16SrRNA sequencing as tool for identification of Salmonella spp isolated from human diarrhea cases

IOP Conf. Series: Journal of Physics: Conf. Series **1294** (2019) 062041 doi:10.1088/1742-6596/1294/6/062041

16SrRNA sequencing as tool for identification of Salmonella spp isolated from human diarrhea cases

Hayder Kamil Jabbar AL Kaabi [1] and Abdul K. Salman AL-Yassari [2]

[1]Nursing college University of Al-Qadisiyah
[2]College of veterinary Medicine/ Al-Qasim Green university

Email: hayderalkaaby@qu.edu.iq

Abstract. This study was conducted in different location of Babylon , (75) clinical specimens were collected, obtained from patients suffering from severe diarrhea were attended to Merjan teaching hospital and Private clinics in Babylon province, and suffer from severe diarrhea that was associated with fever as diagnosed by physician, The period of specimens collection extended from September 2017 to January 2018. Results showed that out of 75 studied specimens of patients, 14 specimens were Salmonella positive (18.66 %) .Salmonella isolates were isolated and identified by using bacterial culturing on selective media and confirming tests such as *16S rRNA* gene amplification, all results of these diagnosis methods referred to all isolates belong to *Salmonella* spp. Depending on sequencing method for nucleotide sequence for 660 bp of *16S rRNA* gene we sent only 10 isolates to registration these sequences in Gene Bank-NCBI for diagnosis of these isolates on species level and obtaining accession numbers then phylogeny. Ten accession numbers were obtained from *16SrRNA* and registration of 10 sequences of *16SrRNA* gene at gene bank-NCBI include one accession number (MH109326.1) for *Salmonella enterica subsp. enterica* serovar Typhimurium , Five accession numbers (MH156032.1, MH109512.1, MH109501.1, MH155972.1 and MH156033.1)belong to *Salmonella enterica sub.sp enterica* serovar Typhi .While *Salmonella enterica sub.sp enterica* serovar Paratyphi recorded under two accession number (MH155974.1 and MH156039) and *Salmonella enterica sub.sp enterica* serovar Enteritidis recorded under two accession number (MH109386.1 and MH155973.1) respectively .

Keyword: Salmonellosis ,*Samonella typhimurium* ,*16SrRNA* ,DNA sequencing.

1. Introduction

Salmonellosis is disease caused by a group of bacteria belong to genus salmonella that can infect human and animals and considered as one of the most common foodborne diseases worldwide, accounting around 93.8 million foodborne illnesses and 155,000 deaths per year worldwide. Salmonella infection is a serious problem to public health and being all of the world that lead to economic loss result from morbidity, mortality and poor growth hazard of transmitting food poisoning with gastroenteritis to human. People at risk for serious complications due to salmonella food poisoning include older adults, pregnant women, infants, children and people who have compromised immune system (1, 2) .

Human stool acts as an important reservoir of salmonella serovars that are the grouping of microorganisms based on their cell surface antigen, the importance of salmonellosis in public health field is growing concern day by day around the world and over the last several decades there have been significant shift in predominant salmonella serovars associated with human infections (3).

Salmonella Typhimurium is the most important frequently isolated from global food – borne outbreaks, poultry are one of the most important reservoirs of salmonella that can be transmitted to human through the food chain. Recent reports of increasing incidence of multiple antibiotics resistant strain of *S.* Typhimurium in human has a major emerging public health issue of international concern and this dramatic increasing in drug resistance will complicate the options available in the treatment of salmonellosis (4). I have recently attempted to isolation and identification of *Salmonella* spp from human stool specimens depend on routine clinical and laboratory techniques such as culture, PCR (*16S rRNA*) and sequencer methods (Phylogenetic tree).

2. Material and methods

2.1. Specimens collection:

Patients specimens: A valid consent was achieved from each patients before their inclusion in the study. Stool specimens were collected from (75) patients suffer from fever and diarrhea after diagnosis by physician ,those patients were attended to Merjan teaching hospital and Private clinics in Babylon province. The stool specimens were received in sterile plastic containers where a loop full of each specimen was immediately inoculated into a sterile tube containing Brian Heart infusion broth .

All specimens transferred to microbiology labrotary in the department of microbiology /Veterinary medicine/ AL-Qasim Green University and unit of zonatic disease /Veterinary medicine/ University of Al-Qadisiyah to investigated and demonstrating the occurrence of *Salmonella* spp in human .

2.2. Isolation and Identification

Isolation

Specimens were collected and transported to the laboratory in Brian Heart infusion broth to allow the multiplication of bacteria; They were incubated for 24–48 hour at 37 °C. The broth culture was aseptically streaked on Salmonella-Shigella Agar (SSA), plates were incubated at 37 °C for 18–48 hour, after which, they were examined for colonies typical of Salmonella. Suspect colonies were streaked on nutrient agar plates to obtain pure cultures which were subjected to another media for confirming the identification (5**).** Identification of Salmonella species was done microscopically by using Gram stain (6). Suspect Salmonella colonies that streaked on nutrient agar plates to obtain pure cultures were subculture on SSA, Bismuth Sulphite Agar (BSA), MacCkonkey agar and Chromogenic agar plates incubated at 37 °C for 18–48 hours for study the phenotypic characters of suspected Salmonella isolates (1).

2.3. Confirming Identification

All suspected isolates were confirmed by using the molecular identification via *16SrRNA* and sequencing method as follows:

2.4. Molecular identification of Salmonella spp.

Separation of genomic DNA from Salmonella spp.

