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IDENTIFICATION OF *ENTEROBACTER* SPP. BY 16SRRNAGENE SEQUENCING IN BASRAH PROVINCE/IRAQ

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ABSTRACT: *Eighty four of Enterobacter spp. were isolated from different specimens (Clinical,environmental ,food)from basrah hospitals that they were identified by traditional characteristics and Vitek 2 compact system were subjected to 16SrDNA sequencing using universal primer after extracted genomic DNA by ExiPrep^{TM}16 fully automated nucleic acid extraction system(Bioneer),where as a single discrete PCR amplicon band of 1500bp of 16SrDNA was gave by all isolates on agarose gel .The isolates showed different similarity levels (97-100%) when compared with reference strains sequences in Genbank, whereas this results indicated the isolates belong to different species for two genera (Enterobacter , Cronobacter), whereas one species belong to Cronobacter (C.sakazakii) seven species belong to Enterobacter (E.cloacae ,E.xiangfangensis ,E.ludwigii ,E.aerogenes , E.cancerogenus,E.hormaechei,E. asburiae) .phylogenetic tree wasdistributed to 15 clusters when it was constructed by neighbor-joining method and some clusters contained species appeared closely related to each other and other clusters contained one species.*

KEYWORDS: *Enterobacter* spp.,16srRNA,Phylogenetic tree

INTRODUCTION

All life- forms could be determined by comparing a stable part of genetic code ;In the late 1970s and beginning of 1980s a breakthrough was achieved by Carl Woese and coworkers when they were able to derive a tree of life consisting of three different branches (bacteria,Archaea and Eukarya)by comparing 16SrRNA gene sequences(Woese,1987).This study revolutionized bacterial taxonomy and the bacteriologists were able to classify prokaryotes on the basis of their phylogenetic relatedness. In previous studies,polymerase chain reaction (PCR)analysis was used to detect pathogens,and many primers have been developed to detect species-specific genes(Kotilanine*et al.*,1998;Backman *etal.*,1999;Skow *etal.*,2005).

The different primers for different species is impractical for routine analysis of cultures that may contain one or more of many possible pathogens,this can be avoided by using a single pair of universal primers designed to amplify conserved stretches of 16SrRNA from any bacterium(Wilson *etal.*,1990;Greisen *etal.*,1994;Khamis *etal.*,2005). Prokaryotic ribosomes consist of three types of RNA 5S molecule with 120 bases ,16S molecule with 1500 bases and 23S molecule with 3000 bases (Harmsen and Karch,2004).5S rRNA gene contained little information due to its short length so that little work has been done to data on 5S molecule.Although the 23S rRNA gene is phylogenetically more discriminatory than the 16SrRNA gene,it is twice the length and therefore it's more difficult to study (Ludwing and Schleifer,1999).Their length and their sizes as well as sequences are conserved between different prokaryotic species(Liao,2000).

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16SrRNA gene is the most common housekeeping genetic marker because the ribosomal small subunit is present universally among bacteria and it consist of both hypervariable regions with species-specific variability where sequences have diverged over evolution and conserved regions ,which makes the 16SrRNA gene sequencing a highly useful tool to study bacterial phylogeny ,ecology and taxonomy (Janda and Abbott,2007;Vetrovsky and Baldrian,2013)).

16SrRNA gene sequence are considered as gold standard for deducing the phylogenetic relationship of prokaryotes(Ciccarelli*etal*.,2006;Nayak *et al*.,2011).This molecular approach has been used for identification and classification of environmental and clinical bacterial isolates (Clarridge,2004). By using 16SrRNA gene sequencing ,the type strain of *Cronobacter* was found to be closer to *Citrobacterkoseri* (97.8%similar)than to *Enterobactercloacae* 97%. Although the latter has been found to share most phenotypic properties with *Cronobacter* (Iversen*etal*.,2004).

Four phylogenetic clusters have been defined among 189 *Cronobacter* strains analysed with partial 16SrRNA gene sequencing and this helped to form the basis of the taxonomic re-classification of these organisms(Iversen*etal*.,2006). Molecular methods have been applied to studying the diversity of the intestinal flora(Vaughan *et al*.,2000). 16SrRNA gene is one commonly used method,the use of 16SrRNA gene sequences has facilitated the study of the microbial flora of intestinal tract,it allows the culture-independent analysis of the microorganisms(Favier *etal*.,2002).

