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Molecular Study of *16SrRNA* Gene in *Enterobacteriaceae* isolated from Iraqi Patients

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Abstract

This study included the collection of 204 clinical and non-clinical samples from Ibn-Al-Baladi childbirth Hospital, Al-Yarmouk Teaching Hospital and Imam Ali Hospital in Baghdad, from both genders of different ages. The collected samples were distributed according to the collection source (urine, wounds, burns, feces, Tigris River water in Baghdad and soil samples). A total of 31 isolates of *Escherichia coli* (48.43 %), 15 isolates *Klebsiella pneumoniae* (23.43 %), 10 isolates of *Enterobacter cloaca*e (15.62 %) and 8 isolates of *proteus mirabilis* (12.50 %) were isolated and identified based on microscopic culture, conventional methods, VITEK 2 and molecular identification of *16 SrRNA* gene. Our investigations indicated *that Escherichia coli and Klebsiella pneumoniae* species represent the most frequent isolates, whereas both *Enterobacter cloaca*e and *Proteus mirabilis* were the less frequent species. The results of the analysis of the sequencing of selected 21 isolates were deposited in the National Center for Biotechnology Information (NCBI) belong to the four species (From LC314477.1 to LC314497.1). [DOI: 10.22401/ANJS.00.1.11]

Keywords: *Enterobacteriaceae*,*16S rRNA*, PCR, Molecular Identification.

Introduction

Members of the family *Enterobacteriaceae* have some common bacteriological features. These individuals are rod-shaped, facultative anaerobes are gram-negative bacteria. They are may be either motile using peritrichous flagella or non-motile without any flagella. The Individuals of this family related with a wide range of disease as a results of infection of urinary tract, gastrointestinal tract and may cause meningitis, [1]. Among the species of this family which has a medical importance, *E. coli* and *Klebseilla pneumoniae*, [2]. While *Proteus mirabilis* and *Enterobactar cloacae* may lead to dangerous wide spread in the hospitals, [3,4]. The identification of bacteria isolated from clinical samples are one of the serious tasks of a clinical microbiology laboratory. The traditional identification of these isolates were achieved using a panel of inoculated biochemical reaction, [5]. At the same period an increasing use of commercial identification kit and semi-automated system were applied. Many researchers have been used API-20E for the identification of gram negative bacterial isolates, [6]. Other investigators were performed of the VITEK 2 system for identification and susceptibility testing of *Enterobacteriaceae* clinical isolates, [7]. Scientists conducted a numerous techniques (PCR, gel electrophoresis, *16S ribosomal RNA* gene sequencing and phylogenetic analysis) to identify the diversity of *Enterobacteriaceae* species, [8,9]. Small rRNA gene sequencing, particularly *16SrRNA* sequencing in bacteria, have led to advances on multiple fronts in microbiology. In this study, we applied many technical methods for the identification of *Enterobacteriaceae* strains isolated from infected Iraqi patients and the significance of identifying the organisms were discussed. The aim of this study was isolation and identification of frequently *Enterobacteriaceae* species in Iraqi patients in a comparison with the reference strains in Gen Bank.

2. Material and Methods

2.1 Samples collection

Two hundred and four samples were collected from Baghdad hospitals (Ibn-Al Baladi; Al-Yarmouk Teaching Hospital and Emam Ali Hospital) during the period from September 2016 to December 2016. These samples were collected from patients of different ages and genders. The collected samples represent clinical and environmental

samples including urine, stool, blood and burns whereas the environmental samples represented by water river and soil. All samples were taken under sterile conditions then sent to laboratory within 1-2 hrs.

2.2 Isolation and Identification of bacterial

Samples inoculated on culture media (EMB agar, blood agar, and MacConkey agar), were incubated at 37 ˚C for 24 hrs. under aerobic conditions, then examined for appearance, morphology of colonies, color and the positive cultures were undergoing to the biochemical tests (catalase, sugar fermentation, oxidase, IMVC).

