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Research Article

Phylogenetic monitoring of *Escherichia coli* in medical wastewater/ Baghdad City

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Abstract
Clean drinking water access is a main factor that supports general health worldwide, where important investment is made to maintain water quality. The gene sequence of 16S rRNA was applied to study bacterial phylogenesis and classification. This study aimed to isolate new bacterial strains from wastewater environments. Thisstudy collected water samples from different wastewater sites for Baghdad Medical City's Hospital. The results of Vitek 2Compact were with a probability of99% for *Escherichia coli* isolates .The bacterial isolates were identified using Polymerase chain reaction (PCR) based on the universal diagnostic gene 16SrRNA, and the PCR product was obtained with a molecular weight of 1250bp. The PCR productsequencing showed 166 isolates of *E. coli* different from the isolates registered in the NCBI database of *E. coli* after alignment between them. The present study reported 16 Iraqi isolates in NCBI with an accession number: OM032663.1; OM 032664.1; OM294659.2; ON724178.1; ON724264.1; ON724331.1; ON725076.1; ON725091.1; ON725139.1; ON725141.1; ON739200.1; ON739201.1; ON739207.1; ON739208.1; ON739209.1; ON739211.1. The identity was then observed(96-100%) with strains in Gene Bank. There were many different sequence position substitutions (transition, deletion, transversionfor the registered local isolates when conducting multiple alignments and comparing themwith the isolates in the Gene Bank. According to the phylogenetic tree building by joining-the neighbor method, the local Iraqi isolates clustered into sub-descents. Moreover, local isolates of *E.coli* appeared more convergent to Saudi Arabia isolates (KY780347.1). This may be due to the geographical proximity of the two countries to the same Arab Gulf region .The present study identified 16 isolates of *E. coli* from the Iraqi aquatic environment (medical wastewater). The novelty of this study was represented to monitoring the evolution of *E. coli* in medical wastewater.

Keywords: Antibiotics, *Escherichia coli*, Genetic detection, Multi-Drug Resistance, Tigris River

INTRODUCTION

Clean drinking water access is a main factor that supports general health worldwide, where important investment is made to keep water quality (Fewtrell and Bartram, 2001). The 16S rRNA sequence is the main one used to survive cells and is greatly preserved all over evolution due to the demand of complicated inter- and intra-molecular reactions to save the mechanism of protein synthesis (Sacchi *et al.*,2002). Therefore, the gene sequence of 16S rRNA was applied to study bacterial phylogenesis and classification, where16S rRNA was the genetic marker of housekeeping utilized for many causes. The causes involve: The 16S rRNA found in almost all bacteria, present as a family of multigene or operons; the role of 16S rRNA gene was not altered at all times, indicating that sequence alterations random are much exact measurement of evolution; also 1500 bp of 16S rRNA gene is great adequate for informatics objective (Janda and Ab-

bott,2007).Phylogenetic indications involve the existence of particular protein-coding or constitutional genes, the compositions of these genes, and their variation, insertion, and frequency of components. Those genetic markers involve 16S rRNA (1500 base pair) gene encoding for catalytic of RNA where it is as a portion of the subunit 30S ribosome ,which has the eligible characteristics that permit it to be employed as a commonly utilized marker. The functional stability of such a gene confirms it is a useful molecular chronometer, and it is major for an accurate estimation of the phylogenetic association of organisms. 16S rRNA is found in all prokaryotes and is preserved and changing sequence parts evolving at various rates, which is important for amplifying and determining distant phylogeny associations. These features permit the employ of 16S rRNA in the assessment of close associations in the genus (Srinivasan *et al.*,2015). Analysis of sequencing of some strains (such as *Escherichia coli* strain BzDS03) showed that its adjacent relative was *E. coli* DH1 (ME8569) with sequence similarity at 99%. It is renowned that strains of *E. coli* are less harmful and survive in the intestines of healthy humans and animals, while some strains generate a potent toxin and cause intense disease. One of the strains *E. coli* (O157:H7) arises to cause food-borne and waterborne disease. *The presence of E. coli* in water is considered an index for fecal pollution and a risk to human health and the environment (Magray *et al.*,2011; Mahdi *et al*.,2023). The present study aimed to isolate new bacterial strains from wastewater environments.

