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Characterization of *Enterobacter cloacae* Strains Isolated from different areas of Basrah Hospital by Multilocus Sequence Typing Method (MLST)

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Abstract

In this study we used seven genes of multilocus sequence typing (MLST) to detect genetic diversity among nine isolates of *Enterobacter*. The results showed that the isolates identified into the species *Enterobacter cloacae* belong to different strains after sequenced and analyzed of seven MLST genes. The phylogenetic trees were drawn for each gene and for all genes based on neighbor-joining method after sequences alignment in Clustal W,whereas the trees divided into groups.

***Keywords*:** Enterobacter cloacae; Phylogenetic tree; MLST.

1. Introduction

Multilocus sequence typing (MLST) is a nucleotide sequence –based method used for characterizing the genetic relationships of strains of bacterial species, or other microbial species via the internet [1,2,3]. Multilocus sequence typing is one of the methods of choice for typing of the isolates of epidemiological or evolutionary importance .This method based on determination of the nucleotide sequences of the internal fragment of a number of a selected house-keeping genes that are subjected to very slow and minor changes resulting in polymorphism and diversity [4,5,6]. MLST involves obtaining the sequences of internal fragments of seven house-keeping genes for each strain of a selected species.

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The sequences of each fragment are compared with all the previously identified sequences(alleles) at that locus(not only to the other strains of the strain collection of the study or nation-wide lineage but also to the global ones) and thereby, are assigned allele numbers at each of the seven loci. The combination of the seven allele numbers defines the allelic profile of the strain and each different allelic profile is assigned as a sequence type(ST),which is used to describe the strain[4,7]. Sequences were assigned as distinct alleles even they differed at a single nucleotide site, no weighing was applied to reflect the number of nucleotide differences between alleles [8]. MLST is a powerful DNA fingerprinting tool that compare the internal regions of 400-600 bp nucleotide sequences of a series of house-keeping genes presenting in a particular species of all isolates [9]. MLST is more appropriate for strain phylogeny and large-scale epidemiology [2]. MLST has been developed, which is practical, provides more discriminatory power, and generates data meaningful for epidemiology studies and understanding the evolution of organisms .applying this method in epidemiological studies of pathogenic organisms such as bacteria, parasites, fungi improves the reliability of their taxonomy [10]. MLST was proposed in 1998 as a portable universal and definitive method for characterizing bacteria and was firstly used to characterize bacterial pathogens such as *Neisseria meningitides* [4,11]. and *Streptococcus pneumoniae* [12]. this method has been applied with pathogenic and nonpathogenic organisms. The study of *N.meningitidis* revealed that with MLST, 470bp sequence variations of internal fragments of eleven loci , six loci reliably identified the major meningococcal lineages associated with invasive disease caused by *N.meningitidis*[4,11] in addition ,the molecular typing data of *Streptococcus pneumoniae* from invasive pneumococcal disease collected from eight countries using MLST implied that ST defined strains showed an increased capacity to cause invasive disease[12]. Other studies using MLST to characterize bacterial pathogens. Forsythe *etal*. [13] proved that the MLST scheme covers all recognized species of *Cronobacter* genus to better quantitate the intraspecific and interspecific diversity of the genus and characterize the strains according to virulence groupings and source.It also enables the retrospective analysis of historic cases and outbreaks following re-identification of those strains which otherwise would have been lost due to taxonomic re-evaluation.A 115 sequence types (STs)have been identified in genus *Cronobacter* ,53 STs have been identified for *C.sakazakii*(The type species of genus),Seventeen STs have been identified for *C.malonaticus*,13 for *C.turicensis* ,10 for *C.muytjensii*,17 for *C. dublinensis*.four STs have been identified for the recently renamed *C.universalis*(previously known as *Cronobacter* genomospecies.and one has been identified for the recently defined species *C.condimenti* [14;15]. The sequences of internal fragments of seven house-keeping genes were obtaind for 155 Staphylococcus aureus isolates from patients with community-acquired and hospital-acquired invasive disease in Oxford,United Kingdom, area [8].The genetic diversity of the Bacillus cereus isolates has been studied by using MLST[16].There are many advantages for MLST include its dependence on the sequence data which are easily comparable ,transferable among laboratories in addition to its reproducibility and its assignment of the nucleotide sequence of each of the selected house-keeping gene as a unit of comparison [4,5,6].

