* and *atpD2* showed that 36 isolates (56%) and 24 (42.8%) isolates were belong to *E.coli* by appearance of amplicon with molecular weight 884 bp while 32 isolates (57.1%) were belong to other *Enterobacteriaceae* (suspected *Citrobacter*) by appearance amplicon with molecular weight 871 bp. DNA sequencing of amplicon resulted from amplification of *tuf*, *atpD1* and *atpD2* was carried out to confirmed identification of *Citrobacter* spp as well as to evaluate the phylogenetic relationship among bacterial isolates. Thirty isolates were selected for DNA sequencing depending one presence and absence of *tuf*, *atpD1* and *atpD2*. The results of DNA sequencing and alignment with other enterobacterial sequences available in public database using MEGA 7 and BLAST showed that 4, 2 and 1 isolates gave strong alignment with *E. coli*, *Klebseilla* and *Citrobacter*.

Key words: Enterobacteriaceae, *Citrobacter*, *tuf* , *atpD*1and *atpD*2.

Introduction:

Citrobacter is a Gram-negative, aerobic or facultative and rod-shaped bacterium which is considered a commensal species of the intestinal tract of humans and other animals (8). They were considered the intestinal inhabitants of human and animals, andhumansonly existed in sewage, water and soil and there are 12 recognized species within the genus *Citrobacter*, of which three were pathogenic bacteria in human. C. ferundii and C. koseri had caused meningitis in immunocompetent patients (6,12).

Elongation factors (EF-Tu) are a set of proteins that are used in protein synthesis in the process of cell cycle and elongation in some cells. In the ribosome, they facilitate translational elongation, from the formation of the first peptide bond to the formation of the last one (7). It is encoded by *tuf* genes, carries aminoacyl- tRNA to the programmed ribosome during protein synthesis. Most enterobacterial genomes possess two copies of the *tuf* gene, *tufA* and *tufB*, in distinct operons, designated str and tRNA *tufB*, respectively (15). *fusA* gene, which encodes elongation factor G, is located upstream of *tufA* in the str operon of gammaproteobacteria (14).

Four tRNA structural genes are located upstream of *tufB* in the tRNA-*tufB* operon, and the secE and nusG genes are downstream of *tufB* in most *Enterobacteriaceae*. *tufA* and *tufB* genes appear to evolve in concert through gene conversion events that maintain the sequence homology (10). A previous analysis of duplicated bacterial *tuf* genes revealed identical or very similar nucleic acid sequences that differ by less than 1.4%. (16). The two copies of the tuf gene (*tufA* and *tufB*) found in *Enterobacteriaceae* share high levels of identity (99 %) in *Salmonella typhimurium* and in *Escherichia coli*. A recombination phenomenon could explain the sequence homogenization between the two copies. (14, 17). Phylogenies based on protein sequences from elongation factor Tu and the F-ATPase b-subunit have shown good agreement with each other and with the rRNA gene sequence data.

FATPase is present on the plasma membranes of eubacteria. It works mainly in ATP synthesis, and the b-subunit contains the catalytic site of the enzyme. The Elongation factors Tu and FATPase have been highly conserved throughout evolution and show functional constancy. Phylogenies based on protein sequences from elongation factor Tu and the F-ATPase b-subunit has shown good agreement with each other and with the rRNA gene sequence data (18).

In order to evaluate the discriminate role of *tuf* and *atpD* in identification of enterobacteriaceae member, this study aimed to investigate the phylogenetic relationships of *Citrobacter* spp with other enterobacteriaceae member isolated from patient with colorectal patients and healthy.

Methods:

Samples collection

A total of 110 stool samples has been collected from healthy people (100 samples) and patients with colorectal cancer (10 samples) during the period from December 2016 to March 2017. All samples were collected in appropriate technique to avoid any possible contamination and cultured on MacConkey agar, Blood Agar Base and SS agar for primary isolation of enterobacteriaceae.