MATERIALS AND METHODS

Samples collection

Water samples (40 samples) were collected from different sites (Adhamiya region, wastewater for Baghdad Medical City's Hospital, and Abu Nuwas region). The period sampling was from February to June 2021. The collection was in 1000ml sterile glass containers. After that, these containers were transferred to the laboratory to identify bacteria and detect 16S rRNA in bacteria.

Isolation and identification of *Escherichia coli*

The serial dilution method in normal saline was applied after the culturing on Nutrient agar, MacConkey agar, and EMB agar. The incubation was for 48 h at 37°C. *E. coli* colonies were diagnosed phenotype by gram stain. Also, *E. coli* was detected by VITEK2 Compact (GN ID :21341. The sub-cultures were carried out several times and were stored for genetic detection.

DNA Isolation and Polymerase Chain Reaction Amplification of 16SrRNA genes from *Escherichia coli*

DNA of bacteria was extracted by Bacterial DNA Mini-Prep™ Zymo Inc. (Catalog No. D6005) and specific primer 16srRNA gene) IDT, Canada) as in Table 1. **(**Miller *et al.,*2013). The polymerase chain reaction (PCR) for 16SrRNA genes was conducted on 40 isolates and was confirmed to be *E. coli*. The reaction contents (25 µl) involved 5µl of 2xTaq PCR PreMix (iNtRON, Korea), 1 µl of forward and reverse primer 10 picomoles/µl (1 µl), 1.5µl of DNA template, and 16.5 µl free nuclease water. The condition of amplification was initial denaturation for 3 min. at 94°C, after the second denaturation for 45 sec.at 94°C for 35 cycles, annealing was for 45 sec. 56°C for 35 cycles, and the extension was for 1 min. at 72°C for 35 cycles. Meanwhile, the final extension was for 7 minutes.at 72°C. The electrophoresis of PCR products was conducted by using 2% agarose gel and was visualized by UV after staining by red safe stain (Intron Korea) and used DNA marker ladder 10000bp (Intron Korea) **(Miller** *et al.,*2013).

DNA sequencing

PCR products were carried out sequencing by DNA sequencer (Applied Biosystem Inc /Macrogen Korea). The outcomes were analyzed using the Basic Local Alignment Search Tool (BLAST) program available on the NCBI (National Center for Biotechnology Information) and BioEdit program website to know the sequence and number of nitrogenous bases.

Phylogenetic tree analysis

The history of evolution was estimated using Neighbor-Joining method. The distances of evolution were estimated using the Jukes-Cantor model for determining phylogenetic distances designed in the MEGA11 program (Tamura *et al.*,2011).

Ethical approval

The research protocol was approved by Ministry of Science and Technology/Environment, Water and Renewable Energy Directorate, Iraq.

RESULTS AND DISCUSSION

The outcomes of E.coli diagnosis by phenotype characteristics showed that it is a gram-negative, rod-shaped stain. The results of Vitek 2Compact were with a probability 99% for *E.coli* isolates. The bacterial isolates were identified by molecular diagnosis using PCR, based on the diagnostic universal gene 16SrRNA, characterized by being a stable gene with intragenomic heterogeneity for a long time in the species. The results showed that the PCR product was obtained with a molecular weight of 1250bp (Fig. 1). Srinivasan *et al.* (2015)showed the PCR method is considered one of the fastest and most effective methods for diagnosing bacteria and the most sensitive in pathogens diagnosing. This gene contains conserved regions that interfere with variable regions that are important in determining

bacterial species and genera due to the existence of gene 16S rRNA gene in all bacterial species (Somerville *et al*.,2020) .