The aim of this study was using MLST technique to detect genetic variation among *Enterobacter cloacae* strains

2. Materials and methods

2.1. Detection of Multilocus Sequence Typing(MLST)by PCR

Nine strains of *Enterobacter cloacae* have been typed using MLST. MLST primers targeted 7 housekeeping genes as recommended by [17,18] which are listed in Table(1).

Table1: Primers sequences for *E.cloacae* MLST

Primers		**Primer Sequences**	**Length bp**	**TA***
Amplification primers	*dnaA-f*	5-AY*AACCCGCTGTTCCTB*TATGGCGGCAC-3	28	62
	dnaA-r	5-K*GCCAGCGCCATCGCCATCTGACGCGG-3	27	62
	fusA-f	5-TCGCGTTCGTTAACAAAATGGACCGTAT-3	28	59
	fusA-r	5-TCGCCAGACGGCCCAGAGCCAGACCCAT-3	28	59
	gyrB-f	5-TCGACGAAGCGCTCGCGGGTCACTGTAA-3	28	62
	gyrB-r	5-GCAGAACCGCCCGCGGAGTCCCCTTCCA-3	28	62
	leuS-f	5-GATCAR*CTSCCGGTK*ATCCTGCCGGAAG-3	28	62
	leuS-r	5-ATAGCCGCAATTGCGGTATTGAAGGTCT-3	28	62
	pyrG-f	5-AY*CCBGAY*GTB*ATTGCRCAY*M*AGGCGAT-3	28	62
	pyrG-r	5-GCR*CGR*ATYTCV*CCCTS*H*TCGTCCCAGC-3	28	62
	rplB-f	5-GTAAACCGACATCTCCGGGTCGTCGCCA-3	28	64
	rplB-r	5-ACCTTTGGTCTGAACGCCCCACGGAGTT-3	28	64
	rpoB-f	5-CCGAACCTTCCGCGAACATCGCGCTGG-3	28	64
	rpoB-r	5-CCAGCAGATCCAGGCTCAGCTCCATGTT-3	28	64
Sequencing primers	*gyrB-r2*	5-GCAGAACCGCCCGCGGAGTCCCCTTCC-3	27	
	gyrB-f2	5-AAAACCGGTACY*ATGGTGCGTTTCTGG-3	27	
	fusA-r2	5-ATCTCTTCACGY*TTGTTAGCGTGCATCT-3	28	

2.2. Reagents

The reagents and their volumes were used for PCR amplification of MLST are described in the Table(2).

Table2: Reagent and volume (50 µl) used in PCR amplification for MLST

NO.	**Reagent**	**Volume per reaction**
1	Forward primer	**2.5 µl**
2	Reverse primer	**2.5 µl**
3	Taq DNA polymerase	**1 µl**
4	DNA template	**1 µl**
5	dNTP mix	**4 µl**
6	Sterile ddH_2O	**30 µl**
7	10×PCR buffer	**5 µl**
8	$MgCl_2$	**4 µl**

2.3. PCR amplification for MLST

The thermal cycling program for amplification of MLST *Enterobacter cloacae* was described in table(3).

Table3: Program used in PCR amplification for MLST reaction

Steps	Temperature	Time	No.of cycles
Initial denaturation	95 ºC	5min	**1**
Denaturation	94 ºC	30sec	**35**
Annealing	62ºC	50sec	
Extension	72 ºC	120 sec	
Finial Extension	72 ºC	10min	**1**

*Annealing temperatures =59 ºC for *fusA* gene primer,64 ºC for *rplB* and *rpoB* genes primers,62 ºC for *dnaA* , *gyrB* ,*leuS*, *pyrG* genes primers.

2.4. Separation of PCR products(MLST bands) by gel electrophoresis

PCR product was separated in the same procedure used with PCR product of 16S rDNA **.** The products were detected and photographed by using gel documentation system.

2.5. Preparation of pure DNA fragment for sending to sequencing

All PCR products were purified by using Gene All Combo kit (Korea). After DNA samples purified all PCR products (7 genes) for each strain were sent to macrogene company(south Korea) for sequencing.

