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Research Article
InvA Gene Sequencing of *Salmonella typhimurium* Isolated from Egyptian Poultry

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Abstract

Background and Objective: *Salmonella typhimurium* is one of the most important zoonotic pathogenic microorganisms affecting man and animals, the traditional phenotypical identification of *Salmonella* involving microbiological enrichment and subsequent identification, usually cannot indicate the genetic determinants of the isolates causing disease. Therefore, the aim of this study was to sequence the invA gene of local isolate which is a very important tool for periodical evaluation of mutagenicity compared with the published sequences on GenBank. **Materials and Methods:** The collected poultry samples were screened for *Salmonella typhimurium* using different biochemical and serological studies. The positive isolates were examined by PCR for detection of invA gene at specific molecular size (2058 bp) using specific sensitized primers (forward and reverse). Sequencing of the invA gene was performed and compared with the published sequences on GenBank. **Results:** The homology percentage of nucleotide sequence (99.2-99.4%) and amino acid sequence (99.6-99.9%) showed high similarity between the local isolates and the other published sequences on GenBank. **Conclusion:** Sequence analysis of the Egyptian *S. typhimurium* revealed that it is mandatory to monitor any changes in the genomic structure, similarity and dissimilarity with other strains in the region and all over the world.

Key words: *Salmonella typhimurium*, InvA gene, sequencing, phylogenetic analysis, homology percentage, PCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salmonellae are widely distributed worldwide affecting man, animals and birds, it is a direct zoonotic disease of great economic and public health concern, poultry and their products usually considered as one of the principal foodborne sources of *Salmonella* species that affect humans. *S. typhimurium* has the most prevalent serotypes isolated from poultry worldwide[1,2]. Salmonellosis is one of an emerging infectious disease in the USA; it causes more than 450 deaths and 1.2 million infections every year[3].

For the effective prevention and control of this foodborne zoonosis, rapid and sensitive detection methods are required, many different conventional media and enrichment regimes have been proposed for isolating *Salmonella* species from the food and environmental samples[2]. Although, isolation of *Salmonella* by growth culture medium followed by serotyping is considered as the gold standard for confirmation of *Salmonella*, it is also time consuming and labor intensive. Therefore techniques like PCR are increasingly being used for rapid detection and confirmation of *Salmonella* and considered as a very important diagnostic tool for detection of *Salmonella* invA targeting gene[4]. Most widely used DNA based technique is PCR, utilizing genus specific primers targeting various genes. For instance, invA gene (marker gene) has been introduced for the effective, rapid and accurate detection of *Salmonella* in foods of animal origin[5].

The invA gene usually codes for protein in the inner bacterial membrane that is responsible for invasion of intestinal cells of the host[6]. The invA gene contains unique sequences specific to the genus *Salmonella* and has been proved as a specific PCR target with important diagnostic applications[5,7]. The invA target gene of *Salmonella* is located on the pathogenicity island 1 (SPI-1), it is important for the invasion of host epithelial cells. This gene is highly specific in most *Salmonella* serotypes and has been used as an important target for detection of *Salmonella*[8,9].This study helps the researcher for periodical monitoring of any changes in the genomic structure of *S. typhimurium* in Egypt.

The study was planned to fulfill the following:

- Detection of invA gene in the local isolates
- Sequencing of invA gene of the most virulence local isolate to detect the homology percentage of the nucleotide and amino acid sequences between local isolate and the published sequences of reference strains and isolates all over the world on GenBank

MATERIALS AND METHODS

***Salmonella* isolates:** Twelve strains of *S. typhimurium* were isolated from 7 chickens, 3 chicken eggs and 2 ducks from different geographical areas.

Colonial morphology: A loop full of the obtained samples were inoculated into selenite-F broth and incubated at 37°C for 16 h then streaked on to the surface of MacConkey's agar, *Salmonella Shigella* agar, XLD and Hektoen enteric agar then incubated at 37°C for 24 h[10].

Microscopical examination: The suspected *Salmonella* colonies were picked up, a film was made on glass slide and stained with Gram's stain to examined under ordinary light microscope (National model 138, China)[11].