Identification of bacterial isolates

All bacterial isolates were identified according to conventional methods using microscopic exam, culturing characteristics and biochemical test (MacFaddin, 2000). Furthermore, all bacterial isolates were re cultured on GHROM agar (Chromagar, France) to confirmed identification of enterobacteriaceae.

Molecular technique

PCR technique was used to confirm identification of bacterial isolates and to detect the phylogenetic relationship between isolates using elongation factors Tu gene (*tuf*) and F-ATPase β-sub unite genes (*atpD1* and *atpD2*).

Extraction of Genomic DNA:

Extraction of bacterial DNA using Boiling method has been followed as described previously (14). Briefly bacterial cell suspension boiled for 5 minutes then incubated in water bath at boiling temperature for 5 minutes, then, ice bath incubation for another 5 minutes. The lyses mixture was centrifuge at 15000 rpm/min then, the DNA was precipitated by mixing with isopropanol for 24 hrs and centrifuged again at 10000 rpm/min.

The DNA precipitate was conserved in TE solution and DNA concentration was measure by DNA-RNA spectrophotometer (Bio-Droop, England).

Amplification Reaction

The oligo-synthesis nucleotide sequences (iNtRON, Korea) that used in PCR technique were mention in table 1. PCR mixture was prepared to the final volume of 20µl by adding 3µl of the forward and revers primers and 6µl of DNA template to the reaction mixture of PCR (Maxime PCR PreMix Kit, iNtRON, Korea), then the volume was completed to 20µl of Nuclease-free water. The thermo-cyclic conditions of each gene were set by thermocycler (Biometra, Germany) as shown in table 2 using gradient PCR. The amplification products were electrophoresing on 1% agarose gel stained with ethidium bromide at 70 V volt for 50 min. Then, the results were record using a gel documentation system (Biometra, Germany).

Table (1) The oligo-synthesis nucleotide sequences.

Genes	Primer sequence (5´- 3´)	Size of amplicon (bp)	Reference
tuf	F-GGGACGCCACTATGTT- R-AACGGTACGGCCGCCTTC-	903	Designed in this study
atpD1	F-TGAGGAGGAAGCTCATGGCCG- R-CGGCCCCCGAGACGA-	866	
atpD2	F-CGCAAGGCATTGAGGAGAAG- R-CGGCCCCCGAGACGA-	876	

Table (2) The Amplification Condition

Genes	Initial Denaturation (ºC/min)	No. of Cycle	The condition of one cycle			Final extension (ºC/min)
			Denaturation (ºC/min)	Annealing (ºC/min)	Extension (ºC/min)	
tuf	95/3	35	95/3	55/1	72/1	72/7
adt1	95/3	35	95/3	50/1	72/1	72/7
adt2	95/3	35	95/3	50/1	72 /1	72/7

DNA Sequencing

A partial sequencing of *tuf, atpD1* and *atpD2* has been carried out. The DNA sequences alignment was done using data available in NCBI depending on BLAST, also, MEGA 7 was used to analyze the phylogenetic relationships between bacterial isolates.

Results and Discussion:

The results of primary isolation and identification of *Citrobacter* spp. as well as other *Enterobacteriaceae* members on MacConkey agar, blood agar base and SS agar showed a wide distribution of *Enterobacteriaceae* members among all collected samples in which all 110 (100%) sample gave +ve culture.

