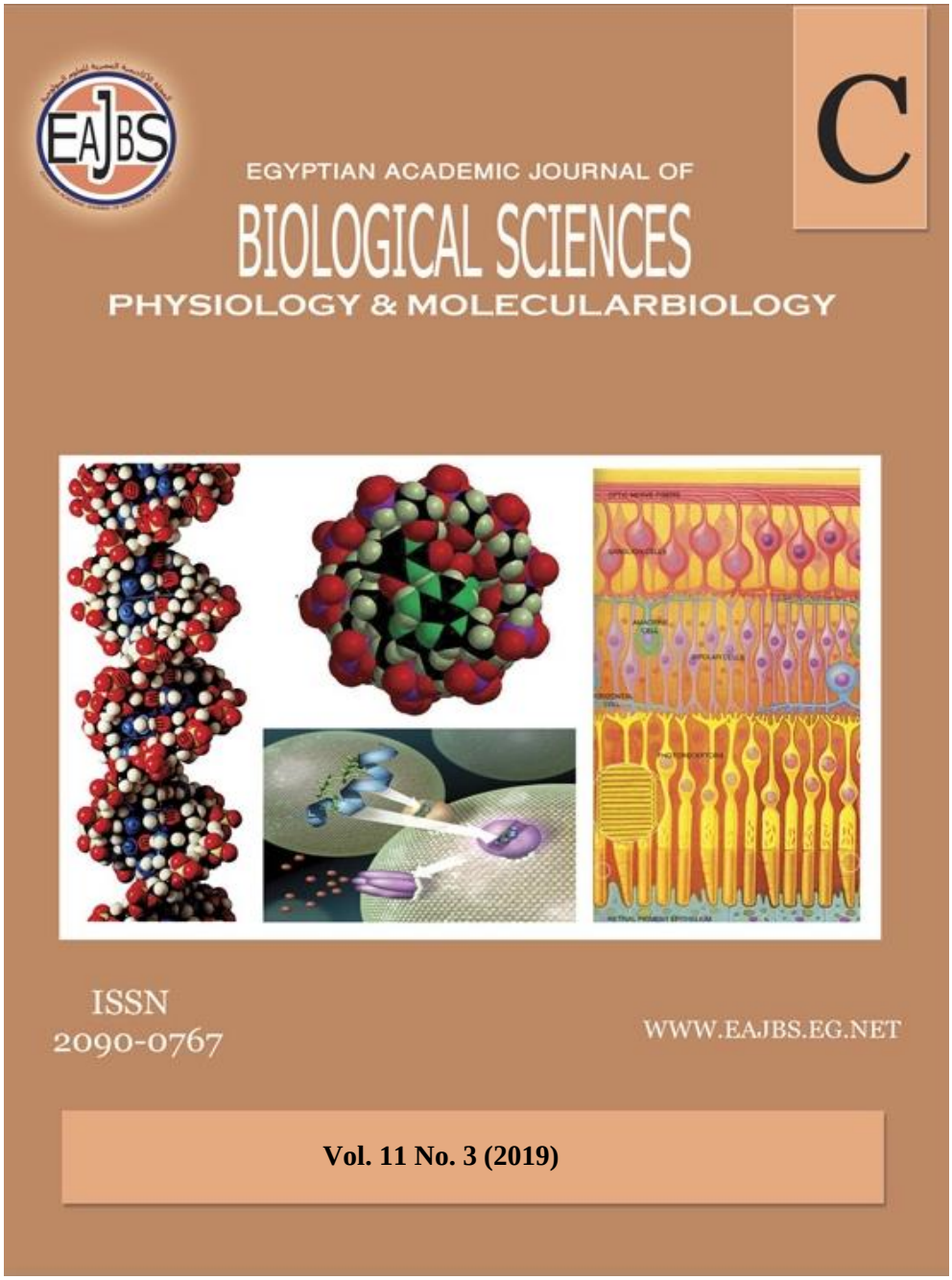

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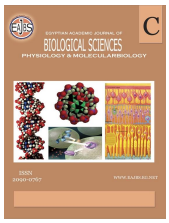

Sequencing and Phylogenetic Analysis of the *Salmonella* Enterotoxin *(stn)* Gene of *Salmonella* spp. Isolated from Egyptian Broiler Breeder Chickens Farms

Mona S. Azab [1,2], Mohamed E. M. Zowail [2], Nassif S. A. [3], Ghada M. Elsadek [3], and Safwa Z. Mohamed [3]

1-Department of Biology, Faculty of Science, Jouf University, Skaka, KSA.
2-Department of Zoology, Faculty of Science, Benha University, Egypt
3-Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo, Egypt
E.Mail: **mona_azab2001@yahoo.com**

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ABSTRACT

Salmonella is a member of *Enterobacteriaceae* family that found to be pathogenic to domestic and wild animals and humans. *Salmonellae* were isolated from three distinct governorates, Giza, Monofia and Qaluobia from broiler breeder chicken farms. Molecular characterization of the *Salmonella* isolates using polymerase chain reaction (PCR) assay as well as Sequencing and phylogenetic analysis of PCR products were conducted to distinguish the collected *Salmonellae* species.