2.6. MLST sequences analysis

All MLST sequences belong to bacterial strains became edited, confirmed and prepare for phylogenetic analysis, BLASTn and polymorphism analysis as before mentioned in [19]. Also, concatenated format of 7 MLST loci have been prepared in Geneious 9.0.5 for assembly analysis. Nucleotide polymorphism of every MLST locus included DNA nucleotide diversity, synonymous and non-synonymous mutation, have been calculated in DnaSp 5.10 [20].

3. Results

3.1. Identification of Enterobacter cloacae based on Seven MLST Genes Sequence

Nine isolates were identified to the different strains belong to the species *Enterobacter cloacae* after the sequences of seven MLST genes of all isolates.

Table(4) showed the characteristics of *E. cloacae* MLST loci,where the sequence target size ranged from 435bp (*pyrG*) gene to 865bp (*rpoB*)gene for all strains ,the *dnaA* gene had the highest dN/dS nonsynonymous to synonymous substitution ratio(1.45040).In contrast , the dN/dS ratio of *rplB* gene was (-1.72651),the percentage of variable sites at each locus ranged from 1.5(*rplB*)to 13.8(*pyrG*).

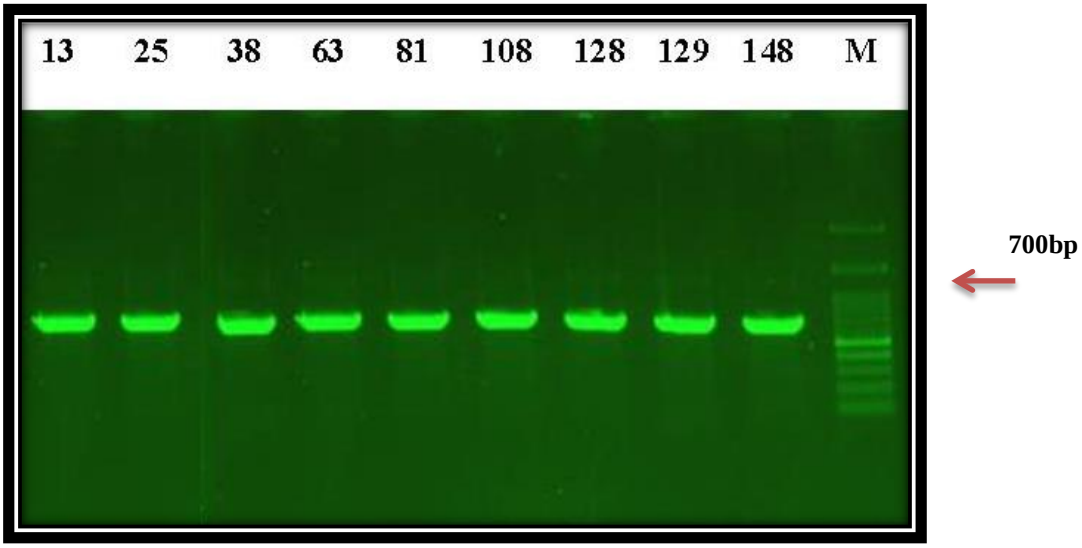

Figure 1: Agarose gel photograph of genomic DNA fragments show PCR amplified products of *dnaA* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

