

Molecular Identification of Five Multidrug-resistant Salmonella Enterica Serovars Isolated from Egyptian Poultry Farms

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Abstract

Salmonella spp is the main cause of foodborne salmonellosis that is considered a public health threat all over the world. The robust usage of antibiotics in Egyptian poultry farms resulted in increasing the prevalence of multi-drug resistant *Salmonella* enterica. In this study, the authors identify five multi-drug resistant *Salmonella* enterica serovars. Multidrug-resistant characteristics of these isolates were detected. The taxonomic evidence of these isolates was investigated based on 16S rRNA gene sequence and pairwise sequence alignment between the isolates' sequence and the nearest sequences in the database. In silico restriction maps and phylogenetic trees were also constructed. The obtained sequences were deposited in the database under accession numbers MW311328.1, MW311371.1, MN820824.1, MN822653.1 and MW310702.1 for *Salmonella* enterica subsp. enterica serovar Enteritidis strains EG.SmE1, EG.SmE2 and *Salmonella* enterica subsp. enterica serovar Typhimurium strains EG.SmT1, EG.SmT2, EG.SmT3, respectively. The five *Salmonella* isolates in this study showed multi-drug resistant characteristics. *Salmonella* Typhimurium isolate EG.SmT3 revealed resistance to more than five antibiotics. Results of pairwise sequence alignment, restriction maps and phylogenetic tree confirmed the close relationship between *S. enteritidis* isolates (EG.SmE1, EG. SmE2) and *S. typhimurium* isolates (EG.SmT1, EG.SmT2). However, the other *S. typhimurium* isolate (EG.SmT3) revealed the lowest identity ratio (98.6%) with the nearest sequence. The relative divergence of this isolate could be attributed to proposed mutations as a result of the vigorous use of antibiotics in Egyptian poultry farms.

Introduction

Salmonella is a Gram-negative bacilliform bacterium, which belongs to the family of *Enterobacteriaceae*. It is a considerable public health threat responsible for one of the most common food-borne illnesses known as Salmonellosis (García-Soto et al. 2020). Globally, it is estimated that *Salmonella* spp. outbreaks are responsible annually for nearly about 85% (80.3 million cases) of diseases associated with foodborne diseases (93.8 million cases), resulting in over 100,000 deaths (Majowicz et al. 2010). Salmonellosis symptoms are abdominal cramps, fever, vomiting, inflammatory diarrhea, and nausea occurring within 12–72 h of infection and last from 2–7 days. Severe invasive *Salmonella* infections such as bacteremia and septicemia, often arise in immunocompromised people, lead to hospitalization and death (Musyoka et al. 2018).

More than 2500 serotypes of *Salmonella* have been identified to date (Grimont and Weill, 2007; Lamas et al. 2018). A great number of these serotypes (1531) are found in subspecies enterica, among which *S. Typhimurium* and *S. Enteritidis* stand out as they are principally responsible for more than 99% of human salmonellosis and therefore, they were widely studied. (Lamas et al. 2018). The adaptability of such subspecies in different environmental stresses augmented the encounters to eradicate them from the food chain (Humphrey, 2004; Spector and Kenyon, 2012). Conventional intervention strategies to control *Salmonella* spp. were predominantly carried out using antibiotics that were considered as an effective method to reduce *Salmonella* propagation. However, later this was showing to lead to the emergence of multidrug-resistant *Salmonella* spp (Agyare et al. 2019; Medeiros et al. 2011).

During the last years, antimicrobial resistance has emerged quickly amongst *Salmonella* spp from different animal sources, creating a serious health hazard worldwide. Increasing prevalence and dissemination of different multidrug-resistant (MDR) subspecies enterica in broiler populations resulted in *Salmonella* spreading in the food chain and via poultry products to humans (García-Soto et al. 2020). In Egypt, the excessive uncontrolled use of antibiotics in poultry farms to treat microbial infections led to expanding of antibiotic resistance subspecies including *Salmonella enterica* serovar Typhi (*S. Typhi*) and other diarrhoeagenic strains (Wasfy et al. 2002; Aouf et al. 2011). However, with no national *Salmonella* surveillance center to provide reliable statistical data, little is known about food-borne salmonellosis in Egypt (Abdel-Maksoud et al. 2015).