The nucleotide sequence of 617 bp PCR products representing the amplified fragment of *stn* gene of seven isolates of *Salmonella enteritidis* has been sequenced. Furthermore, the nucleotide sequence was submitted to the gene bank. The obtained sequences were blasted with the highly similar sequences and the multiple sequence alignments were conducted. Neighbor-joining tree was constructed for the Egyptian *Salmonella* isolates against 30 *Salmonella* spp. from the Gene bank database representing maximum similarity with *stn* gene when subjected to multiple sequence alignment, and the phylogenetic tree was constructed based on the comparative analysis of related sequences at the nucleotide level.

INTRODUCTION

Salmonella is a member of *Enterobacteriaceae* family that found to be pathogenic to domestic and wild animals and humans (Forshell & Wierup, 2006). Salmonellosis is one of the most important bacterial diseases that cause economic loss in poultry due to mortality and the reduction of egg production (Khan *et al*., 1998). Several *salmonella* serotypes were described and classified (Quinn *et al*., 2002; Cortez *et al*., 2006 and Abd El-Ghany *et al*., 2012a). Molecular Characterization and Detection of Virulence Associated Genes of *Salmonella enterica* Serovars was covered throughout an integrated broiler chickens and from Chicken Products (Das *et al*., 2012; Ren *et al*., 2016; Zowail *et al*., 2017; Das *et al*., 2018; Zhou *et al*., 2018; Das *et al*., 2019; Elkenany *et al*., 2019; Wei *et al*., 2019).

The presence of virulence-associated genes *Salmonella* enterotoxin *(stn*) and plasmid-encoded fimbrial *(pef*) was tested for 32 *Salmonella* isolates using PCR protocols. All isolates found to bear the enterotoxin determinant *stn* virulent gene code for *Salmonella* toxin which increases the level of c-AMP in the host, which results in diarrhea and vomiting. Whereas, none of the *Salmonella* isolates was found positive for *pef* gene. This Indicated that the *stn* gene is a widely distributed and highly conserved gene among the *Salmonella* isolates irrespective of the source of sample, species, serovars, and location. So, it may be used as a target gene for the detection of *Salmonella* in different types of field samples *Salmonella* isolates were recovered (Naik *et al*., 2015 and Singh *et al*., 2017). The distribution pattern and molecular identification of *Salmonella* isolate using PCR protocols of distinct genes (*stn*, *sef*, and *pef*) was observed among different serovars of *S. enterica* isolated chicken. While *stn* gene was found in all isolated strains, the *sef* gene was found only among *S. Enteritidis* isolates and *pef* gene was found to be absent in some isolates (Zowail *et al*., 2017).

Sequencing and phylogenetic analysis become important molecular methods for the characterization of pathogens. The occurrence of *Salmonella fimbriae* genes *Salmonella Enteritidis* fimbrial (*sef*) and plasmid-encoded fimbrial (*pef*) was studied among 29 strains of *Salmonella enterica* belonging to seven serovars isolated from human, animals, and birds by PCR amplification technique using their specific primers. All the strains of *S. enteritidis* were found to carry both *sef* and *pef* genes irrespective of the source of isolation. *S. typhimuriam* strains were found to wharf only *pef* genes, while *S. gallinarum* strains harbored only *sef* genes (Rahman *et al.*, 2000; Murugkar *et al*., 2003 and Zowail *et al*., 2017). The results of multiplex PCR for three isolated serotypes of *Salmonella enterica* (*S. typhimurium, S. kentucky* and *S. enteritidis*) could detect the universal gene (*invA*) and virulence genes (*avrA* and *stn*) in all examined serotypes, while (*fliC*) gene was detected in both *S. typhimurium* and *S. kentucky* only but not in *S. enteritidis*, (*stm* 4495) gene was detected as specific gene for *S. typhimurium*, also, (*sefA*) gene was detected as specific gene for *S. enteritidis* (Amin and Abd El-Rahman, 2015).