Also, the PCR product sequencing showed 16 isolates of *E. coli* differ from the isolates registered in the NCBI database of *E. coli* after alignment between them. The present study registered 16 Iraqi isolates in NCBI with an accession number: OM032663.1; OM032664.1; OM294659.2; ON724178.1; ON724264.1; ON724331.1; ON725076.1; ON725091.1; ON725139.1; ON725141.1; ON739200.1; ON739201.1; ON739207.1; ON739208.1; ON739209.1; ON739211.1. The identity was observed to be 96-100% with Gene Bank strains isolated from China, South Africa, Pakistan, Bangladesh, Saudi Arabia, Nigeria, USA. There were many different sequence position substitutions (transition, deletion, transversion) for the registered local isolates when conducting alignment and comparing with the isolates in the Gene Bank by BLAST program (Table2). The sequence similarity values computed by the Clustal Omega program indicated that the similarity matrix for sequencing 16s rRNA among local isolates ranged from 85.26-99.8%. The local isolate (OM032663.1) appeared similar 99% to isolates

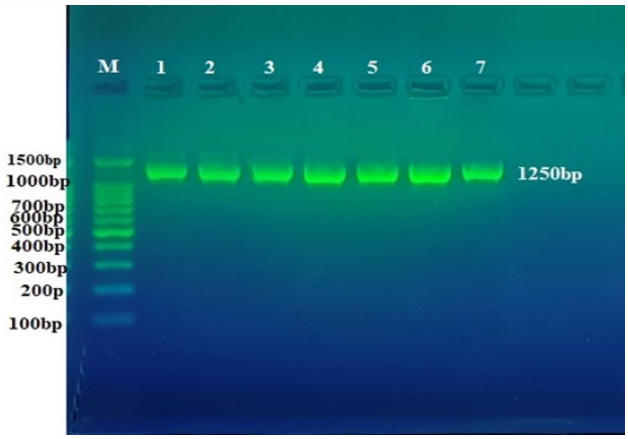

Fig.1. *PCR product of 16SrRNA (125 0bp) for samples (1,2,3,4,5,6,7) using electrophoresis on 1.5% agarose at 75 volt/15cm^2. TBE buffer(1X) for 1:30 hr.DNA ladder (1500bp)*

(ON72 5091.1; ON725141.1; OM294659.2; OM032664.1), while the isolate ON739201.1 had the similarity matrix 85.26% with the isolate ON725139.1 as shown in (Fig.2).

The phylogenetic tree building by the neighbor-joining method (Fig. 3) indicated that the local Iraqi isolates clustered into sub-descents. Moreover, local isolates of

Table 1. Specific sequence primers 16srRNA) Miller *et al.,*2013)

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- AGAGTTTGATCCTGGCTCAG- 3'	54.3	50.0	1250bp
Reverse	5'- GGTTACCTTGTTACGACTT- 3’	49.4	42.1	

Seq->	ON 724264.1	ON 725139.1	ON 724331.1	ON 724178.1	ON 725076.1	ON 725091.1	ON 725141.1	OM 294659.2	OM 032664.1	OM 032663.1	ON 739207.1	ON 739200.1	ON 739211.1	ON 739209.1	ON 739201.1	ON 739208.1
ON724264.1	ID															
ON725139.1	94.77	100.00														
ON724331.1	96.88	95.59	100.00													
ON724178.1	96.78	95.69	96.99	100.00												
ON725076.1	96.28	95.99	97.49	96.89	100.00											
ON725091.1	97.29	96.39	97.59	97.69	97.59	100.00										
ON725141.1	96.88	96.79	97.49	97.59	97.59	98.80	100.00									
OM294659.2	97.19	97.39	97.89	98.09	98.40	98.80	99.20	100.00								
OM032664.1	97.29	97.09	97.99	98.19	98.09	98.90	98.90	99.50	100.00							
OM032663.1	97.49	97.29	98.19	98.40	98.29	99.10	99.10	99.70	99.80	100.00						
ON739207.1	89.01	86.88	89.46	89.35	89.46	89.57	89.46	89.57	89.69	89.80	100.00					
ON739200.1	88.68	86.43	88. 68	89.01	88.57	89.35	89.13	89.35	89.35	89.46	87.87	100.00				
ON739211.1	88.32	86.10	87.99	88.43	88.10	88.54	88.77	88.99	88.43	88.65	89.51	91.91	100.00			
ON739209.1	88.90	86.88	88.57	88.90	88.68	89.24	89.57	89.69	89.24	89.46	91.79	87.10	90.14	100.00		
ON739201.1	87.85	85.26	87.40	87.74	87.85	87.96	87.96	87.96	87.96	88.08	89.18	88.17	88.54	87.10	100.00	
ON739208.1	89.97	87.54	89.86	90.08	89.75	90.41	90.19	90.19	90.52	90.52	91.13	89.81	90.45	88.94	90.41	100.00