Identification of multidrug-resistant bacteria is the first step to understand its nature, structure and characteristics to control its spread in the food chain. Molecular techniques based on PCR amplification targeting conserved regions inside the 16S rRNA gene sequence has proved to be a useful tool for identifying and exploring phylogenetic relationships among bacterial

isolates. The analysis of the 16S rRNA gene sequence provides information about proposed mutation and mobile genetic elements that result in the acquisition of antibiotic resistance in *Salmonella* spp (Abdel-Maksoud et al. 2015).

In this study, the authors identified five *Salmonella* enterica serovars isolates from poultry farms with history of diarrhea in Benha city, Qalubiya governorate, Egypt. Multidrug-resistant characteristics of these isolates were detected applying the antibiotics susceptibility test. Based on 16S rRNA gene sequence, the taxonomic evidence of these isolates was investigated. Single nucleotide polymorphisms (SNPs) between the obtained sequences and the nearest sequences in database were determined. The construction of the phylogenetic trees was also performed.

Materials And Methods

Bacterial strains and growth conditions

Samples were collected from a wide range of broiler farms with a history of diarrhea in Qalubiya governorate, Egypt. Fecal samples were collected and incubated overnight in buffered peptone water at 37°C, 100 µl of the overnight pre-enrichment broth was then inoculate a 10 ml Tetrathionate broth (Müller-Kaufmann) and again was incubated overnight at 37°C. Finally, 10 µl loop from the inoculated Tetrathionate broth was spread on Xylose Lysine Deoxycholate (XLD) agar plates and on Brilliant Green (BGA) agar plates, plates were then incubated overnight at 37°C. *Salmonella* like colonies were selected, subcultured and maintained for further identification. On XLD agar, a typical *Salmonella* colony has a slightly red transparent halo with black centers. On BGA agar, a typical *Salmonella* colony appear red in a red/pink color agar.

Isolated *Salmonella* serotypes were determined by identifying the outermost portion of the lipopolysaccharide “O” antigen and the flagellar protein “H” antigen. Five isolates of *Salmonella* enterica serovars were purified and stored at - 80°C in Brain-Heart-Infusion broth supplemented with 20% (v/v) glycerol.

Antibiotic susceptibility test

Antibiotics susceptibility test was performed using the disc diffusion method (Biemer, 1973) on Mueller-Hinton agar using a collection of ten antibiotics (Oxoid, UK); Ampicillin (AM 10 µg), Amoxicillin + Clavulanic Acid (AMC 20 + 10 µg), Ceftriaxone (CRO 30 µg), Ciprofloxacin (CIP 5 µg), Amikacin (AK 30 µg), Chloramphenicol (CL 30 µg), Gentamycin (Gent 10 µg), Tetracycline (TE 30 µg), Kanamycin (KA 30 µg) and Streptomycin (ST 10 µg). The results were inferred according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2019).

DNA Extraction and PCR amplification

Bacterial genomic DNA from pure isolates was extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA integrity was checked using 1% agarose gel electrophoresis and the image was captured using gel documentation system (Gel Doc. BioRad). Concentration and purity of purified DNA were measured by BioTek Epoch2 Microplate reader (Thermo Scientific, USA). For all samples, DNA purity was $>1.8 \pm 0.20$ under absorbance ratio A260/A280.

PCR amplification was done based on bacterial genomic DNA using universal 16S rRNA primers 27F:5'-AGAGTTTGGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' (Badr et al., 2019). The expected PCR amplicon was almost 1.5 kb. PCR reaction was performed in a 25 µl mixture containing 0.2 µM of each primer with concentration of 10 pM, 200 µM of dNTPs mix, 2.5 µL of 10× PCR reaction buffer, 1.5 µM MgCl₂, 1.25 units of TAKARA Taq DNA polymerase (Cat. #: R001AM), 2 µL of template DNA and the final volume was adjusted with sterilized double distilled water. PCR thermocycler (AriaMx) was used to amplify the reactions consisting of 95 °C for 3 min followed by 35 cycles at 95 °C for 50 s, 55 °C as annealing temperature for 1 min with an extension of 72 °C for 1 min followed by final extension

temperature at 72 °C for 10 min. Amplified PCR products were stored at -20 °C for further purification and downstream application, then 5 µl of PCR amplicons was loaded on 2 % agarose gel electrophoresis stained with Ethidium bromide using GeneRuler™ 1 kb DNA ladder, then visualized using gel documentation system (Gel Doc. BioRad).