Stn is an important virulent gene coding for *Salmonella* toxin. The gene was cloned and sequenced, the sequence was submitted to NCBI Genbank and allotted the Accession No KF032246. Based on the sequence information, the phylogenetic relation was deduced between different serovars of *Salmonella typhimurium.* The sequence was further used for bioinformatics analysis of *Stn* gene, the phylogenetic analysis on *S. typhimurium* exhibited 99% similarity with *Salmonella enterica* sub sp. *enterica* serovar Newport. This degree of similarity confirmed the conservation of *Stn* gene among many serovars of *Salmonella* (Singh *et al*., 2017).

MATERIALS AND METHODS

Samples:

The collection, isolation and serotyping identification of samples were recorded **(Zowail *et al*., 2017).**

Polymerase Chain Reaction:

Oligonucleotide primers sequences encoding for *stn* gene used for PCR protocols were revealed (Zowail *et al*., 2017).

Sequencing Analysis:

QIA quick® PCR Purification kit (Qiagen) used for purification of PCR products. For direct purification of double or single-stranded PCR products from amplification reaction and DNA clean up from other

enzymatic reactions the following kits were used:

QIA quick Spin Columns	50
Buffer PB	30 ml
Buffer PE (concentrate)	2 x 6 ml
Buffer EB	15 ml
PH Indicator I	800 µl
Collection Tubes (2 ml)	50
Loading Dye	110 µl

Buffer PB: Allow efficient binding of single and double-stranded PCR products and removal of primers up to 40 nucleotides.

Buffer EB: Elution buffer which allows DNA elution.

Loading Dye: Provided for analysis of purified DNA samples using electrophoresis that facilitate estimation of DNA migration distance and optimization of agarose gel run time.

Big Dye® Terminator v3.0 Cycle Sequencing kit (Applied Biosystem catalog No.4390242): Provides the required reagent components for sequencing reaction in a ready reaction, pre-mixed format. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on polymerase chain reaction (PCR) fragments, and on large templates. The kits contain the following reagent:

a- Terminator Ready Reaction Mix which consisted of:

A-Big Dye Terminator v3.0

C-Big Dye Terminator v3.0

G-Big Dye Terminator v3.0

T-Big Dye Terminator v3.0

- Deoxynucleoside triphosphates (dATP,dCTP, dITP, dUTP).
- MgCl2.
- Tris-HCl buffer, pH 9.0

b- PGEM®-3Zf (+) double-stranded DNA Control Template, 0.2 µg/µl.

c- 21 M13 Control Primer (forward), 0.8 pmol/µl

Centri-Sep™ spin columns (Applied Biosystems P/N 401762) were used for effective and reliable removal of excess Dye Deoxy ™ terminators from DNA completed sequencing.

GC Content Analysis:

DNA/RNA GC Content Calculator was used to calculate the percentage of GC content in *stn* gene (http://www. endmemo.com/bio/gc.php).

Sequence Similarity and Phylogenetic Analysis:

The sequence obtained was subjected to a homology search using *BIOEDIT version* 7.1.11. The sequences presenting maximum similarity with *stn* gene were subjected to multiple sequence alignment, and the phylogenetic tree was constructed based on the comparative analysis of related sequences using MEGA software (Molecular Evolution Genetics Analysis) tool at the nucleotide level.

RESULTS

The purity of the culture, biochemical characterization, and *Salmonella* specific PCR were recorded by Zowail *et al*. (2017). The nucleotide sequence of 617 bp PCR products representing the amplified fragment of *stn* gene of *Salmonella enteritidis* has been sequenced using (Applied Biosystem, USA, catalog No. 4390242). The nucleotide sequence was submitted to the gene bank, the designation and accession number of each serotype were recorded (Table, 1).

Total GC content of the PCR product of the *stn* gene sequence of the 7 isolated *Salmonella* serotypes was recorded (Table, 2 & Figs. 1-7).

The obtained sequences were blasted with the highly similar sequences which were downloaded and imported in *BIOEDIT version* 7.1.11. Multiple sequence alignments were conducted with *ClustalW* application. Using MEGA 7 software, a

neighbor-joining tree was constructed for the Egyptian *Salmonella* isolates against 30 *Salmonella* spp. from the Gene bank database (Figure, 8).

Salmonella kentucky strains CLEVB1, CLEVB2 and CLEVB3 were identical to *Salmonella kentucky* of accession number NZ_CP022501. Also, *Salmonella enteritidis* strain CLEVB4 was identical to *Salmonella enteritidis* of accession numbers NZ_CP018663, NZ_CP018660, NZ_CP018656, NZ_CP018654, and MTTU01000001. On the other hand, *Salmonella blockly* strain CLEVB5 was in the same bootstrap with *Salmonella manchester* of accession number NZ_CP019414 with a high percentage of similarity. Finally, *Salmonella typhimurium* strains CLEVB6 and CLEVB 7 were similar to a high extent to *Salmonella saintpaul* strain of accession number NZ_CP017727.