CHROM Agar and different selective culture media were used to confirm the primary isolation of *Citrobacter* and other species. The results showed a variable percentage of isolation of *Citrobacter* and other enterobacterial species in which a high percentage was detected to *E. coli* (Table 3)

Table (3) The percentage of bacterial isolates in clinical samples

Bacteria	No. (%) of bacterial isolate on			
	Different culture media to		CHROM Agar to	
	Stool of healthy	Stool of patients with colorectal	Stool of healthy	Stool of patients with colorectal
***Citrobacter* spp.**	13 (7.6)	0	18 (11.1)	2 (9)
E. coli	53 (30.9)	5 (22.7)	56 (34.7)	4 (18)
***Klebseilla* spp**	38 (22.2)	4 (18.1)	46 (28.5)	3(13.6)
***Enterobacter* spp**	29 (16.9)	4 (18.1)	34 (21.1)	2 (9)
***Salamonella* spp**	19 (11.1)	3 (13.6)	0	0
***Shagilla* spp**	14 (8.1)	3 (13.6)	0	0
***Protuse* spp**	5 (2.9)	3 (13.6)	7 (4.3)	0
Total	171(88.6)	22(11.4)	161(93.6)	11(6.3)
Total	193		171	

Sixty bacterial isolates (10 isolates from colorectal cancer and 50 isolates from healthy) from CHROM agar were undergoes PCR technique to amplified *tuf*, *atpD1* and *atpD2* to confirmed identification of bacterial isolates. The results of agarose gel electrophoresis of amplicon resulted from amplification of *tuf* gene showed that 26 isolates (43%) belonged to *E. coli* while 40 isolates (66%) were belonged to other *enterobact-eriaceae*. Also, agarose gel electrophoresis of amplicon resulted from amplification of *atpD1*and *atpD2* showed that 4 (6.6%) isolates and 9 (15%) isolates were belonged to *E. coli* respectively while 11 (18.3%) isolates and 18 (30%) isolates were belonged to other *enterobacteriaceae* respectively (Table4).

Table (4) Comparison between conventional and molecular technique for identification of bacterial isolates.

Bacterial name	**NO. (%) of bacterial isolates identification by**			
	CHROM Agar	***tuf***	***atpD1***	***atpD2***
E. coli	22.1 (36)	22 (44)	4 (6.6)	11(18.3)
other *Enterobacteriaceae*	37.9 (63.1)	38 (63)	10 (16.6)	20 (33.3)
Total	60	60	14	31

The results of DNA sequencing of *tuf*, *atpD1* and *atpD2* amplicon showed a successful result for all amplicons and the results of DNA aligment with other nucleotide sequences available in NCBI using BLAST showed that a high percentage of identity of local DNA sequences of isolates to a DNA sequence available in NCBI data base as mention in table5.

Table (5) The DNA sequences alignment of *tuf, atpD1* and *atpD2*

No. of Local DNA sequences	**Results of alignment using DNA sequences of**			
	tuf	***atpD1***	***atpD2***	***Identities***
1		*Escherichia coli* strain RS571 chromosome		99%
2		*Citrobacter amalonaticus* strain FDAARGOS_122		100%

3		*Escherichia coli* genome assembly FHI90		89%
4		*Shigella sonnei* strain ATCC 29930		84%
5		*Escherichia coli strain* HB-Coli0		92%
6			*Escherichia coli* strain RS571 chromosome	99%
7			*Klebsiella pneumoniae strain BA33875 chromosome*	99%
8			*Escherichia coli strain E62*	100%
9			*Escherichia coli isolate EC-TO75 genome assembly*	99%
10			*Citrobacter sp. CFNIH10 chromosome*	86%
11	*Escherichia coli* strain WCHEC020032 chromosome			98%
12	*Arsenophonus nasoniae whole genome shotgun assembly*			93%

13	*Citrobacter freundii complex sp. CFNIH4 chromosome*			85%
14	*Escherichia coli strain WCHEC050613 chromosome*			81%
15	*Citrobacter murliniae strain KK 10 16S ribosomal*			79%
16	*Yersinia frederiksenii strain FDAARGOS_417 chromosome*			87%
17	*Salmonella enterica subsp. enterica serovar Enteritidis str. SA20094682 genome*			98%
18	*Shigella flexneri strain FDAARGOS_535 chromosome*			98%

Furthermore, Phylogenetic tree between bacterial isolates using *tuf* , *atpD1* and *atpD2* was carried out using MEGA 7 program and depending on construct/ test neighbor – joining Tree. The results of phylogenetic relatedness showed variable consideration which could be explained by the following:

(i) The sequencing *atpD1* separated five bacterial isolates included in this study in to tow group the first one included SAHAR 5 and 4 which is closely related to each other and also related with SAHAR 2 while the other group include

SAHAR 3 and SAHAR 1. Both groups were closely related to *Citrobacter* from AX109531.1(Figure 1-A).