Motility test: Motility was assured by growing the bacteria into semi-solid agar[12].

Biochemical identification: The purified isolates of *Salmonellae* were examined by different biochemical reactions[13].

Serological identification of *Salmonellae*: The isolates preliminary identified biochemically as *Salmonella*, were subjected to serological identification using diagnostic polyvalent (O, H) and monovalent *Salmonella* antisera (Difco) for serological identification of *Salmonella*[14].

Polymerase Chain Reaction (PCR) of invA gene: Extraction of *Salmonella* genome was carried out as recommended by the manufacturer of the Gene JET genomic DNA purification kit (Thermo)[15], the PCR was carried out by using the following specific sensitized primers by Primer 3 software (InvA/F and InvA/R) as shown in Table 1.

The amplified products were separated on agarose gel (1.5%) and examined by electrophoresis after staining with ethidium bromide (0.5 mg mL^{-1}) (Sigma). A 100 bp DNA ladder (Stratagene, USA) was used as a molecular size marker.

Table 1: Specific InvA gene primers

Primer	Sequence
InvA/F	'5-CAC CGT GCT GCT TTC TCT ACT TAA CA-3'
InvA/R	'5-TTA TAT TGT TTT TAT AAC ATT CAC TGA C-3'

F: Forward primer, R: Reverse primer

Table 2: List of isolates and strain with the corresponding identification and accession numbers on GenBank for sequence analysis

Strain name	Accession No.	Identification of *S. typhimurium* isolates and strains
ST. LT2	AE006468.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* str. LT2 chromosome, complete genome, 2001
ST. ATCC 13311	CP009102.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* strain ATCC13311, complete genome, 2014
ST. VNP20009	CP007804.2	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* strain VNP20009, complete genome, 2014
ST. DT2	HG326213.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* str. DT2, complete genome, 2013
ST. DT104	HF937208.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* DT104 chromosome, complete genome, 2013
ST. UK1	CP002614.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* str. UK-1, complete genome, 2011
ST. str.14028S	CP001363.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* str. 14028S, complete genome, 2010
ST. SL1344	FQ312003.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* SL1344 complete genome, 2012
ST. str.ST4/74	CP002487.1	*Salmonella enterica* subsp. *enterica* serovar *typhimurium* str.ST4/74, complete genome, 2011
STYINVA, InvA	M90846.1	*Salmonella typhimurium* InvA (invA) gene, complete cds, STYINVA, 1992

Purification of invA fragment of local *Salmonella* strain: Purification of PCR product for sequence analysis was performed according to the manufacturer instruction using QIA quick gel extraction kit (Qiagen) for extraction of DNA fragment from standard or low-melting agarose gels in TAE buffer (Tris-acetate/EDTA). The kit contains QIA quick spin columns, QG, PE and EB buffers.

Sequence analysis of the DNA fragment: All materials needed for sequence reaction were supplied by the GATC Company, Germany by using (ABI 3730xl DNA) sequencer (Applied Biosystem, USA) and the nucleotide sequencing was done by Sanger sequencing technique[16].

Identification of homologies between nucleotide and amino acid sequences of the *S. typhimurium* isolates published on GenBank was done using BLAST 2.0 and PSI-BLAST search programs, National Center for Biotechnology Information (NCBI). The scores designated in the BLAST search have a well-defined statistical interpretation, making matches easier to distinguish from random background hits. Sequence homology percent and divergence was calculated by MegAlign (DNA STAR, Laser gene, Version 7, USA).

The sequence of the invA gene of local Egyptian isolate was compared with other 10 invA of *S. typhimurium* strains published sequences on GenBank as shown in Table 2.