2.3 Identification of *Enterobacteriaceae* species by Vitek® 2 compact

The isolates were identified by Vitek 2 compact auto analyzer system manufactured by Biomerieux – France. This technique was conducted in Microbiology Laboratory of Ibn-ALbaldi Hospital.

2.4 Genomic DNA preparation

Genomic DNA extraction was performed according to the Instruction Manual of Presto Mini gDNA bacteria kit of Geneaid Company by conducting their protocol procedure.

2.5 PCR-Gel electrophoresis

The 16*SrRNA* gene was amplified by Polymerase Chain Reaction (PCR) from the extracted DNA using the universal primers for *Enterobacteriaceae*, forward primers F (GGA TTA GAT ACC CTG GTA GTCC) and reverse primer R (TCG TTG CGG GAC TTA ACC CAA C).

The reactions were performed of each species in 20 µl containing 2 µl (100 ng) template DNA solution,10µl master mix (Interon corporation), 4 µl DDH_2O, 2 µl (10 Pmol) forward primer and 2 µl (10 Pmol) reverse primer. Nuclease free water was used as negative control. The PCR program were carried out using the following protocol for all bacterial species as follows :initial denaturation at 95 ℃ for 5 min., followed by 35 cycle (denaturation at 95 ℃ for 1min., annealing at 58 ℃ and extension at 72 ℃ for 1min) and final extension 72 ℃ for 7 min.

10 µl of each PCR product was electrophoresed in 1.5 % (w/v) agarose gel with a marker of 100 bp. was performed at 90 volts for 1.5 hour. The gel was stained with gel red 5 µl (0.05 %) and photographed under Ultraviolet light illumination then photographed using Gel photo documentation system.

2.6 Sequencing of the *16SrRNA* gene

Nucleotide sequencing of the PCR products of the studied species (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae*) were sent to Eton Bioscience Inc., USA company for sequencing in forward direction of primer *16 SrRNA.*

2.7 Analysis of DNA sequencing results

The nucleotide sequence of *16SrRNA* was analysed and alignment was done using the BioEdit ver.7.09 program to detect alteration of sequences sites and type, compared with reference sequences strains deposited in the National Center for Biotechnological Information**.**

3. Results

The collected samples (204 samples) included clinical samples (174) and environmental samples (30). The clinical samples were urine (60), wounds (33), burns (36) and stool (45) with environmental samples represented by Water River (15) and soil (15).

3.1 Identification and characterization of *Enterobacteriaceae* species by conventional methods

Table (1) represents different conventional methods (Gram stain, morphological characterization and biochemical tests) were performed in this study in order to identify various isolates.

3.2 Identification of *Enterobacteriaceae* species by Vitek 2 compact

Identification of *Enterobacteriaceae* species that characterized by the conventional methods were confirmed by Vitek 2 compact auto analyzer system. Table (2) showed the results of Vitek 2 which included 64 isolates identified as *Enterobacteriaceae* species as following 31 (48.43 %) isolate verified as *E.coli*, 10 (15.62%) isolate verified as *Enterobacter cloacae*, 8 (12.50 %) isolate verified as *Proteus mirabilis*, 15 (23.43 %)

isolate verified as *Klebsiella pneumonia*. Six isolates were unverified by Vitek 2 compact. Among bacterial species gained only 60 (93.75%) isolates were investigated from clinical samples which verified 29 isolates as *E.coli*, 10 isolates as *Enterobacter cloacae*, 8 isolates as *Proteus mirabilis* and 13 isolates as *Klebsiella pneumoniae.*

While 4 (6.25%) isolates were isolated from environment and verified 2 isolates as *E.coli* and 2 isolates as *Klebsiella pneumoniae* from both water river and soil respectively.

Table (1)
Different conventional methods used to identify various collected isolates.