Fig. 2. *Similarity matrix of sequence 16SrRNA gene among local Escherichia coli isolates*

Table 2. Alignment of local *Escherichia coli* isolates with global isolates

Isolate	Type of substitution	Location subject	Nucleotide (Subject/ Query)
OM032663.1	Deletion	1	-/A
	Transition	38	C/T
	Transition	947	G/A
	Transversion	965	A/T
	Transversion	967	T/G
OM032664.1	Transversion	34	T/G
	Transversion	664	C/G
	Transversion	674	C/G
	Transversion	940	A/T
	Transition	947	G/A
	Transition	951	C/T
	Transition	964	G/A
	Transition	983	T/C
OM294659.2	Transversion	195	A/T
	Transversion	198	A/T
	Transition	846	A/C
ON724178.1	Transition	528	A/G
	Transition	725	A/G
	Transition	760	A/C
	Transition	859	A/C
	Transversion	887	G/C
	Transition	890	G/T
	Transition	895	T/C
	Transversion	904	T/A
	Transition	916	G/T
	Transversion	918	G/C
	Transition	919	A/G
	Transversion	941	T/A
	Transition	948	A/G
	Transition	951	T/G
	Transition	955	C/T
	Transition	957	A/C
ON724264.1	Transition	528	A/G
	Transition	726	A/G
	Transversion	730	G/C
	Transition	769	G/T
	Transversion	797	G/C
	Transition	827	C/A
	Transition	845	A/C
	Transition	850	T/G
	Deletion	851	A/-
	Transition	859	A/C
	Transition	890	G/T
	Transition	892	G/A
	Transition	895	T/C
	Transition	908	G/A
	Transition	914	A/C
	Transversion	917	C/G
	Transition	930	C/T
	Transition	945	A/C
	Deletion	956	C/-
	Transition	958	A/G
	Deletion	964	-/T
	Transition	969	G/T
	Deletion	970	G/-
ON724331.1	Transition	675	G/A
	Transversion	690	G/C
	Transition	726	A/G
	Transition	729	A/C
	Transversion	736	G/C
	Transition	769	G/T

Table 2. *Contd.*

ON724331.1	Transition	791	G/A		
	Transition	806	G/A		
	Transition	829	T/C		
	Transition	841	C/T		
	Transition	845	A/C		
	Transversion	847	G/C		
	Transition	849	T/G		
	Transition	850	A/G		
	Transition	855	C/T		
	Transition	880	G/T		
	Transversion	881	G/C		
	Transition	884	T/C		
ON725076.1	Transversion	195	A/T	ID:MN314217.1	98.28%
	Transition	675	G/A		
	Transversion	678	G/C		
	Transition	687	G/A		
	Transition	690	G/A		
	Transition	791	G/A		
	Transversion	794	G/C		
	Transition	806	G/A		
	Transversion	843	G/C		
	Transition	846	A/C		
	Transversion	871	G/C		
	Transversion	878	G/C		
	Transversion	883	A/T		
	Transition	892	G/A		
	Transition	895	T/C		
	Transversion	968	G/C		
	Transition	971	G/T		
ON725091.1	Transition	746	A/G	ID:KY780351.1	99.6%
	Transition	915	T/G		
	Transversion	961	A/T		
ON725139.1	Transversion	20	A/G	ID:MN314188.1	99.09%
	Deletion	25	-/A		
	Transition	38	C/T		
	Transversion	143	A/T		
	Transversion	163	T/A		
	Transition	171	G/A		
	Transition	203	G/A		
	Transversion	219	A/T		
	Transition	388	G/A		
	Transition	398	G/A		
	Transversion	402	A/T		
	Transition	403	A/G		
	Deletion	411	A/-		
	Transversion	415	T/A		
	Deletion	418	-/T		
	Transition	433	C/T		
	Transition	534	T/C		
	Transition	538	T/C		
	Transition	544	A/G		
	Transition	582	T/C		
	Transversion	586	A/T		
	Transition	594	G/A		
	Transition	605	G/T		
	Transition	689	G/A		
	Transition	717	G/T		
	Transition	755	C/T		
	Transition	947	G/A		
	Transition	950	C/T		
	Transversion	961	A/T		
ON725141.1	Deletion	25	-/A	ID:OP889003.1	99.6%
	Transition	726	A/G		
	Transition	950	C/T		
	Transition	987	G/A		