(ii) In state of *atpD2*, variable results were obtained in which *Citrobacter* for cure closely related with SAHAR 10 while SAHAR 7 and 11 gave high similarity to each one. Whereas other isolates gave variable relatedness with both groups (Figure 1-B).

(iii) Five groups were obtained when phylogenetic tree was depended on *atpD1- atpD2*, each group consist of 2 bacterial isolates that showed closely related to each other except one group that involved SAHAR 3 and 9 which appear the same isolates and gave relatedness with SAHAR 4 (Figure 2-A) .

(iv) The results of sequencing of *tuf* segregate the bacterial isolates in to 3 group consist of 2 isolates in which were closely related together and SAHAR 18 and 19 appear as the same isolates. The relatedness between these isolates was more than that occur in *atpD* (Figure 2-B).

(v) The results of phylogenetic tree depending on *atpD-tuf* sequences showed that the isolates were segregated into four groups with low distances between each group (Figure 3).

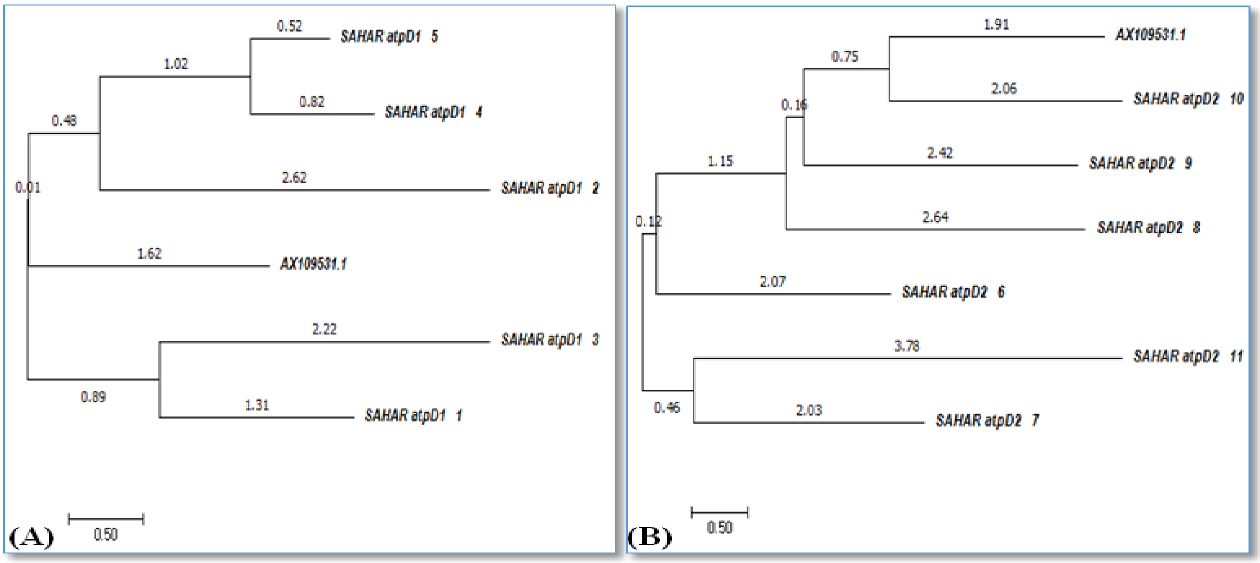

Figure (1) phylogenetic trees based on sequence data from (A) *atpD1* (B) *atpD2.* The phylogenetic analysis was performed with the neighbor-joining test. Values on each branch indicate the display branch length. The percentage of identity between each branch was 100%. AX109531.1refer to *Citobacter ferundi.*