Transferred 1 ml of bacteria cell (up to 1 x 10^9 cfu/ml) that grow on Luria-Bertoni broth media and incubated for 18 hours to a 1.5 ml micro centrifuge tube and DNA extracted according to the manufacturer's instructions(Anatolia/Turky).

Estimation of DNA extracts

The extracted DNA was checked by using nanodrop that measured DNA concentration (ng/µL) and checked the DNA purity by reading the absorbance at (260/280nm) According to the device instructions(Thermol / U.S.A).

Polymerase Chain Reaction amplification

Amplification of 16S rRNA gene via PCR technique

The full length of *16S rRNA* gene was amplified via conventional PCR using the universal *16S rRNA* primer set (F-5'-GGAACTGAGACACGGTCCAG -3' and R-5'-CCAGGTAAGGTTCTTCGCGT-3'). PCR reaction volume was set to be 25 µL. It contained 3µL (30ng) of genomic DNA, 1.5 µL (15 pmol) of each forward and reverse primer, 12.5 µL of 2X Master Mix and 6.5 µL of nuclease free water. Then reaction tubes were put in the PCR thermocycler. PCR conditions were set to be as follows: 5 minute at 95^{o}C for initial denaturation, 30 cycles each 1 minute at 94^{o}C for denaturation, 1 minute at 60^{o}C for annealing and 30 second at 72^{o}C for extension and final extension at 72^{o}C for 10 minute, after termination of PCR, the PCR product was run on 1% agarose along with DNA ladder. Gel document was used at 320-336 nm for the observation of DNA bands, and the gel was photographed using digital camera.

Purification and sequencing of PCR product

The amplified fragment of *16S rRNA* gene was purified using PCR Clean UP-kit following the manufacturer instructions. The purified PCR product was sequenced along with the aforementioned universal primer set. The *16SrRNA* sequence was determined with a model 373A automated fluorescent- DNA sequencer.(Applied Biosystem / U.S.A)

Analysis of the PCR product sequence

The obtained nucleotide sequence of 16S *rRNA* gene was processed through Finch TV software. Analysis of the PCR product sequence was analyzed using BLAST N (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) that is an online programme belonging to NCBI (National Center for Biotechnology Information) to determine the hits of subjects sequences deposited in the international nucleotide databases (e.g., GenBank, EMBL, DDJD, etc.) giving the best matching with the query sequence.

3. Results and Discussion

3.1. Incidence of Salmonella according to specimen's types:

The results showed that of out of 75 studied specimen of human, 14 specimens were *Salmonella* positive 14/75 (18.66%) table (1).

Table (1): Isolation rates of *Salmonella* spp. from collected specimens.

Source of specimens	No. of examined specimens	No. of positive specimens	% of positive specimens
Human	75	14	18.66
X2 Calculated =1.368	df = 1		P < 0.01

3.2. Morphological and Microscopically Characterization of Salmonella spp.

Since the isolation and correct identification of Salmonella are very crucial for the characterization purpose, the colonies having typical cultural characteristics were selected as presumptive for *Salmonella* spp. (7).

The colony characteristics of *Salmonella* spp. found in this study was black, smooth, small round colonies on SSA. (Figures 1, A). On the MacConkey Agar, colonies appeared pale yellow or nearly colorless, 1-3 mm in diameter and non-lactose fermented. (Figures 1, B). While growth of these bacteria on BSA more colonies grow as black colonies with a surrounding metallic sheen resulting from H_2S production and reduction of sulphite to black ferric sulphide and some colonies grow as light green colonies (Figures 1, C). Colonies features on SSA and BSA plates revealed that it is a member of family enterobacteriaceace particularly *Salmonella* spp. On the other hand, the appearance of colonies on chromogenic agar were variable in size convex and mauve in color as shown in (Figure 1, D). This finding were similar to the findings of other authors (8, 9).

In Gram's staining the organism appeared as gram negative, short rod shaped bacteria, arranged in single and paired under light microscope, these characteristics corresponding to *Salmonella* spp that mentioned by (10). As a result, 14 isolates from human which showed above features on SSA, MacConkey agar, BSA and chromogenic agar were suspected as *Salmonella* spp. and subjected for further confirming tests such as *16S rRNA* gene amplification and DNA sequencing.