Type of test	*Gram Stain*	*Motility*	*Indole*	*Methyl red*	*Vogas Proskaure*	*Simmon citrate*	*Catalase*	*Oxidase*	*Urease*	*H_2S*	*Gelatin liquefication*	*Glucose*	*Lactose*	*Co_2*	*Morphology shape*	*Triple sugar iron*	*Phenyl alanine deaminase*	*Maltose fermentation*	*D. Nase test*	*Utilization of raffinose*	*esculin hydrolyzed*	*ODC*	*ONPG*	*Sucrose*	*mannitol*
Escherichia coli	-	+	+	+	-	-	+	-	-	-	-	+	+	+	Rod	A/K A/A	NT	NT	NT	NT	NT	+	+	NT	+
Klebsiella pneumoniae	-	-	-	-	+	+	+	-	+	-	-	+	+	+	Rod	A/A	NT	NT	NT	NT	NT	-	+	+	+
Enterobacter cloacae	-	+	-	-	+	+	+	-	V	-	-	+	+	+	Rod	A/A	NT	NT	NT	NT	-	+	+	+	+
Proteus mirabilis	-	+	-	+	-	+	+	-	+	+	+	+	-	+	Rod	K/A	+	-	-	V	V	+	-	-	-
	(-) Negative , (+) Positive, A/A=Acid/Acid, K/A = Alkaline / Acid, V= Variable, NT= None Tested, ODC= Ornithine decarboxylase, ONPG= Orthonitrophenyl-B-D-galactopyranoide.																								

Table (2)
The distribution of Enterobacteriaceae species in various samples.

Sample	*% Samples*	*Distribution species of Enterobacteriaceae*				*Total number*	
		E. coli	*Enterobacter cloacae*	*Proteus mirabilis*	*Klebsiella pneumoniae*	N	%
Clinical samples **Urine 60**	29.41%	16	3	7	8	34	53.12%
Stool 45	22.05%	11	0	1	1	13	20.31%
Wounds 33	16.17%	1	2	0	4	7	10.93%
burns 36	17.64%	1	5	0	0	6	9.37%
Environmental samples **Water river 15**	7.35%	1	0	0	1	2	3.17%
Soil 15	7.35%	1	0	0	1	2	3.17%
		31 48.43%	10 15.62%	8 12.50%	15 23.43%	64	

3.3 Molecular Identification of *Enterobacteriaceae* spp. based on *16SrRNA* gene sequencing.

Amplification and melting conditions were optimized for the PCR assay, using universal primer sequences of *16SrRNA* (331bp) Fig.(1) sequencing of *16SrRNA* gene of 21 isolates were compared with the highly similar reference strains of the same species in GenBank using BLAST program in NCBI database then deposited in GenBank by accession no. from LC314477.1 to LC314497.1

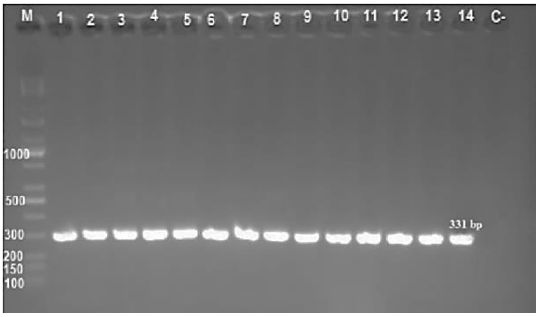

Fig.(1) Gel electrophoresis of PCR product of 16 SrRNA gene (331bp) for Enterobacteriaceae species using 1.5 % agarose gel at 5 Volt/ cm for 2 hour. Lane 1-14: PCR product positive for gene. C-: control negative (DNA free) .M: 100 bp DNA marker.

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                        310        320        330        340        350
                ...|....|....|....|....|....|....|....|....|....|....|.
KU981210.1(REF)  ATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGC
LC314477.1 E-41  .............................................
LC314478.1 E-42  .............................................
LC314479.1 E-44  .............................................
LC314480.1 E-51  .............................................
LC314481.1 E-36  ..........C..................................

                        360        370        380        390        400
                ...|....|....|....|....|....|....|....|....|....|....|.
KU981210.1(REF)  GTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
LC314477.1 E-41  .............................................
LC314478.1 E-42  .....G.......................................
LC314479.1 E-44  .....G.......................................
LC314480.1 E-51  .....G.......................................
LC314481.1 E-36  .....G.......................................