The general conservation of ribosomal RNA genes amongst bacteria,and certain hypervariable sequences presence in the genes within different species,can permit discrimination of bacteria to the genera,species and sometimes sub-species level(Woese,1987;Ge and Taylor,1998).

The variable portions of the 16SrRNA gene provide unique signatures that can be analyzed to provide identification of the bacteria species as showed by (James,2010),where he used primers are broad range primers which recognize conserved sequences within the 16S rRNA gene and amplify the intervening variable regions. 16SrRNAgene of bacteria contain nine hypervariable regions that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification (Van de Peer *etal.*,1996). Numerous studies have identified 16SrRNA hypervariable region sequences that identify a single bacterial species or differentiate among a limited number of different species or genera (Rothman *etal.*,2002;Becker *etal.*,2004;Maynard *etal*.,2005).

Unfortunately,16SrRNA hypervariable regions exhibit different degrees of sequence diversity,and no single hypervariable region is able to distinguish among all bacteria(Varma-Basil *et al*.,2004;Selim *etal*.,2005). The aim of this study was to determine the phylogenetic tree of *Enterobacter* spp. based on 16S rDNA sequencing

MATERIALS AND METHODS

Isolation and Identification of *Enterobacter* spp.

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Eighty four *Enterobacter* spp. were isolated from different specimens (Clinical,environmental,food) from hospitals of basrah/Iraq and identification by conventional methods and confirmed by vitek 2 compact system as mentioned in (Abbas and Radhi,2016).

Genotypic identification of *Enterobacter* spp.

DNA Extraction

Genomic DNA of 84 isolates was extracted by ExiPrep™ Bacteria Genomic DNA kit using ExiPrep™ 16 Fully Automated Nucleic acid Extraction System (Bioneer).

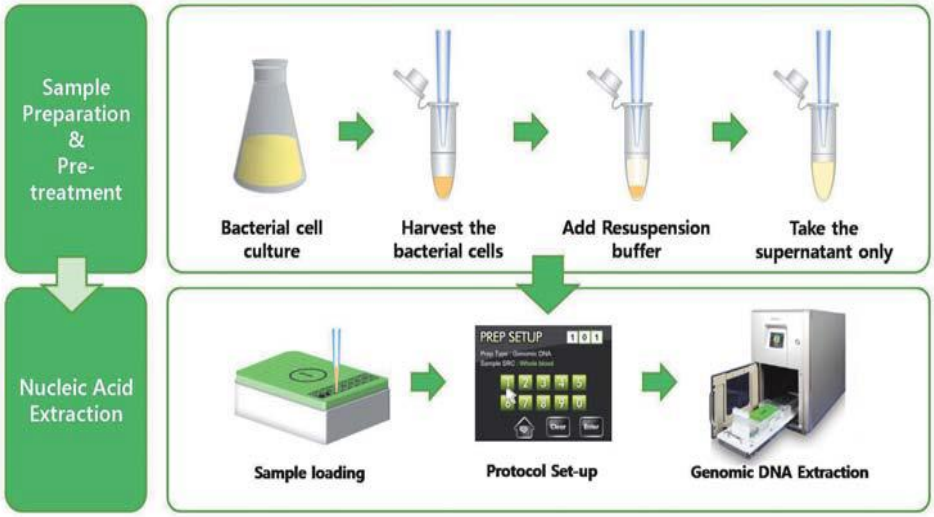
Procedure:

1-Bacterial cells were cultivated in tubes each one contain 5ml LB broth medium and incubated at 37°C for 24hrs.

2- Broth culture (1.5 ml) was transferred to Eppendrof tubeand centrifuged at 13000rpm for 5min in microcentrifuge after thensupernatant was discarded by pipette tipe to pellet the cells.

3-The pellet cells was resuspended in 200µl of resuspension buffer and mix well gently by pipette.