Table (1): The designation and accession number of the isolated *salmonella* serotypes.

Strain	Accession No.	Designation
Kentucky	ZX079693	CLEVB-1/EGY013 enterotoxin (*stn*) gene
Kentucky	ZX079694	CLEVB-2/EGY013 enterotoxin (*stn*) gene
Kentucky	ZX079695	CLEVB-3/EGY013 enterotoxin (*stn*) gene
Enteritidis	ZX079696	CLEVB-4/EGY013 enterotoxin (*stn*) gene
Blockley	ZX079697	CLEVB-5/EGY013 enterotoxin (*stn*) gene
Typhimurium	ZX079698	CLEVB-6/EGY013 enterotoxin (*stn*) gene
Typhimurium	ZX07969	CLEVB-71/EGY013 enterotoxin (*stn*) gene

Table (2): GC content of the *stn* gene in the 7 isolated *salmonella* serotypes.

Stain	GC Content	DNA length
CLEVB-1/EGY013	53.9%	568
CLEVB-2/EGY013	53.8%	565
CLEVB-3/EGY013	54.1%	566
CLEVB-4/EGY013	53.5%	565
CLEVB-5/EGY013	54.2%	566
CLEVB-6/EGY013	54.1%	566
CLEVB-71/EGY013	54%	565

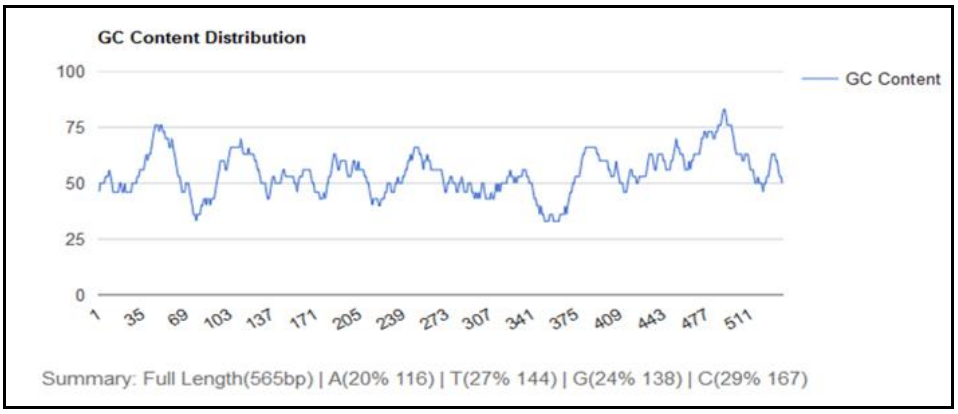

Fig. 1: Total GC content in the *stn* gene sequence of *Salmonella KX079693 Salmonella enterica* subsp. *enterica* strain *CLEVB-1/EGY013.*

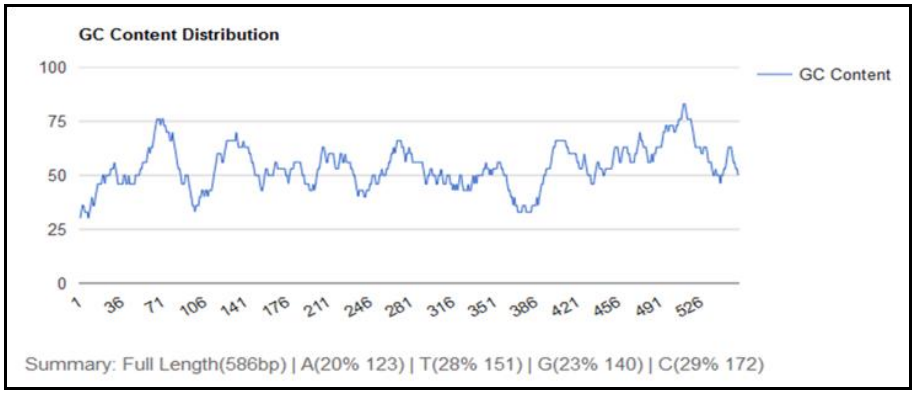

Fig. 2: Total GC content in the *stn* gene sequence of *Salmonella KX079694 Salmonella enterica* subsp. *enterica* strain *CLEVB-2/EGY013.*