                        410        420        430        440        450
                ...|....|....|....|....|....|....|....|....|....|....|.
KU981210.1(REF)  ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA
LC314477.1 E-41  .............................................
LC314478.1 E-42  .............................................
LC314479.1 E-44  .............................................
LC314480.1 E-51  .............................................
LC314481.1 E-36  .............................................

                        460        470        480        490        500
                ...|....|....|....|....|....|....|....|....|....|....|.
KU981210.1(REF)  ACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATG
LC314477.1 E-41  .............................................
LC314478.1 E-42  ..........................................G..
LC314479.1 E-44  .............................................
LC314480.1 E-51  .............................................
LC314481.1 E-36  ..........................................G..

                        510        520        530        540        550
                ...|....|....|....|....|....|....|....|....|....|....|.
KU981210.1(REF)  GATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAG
LC314477.1 E-41  .T...........................................
LC314478.1 E-42  .............................................
LC314479.1 E-44  .............................................
LC314480.1 E-51  .T.................C...........................
LC314481.1 E-36  ..................C...........................

                        560        570        580        590
                ...|....|....|....|....|....|....|....|...
KU981210.1(REF)  CTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGA
LC314477.1 E-41  ...................................
LC314478.1 E-42  ...................................
LC314479.1 E-44  ...........................C.......
LC314480.1 E-51  ...........................C.......
LC314481.1 E-36  ...................................
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Fig.(2) The alignment part of the 16 SrRNA sequences of five isolates of E. coli bacteria with standard isolate of E. coli (KU981210.1).

Fig. (2) showed the results of the 16SrRNA gene sequence analysis of E. coli were summarized in Table (3) indicating the number and location of alterations and showed that there were 7 alterations in 5 samples in this gene with more than one alterations in each sample.

Table (3)
Alterations in the gene sequence of 16 SrRNA in E. coli bacteria isolated from different patient samples.

The sample number	*(Acc.NO.)*	*The location of alterations*
36 Sh	LC314481.1	318
Sh42,Sh44,Sh51,Sh36	LC314478.1- LC314481.1	362
42, Sh36 Sh	LC314478.1, LC314481.1	494
41,Sh 51 Sh	LC314477.1, LC314480.1	508
51 Sh	LC314480.1	527
36 Sh	LC314481.1	526
44, Sh51 Sh	LC314479.1**,** LC314480.1	583

