How to Cite:
Al-janabi, S. S. (2022). Detection of 16SrRNA gene Polymorphism using PCR for some bacterial species of clinical samples. *International Journal of Health Sciences*, *6*(S9), 2916–2926.
https://doi.org/10.53730/ijhs.v6nS9.13157

Detection of *16SrRNA* gene Polymorphism using PCR for some bacterial species of clinical samples

Saif Saad Al-janabi
Medical Laboratory techniques department, Al-maarif University College, Iraq
Email: saifsaad1989aljanbi@gmail.com

***Abstract*---**Fifty samples were collected from patients suffering from the presence of infections in the skin, including wounds and burns, from inside the Fallujah Teaching Hospital. The samples were collected in the period from September to December 2021, and after isolation, transplantation, and microscopic and molecular diagnosis, 30 samples were obtained on which the bacteria appeared Including (*E. coli and K. pneumoniae*), which constitute about 60 % of the total samples. The microscopic test showed its development on different culture media. These isolates were cultured on Chrom agar, MacConkey agar, and Blood agar. It was found that there was a variation of the species, where the highest percentage of bacteria (*E. coli and K. pneumoniae*) appeared, and biochemical tests were also used. By using the diagnosis of the 16srRNA gene to detect the variation between the isolates by the PCR technique. The results of the sequence analysis were examined by blast in the National Center for Biotechnology Information (NCBI) and BioEdit program to detect polymorphism. Eight variations between three transversion and five transition nucleotides were discovered, and 99 percent under the sequence IDs MT453882.1 and KJ803927.1 from 53 to 902 and from 46 to 904 from Klebsiella pneumoniae strain CCFM8370 of Gene Bank were shown. These variations had scores of (1463), (1545 (1538).

Keywords*---Klebsiella pneumoniae*, *Escherichia coli, 16s rRNA* gene, Transversion, Transition.

Introduction

There are numerous species of *Klebsiella* in nature. This is believed to be the result of different sublineages generating niche-specific adaptations and the corresponding metabolic adaptations that make them more adapted to a given environment. They can be found in humans, as well as in water, soil, plants, insects, and other creatures (1,2).

Compared to other members of the Enterobacteriaceae family, *Klebsiella* bacteria are typically rounder and thicker. Most often, they take the form of straight rods with rounded or slightly pointed ends. They come alone, in pairs, or in brief chains. In vivo, diplobacillary forms are frequently observed (3).

The lower intestine of warm-blooded species (endotherms) is frequently home to the Gram-negative, facultatively anaerobic, rod-shaped coliform bacterium *Escherichia coli* (4). Although the majority of *E. coli* strains are benign, some serotypes (EPEC, ETEC, etc.) can seriously poison their hosts, and they occasionally cause food contamination situations that force product recalls (5,6). A symbiotic relationship between the innocuous strains, which are a typical component of the gut's microbiota, and their hosts allows them to produce vitamin K2 (which aids in blood clotting) and prevent the colonization of the intestine with dangerous bacteria (8,9). Within fecal matter, *E. coli* gets released into the environment. For three days, the bacterium thrives rapidly in fresh feces under aerobic circumstances, but after that, it gradually starts to drop in numbers (10,11).

The 1550 base pair *16S rRNA* gene has numerous polymorphisms that are utilized to distinguish it. While the variable area sequence is employed for taxonomy, the used primers are designed to complement the target region at a specific area at the beginning or end of the gene (12). Despite the fact that (500) and (1500) base pairs are frequently employed for sequencing and comparison. Many strains, including Genbank, which has more than 20 million of the deposited sequences, had their 16S rRNA sequences determined (13,14). The sequences that were deposited were utilized to compare the sequencing. Since *16S rRNA* is a gene found in all bacteria, it is utilized to examine the relationships among them. The comparison of bacteria's *16S rRNA* sequences is extremely helpful for detecting bacteria. It is also used to categorize bacteria's strains and build a relationship tree among bacteria (15). However, there are some rare instances where the 16S rRNA sequence is rendered meaningless by other sequences (13). The bacterial isolates that used in this study *Klebsiella pneumoniae* and *Escherichia coli.*

Methods

Samples collection

Fifty samples were collected from patients suffering from the presence of infections in the skin, including wounds and burns, from inside the Al-Ramadi Teaching Hospital. Including (*Klebsiella pneumoniae and Escherichia coli*), which constitute about 60% of the total samples. The microscopic test and its development on different culture media showed that this bacterial colony is Gram-negative and Gram positive colonies and are unable to move.