Table 2. *Contd.*

ON739200.1	Deletion	5	A/-	**ID:FN356960.1**	**95.48%**
	Transversion	14	A/T		
	Transition	25	G/C		
	Transition	26	A/C		
	Transition	669	C/T		
	Deletion	676	-/C		
	Deletion	680	-/A		
	Transversion	686	C/G		
	Transition	689	C/T		
	Transition	690	C/T		
	Deletion	691	-/T		
	Deletion	707	-/T		
	Deletion	744	-/C		
	Deletion	764	A/-		
	Deletion	774	-/A		
	Deletion	780	-/G		
	Deletion	781	-/A		
	Transition	792	C/T		
	Deletion	794	G/-		
	Transition	798	G/T		
	Deletion	806	-/T		
	Deletion	821	A/-		
	Transition	829	C/T		
	Transition	832	C/T		
	Deletion	838	-/G		
	Deletion	839	-/G		
	Transition	845	G/T		
	Transition	847	C/A		
	Deletion	849	-/T		
	Transition	853	A/C		
	Deletion	854	G/-		
	Deletion	855	G/-		
	Deletion	860	A/-		
	Deletion	861	A/-		
	Deletion	873	-/T		
	Deletion	877	-/T		
	Transition	882	C/T		
	Deletion	884	-/T		
	Deletion	889	-/C		
	Deletion	911	-/G		
ON739201.1	Transversion	9	A/T	ID:MN314217.1	95.73%
	Transition	20	G/C		
	Transition	21	A/C		
	Deletion	24	A/-		
	Transition	37	T/C		
	Transversion	198	A/T		
	Deletion	511	-/G		
	Deletion	656	-/G		
	Deletion	662	-/A		
	Deletion	668	-/G		
	Transversion	673	C/G		
	Transition	675	C/T		
	Deletion	677	-/T		
	Deletion	705	G/-		
	Deletion	760	A/-		
	Deletion	769	-/C		
	Transversion	786	C/T		
	Deletion	790	G/-		
	Deletion	808	-/G		
	Deletion	823	A/-		
	Deletion	834	-/G		
	Deletion	841	G/-		
	Transition	943	C/G		
	Deletion	847	A/-		

Table 2. *Contd.*

	Deletion	849	G/A		
	Transition	851	T/-		
	Deletion	854	A/-		
	Transition	855	A/-		
	Deletion	860	A/-		
	Deletion	866	T/-		
	Deletion	870	G/C		
	Deletion	877	-/T		
	Deletion	884	A/-		
	Transversion	891	-/G		
	Deletion	893	-/A		
	Deletion	896	-/C		
ON739207.1	Deletion	8	-/C	ID:KR080743.1	96.68%
	Transition	12	A/G		
	Transition	13	G/T		
	Transition	27	T/C		
	Deletion	392	A/-		
	Transition	653	C/T		
	Deletion	660	-/C		
	Transition	674	C/T		
	Deletion	704	-/G		
	Deletion	710	-/G		
	Deletion	714	A/-		
	Transition	760	C/T		
	Transition	762	T/G		
	Transition	776	C/T		
	Deletion	782	G/-		
	Deletion	786	G/-		
	Transition	792	C/G		
	Transition	814	C/T		
	Deletion	815	C/-		
	Transition	818	C/T		
	Transition	819	T/G		
	Transition	830	G/T		
	Transition	831	C/T		
	Transition	832	C/-		
	Deletion	840	A/-		
	Deletion	850	T/-		
	Transversion	857	G/C		
	Deletion	861	-/C		
	Deletion	876	-/A		
ON739208.1	Transition	15	G/T	ID:KY780343.1	95.76%
	Deletion	22	A/-		
	Transition	31	A/T		
	Transversion	42	G/C		
	Transition	43	A/C		
	Deletion	550	-/G		
	Transition	580	C/T		
	Deletion	593	-/G		
	Transition	686	C/T		
	Deletion	693	-/C		
	Deletion	697	-/A		
	Transition	703	C/G		
	Deletion	706	-/T		
	Deletion	710	-/G		
	Deletion	726	G/-		
	Deletion	780	A/-		
	Deletion	793	-/T		
	Transition	808	C/T		
	Transversion	819	G/C		
	Deletion	852	-/G		
	Deletion	853	-/G		
	Deletion	862	C/-		
	Transition	866	A/C		
	Transition	867	G/C		