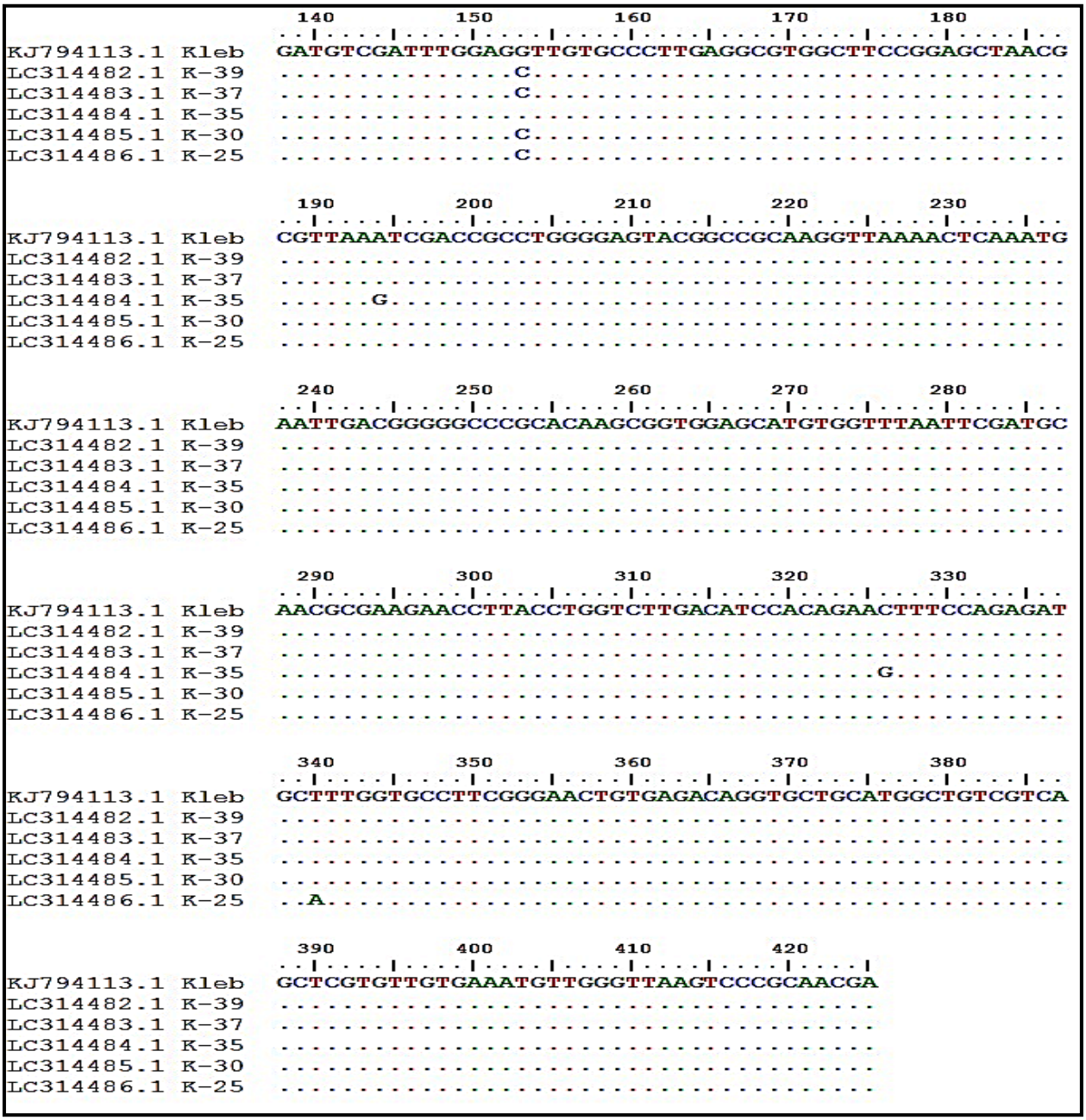

Fig.(3) The alignment of part of the 16 SrRNA sequences of five isolates of K. pneumoniae bacteria with standard isolate (KJ794113.1)) of K.pneumoniae was recorded in GenBank.

The results of the analysis of the *16 SrRNA* sequences of *K. pneumoniae* shown in Fig.(3) and Table (4) showed that there were 3 alterations in 5 samples and the location of the alterations found in them.

Table (4)
Alterations in the gene sequence 16 SrRNA in K. pneumoniae isolated from different pathological samples.

The sample number	(Acc.NO.)	The location of alterations
Sh 39,Sh 37 Sh 30,Sh 25	LC314482.1, LC314483.1 LC314485.1, LC314486.1	53
Sh 35	LC314484.1	194
Sh 25	LC314486.1	340