16S rDNA sequencing

PCR amplicons were purified according to the manufacturer's QIAquick Gel Extraction Kit (Cat. #: 28704). The purified PCR fragments were sequenced by Macrogen Company, South Korea applying automated Sanger Sequencing method. The obtained sequences for 16S rRNA gene were surveyed using Standard Nucleotide BLAST tool and registered at NCBI database under accession numbers [1], MW311328.1, MW310702.1, MN822653.1 and MN820824.1 for *Salmonella enterica* subsp. *enterica* serovar Enteritidis strains EG.SmE2, EG.SmE1 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strains EG.SmT3, EG.SmT2, EG.SmT1, respectively, (<http://www.ncbi.nlm.nih.gov>).

Pairwise alignment and Phylogenetic construction

NEBcutter software V2.0 was used to create a restriction map and to identify the GC content of the obtained sequences (Vincze et al. 2003, (<http://nc2.neb.com/NEBcutter2/>)). Jalview software (Waterhouse et al. 2009) was used to show single nucleotide polymorphisms (SNPs) and consensus resulted from the alignment of the obtained sequence and the nearest strains in NCBI database (<http://www.jalview.org/>). Construction of the phylogenetic tree was done using Clustal Omega tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and MEGA X software (Kumar et al. 2018).

Results

Bacteria and Antibiotic sensitivity test

Five isolates of *Salmonella enterica* serovars were obtained. Among them, three isolates were detected as *Salmonella enterica* serovar Typhimurium and two isolates were identified as *Salmonella enterica* serovar Enteritidis.

Antibiotic susceptibility patterns of the five obtained isolates demonstrated a susceptibility and resistant profile characteristic (Table 1). Qualitative results from the antibiograms showed that all isolates were susceptible to Ciprofloxacin. Although *Salmonella* Typhimurium isolates were resistant to Amoxicillin + Clavulanic Acid 20, *Salmonella* Enteritidis isolates were susceptible to it. On another hand, all isolates were susceptible to Tetracycline except *S. Typhimurium* EG.SmT3 that was resistant to Tetracycline. This isolate revealed the highest resistant pattern as it was resistant to five antibiotics (Ampicillin, Amoxicillin + Clavulanic Acid, Ceftriaxone, Chloramphenicol, Tetracycline). Also, it was categorized as intermediate resistant to Amikacin and Gentamycin. While, the other four isolates were more susceptible to the tested antibiotics.

Table 1

Antibiotic susceptibility pattern of five *Salmonella* enterica serovars against a selection of ten antibiotics.

Antibiotic tested (Category)	<i>S. Typhimurium</i>			<i>S. Enteritidis</i>	
	EG.SmT1	EG.SmT2	EG.SmT3	EG.SmE1	EG.SmE2
AM (Penicillins)	S*	S	R	S	S
AMC (Penicillins + β lactamase inhibitors)	R*	R	R	S	S
CRO (Cephalosporins)	I*	S	R	I	I
CIP (Fluoroquinolones)	S	S	S	S	S
AK (Aminoglycosides)	S	R	I	S	R
Gent (Aminoglycosides)	I	S	I	R	R
Tet (Tetracyclines)	S	S	R	S	S
Chl (phenolics)	R	S	R	S	S
AM (Ampicillin 10 μ g); AMC (Amoxicillin + Clavulanic Acid 20 μ g + 10 μ g);					
CRO (Ceftriaxone 30 μ g); CIP (Ciprofloxacin 5 μ g);					
AK (Amikacin 30 μ g); Chl (Chloramphenicol 30 μ g);					
Gent (Gentamycin 10 μ g) Tet (Tetracycline 30 μ g);					
* Denotes for Resistant (R), Intermediate (I), and Susceptible (S).					

Molecular characterization

The amplified PCR products of 16S rRNA gene of five isolates were sequenced and deposited in NCBI database under accession numbers MW311371.1, MW311328.1, MW310702.1, MN822653.1 and MN820824.1 for *Salmonella* enterica subsp. enterica serovar Enteritidis strains EG.SmE2, EG.SmE1 and *Salmonella* enterica subsp. enterica serovar Typhimurium strains EG.SmT3, EG.SmT2, EG.SmT1, respectively.

BlastN and Jalview alignment results revealed that, the two isolates EG.SmE2, EG.SmE1 were closely similar to *Salmonella* sp. strain Enteritidis_S85 with identity ratio of 98.9 % and 99.66%, respectively. As similar, the two isolates EG.SmT2 and EG.SmT1 were closely similar to *Salmonella* enterica subsp. enterica serovar Typhimurium strain OLF-FSR1 with identity ratio of 98.9% and 99.2%, respectively. However the isolate EG.SmT3 revealed the lowest identity ratio (98.6%) with the nearest deposited sequence in database of *Salmonella* sp. strain Ohio_S72.