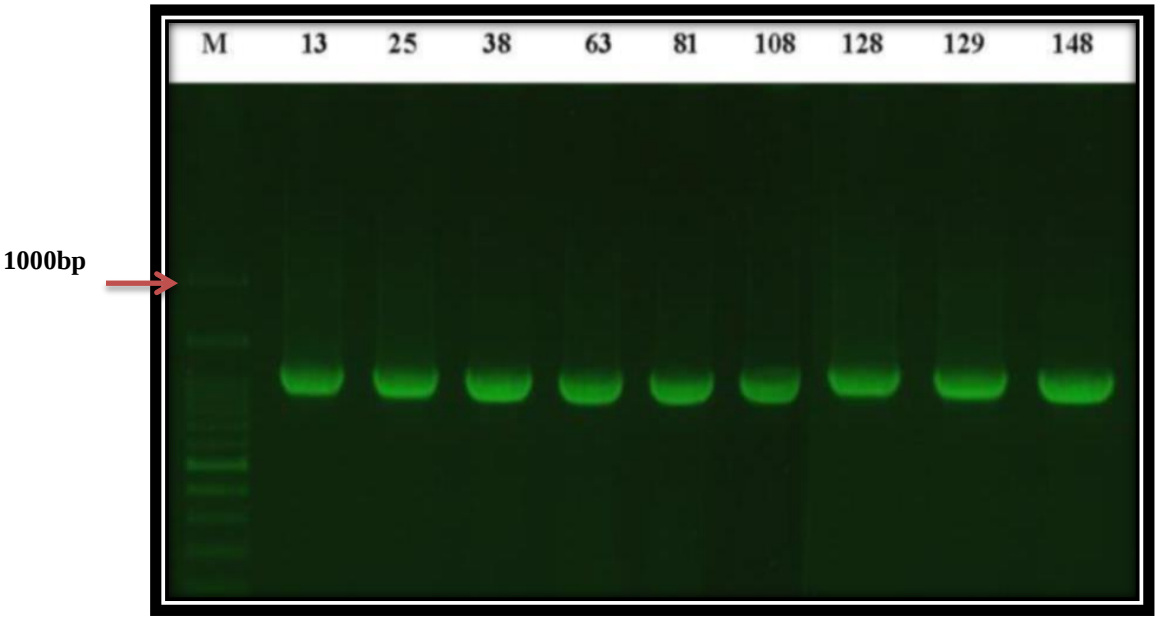

Figure 2: Agarose gel photograph of genomic DNA fragments show PCR amplified products of *fusA* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

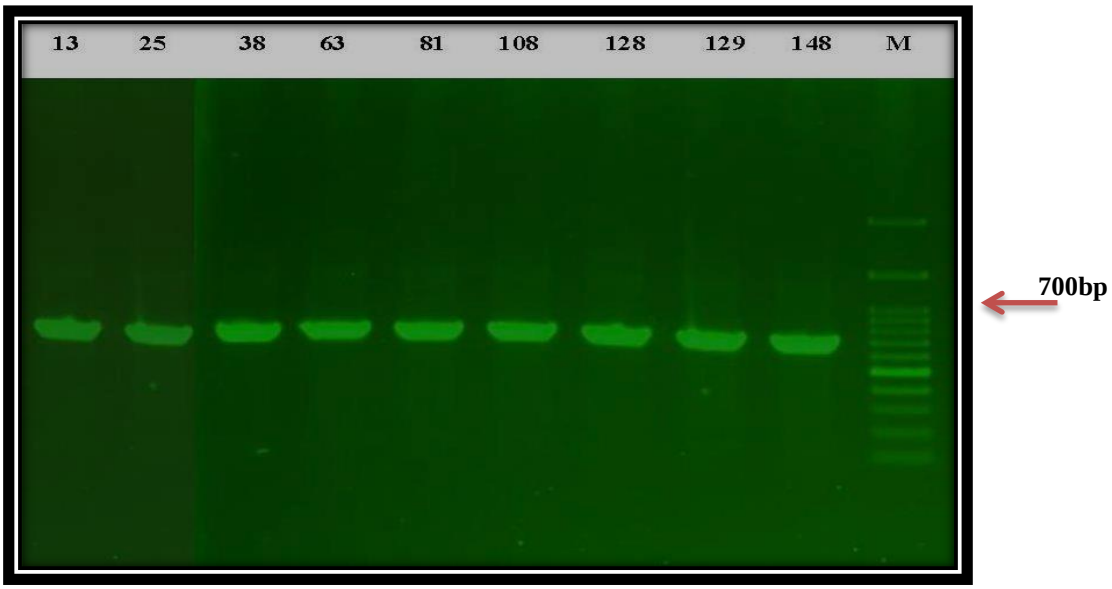

Figure 3: Agarose gel photograph of genomic DNA fragments show PCR amplified products of *gyrB* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