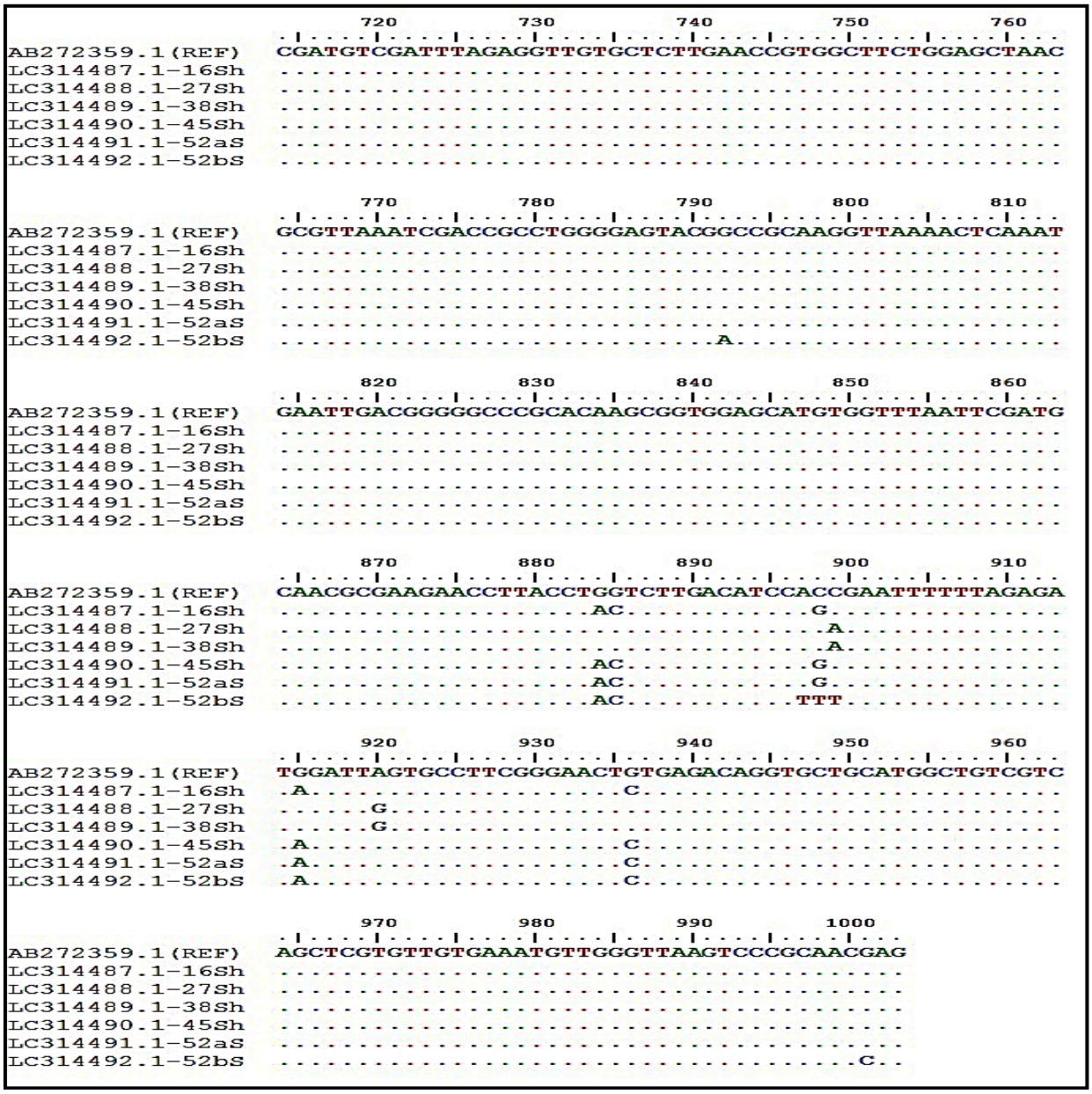

Fig.(4) The alignment of part of the 16 SrRNA sequences of five isolates of Proteus mirabilis bacteria with standard isolate (AB272359.1) of Proteus mirabilis was recorded in GenBank.

The results of the *16 SrRNA* analysis of *Proteus mirabilis* shown in Fig.(4) and Table (5) showed 5 alterations in 5 samples of the same gene.

Table (5)
Alterations in the gene sequence 16 SrRNA in Proteus mirabilis isolated from different pathological samples.