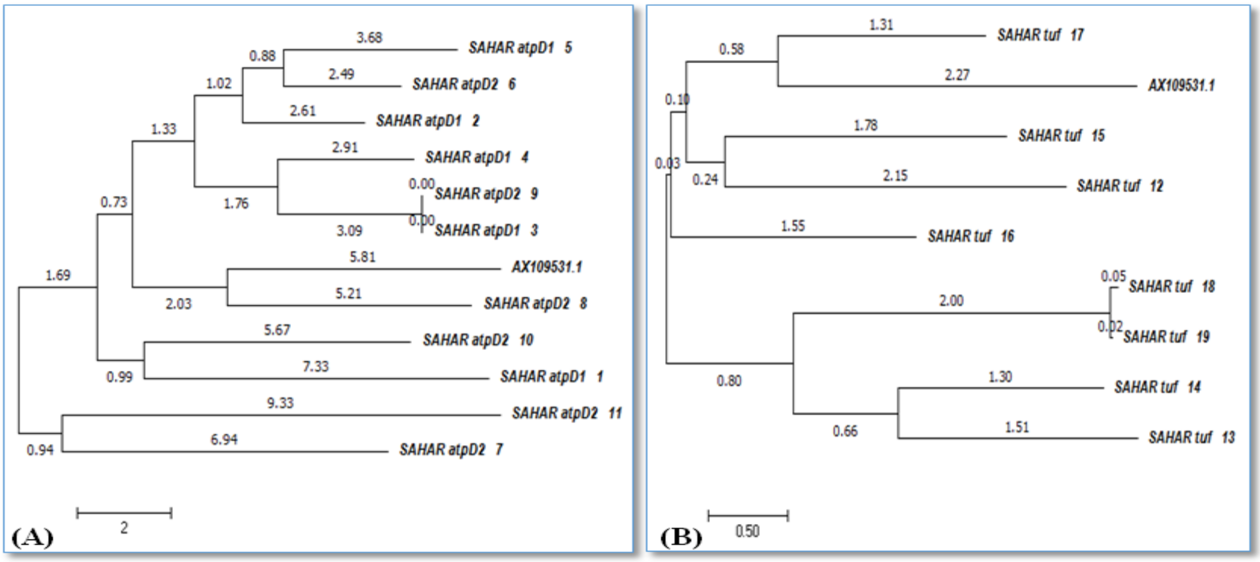

Figure (2) Phylogenetic trees based on sequence data from: (A) *atpD1- atpD2* and (B) *tuf*. The phylogenetic analysis was performed with the neighbor-joining test. Values on each branch indicate the display branch length. The percentage of identity between each branch was 100%. AX109531.1refer to *Citobacter ferundi*.