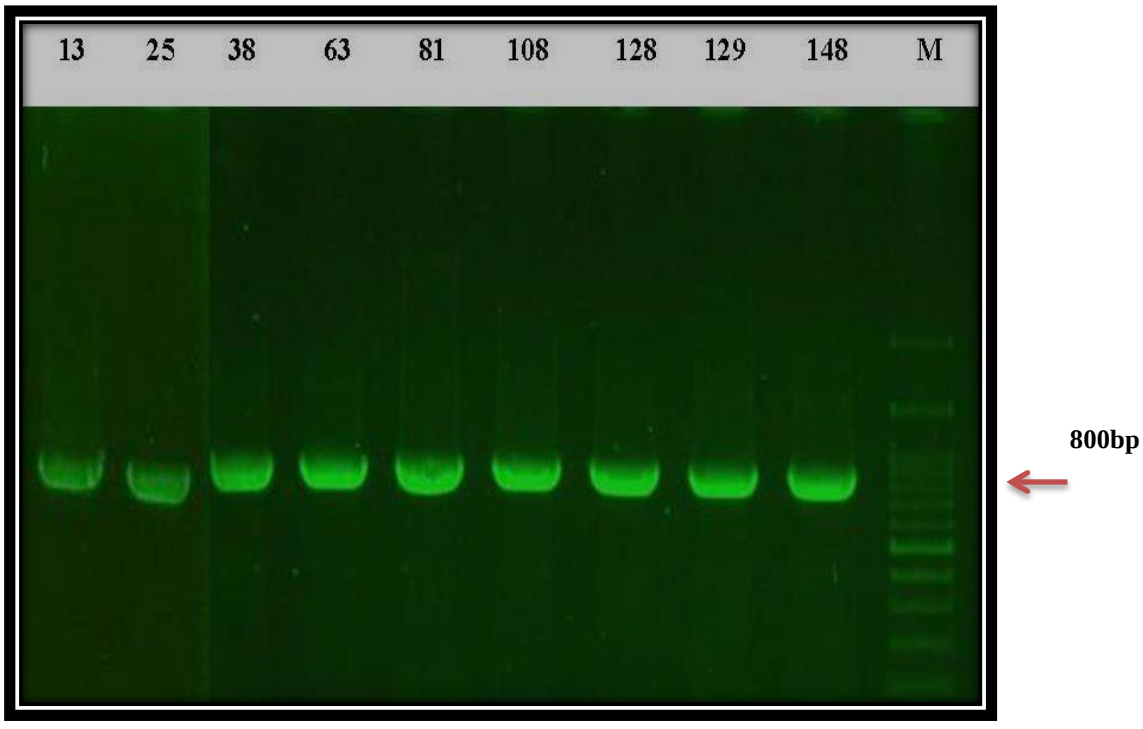

Figure 4: Agarose gel photograph of genomic DNA fragments show PCR amplified products of *leuS* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

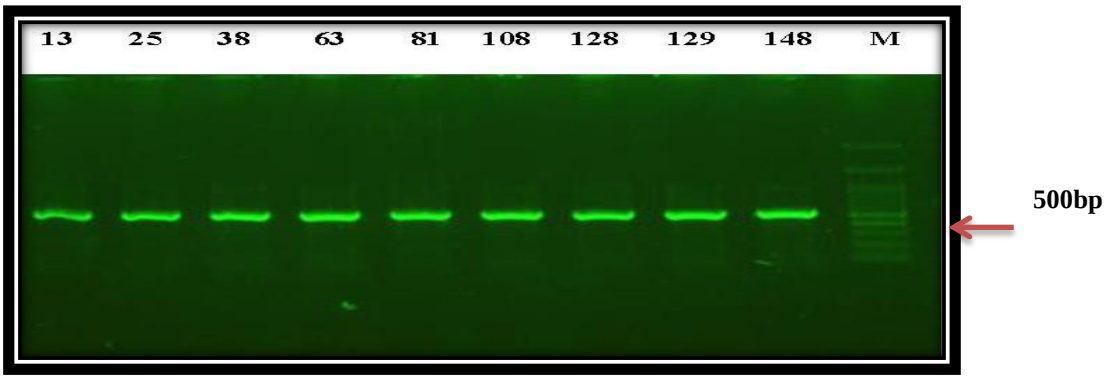

Figure 5 : Agarose gel photograph of genomic DNA fragments show PCR amplified products of *pyrG* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