Isolation and Identification of bacterial isolates

After collecting the samples have been cultured directly on the Blood agar, MacConkey agar and EMB, even activated and allowed most of the bacterial genus to grow and incubated at 37^{o}C for 24 hrs.

DNA Extraction

In accordance with the manufacturer's instructions for the Genomic DNA Mini Kit, DNA was isolated from bacterial broth (Intron -Korea). To verify that DNA was present in each sample, the extracted DNA was electrophoresed on an agarose gel (0.5 percent agarose stained with 5 l of ethidium bromide), after which DNA-containing microcentrifuge tubes were stored at -20 oC and even employed in PCR (16).

PCR Reaction

According to the manufacturer's instructions, specific primers for the *16S rRNA* gene (F-5 AGAGTTTGATCCTGGCTCA 3 and R-5 GGTTACCTTGTTACGCTT - 3), are prepared by combining the primers with water to generate a stock solution at (100) pmol/ µl (17). Using the formula (C1V1 = C2V2), the stock of the primer is diluted with water to create the final working concentration of (10) pmol/ µl. The master mix done in accordance with (Bioneer Company, made in Korea). The source of the master mix was (MgCl2, dNTPs, KCl, polymerase, Tris-HCl, stain, and stabilizer). DNA was required for the reaction, and F and R primer were added to create the mixture. The mixture was then brought to volume (25 µl), mixed, and then vortexed (18) and program of PCR for detect the *16SrRNA* gene show in table 2.

Table 1: Mixture of the specific interaction for diagnosis *16srRNA* gene

Components	Concentration
Green Master Mix	12.5µl
Primer Forward	1 µl
Primer Reverse	1µl
DNA	1.5µl
Distill water	9 µl
Final volume	25µl

Table 2: The optimum condition of detection *16SrRNA* gene

No.	Phase	Tm (˚C)	Time	No. of cycle
1 -	Initial Denaturation	94°C	5 min.	1 cycle
2 -	Denaturation -2	94°C	30Sec	35 cycle
3 -	Annealing	55°C	30Sec.	
4 -	Extension-1	72°C	30 Sec.	
5 -	Extension -2	72°C	10 min.	1 cycle

The final products were put through an electrophoresis using Agarose gel (1.5%) and dyed with 3 µl of Red safe. The Tris-borate-EDTA buffer used in electrophoresis is made up of boric acid, Tris-base, and EDTA. The DNA ladder ranges in size from 100 to 1500 bp (Roche, US). In the gel, PCR byproducts were employed. 110 V was present for (60) minutes. Red Safe is a stain developed by the Paisley firm in the UK that is used to stain DNA before being observed under ultraviolet light at a (1500 bp band) (17).

Sequencing and Sequence Alignment

After red safe staining, PCR products were separated on a 1.5% agarose gel and examined using UV light (336 nm). National Instrumentation Center for Environmental Management (NICEM) online at (http://nicem.snu.ac.kr/main/?en skin=index.html), biotechnology lab, DNA sequencer 3730XL, Applied Biosystem) sequenced 16srRNA gene. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) and BioEdit programs.

Results and Discussion

Bacterial Isolate

Out of the 50 total samples, the first round of isolation revealed 30 isolates, of which 11 had *Klebsiella* sp. colony characteristics and 19 had *E. coli* colony characteristics. These samples contained pink colonies on chrom agar and blue beautiful colonies with a mucus texture, which is one of the key characteristics of *Klebsiella pneumoniae*.