4-200µl of each sample was loaded into sample loading wells as show in fig(1).



Measurment of DNA concentration by using Nanodrop

DNA concentrations and purification were determined by measuring the absorbance and purity at 260/280nm, using the nanodrop (thermo2000C).

Visualization of total DNA on agarose gel

The extracted genomic DNA for all isolates were visualized by subjected to electrophoresis (Sambrook andRussel, 2001)

Preparation of Agarose gel and DNA loading as follow.

1-Agarose powder (0.5gm) was dissolved in (50ml)of 1x TAE buffer.in beaker,the mixture was heated by hot plate until the appearance of bubbles and all agarose powder was dissolved.

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2-DNA green viewer dye(5µl) was added to the agarose solution and mixed gently when agarose temperature reach to 50°C.
3-Before the agarose gel poured into the casting tray of electrophoresis apparatus both edge of the tray were sealed and comb was placed at one end of the tray to form wells for loading.
4-Gel was poured into tray and left solidifying at room temperature.
5-The combs and the seal were removed gently from the tray and the gel tray replaced in electrophoresis chamber.
6-The electrophoresis apparatus was filled with 1x TAE buffer until the entire gel surface was covered with the buffer.
7- DNA sample(4µl) was mixed with (2µl) of loading dye on parafilmpaper,then the mixture transferred to the wells in agarose gel carefully.
8-The gel was subjected to equal electric current by connecting to a power supplier.
9-The cathode was connected to the well sides of the tray while the anode on the other side and the gel was ran at (120V)for(30min).
10-The DNA bands was detected and photographed by gel documentation system.Then the extracted DNA was stored at -20°C until use.

Detection of 16S Ribosomal DNA (16S rDNA)
84 bacterial isolates(clinical,environmental,food) were identified by using PCR and universal bacterial 16S rDNA primers(Jin-Kyung and Cho,2012) which were listed in Table(1)

Table(1)Universal primers sequence of 16S rDNA used in PCR.

Primers	Sequence	TA	length
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	60ºC	20
1492R	5'-AAGGAGGTGATCCAGCCGCA-3'	60ºC	20

TA= Annealing temperature,F=Forward primer,R=Reverse primer

Reagents:-
The reagents and their volumes were used for PCR amplification are described in the Table(2)

Table(2)Reagent and volume (50µl)used in PCR amplification for 16s rDNA

No.	Reagent	Volume per reaction
1	27 Forward primer	2.5 µl
2	1492 Reverse primer	2.5 µl
3	Taq DNA polymerase	1 µl
4	DNA template	1 µl
5	dNTP mix	4 µl
6	Sterile dH_2O	30 µl
7	10×PCR buffer	5 µl
8	$MgCl_2$	4 µl

PCR Amplification of 16S rDNA:
The thermal cycling program for amplification of 16S rDNA *Enterobacter* was described in Table(3)

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Table(3)Program used in PCR amplification for 16S rDNA

Steps	Temperature	Time	No.of cycles
Initial denaturation	95ºC	5min	1
Denaturation	95ºC	30sec	35
Annealing	60ºC	40sec	
Extension	72ºC	120 sec	
Finial Extension	72ºC	10min	1

Separation of PCR products by gelelectrophoresis

PCR products were separated on agarose gel by the same procedure for DNA genome electrophoresis with some exceptions

Procedure:

DNA ladder(100bp-3kb)(2µl) and 4µl of 16S rDNA produced from PCR were electrophoresis for (20min) in (150V) in casting tray with (1%) agarose gel prepared in 1x TBE ,containing 5µl DNA green viewer stain in 50 ml of agarose solution .the products were detected and photographed by using gel documentation system.

Preparation of pure DNA fragment for sending to sequencing.

16S rDNA PCR product was purified by using Gene All Combo kit (Korea)according to manufacturer's recommendations the following procedure:

1-16S rDNA bands (1500bp) of the PCR fragments were excised with a sharp sterile scalpel blade under UV transilluminator.

2-The agarose gel slice containing the fragment was collected in a sterile pre-weighted microcentrifuge tube and added(3µl) 0f buffer GB to(1mg) of gel.