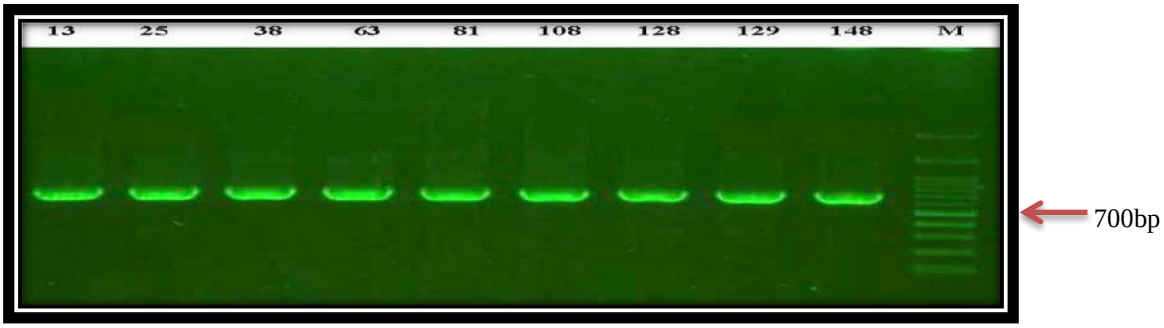

Figure 6 : Agarose gel photograph of genomic DNA fragments show PCR amplified products of *rplB* gene of *Enterobacter cloacae* strains,M=3kb DNA ladder ,Lanes:**13-148** =*E. cloacae* strains

Table4: Characteristics of *Enterobacter cloacae* MLST loci

Locus	***dnaA***	***fusA***	***gyrB***	***leuS***	***pyrG***	***rplB***	***rpoB***
Sequence target size (bp)	700	858	688	752	435	613	865
dN/dS ratio*	1.45040	1.09559	0.91151	0.69762	1.29933	-1.72651	1.25560
Number of variable sites	61	58	52	77	60	9	103
Percentage of variable sites	8.7	6.8	7.6	10.2	13.8	1.5	11.9

*Nonsynonymous(dN) to synonymous(dS) substitution ratio.

3.2. Phylogenetic trees of seven genes MLST

Phylogenetic tree were drawn for each of the seven genes based on neighbor-joining method after sequences alignment in Clustal W program.

Figure(8)showed the phylogenetic tree of *dnaA* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into five groups,group(1) consisted of three isolates (D128,D81,D129),groups(2,4) consisted of one isolate(D38,D108)respectively,groups (3,5)contained two isolates(D148,D63)(D13,D25)respectively.

Figure(9)showed the phylogenetic tree of *fusA* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into five groups , group(1) consisted of three isolates (F25,F108,F128),groups(2,4) consisted of two isolates(F81,F129),(F38,F148) respectively ,groups (3,5)contained one isolate (F63,F13) respectively.

Figure(10)showed the phylogenetic tree of *gyrB* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into three groups, group(1) consisted of five isolates (G128,G108,G81,G25,G129),group (2) consisted of three isolates(G38,G63,G148), group (3)contained one isolate (G13).

Figure(11)showed the phylogenetic tree of *leuS* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into four groups, groups(1,4) consisted of three isolates (L81,L108,L25) ,(L63,L38,L148) respectively, group (2) consisted of two isolates(L128,L129),group (3)contained one isolate (L13).

Figure(12)showed the phylogenetic tree of *pyrG* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into five groups, groups (1,5) consisted of three isolates (P38,P148,P63),(P25,P81,P129) respectively, groups (2,3,4) consisted of one isolate (P108,P128,P13) respectively.

Figure(13)showed the phylogenetic tree of *rplB* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into four groups, group (1) consisted of five isolates (R129,R148,R128,R38,R81), group (2) consisted of two isolates(R13,R63),groups (3,4)contained one isolate (R25,R108).

Figure(14)showed the phylogenetic tree of *rpoB* gene for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into four groups, group (1) consisted of five isolates (RP38,RP148,RP108,RP128,RP81), groups (2,4) consisted of one isolate (RP63,RP13)respectively, group (3)contained two isolates (RP25,RP129).

Figure(15)showed the phylogenetic tree of seven genes (*dnaA,fusA ,gyrB,leuS,pyrG,rplB,rpoB*) for nine *Enterobacter cloacae* strains .Observed from the results these strains clustered into three groups, group (1) consisted of five isolates (81,128,129,108,25), group (2) consisted of three isolates(63,38,148),group

(3)contained one isolate (13).