As shown in Table 2, the isolate EG.SmT3 exhibited unique pattern in SNPs and GAPS positions and numbers compared to the other isolates. Pairwise alignment analysis of this isolate and nearest sequence in database exhibited 4 SNPs and 13 GAPS. While both isolates EG.SmE2, EG.SmE1 revealed 2 SNPs one of them at the same position 67 with the same substitution reflecting the close similarity between the two isolates. Moreover the isolates EG.SmT2 and EG.SmT1 displayed relatively similar pattern of GAPS position with three identical GAPS in the same positions (477,1114,1340) Fig S1.

Table 2

Pairwise sequence alignment data of partial 16S rRNA sequence between obtained isolates and nearest sequences in database.

Isolate/ accession	Nearest accession	Identity	Coverage	SNPs/ Positions	GAPs/ Positions
EG.SmE2/ MW311371.1	MT621365.1	98.9%	100%	2 SNPs 67,1375	12 GAPs/ 145,146,147,247,248,274,316,502,716, 744,1008,1343
EG.SmE1/ MW311328.1	MT621365.1	99.66%	100%	2 SNPs 67,144	3 GAPs/ 147,244,245
EG.SmT3/ MW310702.1	MT621352.1	98.67%	99%	4 SNPs 706,1271,1273,1274	13 GAPs/ 2,3,5,8,14,579,589,807,829,1012,1034, 1218,1252,
EG.SmT2/ MN822653.1	CP051276.1	98.9%	99%	2 SNPs 3,333	14 GAPs/ 118,331,337,476,477,758,759,1113,1114 1119,1120,1340,1341,1435
EG.SmT1/ MN820824.1	CP051276.1	99.2%	100%	1 SNPs 334	11 GAPs/ 119,332,338,477,478,759,760,1114,1115, 1339,1340

The restriction map of the obtained sequences provided predicted data of restriction sites that found in the five isolates (Fig. 1). Totally, 46 restriction enzyme site were observed in both isolates EG.SmE2, EG.SmE1 with similar restriction sites pattern. However, there was one specific restriction site for each isolate that was ECOP15I in EG.SmE2 and BSrG1 in EG.SmE1. Additionally, the isolates EG.SmT2 and EG.SmT1 displayed relatively similar restriction sites pattern with total number of 48 and 46 restriction enzyme site in both isolates, respectively. On another hand, A completely different restriction sites pattern was observed in isolate EG.SmT3 reflecting the divergence of this isolates compared to other isolates. The GC, AT contents of all isolates was 55% GC and 45% AT.

Phylogenetic construction

From the phylogenetic tree (Fig. 2), it can be inferred that the isolates EG.SmE2, EG.SmE1 were clustered together in the same clade as similar the isolates EG.SmT2 and EG.SmT1 were clustered together. While the isolate EG.SmT3 diverged from them and clustered with the nearest deposited sequence in the database. However, all obtained isolates and nearest sequences in database shared the same ancestor of *Salmonella* sp that diverged from E-coli ancestor.

Discussion

Antimicrobial-resistant strains of *Salmonella* spp are now widespread all over the world constituting a major threat to human health. In Egypt, some reports on *Salmonella* serovars dispersion on poultry farms were documented by (Ammar et al., 2010; Abd El-Ghany et al., 2012). In this study, antibiotic sensitivity test of five *Salmonella* enterica serovars proved the multidrug-resistant characteristics of these isolates. Three isolates (EG.SmT1, EG.SmT2, EG.SmE2) were resistant to more than two antibiotics while one isolate (EG.SmT3) revealed resistance to more than five antibiotics. MDR *Salmonella* serovars were

previously isolated from Egypt (Abdel-Maksoud et al., 2015; **Abdelhakim et al., 2011**; Mahmoud et al., 2018; Merwad and Abdel-Haliem, 2018; Wasfy et al., 2002). However, most of these studies based on microbiological identification with little information about molecular characterization that provides information about proposed mutation and mobile genetic elements that result in the acquisition of antibiotic resistance in *Salmonella* spp (**Abdel-Maksoud et al.,2015**).