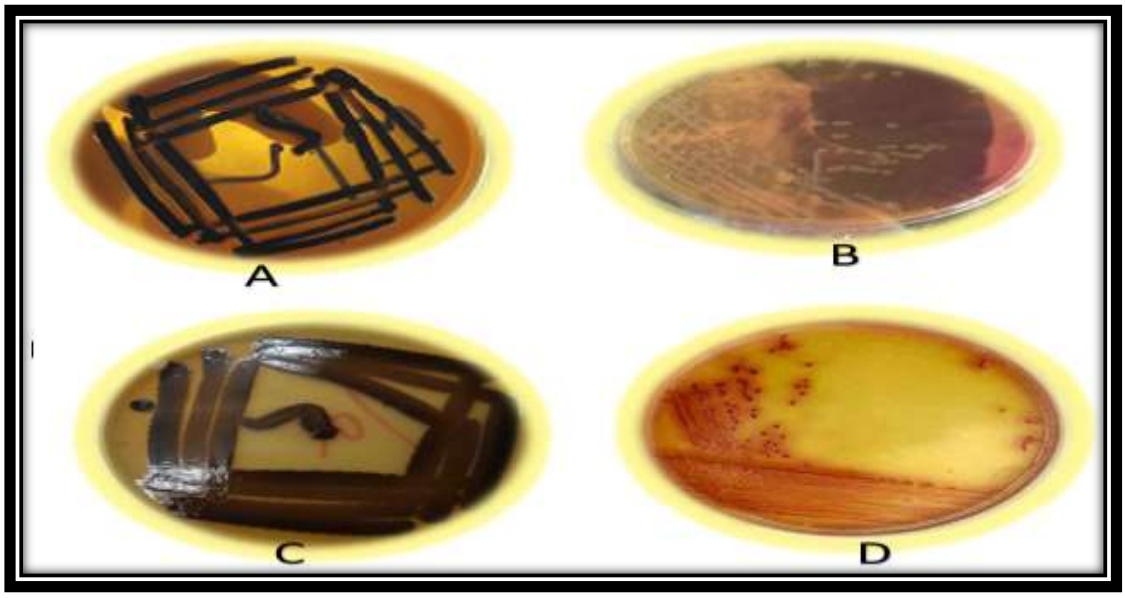


Figure (1) Colonies of *Salmonella* spp. isolate on: (A) SSA (B) MacConkey agar (C) BSA (D) Chromogenic agar.

3.3. DNA Amplification by PCR technique

Due to the rapid increasing nature of Salmonella infections, there is a need for the development of fast and reliable techniques for the immediate detection of these infections in order to initialize appropriate control measures. One of the limitations of phenotypic methods for bacterial identification is inability to identify the bacterium on a species level in some cases (11). Mostly biochemical profile didn't lead to accurate bacterial identification in most cases, reproducibility of result is not guaranteed, it depend mainly on metabolic fingerprint of the isolates that in turn varies based on the physiological status of isolate in the time of carrying out the assay (12). PCR is one of the most widely used

molecular tools for the rapid detection of several pathogens, consequently it was necessarily to carry out molecular identification of bacterial isolates ,in this regard molecular identification was carried out firstly: extracted DNA of all isolates with purified using genome DNA purification kit .The results were detected by nanodrop showed different DNA concentration ranged (98.8 to 125)ng/ml in addition to electrophoresis on 1% agarose gel and exposed to U.V light in which the DNA appeared as compact bands figure (2).

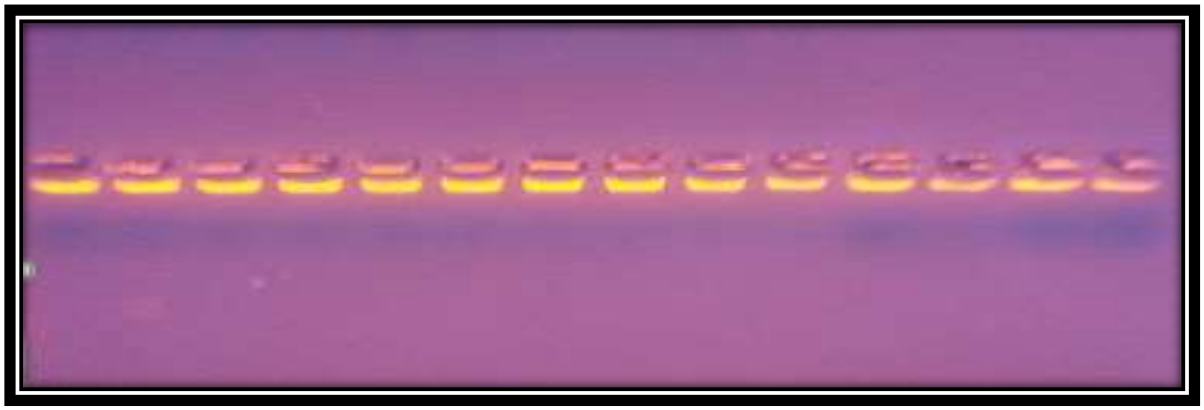


Figure (2): Total genomic DNA extracted from isolates using 1 % agarose gel electrophoresis (purified DNA of *Salmonella* spp.).

Secondly by using *16SrRNA* approach a gold standard technique in identification bacteria on genus levels (13). The full length 660 bp of *16S rRNA* gene as shown in (Figure 3) were performed on the DNA extracted from isolates and confirmed by electrophoresis analysis. By this analysis, the bands of DNA which resulted from the successful binding between specific primers and isolates from extracted DNA,these successful bindings appeared as single bands under the U.V. light using ethidium bromide as a specific DNA stain. The electrophoresis was also used to estimate DNA molecular weight depending on DNA marker (10000 bp DNA ladder). The results showed all isolates contain *16S rRNA* in molecular weight 660 bp. This study was closely related with the results of several authors such as (14 and 15) whom found that all isolates of Salmonella species were positive to *16s rRNA* gene. Targeting genes for the detection of Salmonella species is a promising tool for the rapid identification of these microorganisms (14).