Microscopic test

Microscopic test has shown that the cells form of short bacilli negative to Gram stain, non-motile and is arrangement form single or pairs or short chain (19).

Biochemical tests

Upon microscopic examination, culture diagnosis, and biochemical testing, it was determined that 30 isolates out of the total of 50 samples 11 that underwent these tests were positive for *Klebsiella* spp. and 19 isolates were positive for *E. coli*. In applying the motility test, it is shown that *Klebsiella* spp. non motile by making a twinge in semi solid medium where it was noted the non-proliferation growth outside the twinge. This is considered an important test for being able to differentiate between Klebsiella spp. and other Enterobacteriaceae species that are similar *Klebsiella* spp. in many from culture qualities. This motility test and Indol test are important tests for recognizing the *Klebsiella* spp. and *E.coli*, by which K. pneumoniae has been recognized so has *K.oxytoca* by producing Indol ring in which *K.oxytoca* is positive to this test and both species are non-motile, while *E.coli* gives the positive result to Indol test and which it resembles *K.oxytoca* and differs from *E.coli* is motile table 3.

Table 3: Biochemical tests used for identification and differentiation between *K. pneumonia and E.coli*

Tests	*K. pneumonia*	*E.coli*
Indole	-	+
Motility	-	-
Capsule	+	+
Oxidase	-	-
Catalase	+	+
Methyl red	-	+
Voges proskauer	+	-
Citrate utilization	+	-
Urease production	+	+

Extraction of DNA

Using an extraction kit provided by Intron Company, DNA was extracted from S. aureus. All of the isolates (17) from ear infections were initially grown on Mannitol

Salt Agar Medium in order to activate bacteria that had been kept on the Maintenance Medium before being transferred to the Nutrient Broth. Measure the concentration and purity of samples when the extraction procedure is finished (figure 1).

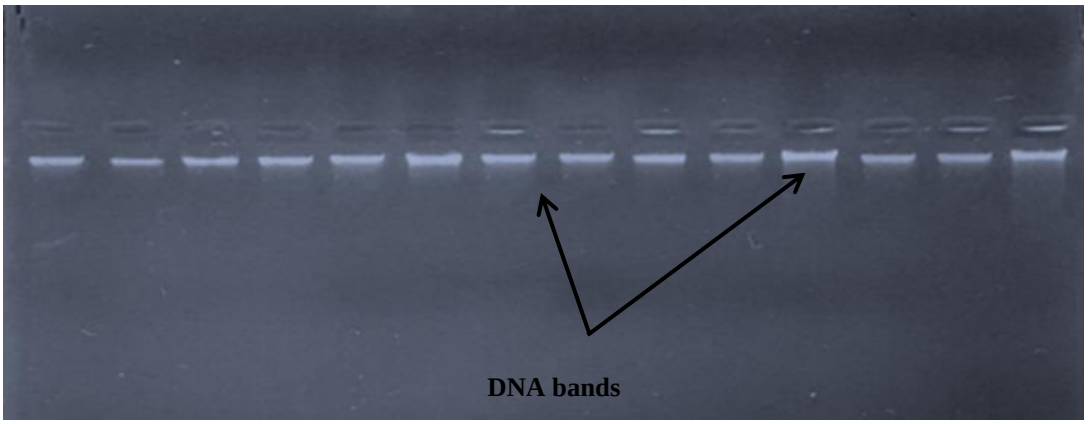

Figure 1: Genomic DNA extraction by gel electrophoresis Gel at 6 vols/cm at 1 percent agarose for one hour

Diagnosis via PCR

The reaction was carried out in volume 25 µl using a PCR kit in accordance with Promega's instructions. Table 1 displays the particular compound for *16SrRNA* gene diagnosis (1500bp). By adjusting annealing temperature, time, and cycle number, conditions can be changed to provide the best possible result for each primer (20,21).