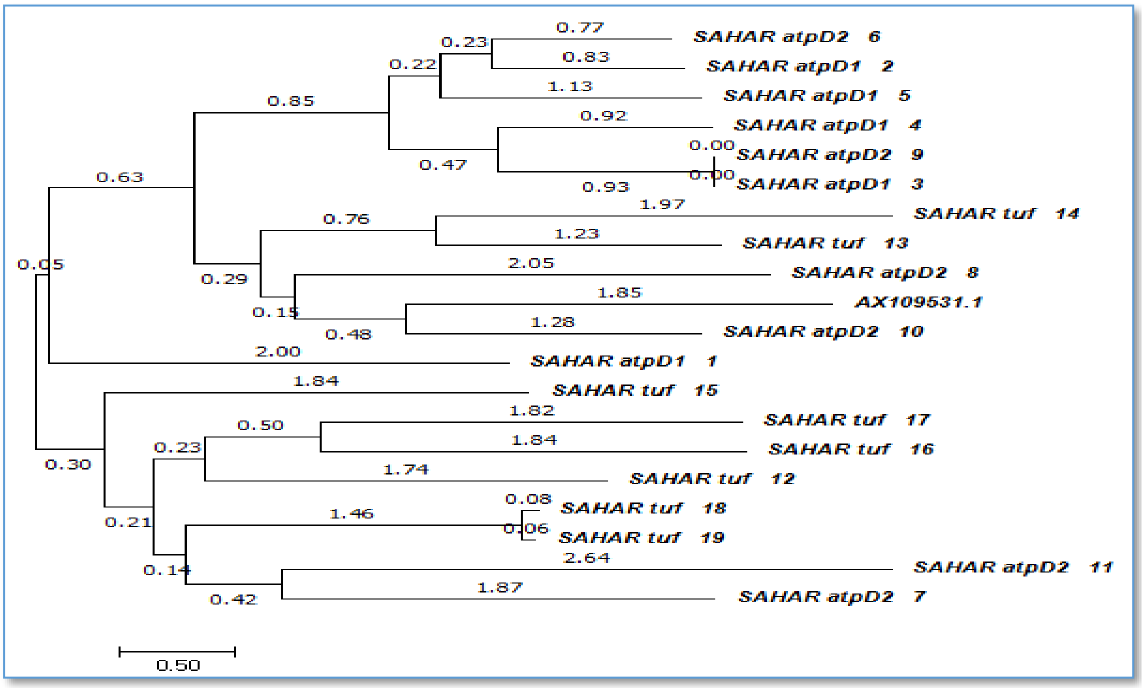

Figure (3) Phylogenetic trees based on sequence data from *atpD- tuf*. The phylogenetic analysis was performed with the neighbor-joining test. Values on each branch indicate the display branch length. The percentage of identity between each branch was 100%. AX109531.1refer to *Citobacter ferundi*.

Most *Enterobacteriaceae* are opportunistic pathogens, especially *Escherichia coli, Klebsiella* spp., *Serratia* spp., *Enterobacter* spp. and *Salmonella*, and are associated with significant morbidity and mortality (2,5). These bacteria can cause many different kinds of infections in wounds (sepsis), brain (meningitis) and diarrhea (4). (9) showed that 42.3% of isolates belonged to *Citrobacter* isolated from animals and other studies have been confirmed that Citrobacter, associated with bacteremia most commonly occurred in patients with malignancies (48.9%) or hepatobiliary stones (22.2%) or Intra-abdominal tumors (59.1%) (3, 21). (16) reported that the percentage of *Citrobacter* isolation from intraabdominal infection was 61.1% while from urinary tract was 8.3% and intravascular catheter was 5.6%, whereas soft tissue was 2.8%, in comparison with (19) pointed out the percentage of isolation of *Citrobacter* from stool sample of children with diarrhea was 14%. Bacteremia, which commonly originated from sites of infection with *Citrobacter* such as the abdominal cavity, urinary tract and lung, consist of 51.1%, 20%, 11.1% respectively (11,17, 20).

A single or dinucleotide polymorphism made *tuf, atpD1* and *atpD2* more sufficiently in identification of bacterial isolates to species level. *tuf* and *atpD* tree exhibit very short genetic distances between taxa belonging to same genetic species including species segregated on the basis of clinical considerations (2) . The variation in the results of phylogenetic trees referred to the presence of SNP in *atpD* and *tuf* that lead to identification of bacterial isolates to sub species levels. One to three of SNP have been found in *tuf* and *atpD*. This SNP may result from hybridization between closely related species. Many studies referred to a closer relationship between *Salmonella* and *C. freundii* (10,19). (7) reported that *tuf* and *atpD* distances provide higher discriminating power at the species level.

Conclusions:

Elongation factor Tu and FATPase have been highly conserved throughout evolution and show functional constancy. Phylogenies based on protein sequences from elongation factor Tu and the F-ATPase b-subunit have shown good agreement with each other and with the rRNA gene sequence data. (19)

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