3- Microcentrifuge tube was incubated in water bath at 50ºCfor(5-10)min until the agarose gel is completely melted to help the efficient dissolving of gel.vortex the tube every 2-3 min during the incubation.

4-After the gel slice has dissolved completely,and checked that the color of the mixture is yellow(similar to Buffer GB)

5-(1µl) of isopropanol was added to(1mg) of gel and vortex to mix.

6- The mixture was transferred to a SV column and centrifuged at (10000)rpm for 1 min , the pass-through was discarded and the SV column placed back in the same tube.

7- 500µl of buffer GB was added to SV column and centrifuged at (10000)rpm for 30Sec and the pass-through was discarded and the SV column placed back in the same tube.

8- 700µl of buffer NW was added to the SV column and centrifuged at (10000)rpm for 30Sec.after then the pass-through discarded and SV column placed back in the same tube.

9-The SV column was centrifuged (10000)rpm for 1min once more to removed residual wash buffer and SV column placed in a clean(1.5 ml)microcentrifuge tube.

10-(50µl) of buffer EB was added to eluted DNA in center of SV column membrane and centrifuged at 13000 rpm for 1min.

After DNA samples purified the tube of each sample was labeled,then they were sent to macrogene company(south Korea) for sequencing.

16s rDNA analysis

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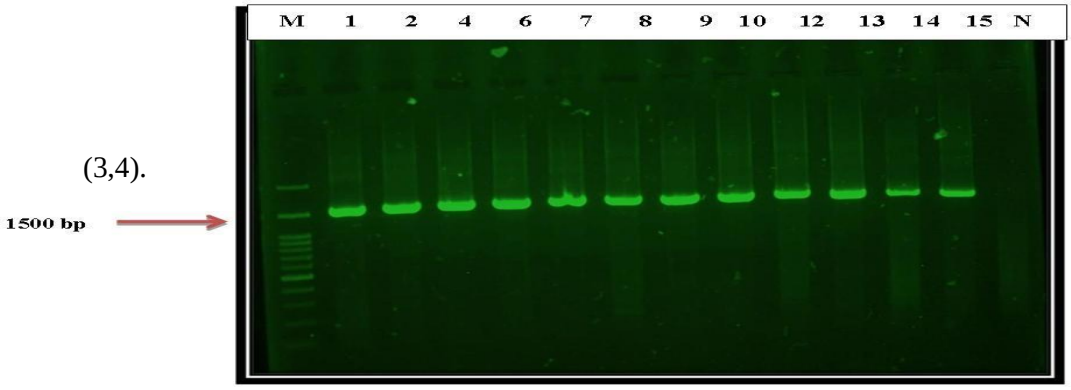
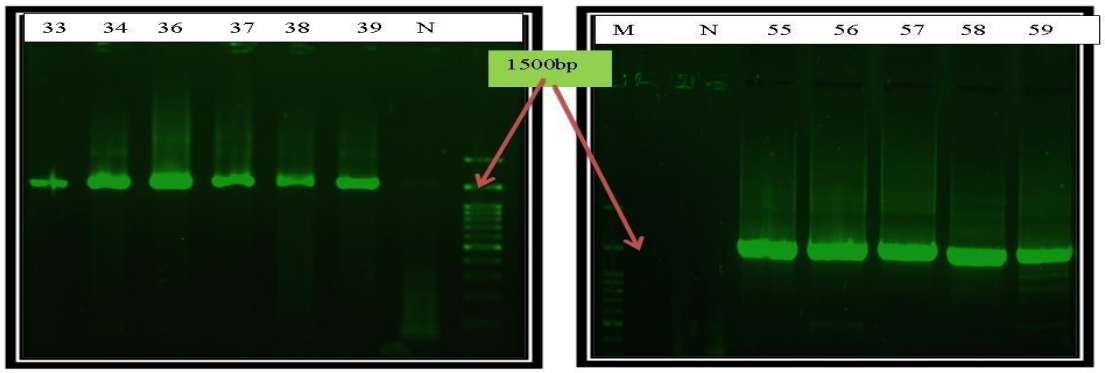
Analysis data accomplished by Centeral lab /Karaj /Tahran /IranWhen the sequence chromatograms received from macrogene company/South Korea , the data were edited manually in chromas version 2.01 to treat the sequencer. Similarity analysis of samples were performed in BLASTn software implemented in National center for biotechnology information (NCBI)homepage(http:// www .ncbi .nlm.nih.gov) and Ribosomal database project II(Cole *etal*.,2005).Sequences with similarity percentage greater than 97% were selected as a species (Stackebrandt*etal*.,2002).Alignment of sequences constructed with Clustal W http:// www.ebi.ac.uk/clustalW(Thompson *et al*.,2002) and used for phylogenetic analysis in PAUP4 in Geneious program (Drummond *et al*.,2011).