4.4.4 Detection of *16SrRNA* gene

The optimal conditions (Initial denaturation and annealing) have been identified after several experiments. The temperature has been changed through gradient PCR for all samples to select the optimal condition, and the DNA template concentration has been changed between (1.5-2µl). These two factors are important in primer annealing with complement. It has been added 1l from Forward primer (F) 10 Pico mole concentration and the same amount from Reverse primer (R) and the same concentration and 1.5µl from DNA template, the temperature has used in PCR steps (94^{o}C)to initial denaturation for 5 minutes to one cycle as for annealing temperature has adopted on gradient PCR where used several temperature in the same time to shorten the time (50,52,54,56,58 and 60^{o}C) for 30 second.

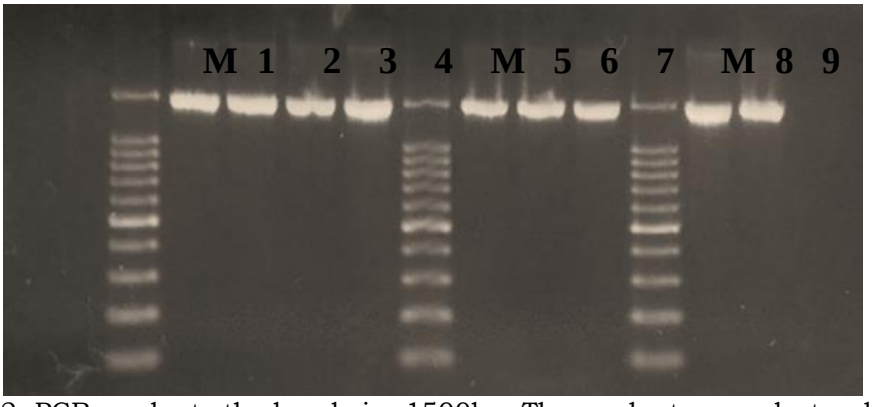

Figure 2: PCR products the band size 1500bp. The products was electrophoresis on 1.5% agarose at 5 volt/cm2 . 1x TBE buffer for 1:30 hours. M: DNA ladder (100-1500 bp), lane (1-9) PCR product of band size 1500bp. visualized under U.V light

Sequencing and alignment of NCBI

The results shown in (Figure 3 and Figure 4) indicated that a yield of single band of the desired product with a molecular weight of 1500 bp for *16srRNA* gene of *K. pneumoniae* and (Figure 5) of *E.coli* were obtained from 3 samples. Four PCR product samples were sent for sequence analysis; and 25 µl (10 pmol) from the forward (F) primer. The samples were treated with AB13730XL Applied Biosystems machine in National Instrumentation Center for Environmental Management NICM/USA company online at (http:// nicem.snu.ac. kr/main/?en_skin= index. html) (22). The results of the sequence analysis was analyzed by blastn in the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program to detect polymorphism in 16srRNA, found 8 variation between 3 transversion (refers to the substitution of a (two ring) purine for a (one ring) pyrimidine) and 5 transition (a point mutation that changes a purine nucleotide to another purine (A ↔ G) or a pyrimidine nucleotide to another pyrimidine (C ↔ T), showed 99% compatibility as shown in (table 4), under sequence **ID: MT453882.1**| and **KJ803927.1** from 53 -902, number of nucleotide from *Escherichia coli* strain EC87E and from 46 – 904 from *Klebsiella pneumoniae* strain CCFM8370 of Gene Bank, and score (1463), (1545) and (1538).