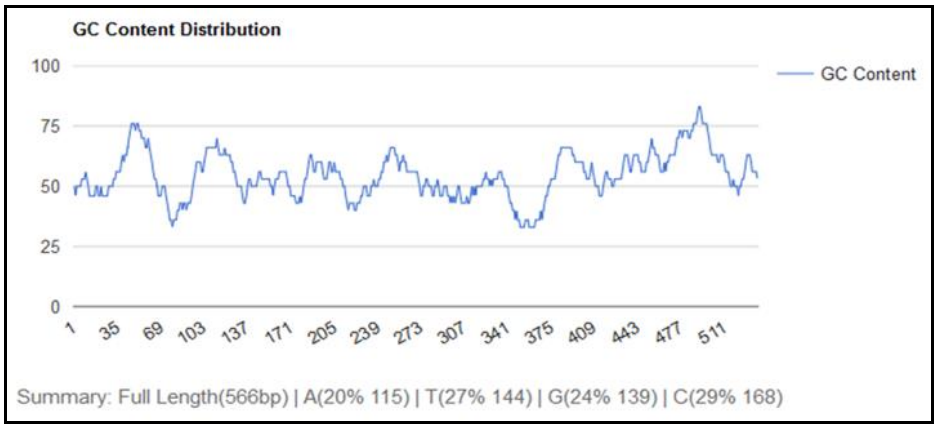

Fig. 3: Total GC content in the *stn* gene sequence of *Salmonella KX079695 Salmonella enterica* subsp. *enterica* strain *CLEVB-3/EGY013.*

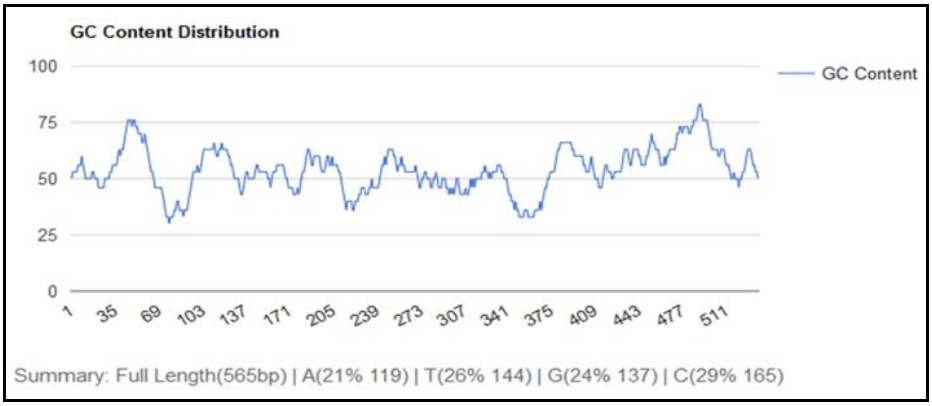

Fig. 4: Total GC content in the *stn* gene sequence of *Salmonella KX079696 Salmonella enterica* subsp. *enterica* strain *CLEVB-4/EGY013.*

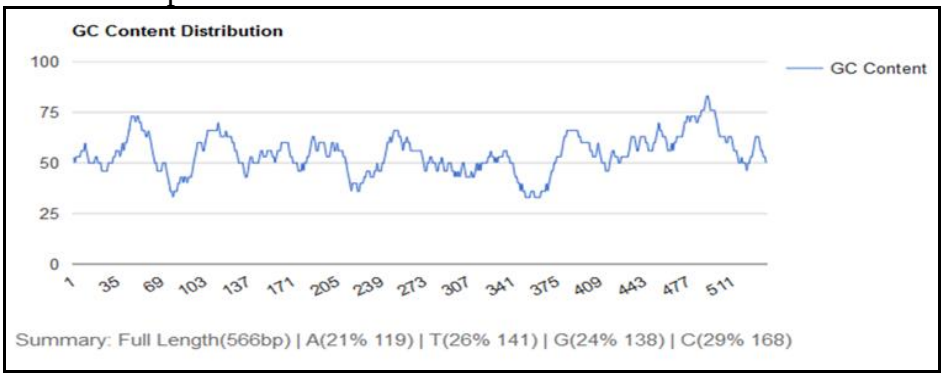

Fig. 5: Total GC content in the *stn* gene sequence of *Salmonella KX07967 Salmonella enterica* subsp. *enterica* strain *CLEVB-5/EGY013*