The prevalence of *Salmonella* serovars recovered from the Egyptian poultry farms were two isolates of *S. enteritidis* (EG.SmE2, EG.SmE1) and three isolates of *S. typhimurium* (EG.SmT1, EG.SmT2, EG.SmT3). The pairwise sequence alignment confirmed the close relationship between the *S. enteritidis* isolates that displayed the same nearest sequence in the database of *Salmonella* sp. strain Enteritidis_S85 (MT621365.1). The similar pattern of SNPs and GAPs position (67, 147) reflects a fixed change in both isolates that could be attributed to a proposed mutation at this position. In the same manner, two *S. typhimurium* isolates (EG.SmT1, EG.SmT2) were closely similar to each other with the same nearest sequence in the database of *Salmonella* enterica subsp. enterica serovar Typhimurium strain OLF-FSR1 (CP051276.1). While, the other *S. typhimurium* isolate (EG.SmT3) revealed the lowest identity ratio with the nearest sequence and with other *S. typhimurium* isolates. The divergence of this isolate among others confirmed not only by the relatively low identity ratio but also by the unique pattern of SNPs and GAPs position. These molecular changes in the conserved sequence of 16S rRNA gene could be proposed mutations that affect the multi-drug resistant characters of this isolate.

From restriction maps, the specific restriction enzymes ECOP15I and BSrG1 could be used to differentiate between the two isolates EG.SmE2 and EG.SmE1 respectively. The different restriction map of EG.SmT3 compared to other *S. typhimurium* isolates confirmed the variability in its 16S RNA sequence and consequently its exceptional characteristics as a strong multi-drug resistant bacteria.

The phylogenetic tree confirmed the same identity ratios on the roots of clades. It concluded the taxonomic evidence of these isolates and the relative divergence of EG.SmT3 isolate that could attribute to proposed mutations caused by the vigorous use of antibiotics in Egyptian poultry farms. Hence, the continual usage of antibiotics could simulate changes in conserved genomic regions such as 16S rRNA sequence.

Conclusion

The five *Salmonella* enterica serovars isolates showed multi-drug resistant characteristics. The isolate EG.SmT3 showed the most multi-drug resistant profile with relative divergence in sequence similarity. The vigorous use of antibiotics could stimulate mutations in 16S rRNA gene sequence. In further investigation, the isolate EG.SmT3 could be used as a good and strong model for multi-drug resistant *Salmonella* Typhimurium to assess biocontrol agents against foodborne salmonellosis.

Declarations

Acknowledgments

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Conflict of interest

The authors declare that there are no conflict of interests.