Klebsiella pneumoniae strain CCFM8370 16S ribosomal RNA gene, partial sequence

Sequence ID: KJ803927.1Length: 1429Number of Matches: 1
Range 1: 46 to 905GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1538 bits(1705)	0.0	857/860(99%)	0/860(0%)	Plus/Plus

Query 661
CTGACGCTCAGGTGCGAAAGCGTGGGGAGCAGACGGGATTAGATACCCTGGTAGTC
CACG 720

Sbjct 706 .....**A**..**A**..... 765

Query 721
CCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAAC
GCG 780
Sbjct 766 825

Query 781
TTAAGTCGACCGCCTGGGGAGTACGGCCGCCAGGTTAAAACTCAAATGAATTGACGG
GGG 840
Sbjct 826 .....**A**..... 885

Figure 3: Sequencing of sense flanking the partial *16srRNA* gene for *K. pneumoniae*

Klebsiella pneumoniae strain CCFM8370 16S ribosomal RNA gene, partial sequence

Sequence ID: KJ803927.1Length: 1429Number of Matches: 1
Range 1: 46 to 904GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1545 bits(1713)	0.0	858/859(99%)	0/859(0%)	Plus/Plus

Query 781
TTAAGTCGACCGCCTGGGGAGTACGGCCGCCAGGTTAAAACTCAAATGAATTGACGG
GGG 840
Sbjct 826 .....**A**..... 885

Query 841 CCCGCACAAGCGGTGGAGC 859
Sbjct 886 904

Figure 4: Sequencing of sense flanking the partial *16srRNA* gene for *Klebsiella pneumoniae.*

Escherichia coli strain EC87E 16S ribosomal RNA gene, partial sequence

Sequence ID: MT453882.1Length: 1426Number of Matches: 1
Range 1: 36 to 856GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1463 bits(1622)	0.0	817/821(99%)	0/821(0%)	Plus/Plus

Query 661
CCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAGACGGGATTAGATACCC
720
Sbjct 696 .....**A**..**A**..... 755
Query 721
GGGTAGTCCACGCCGTAAACAATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCC
780
Sbjct 756 **T**.....**G**..... 815

Figure 5: Sequencing of sense flanking the partial *16srRNA* gene for *E.coli*

Table 4: Represent type of polymorphism in of sense flanking the partial *16srRNA* gene of *E. coli* strain EC87E *K. pneumoniae* strain CCFM8370

Gene: 16S ribosomal RNA gene						
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Source**	**Identities**
1	Transition	737	A\G	ID: KJ803927.1	*Klebsiella pneumoniae*	99%
	Transition	740	A\G			
	Transvertion	856	A\C			
2	Transvertion	856	A\C	ID: KJ803927.1	*Klebsiella pneumoniae*	99%
3	Transition	739	A\G	ID: MT453882.1	*Escherichia coli*	99%
	Transition	742	A\G			
	Transvertion	756	T\G			
	Transition	776	G\A			

Conclusions

The more antibiotics do not have effect toward *K. pneumoniae and E.coli* which was resistance to them. *Klebsiella pneumoniae* that was more *Klebsiella* species spread from *Klebsiella* species. Analyzed results of the sequence by (NCBI) found 8 variations between 3 transversions , 5 transitions nucleotide, showed 99% under sequence **ID: MT453882.1** | and **KJ803927.1** from 53 -902, number of nucleotide from *Escherichia coli* strain EC87E and from 46 – 904 from *Klebsiella pneumoniae* strain CCFM8370 of Gene Bank, and score (1463), (1545) and (1538).

References

1. Bagley S (1985). "Habitat association of Klebsiella species". Infect Control. 6 (2): 52–8.
2. Brisse S, Grimont F, Grimont PD (2006). Prokaryotes. New York, NY: Springer New York. pp. 159 - 196.
3. Ristuccia, Patricia A; Cunha Burke A (1984). "Klebsiella". Topics in Clinical Microbiology. 5 (7): 343–348.