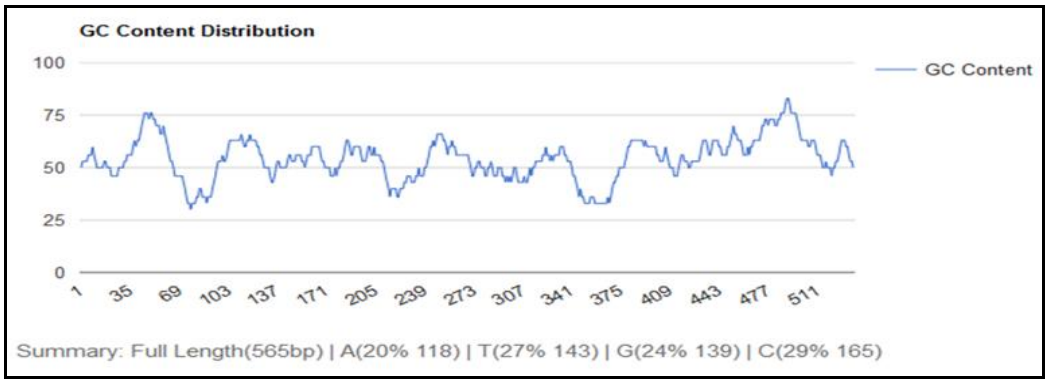

Fig. 6: Total GC content in the *stn* gene sequence of *Salmonella KX07968 Salmonella enterica* subsp. *enterica* strain *CLEVB-6/EGY013*

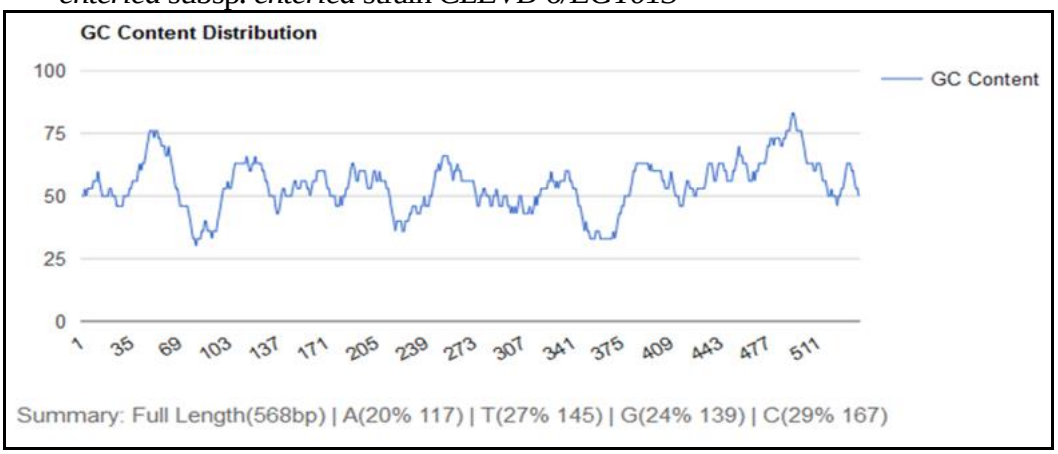

Fig. 7: Total GC content in the *stn* gene sequence of *Salmonella KX079699 Salmonella enterica* subsp. *enterica* strain *CLEVB-71/EGY013.*