Data availability statement

All data are available on request from the corresponding author

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Figures

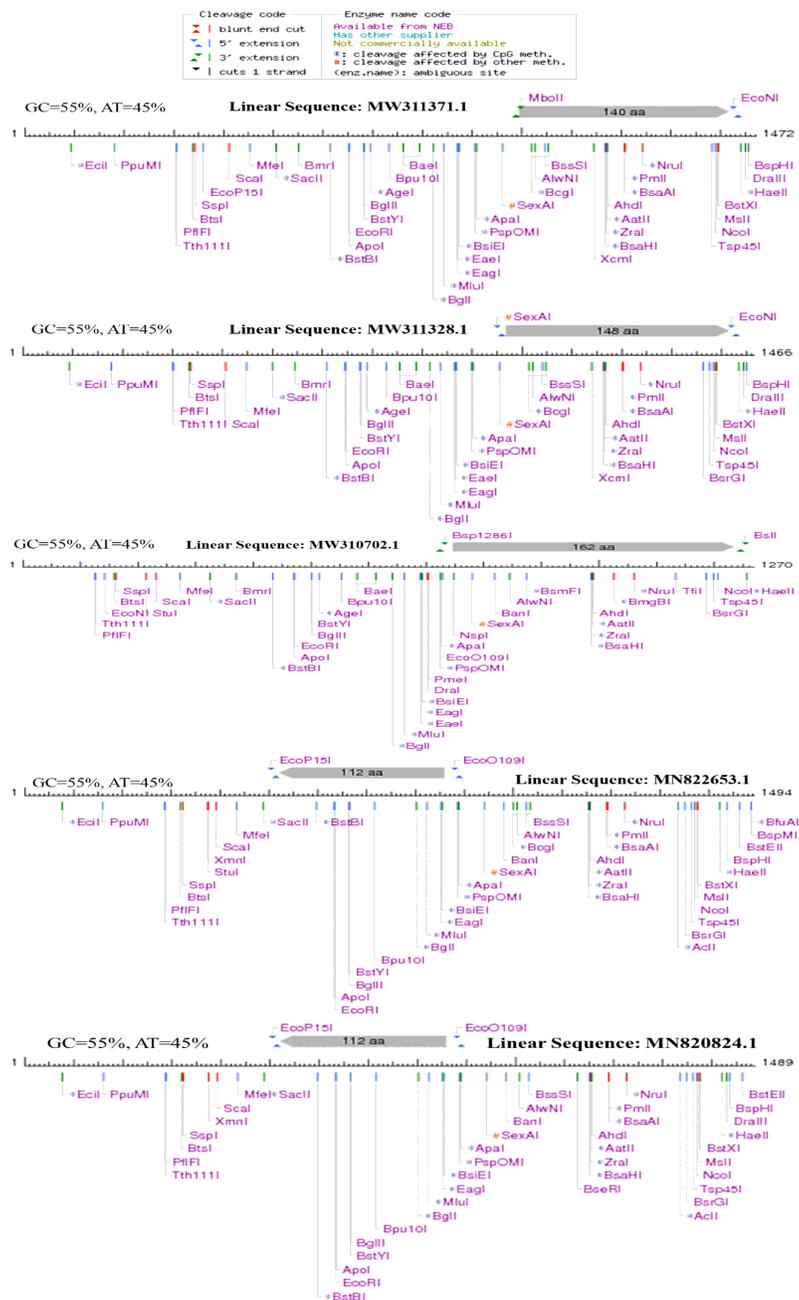


Figure 1

Restriction map of the partial sequence of 16S rRNA gene of obtained strains with available commercially restriction enzymes.

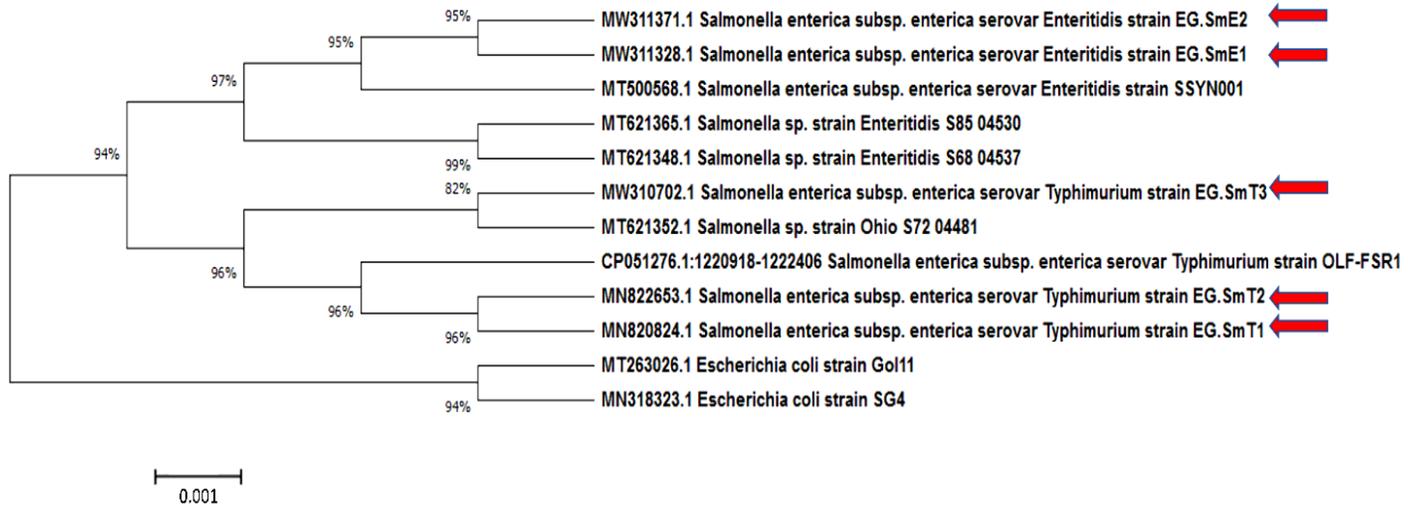


Figure 2

Phylogenetic tree shows the evolutionary relationships between the 16s rRNA sequences of five Salmonella enterica serovars against their bacterial concatenated nucleotide sequences of their 16s rRNA. The Maximum Likelihood tree was constructed using the MEGA X software with the Maximum Likelihood algorithm and default setting. The bar length represents 0.001 substitutions per nucleotide site. Isolates in the current study are indicated by red arrows. Branch support was estimated from 1000 bootstrap replicates. Two E. coli 16s rRNA sequences serve as outgroups to root the tree.

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