Table 2. *Contd.*

	Deletion	868	G/-		
	Deletion	870	T/-		
	Deletion	871	A/-		
	Transition	880	A/T		
	Transition	890	G/T		
	Transition	895	C/T		
	Deletion	897	-/T		
	Deletion	903	A/-		
	Deletion	917	-/T		
	Transition	921	G/A		
	Deletion	924	T/-		
	Deletion	926	A/-		
	Deletion	934	-/G		
	Transition	937	A/C		
ON739209.1	Transversion	9	A/T	ID:LC270238.1	96.27%
	Transversion	20	G/C		
	Transition	21	A/C		
	Transversion	625	C/G		
	Deletion	668	-/G		
	Deletion	672	A/-		
	Deletion	775	G/-		
	Transition	784	C/T		
	Deletion	794	G/-		
	Deletion	819	-/C		
	Transversion	823	C/G		
	Transition	826	C/T		
	Deletion	831	G/-		
	Deletion	838	G/-		
	Deletion	840	C/-		
	Deletion	844	A/-		
	Deletion	848	G/-		
	Transition	852	A/G		
	Deletion	854	A/-		
	Deletion	862	-/T		
	Deletion	866	T/A		
	Deletion	868	-/G		
	Transversion	878	C/G		
	Deletion	884	A/-		
	Transition	889	T/G		
	Deletion	892	-/G		
	Deletion	895	-/T		
ON739211.1.	Transversion	10	G/C	ID:MK156322.1	96.87%
	Deletion	13	-/T		
	Deletion	20	A/-		
	Transition	29	A/T		
	Transversion	40	G/C		
	Transition	41	A/C		
	Deletion	468	G/-		
	Transition	577	C/T		
	Transition	685	C/T		
	Deletion	692	-/C		
	Deletion	696	-/A		
	Transversion	702	C/G		
	Transition	704	C/T		
	Deletion	706	-/T		
	Deletion	782	A/-		
	Deletion	792	-/A		
	Deletion	796	-/T		
	Deletion	797	-/G		
	Deletion	813	-/T		
	Deletion	824	-/C		
	Deletion	827	-/T		
	Deletion	828	-/C		
	Deletion	845	-/T		
	Transition	863	C/T		

Table 2. *Contd.*

Transition	863	C/T
Deletion	881	-/T
Deletion	895	G/-
Transition	904	A/G
Deletion	905	G/C
Deletion	912	-/G

E.coli appeared more convergent to Saudi Arabia isolates (KY780347.1) with 65bot strap. This may be due to the geographical proximity of the two countries to the same Arab Gulf region. Also, it was observed some local isolates were divergent from their ancestors, as in local isolates ON725139.1, ON739209.1, and ON739200.1

Sequencing and establishing the phylogeny tree have a major role in improving bacterial recognition, structure, and epidemiological prevalence (Humphreys *et al.*,2019). The previous studies confirmed the successful employment of the sequences of the 16S rRNA gene for the detection of clinical pathogenic bacterial isolates (Patel,2001). Also, Nakano *et al.* (2023) showed in their study that it can monitor evolution bacteria by using sequencing of 16S rRNA gene can distinguish among *Escherichia coli*, *Shigella*, Yersinia, *Klebsiella*, and *Neisseria* spp. Another study identified *E. coli* in Rainbow Trout using 16S rRNA (Fattahi *et al.*, 2013). The evolution speed theory illustrated the high speciation percentage in tropical area, i.e., high temperatures caused the rapid evolution process due to short proliferation times, raised mutation, and increased natural selection. The recent research has also aimed to find the association between the rate of genetic evolution and temperature depending on a hypothesis that the rate of metabolism sets the rate of mutation (Chu *et al.*,2018). Several factors impact *E. coli* growth, such as nutrients, temperature, solar radiation, pH, water availability, completion with other organisms, and biofilm formation in environments (Jang *et al.*,2017).