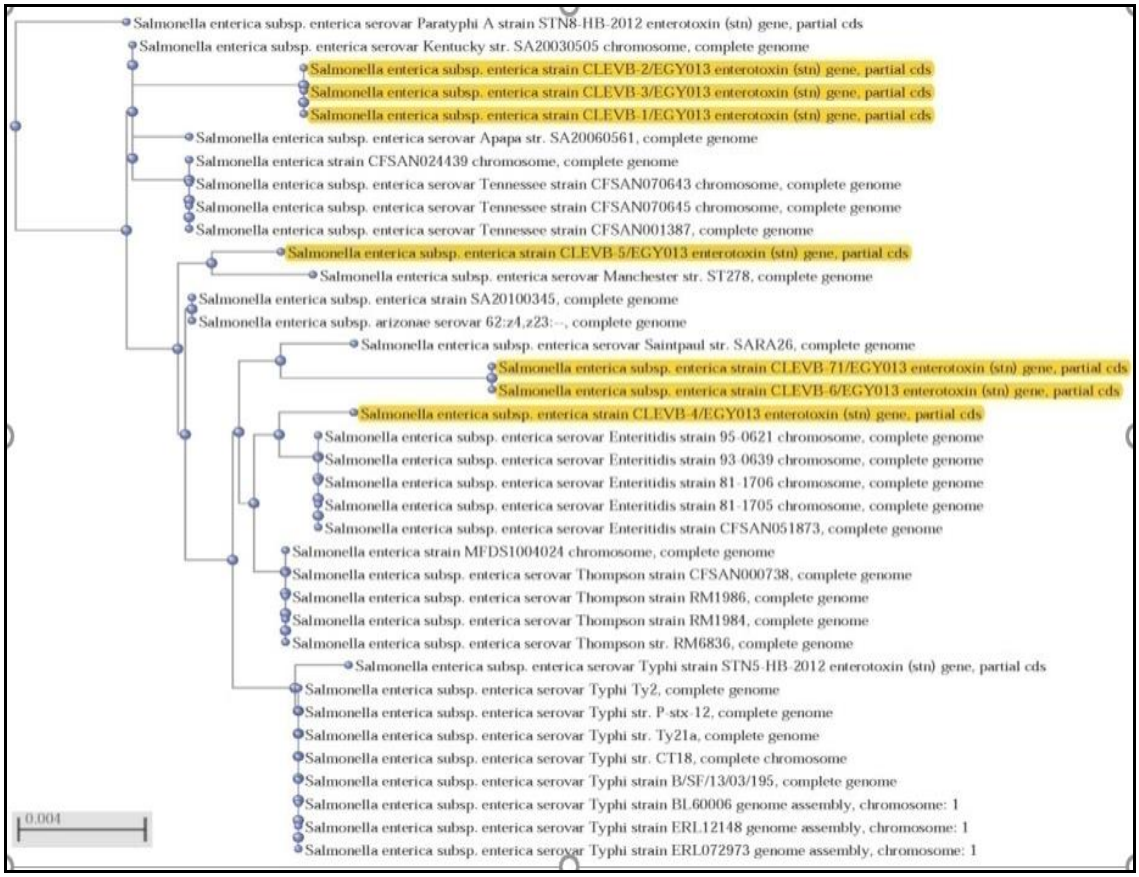

Fig. 8: The phylogenetic tree of the obtained nucleotide sequence in comparison with reference strains.

DISCUSSION

Recently conformist PCR based assays for *Salmonella* detection in foods have been widely reported (Moreira *et al.,* 2008; Ammar *et al*., 2010 and Zowail *et al*., 2017). The *stn* gene is in attendance in all *Salmonella* serotypes and contained a unique sequence that considered as suitable PCR target for detection of *Salmonella* strains in field samples (Moore *et al*., 2007; Singh *et al*., 2017 and Ammar *et al*., 2019)

The sequencing analysis and GC content of the *stn* gene of *Salmonella* isolates were an average of 53.3% which is a good prediction (Singh *et al.*, 2017). In the present study, the total GC content of the PCR product of the *stn* gene sequence of the 7 isolated *salmonella* serotypes is an average of 54%, i.e. good prediction. Concerning the phylogenetic analysis of *stn* gene of the presented 7 *Salmonella* strains, *Salmonella kentucky* strains CLEVB1, CLEVB2 and CLEVB3 were identical to *Salmonella kentucky* of accession number NZ_CP022501. Also, *Salmonella enteritidis* strain CLEVB4 was identical to *Salmonella enteritidis* of accession numbers NZ_CP018663, NZ_CP018660, NZ_CP018656, NZ_CP018654, and MTTU01000001.

On the other hand, *Salmonella blockly* strain CLEVB5 was in the same bootstrap with *Salmonella manchester* of accession number NZ_CP019414 with a high percentage of similarity. Finally, *Salmonella typhimurium* strains CLEVB6 and CLEVB 7 were similar to a high extent to *Salmonella saintpaul* strain of accession number NZ_CP017727. These data indicate that the sequence of the *stn* gene could be nearly conserved between different pathogenic *salmonella* spp. with no more than 1% difference, the matching results were recorded (Singh *et al*., 2017).

REFERENCES

Abd El-Ghany, W. A.; El-Shafii, S. S. A. and Hatem, M. E. (2012a): A Survey on *Salmonella* species isolated from chicken flocks in Egypt. Asi. J. of anim. and vet. Adv.; 7(6): 489-501.

Amin, H. S. and Abd El-Rahman, A. A. (2015): Detection of Molecular Characterization of *Salmonella enterica* isolated from Chicken Meat and Its Products by Using Multiplex PCR. Alex. J. Vet. Sci.; 46 (1): 155 - 160.

Ammar, A. M. A.; Ahmed, Y. A. E.; Asawy, A. M. I. and Ibrahim, A. A. (2010): Bacteriological studies on *Salmonella entertidis* isolated from different sources in Dakhlia governorate. Assiut Vet. Med. J.; 56: 125 - 135.

Ammar, A. M.; Abdeen, E. E.; Abo-Shama, U. H.; Fekry, E. and Kotb Elmahallawy, E. (2019): Molecular characterization of virulence and antibiotic resistance genes among *Salmonella* serovars isolated from broilers in Egypt. Lett. Appl. Microbiol.;68 (2): 188 - 195.

Cortez, A. L.; Carvalho, A. C.; Ikuno, A. A.; Burger, K. P. and Vidal-Martins, A. M. (2006): Identification of *Salmonella* spp. isolates from chicken abattoirs by multiplex-PCR. Res. Vet. Sci.; 81(3): 340 - 344.