(3,4).



Figure(3): Agarose gel electrophoresis photograph show PCR products of 16SrDNA,Lane M=3Kb DNA ladder,Lane 1-15= 16S rDNA bands of bacterial isolates at 1500bp ,Lane N=negative control



Figure(4): Agarose gel electrophoresis photographs show PCR products of 16SrDNA,Lane M=3Kb DNA ladder,Lanes (33-39) ,(55-59)-= 16S rDNA bands ofbacterial isolates at 1500bp ,Lane N=negative control

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Molecular Identification of *Enterobacter* spp. based on 16SrDNA gene sequence

Eighty four isolates were identified to the strain level after the sequences of 16SrDNA gene of all isolates were compared with the highly similar DNA sequences of Enterobacteriaceae in GenBank database using BLAST(Basic Local Alignment Search Tool) program in NCBI database .The results showed that the sequences of the 16SrDNA gene of twenty three isolates (6,9,13,25 ,33,37,38,39 ,41,52,56,64,65 ,71,87,108,109 ,111,116,132,138 ,146,148) had high similarity level(100%) with sequences of 16SrDNA gene of different strains belong to the species(*Enterobacter cloacae* ,*Enterobacter hormaechei*),which the isolate (6)was similar to the strain *E. cloacae* VRBG-74 in genbank , the isolates(9,41,65) were similar to strain *E. cloacae* WL1310 in genbank,The isolates(13,109) were similar to strain *E.cloacae* ECNIH5,The isolates(25,37,56,148)were similar to strain *E. cloacae* 34998,The isolates(39,132)were similar to the strain *E.cloacae* VRBG-62,The isolates (52,71)were similar to strain *E.cloacae* KLHD10,other isolates(38,64,108,111,116,138,146)were similar to the strains(*E.cloacae* 34399,*E.cloacae*subspdissolvens SB3013,*E.cloacae* 34978,*E.cloacae* RCB77,*E.cloacae* KLHD12,*E.cloacae* T137,*E.cloacae*subsp cloacae ENHKV01)respectively, while the isolates(33,87)were similar to the species(*E.hormaechei*) and the strain(SBANHCA2) deposited in GenBank.