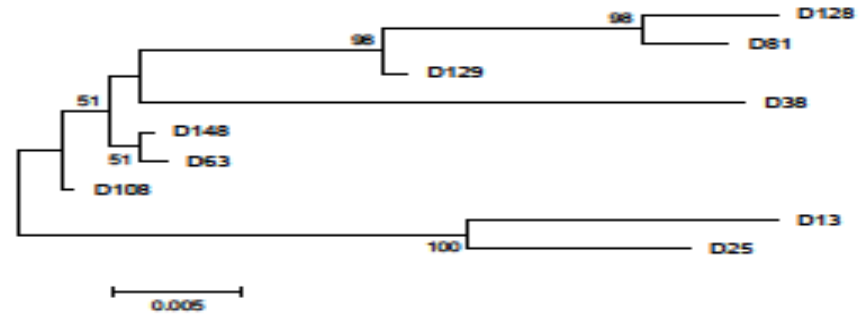

Figure 7: Phylogenetic tree was constructed using neighbor-joining method for *dnaA* gene for nine *Enterobacter cloacae* strains

Figure 8: Phylogenetic tree was constructed using neighbor-joining method for *fusA* gene for nine *Enterobacter cloacae* strains

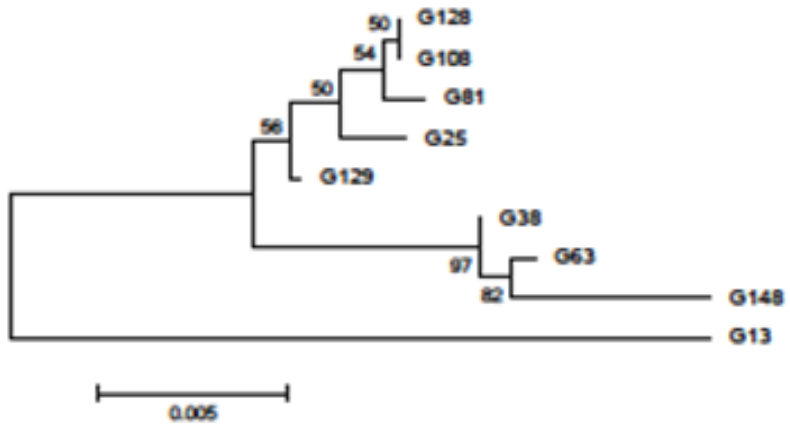

Figure 9: Phylogenetic tree was constructed using neighbor-joining method for *gyrB* gene for nine *Enterobacter cloacae* strains

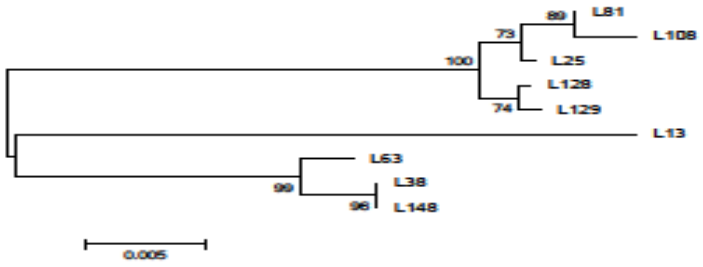

Figure 10: Phylogenetic tree was constructed using neighbor-joining method for *leuS* gene for nine *Enterobacter cloacae* strains

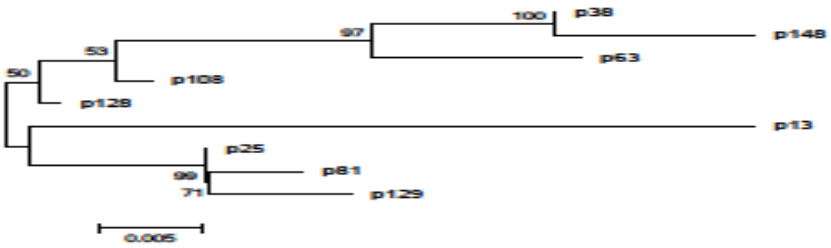

Figure 11: Phylogenetic tree was constructed using neighbor-joining method for *pyrG* gene for nine *Enterobacter cloacae* strains