4. Tenaillon O, Skurnik D, Picard B, Denamur E (March 2010). "The population genetics of commensal Escherichia coli". Nature Reviews. Microbiology.
5. Singleton P (1999). Bacteria in Biology, Biotechnology and Medicine (5th ed.). Wiley. pp. 444–54.
6. CDC National Center for Emerging and Zoonotic Infectious Diseases. Retrieved 2 October 2012.
7. Vogt RL, Dippold L (2005). "Escherichia coli O157:H7 outbreak associated with consumption of ground beef, June–July 2002" . Public Health Reports. 120 (2): 174–78.
8. Bentley R, Meganathan R (September 1982). "Biosynthesis of vitamin K (menaquinone) in bacteria" . Microbiological Reviews. 46 (3): 241–80.
9. Hudault S, Guignot J, Servin AL (July 2001). "Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection" . Gut. 49 (1): 47–55.
10. Al-Hadeithi, Z. S., Al-Kazaz, A. K. A., & Al-Obaidi, B. K. (2012). Genetic diversity and relationships among Iraqi barley cultivars using RAPD–PCR technique. *The Iraqi Journal of Agricultural Science*, *43*, 117-124.
11. Russell JB, Jarvis GN (April 2001). "Practical mechanisms for interrupting the oral-fecal lifecycle of Escherichia coli". Journal of Molecular Microbiology and Biotechnology. 3 (2): 265–72.
12. Al-Awsi, G. R. L., Al-Hadeithi, Z. S., Jasim, S. A., Alkhudhairy, M. K., & Ghasemian, A. (2022). Virulence traits and plasmid-mediated quinolone resistance among Aggregatibacter actinomycetemcomitans from Iraq: Low rate of highly virulent JP2 genotype. Microbial Pathogenesis, 164, 105438.
13. Gee, J. E., Sacchi, C. T., Glass, M. B., De, B. K., Weyant, R. S., Levett, P. N., ... & Popovic, T. (2003). Use of 16S rRNA gene sequencing for rapid identification and differentiation of Burkholderia pseudomallei and B. mallei. *Journal of clinical microbiology*, *41*(10), 4647-4654.
14. Dewhirst, F. E., Paster, B. J., Tzellas, N., Coleman, B., Downes, J., Spratt, D. A., & Wade, W. G. (2001). Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family Coriobacteriaceae: description of Olsenella gen. nov., reclassification of Lactobacillus uli as Olsenella uli comb. nov. and description of Olsenella profusa sp. nov. *International journal of systematic and evolutionary microbiology*, *51*(5), 1797-1804.
15. Clarridge III, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical microbiology reviews*, *17*(4), 840-862.
16. Panova, M., Aronsson, H., Cameron, R. A., Dahl, P., Godhe, A., Lind, U., ... & Johannesson, K. (2016). DNA extraction protocols for whole-genome sequencing in marine organisms. In *Marine genomics* (pp. 13-44). Humana Press, New York, NY.
17. Klaif, S. F., Naser, H., & Sadeq, J. N. (2019). The genetic relationship for Klebsiella pneumoniae isolated from human urinary tract and beef. *Iraqi Journal of Veterinary Sciences*, *33*(1), 75-80.
18. Jasim, S. A., & Farhan, S. M. (2020). Variation of 16SrRNA gene for Some Nosocomial Bacteria Isolated from Ramadi Teaching Hospital for Women and Children. *Indian Journal of Forensic Medicine & Toxicology*, *14*(4).

19. Facklam, R., & Elliott, J. A. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clinical microbiology reviews*, *8*(4), 479-495.
20. Al-Hadeithi, Z. S. (2016). Detection of genetic polymorphism in Iraqi barley using SSR-PCR Analysis. *Iraqi Journal of Science*, *57*(2B), 1158-1164.
21. Abed, S. M., Raoof, W. M., Assie, A. H., Al-Hadeithi, Z. S., & Ibrahim, F. (2018). Detection of Some Staphylococcal Enterotoxin Genes in MRSA Strains Using PCR Techniques. *Tikrit Journal of Pure Science*, *21*(3), 33-38.
22. Dheeb, B. I., Al-Judy, N. J., Al-Hadeithi, Z. S., Abdulateef, S. M., Mohammad, F. I., & Hasan, G. M. (2014). Genotyping based on aflr1 gene sequencing in local Iraqi isolates of phylopathoginic-aflatoxigenic Aspergillus flavus. *International Journal for Sciences and Technology*, *143*(1735), 1-12.