Fifty six isolates(1 , 2 , 4 , 7 , 8 , 12 , 14 , 15 , 16 , 22 , 23 , 24 , 29 , 31 , 34 , 36 , 43 ,44 , 45 , 48 , 50 , 55 , 57 , 58 , 59 , 63 ,68, 69 , 73 , 80 , 81 , 82 , 83 , 85 , 94 , 98 , 107 , 112 , 114 , 121 , 123 , 124 , 127 , 128 , 129 , 130 , 133 , 134 , 135 , 142 ,144,145,149,150,152,153) had similarity level (99%) with different strains belong to two genera of *Enterobacter* and *Cronobacter* ,where the isolates (1,50) were similar to the species *E. cloacae* strain VRBG-74,The isolates (4,73) were similar to species *E. cloacae* strain Y219,the isolates (7,63,81) were similar to the species *E. cloacae* strain 34399,the isolates (8,14,45,48,58,69,85,124,144,145) were similar to the species *E. cloacae* strain 34998 ,the isolates(34,153)were similar to species *E. cloacae* strain AV2 ,the isolates (68,133) were similar to *E. cloacae* strain KLHD10,the isolates(82,128) were similar to *E. cloacae* strain 34978,the isolates(129,135) were similar to species *E.cloacae* strain RCB279,the isolates (23,43,44,55, 114,127) were similar to the species *E.cloacae* strains(WL1310,VRBG-62,R27091,S20504,ECNIH5,EC7) respectively, the isolates (12,22,24,29,57,80,130,134)were similar to the species *E. aerogenes* and strain HC050612-1,the isolates (15) was similar to the species *E. xiangfangensis* Ps21,the isolate(59)was similar to the species *E. asburiae* strain ATCC35953, the isolates (83,98,107)were similar to the species *E.aerogenes* strains (*E.aerogenes* BXCC-49,*E.aerogenes* 165(C4(plas(B)),*E. aerogenes* HK20-1)respectively, the isolates (31,36,94,112,121,123,152)were similar to the species *E.hormaechei*, where the isolates (31,112) were belong to the strain (10(C4plas(D)),the isolates(36,152) were belong to the strain (D20) and the isolates (94,121,123) belong to the strains(SBANHCA2,D15,1-2-b-1)respectively in genbank.The isolate (16) was similar to the genus *Enterobacter* strain UIWRF0949,the isolate (142) was similar to the species *E. cancerogenus* strain M119,,The isolate(149) was similar to the species *E. ludwigii* strain WAB1946,the isolate(2) was similar to the genus *Cronobacter* species *C.sakazakii* strain CCFM8312 ,the isolate(150) was similar to the genus *Cronobacter*strain 1044in genbank.

Three isolates(10,21,137) had similarity level (98%),where the isolates(10,21)were similar to the species *E.cloacae* strains (VRBG-62,WL1310) respectively while the isolate(137) was similar to species *E.aerogenes* strain B4in genbank. Two isolates(67,78) had similarity level

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(97%),where the isolate (67) was similar to the species *E. aerogenes* strain EGU16 while the isolate (78) was similar to the genus *Enterobacter* strain 312502 from the comparison of its 16S rDNA sequence data with reference strains in GenBank using NCBI database.

16SrDNA gene phylogenetic tree of *Enterobacter* spp. and related genus

The results showed the phylogenetic relatedness of *Enterobacter* spp. to each other and other related genus constructed using the Neighbor-Joining method. the sequence alignment of 16SrDNA genes of isolated *Enterobacter*spp,and some other related species was performed with ClustalW software as represented in Figure(5).

Eighty four isolate were found in 15 different group in the phylogenetic tree.The groups one,two,three and nine contain more than one species belong to the genus *Enterobacter* and phylogenetically closely linked with each other, where group one contain species(*E.cloacae* ,*E.xiangfangensis* ,*E.ludwigii* ,*E.aerogenes* , *E.cancerogenus* , *E.hormaechei*,*E.asburiae*),group two contain species (*E.hormaechei*,*E.aerogenes*),group three and nine contain species (*E.aerogenes* ,*E.cloacae* , *E.hormaechei*) .Other groups contain one species ,where the groups(5 ,6 ,7 ,14) contain the species *E.cloacae* ,groups(4,12) contain species *E. aerogenes*and group (8) contain species (*E.hormaechei*). The groups(10,13) contain isolates belong to the genus *Enterobacter* but unidentified species and belong to the strains (UIWRF0949 ,312502) respectively ,while the group (11) contain isolate belong to the genus *Cronobacter* species *sakazakii* strain CCFM8312 and group (15) contain *Enterobacter cloacae* Y219 are phylogenetically closely linking with *Cronobacter*sp.