The sample number	(Acc.NO.	The location of alterations
52bS	LC314492.1	792
16Sh,45Sh 52aS,52bS	LC314487.1, LC314490.1 LC314491.1, LC314492.1	885
16Sh,45Sh 52aS, 52bS	LC314487.1, LC314490.1 LC314491.1, LC314492.1	898
16Sh,45Sh 52aS,52bS	LC314487.1, LC314490.1 LC314491.1, LC314492.1	915
16Sh,45Sh 52aS,52bS	LC314487.1, LC314490.1 LC314491.1, LC314492.1	936

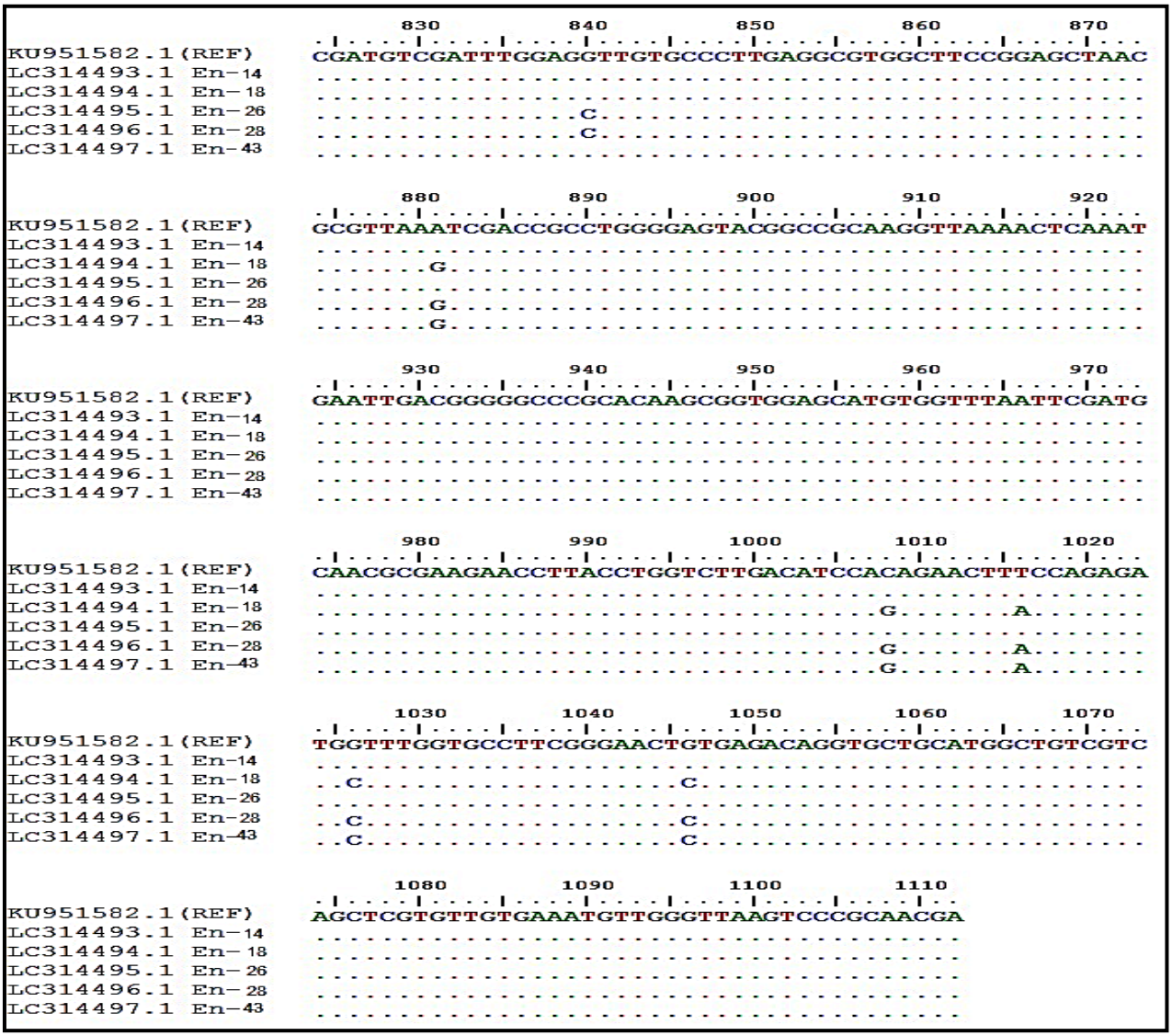

Fig.(5) The alignment of part of the 16 SrRNA sequences of five isolates of Enterobacter cloacae bacteria with standard isolate (KU951582.1) of Enterobacter cloacae was recorded in GenBank.