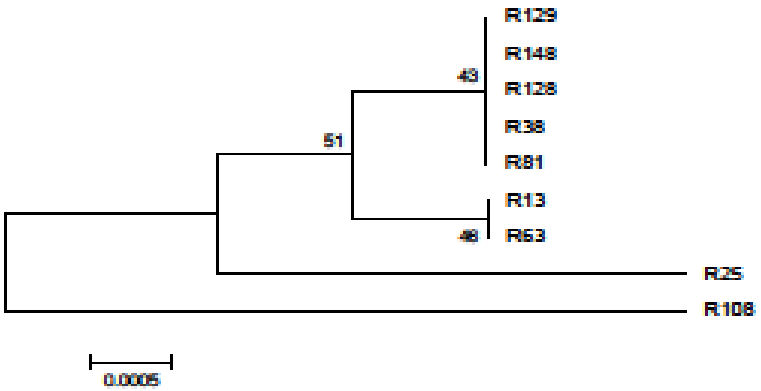

Figure 12: Phylogenetic tree was constructed using neighbor-joining method for *rplB* gene for nine *Enterobacter cloacae* strains

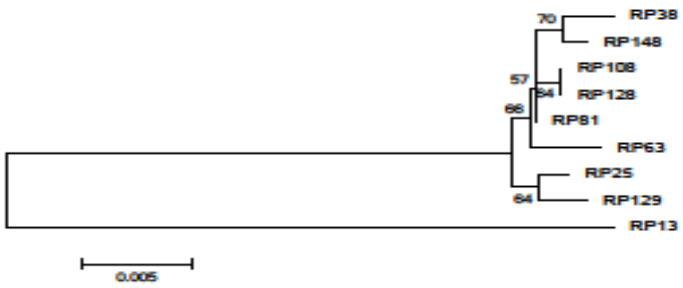

Figure 13: Phylogenetic tree was constructed using neighbor-joining method for *rpoB* gene for nine *Enterobacter cloacae* strains

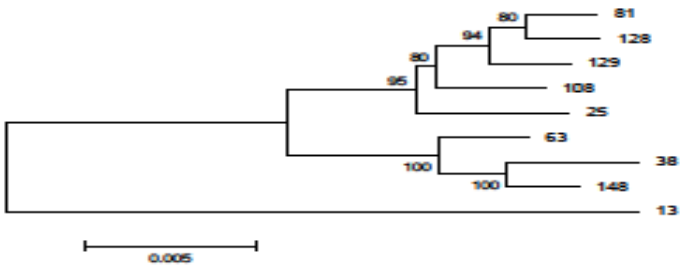

Figure 14: Phylogenetic tree was constructed using neighbor-joining method for seven (*dnaA,fusA,gyrB,leuS,pyrG,rplB,rpoB*) genes for nine *Enterobacter cloacae* strains

4. Discussion

MLST studies that employ nucleotide sequence analysis to identify genetic variation have been highly successful for characterization bacterial genetic variation and for developing evolutionary framework that interprets this diversity [7].

In this study MLST performed with seven housekeeping genes for nine *Enterobacter cloacae* strains .The amplicon sizes for seven genes ranged from 500bp (*pyrG*) to 900bp(*rpoB*).The results showed that the isolates identified into the species *Enterobacter cloacae* belong to different strains after sequenced and analyzed of seven MLST genes as showed in Table(34).The sequences had the percentage of variable sites at each locus range from 1.5%(*rplB*) to 13.8%(*pyrG*),*dnaA* gene had the highest dN/dS nonsynonymous (change of amino acid) to synonymous (no change of amino acid)substitution ratio(1.45040).In contrast *rplB* had the lower dN/dS(-1.72651).similar results observed in study of [17].

Figures(63,64,65,66,67,68,69,70) showed that the phylogenetic trees were drawn for each gene and for all genes based on neighbor-joining method after sequences alignment in Clustal W, whereas the trees divided into groups

,each group consisted of isolates this may belong to that the nine isolates isolated from different areas so that divided to groups [21].

5. Conclusions

The genetic variation of *Enterobacter cloacae* strains had been demonstrated by using seven genes of MLST.

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