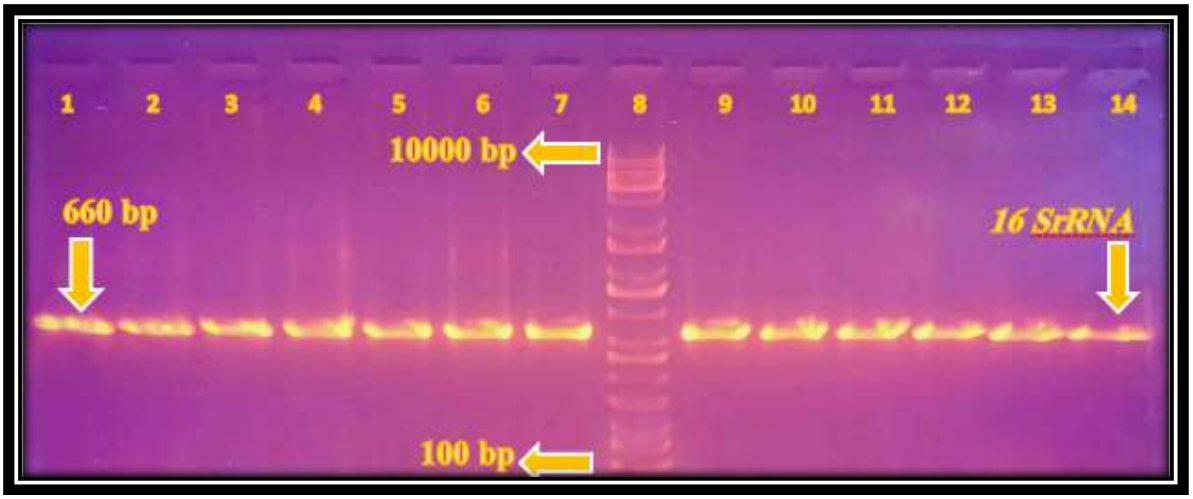


Figure (3) DNA amplification of 660 bp of *Salmonella* spp. detecting *16srRNA* gene using PCR. Lane 8= Ladder, lane,1,2,3,4,5,6 ,7,9,10,11,12 ,13,14 positive results.

3.4. Nucleotides sequencing sets

In the present work, the identification key in culture and *16SrRNA* mentioned above led to an assignment of the bacterial isolates on genus level only. This in turn addressed the indispensable need to identify the bacterial isolates on a species level via a molecular approach by sequencing method and to get more emphasis into the identification of the isolates, the amplified partial fragment of 660 bp was sequenced directly for 10 isolates. The sequencing was carried out (Macrogen Company, S. Korea) which uses Dye-terminator sequencing method. The sequences results were sent by e-mail as text fasta and waves as Pdf files, they showed different colors (red T base, green A base, black G base and blue C base).

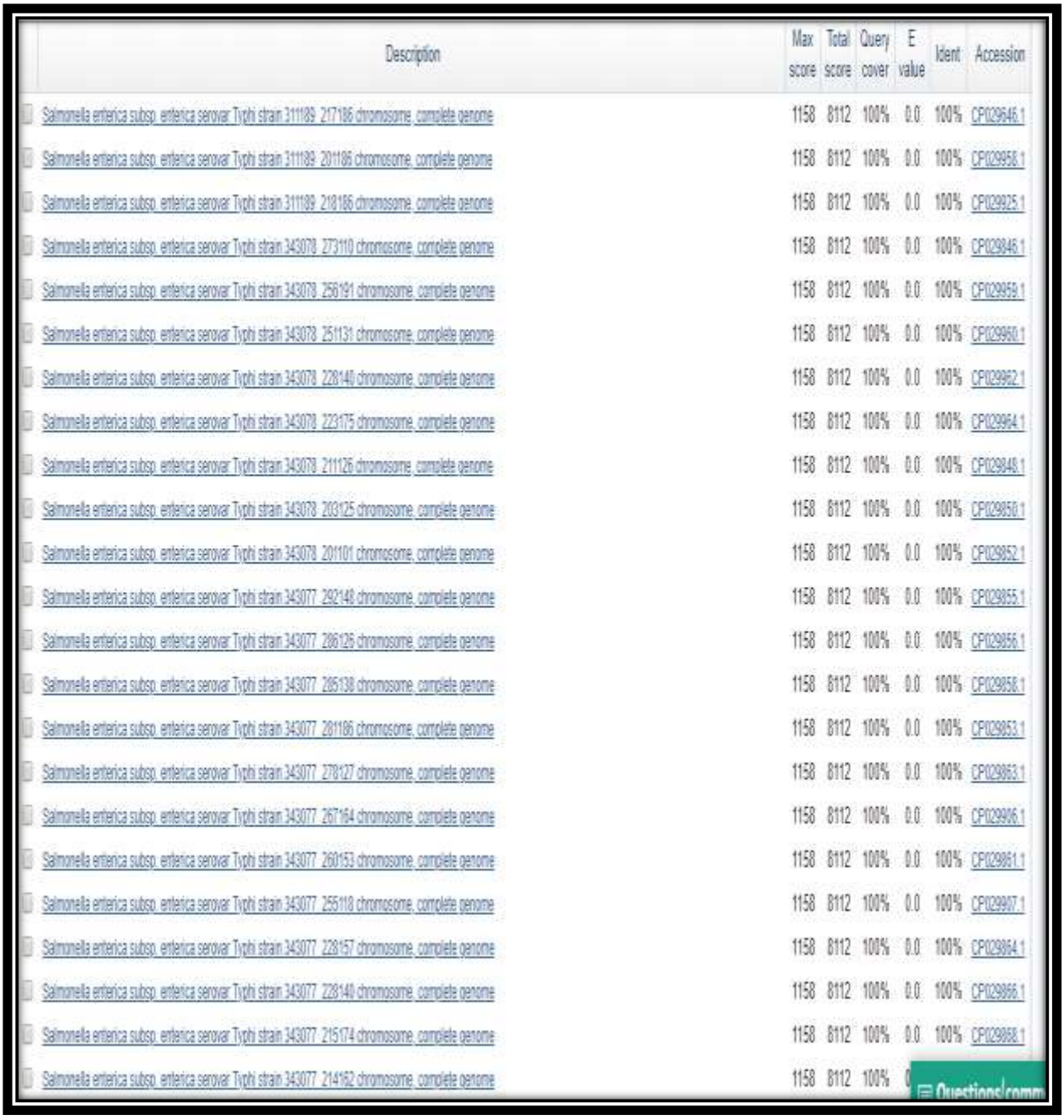
Each sequence data was trimmed from beginning to end corresponding with normal waves to produce another trimmed sequence, this sequence gives high identity to another global sequence data when compared to NCBI- Blast. These trimmed sequences give other definition names to inter Bankit Program for registration at gene bank.