Conclusion

The current study showed there are 16 isolates of *E. coli* in medical wastewater that differ from the isolates registered in the NCBI database of E. coli when it was

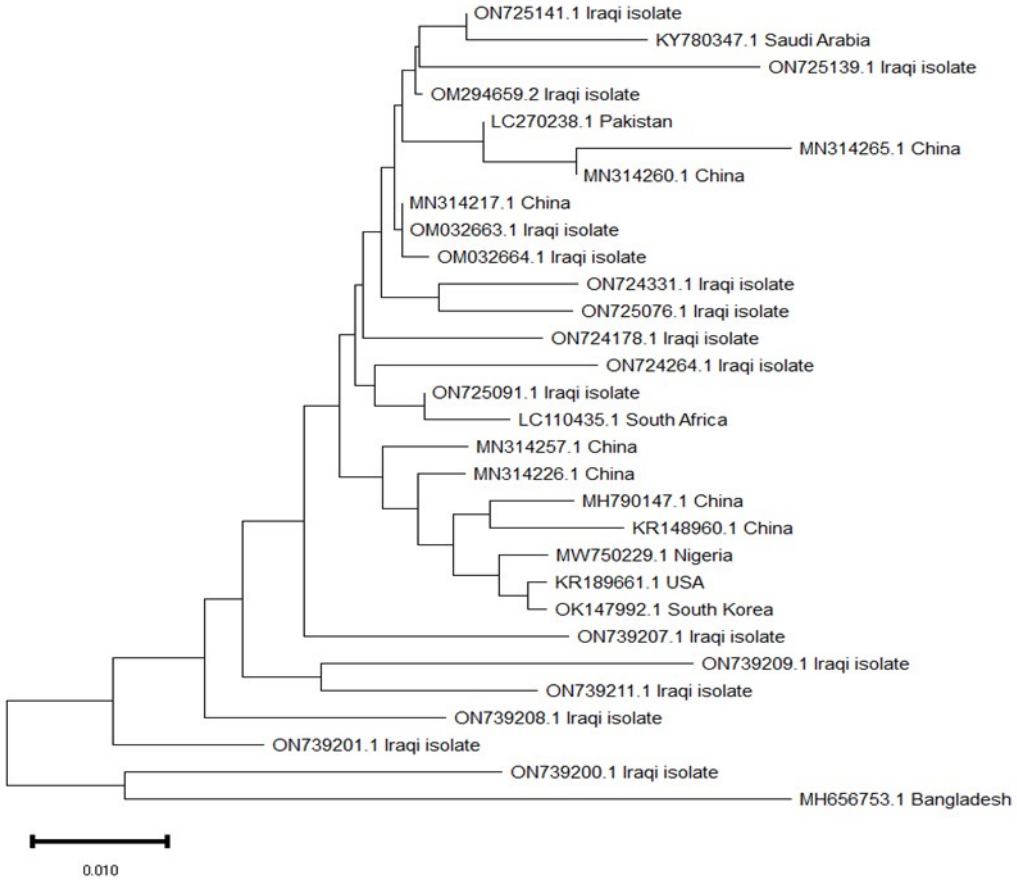

Fig. 3. *Phylogenetic relationships for local Escherichia coli isolates in phylogenetic tree. Historical evolution deduced by the Neighbour-Joining method*

conducted PCR product sequencing and alignment with NCBI data. Moreover, local isolates of *E. coli* appeared more convergent to Saudi Arabian isolates. Therefore, this study demonstrated novelty by monitoring the evolution of *E. coli* in medical wastewater.

Conflict of interest

The authors declare that they have no conflict of interest.

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