Das, A.; Hari, S. S.; Shalini, U.; Ganeshkumar, A. and Karthikeyan, M. (2012): Molecular screening of Virulence Genes from *Salmonella enterica* isolated from Commercial Food Stuffs. Biosci. Biotech. Res. Asia.; 9 (1): 363 - 369.

Das, M.; Motina, E.; Deka, D.; Dutta, T. K.; Roychowdhury, P.; De, A. and Chakraborty, S. (2019): Molecular Detection of

Virulence Associated Genes in *Salmonella* Serovars Isolated from Raw Pork of Aizawl and Imphal. Int. J. Curr. Microbiol. App. Sci.; 8 (7): 23 - 31.

Das, M.; Motina, E.; Deka, D.; Singh, N. S.; Dutta, T. K.; Roychoudhury, P. and Chakraborty, S. (2018): Bacteriological quality of raw pork sold in retailed butcher shops of Aizawl and Imphal. Int. J. Curr. Microbiol. Appl. Sci.; 7 (5): 1189 - 1195.

Elkenany, R.; Elsayed, M. M.; Zakaria, A. I.; El-sayed, S. A. and Rizk, M. A. (2019): Antimicrobial resistance profiles and virulence genotyping of *Salmonella enterica* serovars recovered from broiler chickens and chicken carcasses in Egypt. BMC Vet. Res., (15):124.

Forshell, L. P. and Wierup, M. (2006): *Salmonella* contamination: a significant challenge to the global marketing of animal foods products. Revue Scientifique Techinique Office International des Epizooties, Paris; 25 (2): 541 - 554.

Khan, M. A.; Bari, A. S. M.; Islam, M. R.; Das, P. M. and Ali, M. Y. (1998): Pullorum disease in semi mature chicks and its experimental Pathology Bang. Vet. J.; 32: 124 - 128.

Moore, M. M. and Feist, M. D. (2007): Real-time PCR method for *Salmonella* spp. targeting the stn gene. J. Appl. Microbiol.; 102 (2): 516 - 30.

Moreira, A. N.; Conceicao, F. R.; ConceicaoRde, C.; Ramos, R. J.; Carvalhal, J. B.; Dellagostin, O. A. and Aleixo, J. A. (2008): Detection of *Salmonella typhimurium* in raw meats using in-house prepared monoclonal antibody coated magnetic beads and PCR assay of the fimA gene. J. Immunoassay Immunochem.;29(1): 58 - 69.

Murugkar, H. V.; Rahman, H. and Dutta, P. K. (2003); Distribution of virulence genes in *Salmonella* serovars isolated from man and animals. Indian Med. Res.; 177, 66 – 70.

Naik, V. K.; Shakya, S.; Patyal A. and Gade, N. E. (2015): Detection of Virulence Genes in *Salmonella* Species Isolated from Chevon and chicken Meat. J. Anim. Res.; 5 (1): 115-118.

Quinn, P. J.; Markey, B. K.; Carter, M. E.; Donnelly, W. J. and Leonard, F. C. (2002): Veterinary Microbiology and Microbial Diseases. Black well scientific publications, Oxford, London.

Rahman, H.; Prager, R. and Tschape, H. (2000): Occurrence of sef and pef genes among different serovars of *Salmonella*. Indian J. Med. Res.; 111: 40 - 42.

Ren, X.; Li, M.; Xu, C.; Cui, K.; Feng, Z.; Fu, Y.; Zhang, J. and Liao, M. (2016): Prevalence and molecular characterization of *Salmonella enterica* isolates throughout an integrated broiler supply chain in China. Epidemiol. Infect., 144 (14): 2989-2999.

Singh, Y.; Tiwari, A.; Kumar R. and Saxena, M. K. (2017): Cloning, Sequencing and Phylogenetic Analysis of stn gene of *Salmonella Typhimurium*. J. Bioscie. Biotech. Res. Asia.; 14 (4): 1387 - 1393.

Wei, X.; You, L.; Wang, D.; Huang, H.; Li, S. and Wang, D. (2019): Antimicrobial resistance and molecular genotyping of *Salmonella enterica* serovar Enteritidis clinical isolates from Guizhou province of South western China. PLoS One.;14 (9).

Zhou, X.; Xu, L.; Xu, X.; Zhu, Y.; Suo, Y.; Shi, C. and Shi, X.

(2018): Antimicrobial Resistance and Molecular Characterization of *Salmonella enterica* Serovar Enteritidis from Retail Chicken Products in Shanghai, China. Food borne Pathog. Dis.; 15 (6): 346 - 352.

Zowail, M. E. M.; Azab, M. S.; Nassif, S. A.; Elsadek, G. M. and Mohamed, S. Z. (2017): Some Molecular Studies on *Salmonella* on Chickens. Int. J. of Sci. and Res.; 6 (11): 349-353.