Recently, due to the wide steps of development in researches and the widespread use of PCR, cloning and DNA sequencing, the *16S rRNA* gene sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel species. The*16S rRNA* gene is important for bacterial identification because it of its presence almost in all types of bacteria. Not only the presence of the *16S rRNA* gene in all bacteria but also the large size, candidates it is a universal target for bacterial identification and offers a wide scope of analysis in addition, *16S rRNA* gene sequencing is a fast method for identification of unusual phenotypic bacteria or slow growing bacteria (16).

3.5. Results of examination and confirmation of received nucleotides sets.

The results of nucleotides sets checked and confirmed by using (NCBI) Basic Local Alignment Search Tool (BLAST analysis) nucleotide blast Search a nucleotide database using a nucleotide query online. Sequences alignment must be perform by using references *16srRNA* gene of *S.*Typhimurium, *S.*Typhi, *S.*Paratyphi *and S.*Enteritidis sequences databases information recorded in Gene Bank to find identity and similarity score degrees of *16srRNA* gene and compared with our isolates .The results showed high identity, highly query cover, max score , total score while zero for E-value with other world *S.*Typhimurium, *S.*Typhi,*S.*Paratyphi and *S.*Enteritidis.(Figure 4).

The *16S rRNA* gene is important landmark in the study of the evolution and classification of living organisms. Because of the wide spread of the *16S rRNA* gene, it has served as base molecular identification tool for study of evolutionary relationships among groups of bacteria The sequencing of *16S rRNA* gene has been widely used for bacterial identification using the*16S rRNA* gene sequence databases at GenBank, which facilitate the identification of unknown bacteria up to the genus or species level, and the generating of information on phylogenetic relation between different bacteria (16*).*



Description	Max score	Total score	Query cover	E value	Ident	Accession
Salmonella enterica subsp. enterica serovar Typhi strain 311189_217186 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029646.1
Salmonella enterica subsp. enterica serovar Typhi strain 311189_201186 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029958.1
Salmonella enterica subsp. enterica serovar Typhi strain 311189_218186 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029925.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_273110 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029845.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_256191 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029959.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_251131 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029960.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_228140 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029962.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_223175 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029964.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_211126 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029849.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_203125 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029850.1
Salmonella enterica subsp. enterica serovar Typhi strain 343078_201101 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029852.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_292148 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029855.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_286125 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029856.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_285138 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029858.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_281186 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029853.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_278127 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029863.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_267164 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029966.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_260153 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029861.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_255118 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029967.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_228157 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029864.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_228140 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029866.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_215174 chromosome, complete genome	1158	8112	100%	0.0	100%	CP029868.1
Salmonella enterica subsp. enterica serovar Typhi strain 343077_214162 chromosome, complete genome	1158	8112	100%	0		

Figure:(4):gb-NCBI blasting of local sequence *16S rRNA* with world sequences of *16S rRNA***.**

3.6. Results of recording Iraqi Salmonella spp isolates based on 16SrRNA gene.

After DNA sequencing partial nucleotide of *16SrRNA* from each bacterial isolate was obtained and their lengths were as follows: Twenty-one sequences of *Salmonella* spp were isolated from human and chicken sources in Babylon Province and each sequence has symbol code (HK^1, HK11, HK12, HK15, HK^24, HK25, HK10, HK17, HK19 and HK20), and then submitted to Gen Bank,The results of these sequences analyzed and examined by professional staff in gene bank . All these sequences accepted in gene bank and each sequence takes accession number (MH156040.1, MH109501.1,

MH109512.1, MH155972.1, MH156033.1, MH156032.1, MH109386.1, MH155973.1, MH156039.1 , MH155974.1, ,) respectively and these results recorded and published in the International Nucleotide Sequence Database Collaboration (INSDC) this location contains the database of National Center for Biotechnology Information (NCBI).(Figure 5).

Several studies of diverse taxa showed that the majority of the identified species that have been examined to date differ in their *16S rRNA* gene sequences from related species of the same genus in at least 1% of the sequence positions and typically by more (17). (18) defined the cutoff values of *16S rRNA*-based bacterial identification. A value of ≥ 99% similarity of *16S rRNA* gene sequence should be a suitable cutoff for bacterial species identification and ≥ 97% for bacterial identification at genus level.