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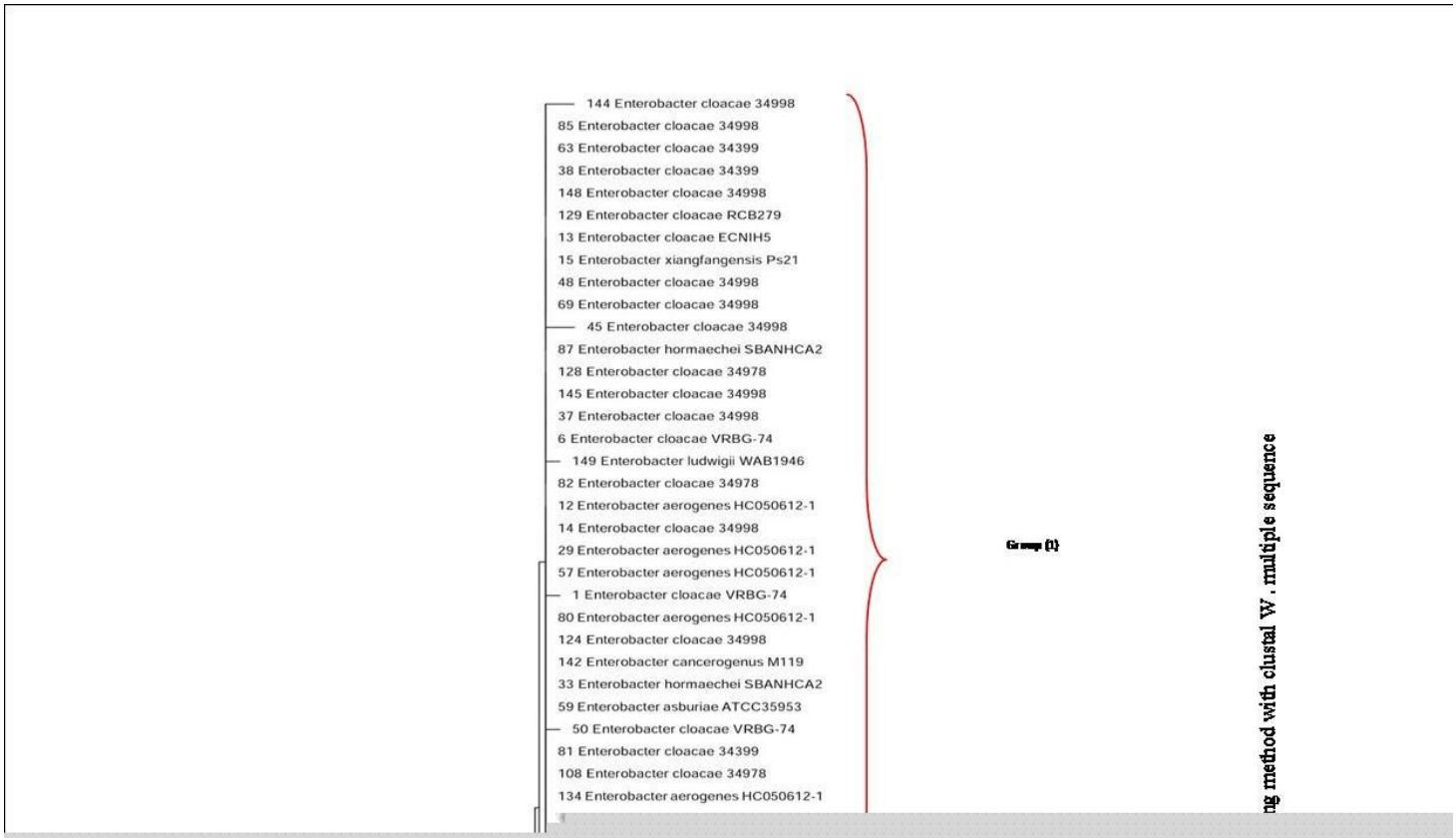
144 Enterobacter cloacae 34998
85 Enterobacter cloacae 34998
63 Enterobacter cloacae 34399
38 Enterobacter cloacae 34399
148 Enterobacter cloacae 34998
129 Enterobacter cloacae RCB279
13 Enterobacter cloacae ECNIH5
15 Enterobacter xiangfangensis Ps21
48 Enterobacter cloacae 34998
69 Enterobacter cloacae 34998
45 Enterobacter cloacae 34998
87 Enterobacter hormaechei SBANHCA2
128 Enterobacter cloacae 34978
145 Enterobacter cloacae 34998
37 Enterobacter cloacae 34998
6 Enterobacter cloacae VRBG-74
149 Enterobacter ludwigii WAB1946
82 Enterobacter cloacae 34978
12 Enterobacter aerogenes HC050612-1
14 Enterobacter cloacae 34998
29 Enterobacter aerogenes HC050612-1
57 Enterobacter aerogenes HC050612-1
1 Enterobacter cloacae VRBG-74
80 Enterobacter aerogenes HC050612-1
124 Enterobacter cloacae 34998
142 Enterobacter cancerogenus M119
33 Enterobacter hormaechei SBANHCA2
59 Enterobacter asburiae ATCC35953
50 Enterobacter cloacae VRBG-74
81 Enterobacter cloacae 34399
108 Enterobacter cloacae 34978
134 Enterobacter aerogenes HC050612-1
ng method with clustal W . multiple sequence