The results of the *16 SrRNA* gene sequence analysis were summarized in Fig.(5) and Table (6), showing that there were 6 alterations in 5 samples of the *16 SrRNA* gene, and that there was a single alteration in one sample or more than one alteration in other samples.

Table (6)
Alterations in the gene sequence 16 SrRNA in Enterobacter cloacae isolated from different pathological samples.

The sample number	(Acc.NO.)	The location of alterations
26 Sh, 28Sh	LC314495.1, LC314496.1	840
18 Sh, 28Sh, 43Sh	LC314494.1, LC314496.1, LC314497.1	881
18Sh, 28Sh, 43Sh	LC314494.1, LC314496.1, LC314497.1	1008
18Sh, 28Sh, 43Sh	LC314494.1, LC314496.1, LC314497.1	1016
18Sh, 28Sh, 43 Sh	LC314494.1, LC314496.1, LC314497.1	1026
18Sh, 28Sh, 43Sh	LC314494.1, LC314496.1, LC314497.1	1046

Discussion

Our results revealed that *Enterobacteriaceae* species (*E. coli, Klebsiella pneumonia, Enterobacter cloacae, Proteus mirabilis*) were found in urine (53.12 %), stool (20.31 %), blood (10.93 %), burn (9.37 %), and (3.17 %) for both river water and soil as shown in Table (2). The present results showed that urine samples of a higher percentage of species isolates *E. coli* (48.43 %) and *Klebsiella pneumoniae* (23.43 %) which represent the most frequent isolates gained from Ibn – ALBaladi Hospital for children. So, most urine samples were taken from pregnant women infected with UTI and diabetics and their new borns have also UTI. Pregnant women normally undergo many physiological changes such as high level of glucose in their urine, which will be suitable environment for UTI infection. The higher level of *E. coli* (48.43 %) and *Klebsiella pneumoniae* (23.43 %) may due to their multidrug resistance and nominated for dissemination in the clinical and environment samples in Iraq, [10]. Meanwhile the less frequent isolates *Proteus* mirabilis (12.50 %) and *Enterobacter cloacae* (15.62 %) may be due to absence of *Proteus mirabilis* in blood and burns samples whereas *Enterobacter cloacae* disappeared in stool samples.

Enterobacter cloacae can be considered one of the important opportunistic community infections. This bacteria regarded as one of gram negative has been largely described during several outbreaks of hospital acquired infection in Europe and particularly in France, [11]. On sight of the results obtained the percentage of *Enterobacter cloacae* isolated from various samples occupied the third sequence of gram negative bacteria distributed in Baghdad. Whereas other local study [12] their results indicated the wide distribution *Enterobacter cloacae* with other *Enterobacter spp*. in Basra hospital environment. The difference between our findings and the conclusion of the last investigators attributed to the number of samples taken from the hospital environment with few numbers of the clinical samples. So, they got higher percentage of bacterial contamination in the hospital environment with less percentage for the clinical samples. This study showed that the isolates of *Proteus mirabilis* represents the fourth sequence of less frequent bacteria as indicated in Table (1). The total percentage of isolation of this species was slightly differing from Egyptian study, [13]. They obtained 8.2 % from clinical samples of less frequent bacteria in Egypt.

Conclusion

DNA sequencing of the samples revealed the first record of five selected 16*SrRNA* sequencing isolated from each species (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae*) participating the enrichment of international library with *16 SrRNA* sequencing of *Enterobacteriaceae* species in Iraq. *E.coli* and *Klebsiella pneumoniae* represent the most frequent isolates gained in Iraq. While the less frequent isolates *Proteus mirabilis* and *Enterobacter cloacae.*

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