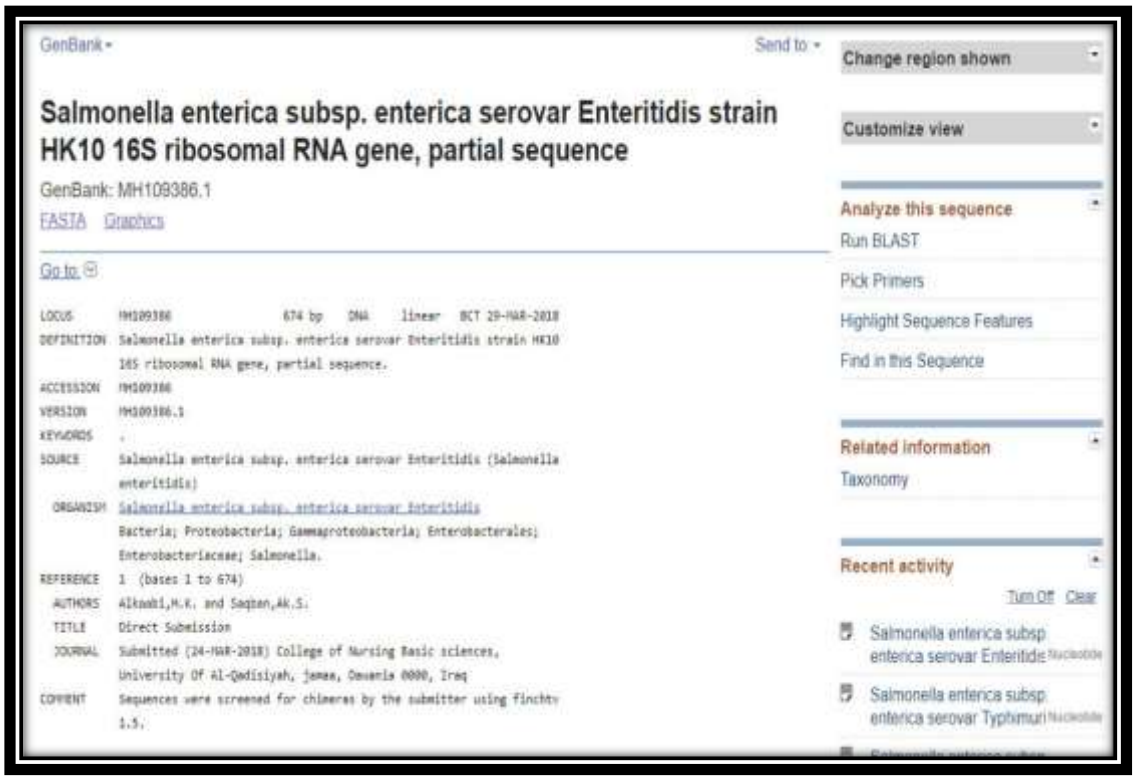


Figure (5): Accession number report of local sequence MH109386.1 at gb-NCBI.

Result of BLAST analysis, multiple sequence aliment (MSA) and phylogenetic trees figures (6, 7 and 8) conferred that *16SrRNA* nucleotide sequence of 21 bacterial deposited in international nucleotide databases with accession numbers and similarity as follows: *Salmonella* Typhimurium isolates which include HK1 under accession numbers MH156040.1 similar to (HM007581.1 in Germany; JQ694621.1in USA; ABB55734.1, in Saudia Arabia and KY50226.1 in India. So *Salmonella* Typhi. That include HK11, HK12, HK15, HK^24 and HK25 under accession number MH109501.1, MH109512.1, MH155972.1, MH156033.1 and MH156032.1 respectively similar to KJ740151.1 and GU826689.1 in India, While *Salmonella* Paratyphi which include, HK19 and HK20 under accession number MH156039.1 and MH155974.1 respectively, similar to EU118080.1 in Iran and MF772485.1 in China. Regarded to *Salmonella* Enteritidis that include HK10 and HK17 under accession number MH109386.1 and MH155973.1 respectively, similar to MF773880 in China.

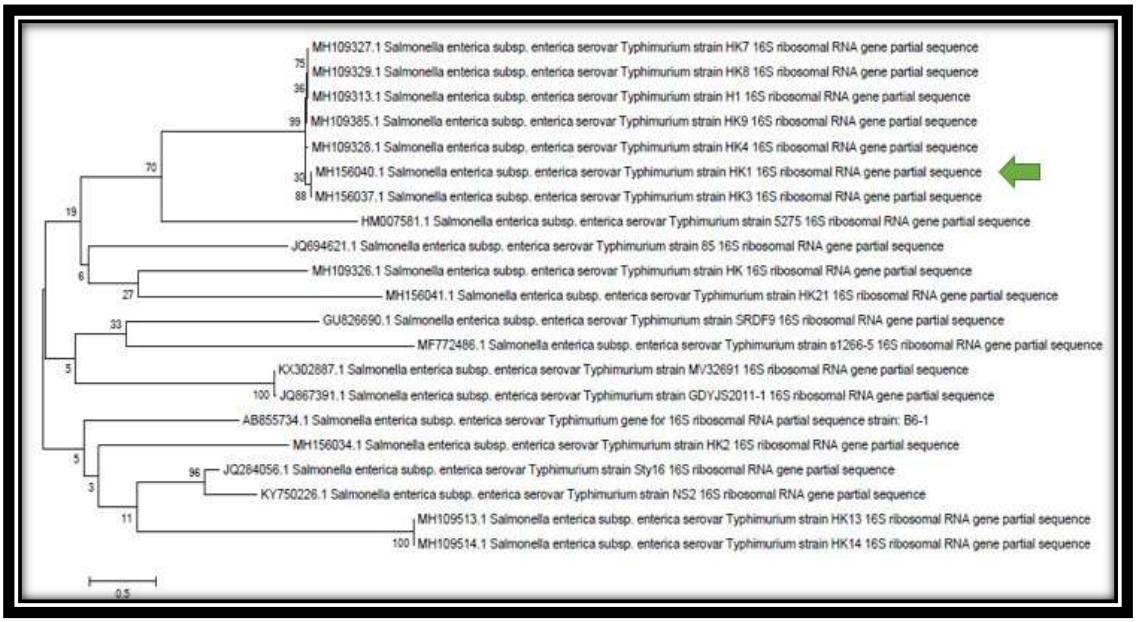


Figure (6): Neighbor- joining tree shows the phylogenetic relationship among *16S rRNA* sequences of 1 bacterial isolate (isolated from local human in Babylon- Iraq) belonging to *Salmonella enterica subsp. enterica* serovar Typhmurium and other *16S rRNA* sequences belong to closely related bacteria. They are expressed by their accession numbers in international nucleotide databases. Phylogenetic tree was constructed via MEGA 6 sequence viewer 6.5 software. Numbers on branch nodes represent bootstrap values (500 re-samplings). The twelfth bacterial isolates were indicated by green solid shape. ( ) .