All isolates(84) that obtained from different sources (Clinical,environmental and food specimens))in hospitals of Basrah were subjected to 16SrDNA sequencingand all isolates showed different similarity levels(97-100%) when compared with reference strains sequences in GenBank,whereas this results indicated the isolates belong to different species (*E.cloacae*,*E.aerogenes*,*E.hormaechei*,*E.asburiae*,*E.cancerogenus*,*E.sakazakii*,*E.xiangfangensis*) and different strains .Simillar studies used 16SrDNA to identify *Enterobacter* spp. (Neto*etal*.,2003;Ramin *etal*.,2009;Fakruddin *etal*.,2014;Chandrika *etal*.,2015;Zhang *etal*.,2015). Phylogenetic tree that constructed by neighbor-joining method wasdistributed to 15 clusters with two genera (*Enterobacter* ,*Cronobacter)*,whereas one species belong to *Cronobacter* (*C.sakazakii*) ,seven species belong to *Enterobacter* (*E.cloacae* ,*E.xiangfangensis* ,*E.ludwigii* ,*E.aerogenes* ,*E.cancerogenus*,*E.hormaechei*,*E.asburiae*).

The groups one,two,three and nine contain more than one species belong to the genus *Enterobacter* and phylogenetically closely linked with each other, where group one contain species(*E.cloacae* ,*E.xiangfangensis* ,*E.ludwigii* ,*E.aerogenes* , *E.cancerogenus* , *E.hormaechei*),group two contain species (*E.hormaechei*,*E.aerogenes*),groups three and nine contain species (*E.aerogenes* ,*E.cloacae* , *E.hormaechei*).Other groups contain one species ,where the groups(5 ,6 ,7 ,14) contain the species *E.cloacae* ,groups(4,12) contain species *E. aerogenes* and group (8) contain species (*E.hormaechei*).this observation is consistent with previous studies of the phylogenetic relationships of *Enterobacter* spp.(Paauw*etal*.,2008;Liu *etal*.,2013).The study of (Taghavi*etal*.,2010) revealed that *E.cloacae* strains,similar to other *Enterobacter* ,are characterized by their ability to use a wide range of carbon sources through their diverse carbohydrate metabolic pathways and transport systems.The result indicated that closely related species can not be distinguished solely on the basis of 16S rRNA gene as showed in studies (Porwal*etal*.,2009;Kalia *etal*.,2011;Lal *etal*.,2011).Study of (Nayak*etal*.,2011)proved this,where as they found that the 16S rRNA gene sequences of *Enterococcus faecium* and *Enterococcus mundtii* were approximately 98% similar and formed a single cluster in phylogenetic tree.Incontrast,the BOX-PCR patterns of *E.faecium* and *E.mundtii* exhibited less than 60% similarity.

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he groups(10,13) contain isolates belong to the genus *Enterobacter* but unidentified species and belong to the strains (UIWRF0949 ,312502) respectively ,while the group (11) contain isolate belong to the genus *Cronobacter* species *sakazakii* strain CCFM8312 and group (15) contain *Enterobacter cloacae* Y219 are phylogenetically closely linking with *Cronobacter* sp..

Iversen *etal*.(2004) investigated the phylogenetic relationships of *Enterobacter sakazakii* using 16SrDNA they found that strains were distributed among four clusters,indicating taxonomic heterogenety.Loc-Corrillo*etal*.(2004) showed that *Enterobacter* genus is polyphyletic.

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