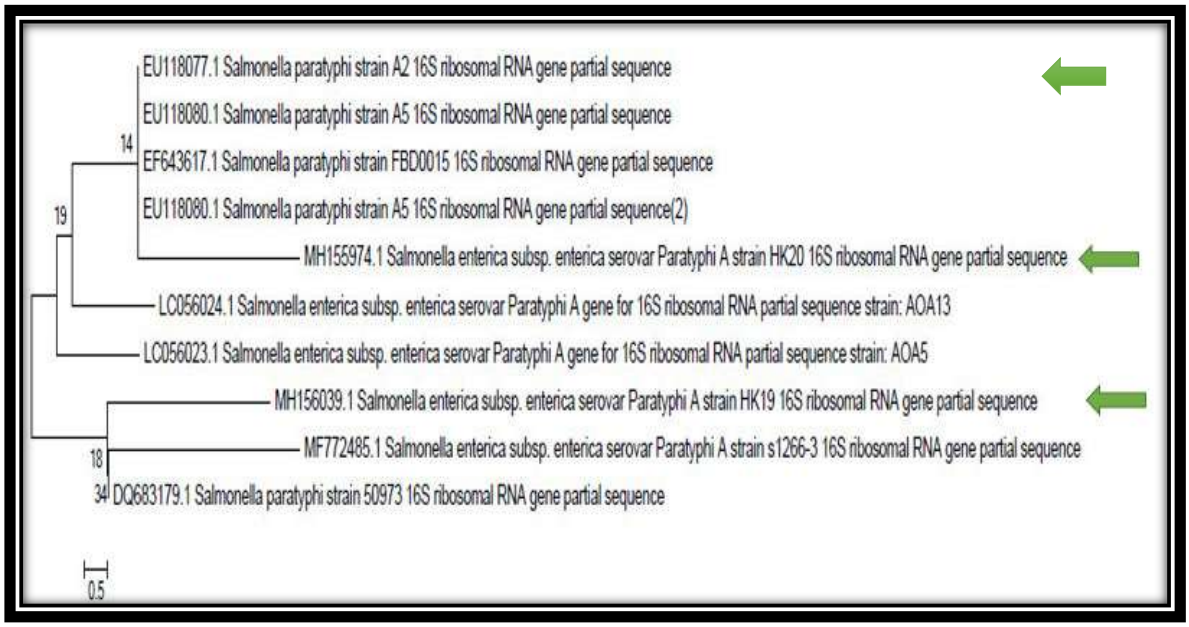


Figure (7): Neighbor- joining tree shows the phylogenetic relationship among *16S rRNA* sequences of 2 bacterial isolates (isolated from human in Babylon- Iraq) belonging to *Salmonella enterica subsp. enterica* serovar Paratyphi and other *16S rRNA* sequences belonging to closely related bacteria. they are expressed by their accession numbers in international nucleotide databases. Phylogenetic tree was constructed via MEGA 6 sequence viewer 6.5 software. Numbers on branch nodes represent bootstrap values (500 re-samplings). The tow bacterial isolates were indicated by green Row. ( )

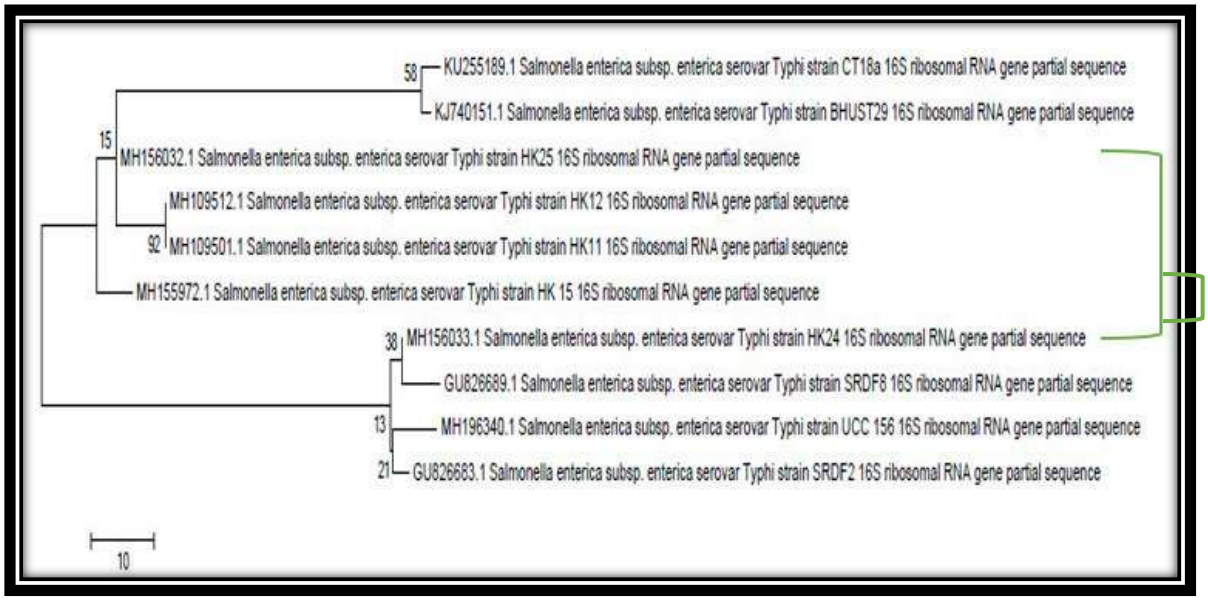


Figure (8): Neighbor- joining tree shows the phylogenetic relationship among *16S rRNA* sequences of 5 bacterial isolates (isolated from human in Babylon- Iraq) belonging to *Salmonella enterica subsp. enterica* serovar Typhi and other *16S rRNA* sequences belonging to closely related bacteria. they are expressed by their accession numbers in international nucleotide databases. Phylogenetic tree was constructed via MEGA 6 sequence viewer 6.5 software. Numbers on branch nodes represent bootstrap values (500 re-samplings). The five bacterial isolates were indicated by green solid shape ( ).

4. References

[1] **Nesa ,M.K ; Khan, M.S.R ; Alam ,M . (2011**) .Isolation ,Identification and Characterization of Salmonella serovars from diarrheic stool samples of human. *Bangl. J. Vet. Med*. 9(1): 85 – 93.

[2] Eng,S.K.; . Pusparajah, P.N.; Mutalib,S.A.; Ser,H.; Chan, K.L.; Lee,H. (2015). Salmonella: a review on pathogenesis, epidemiology and antibiotic resistance .Front Life Sci. 8 , pp. 284-293

[3] Steven, L.F.; Rajesh, N.;Irene, B.H.; Timothy, J.J.; Jing, H. and Steven, C.R. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied Environmental Microbiology* 77:4273-4279.

[4] Mohammed, M. (2017).Phage typing or CRISPR typing for epidemiological surveillance of Salmonella Typhimurium? BMC Res Notes;10:578

[5] **Cheesbrough, M. (2002).** District laboratory practice in tropical countries. E.C.B.S edition Combridge University Press **2:**97-182.

[6] **Collee, J. G.; Miles, R. S. and Watt, B. (1996).** Test for the identification of bacteria. In: Collee. J. G., Fraser, A. G., Marmion, B. P. and Simmons, A. Eds: Practical Medical Microbiology.14th Ed. Churchill Livingstone New York. pp. 131-146.

[7] **Murray, P.; Baron, E.; Jorgensen, J.; Landry, M.; Pfaller, M. (2007).** Manual of clinical microbiology, 9th.Ed, ASM Press. Chapter 6; the Enterobacteriaceae

[8] **Li ,R.; Lai ,J.;Wang, Y.; Liu, S.; Li ,Y.; Liu, K.; Shen, J.; Wu ,C**. (**2013).** Prevalence and characterization of Salmonella species isolated from pigs, ducks andchickens in Sichuan Province, China.Pubmed. *Int. J .Food Microbiol.*15(1):14-

[9] **Parvej,M.SH. (2013). Isolation ,Identification and Molecular characterization of Poultry Salmonella**.Thesis ,Bangladesh Agricultural University, Mymensingh .p:1-68

[10] **Bae, D.H.; Dessie, H.K.; Baek, H.J.; Kim, S.G.; Lee, H.S.; Lee, Y.J**.; (**2013).** Prevalence and Characteristics of *Salmonella spp.* isolated from Poultr Slaughterhouses in Korea. *J. Vet. Med. Sci*. 13:13-93.

[11] **Raman, R.; Colavecchio, A.; Barrere, V.; Levesque, C.; Roger; G.; Lawrence,D. (2017).** "Molecular Characterization of bacteria that causefalse-positive and false-negative test results on the VIDAS UP *Salmonella* (SPT) affinity assay"**.MSC. Thesis ,** Faculty of Agriculture and Environmental Sciences**;** McGill University,Sainte-Anne-de-Bellevue, Québec, Canada.P:1-76.

[12] **Zhang, G.;Brown, E. W.; González-Escalona, N. (2011).** Comparison of real-timePCR, reverse transcriptase real-time PCR, loop-mediated isothermal amplification, and the FDA conventional microbiological method for the detection of *Salmonella*spp. in produce. *Applied and Environmental Microbiology, 77*(18), 6495-6501

[13] -El-Sebay ,N.A.; Abd Shady ,H.M.;EL-Zeedy ,S.A. and Sammy,A.A.(2017).InvA gene sequencing of *Salmonella typhimurium* isolated from Egyptain Poultry .Asian Journal of Scientific Research,10:194-202.

[14] Taddele, M.H.; Rathore,R.; Dhama,K.(2011). Application of PCR for the detection of *Salmonella* species isolated from poultry targeting 16s rRNA and *Fim*H genes. *Afri. J. Anim. Biomed. Sci*., 6(1): 129-134.

[15] Al -Mamun ,M.D.A.; Lutful, K. S.M,. Mehedu, I. M. ; Mostary L. ; Shaheenur, I S.K , Taslima A.H.M. and Mehedi, H.M.D. (2017). Molecular identification and characterization of Salmonella species isolated from poultry value chains of Gazipur and Tangail districts of Bangladesh. African Journal of Microbiology Research Vol. 11(11), pp. 474-481.

[16] **Yang,B.;Wang,Y.;Yuan Qia,P.(2016).** Sensitivity and correlation of hypervariable regions in *16S rRNA* genes in phylogenetic analysis.BMC Bioinformatics 17:135.

[17] 17- **Song, Y., Liu, C.; Bolanos, M.; Lee, J.; McTeague, M.; Finegold, S. M. (2005).** Evaluation of *16S rRNA* sequencing and reevaluation of a short biochemical scheme for identification of clinically significant *Bacteroides* species. J. Clin. Microbiol. 43, 1531-1537.

[18] **Drancourt, M.;Bollet, C.; Carlioz, A.; Martelin, R.;Gayral, J. P.; Raoult, D.(** 2000).16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol. 38, 3623-3630.