RESULTS AND DISCUSSION

Salmonella typhimurium is a leading cause of enteric diseases in humans and animals with millions of illness reported worldwide, therefore, periodical monitoring of any changes in the genomic structure of local *S. typhimurium* is mandatory. Non-typhoid *Salmonella* serovars are predominantly associated with animal origin such as eggs, milk, poultry and beef responsible for zoonotic transmission[17]. *Salmonella enteric* serovar *typhimurium* and *S. enterica* serovar *enteritidis* are the most frequently encountered species from foods like poultry, pork and beef products[18]. The incidence of salmonellosis has been reported in many developing countries including India, Egypt, Brazil and Zimbabwe[19].

Twelve strains of *S. typhimurium* isolated from 7 chickens, 3 chicken eggs and 2 ducks from different geographical areas, all examined *Salmonella* strains were Gram negative, non-spore forming straight rods and highly motile, the isolates were appeared as non-lactose fermenting colonies on MacConkey agar medium. On S.S agar, a colorless colony with black center was formed. On Hekton enteric agar, blue-green colonies with black center was described onto the medium. On Xylose-Lysine Deoxycholate (XLD) agar medium, red colonies with black center were formed. All strains were oxidase negative and H2S positive. *Salmonella* colonies were tested for serotyping and all 12 isolates are *Salmonella typhimurium*. These results agree with El-Zeedy *et al.*[20] who isolated 12 isolates from egg samples and 67 isolates from poultry and serotyped as *S. rubislaw*, *S. virginia*, *S. poona*, *S. typhimurium*, *S. montevideo*, *S. enteritidis*, *S. infantis*, *S. cerro*, *S. agona*, *S. derby* and *S. kentucky*. The same results obtained by Abdellah *et al.*[21] who recovered *S. typhimurium* as most predominant *Salmonella* isolated from contaminated chicken samples. While Abd El-Aziz[19] found that the prevalence of *S. typhimurium* in raw chicken meat, heart and liver was 44, 48 and 40%, respectively. Also, Andino and Hanning[22] mentioned that the data from food born outbreak in USA related to human illness showed that serovar *enteritidis* was the most frequently isolated followed by *typhimurium*, *newport*, *heidelberg* and *montevideo* by percentage 27, 14, 7, 10 and 3%, respectively. While in the study of Parvej *et al.*[23] the *S. enteric* serovar *typhimurium* was isolated and characterized from commercial poultry farms in Bangladesh.

The usefulness of PCR for rapid detection of *Salmonella typhimurium* from poultry carcasses was proved by many recent studies[24,25]. Therefore, the polymerase chain reaction (PCR) was performed on genomic DNA extracted from 12 local *Salmonella typhimurium* isolates for amplification of 2058 bp

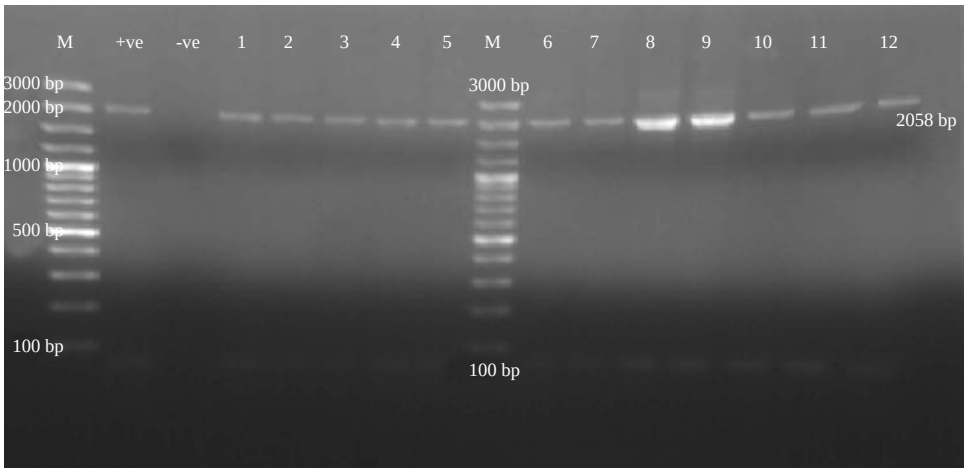

Fig. 1: PCR products of 2058 bp fragment of InvA gene

Lane M: Gene ruler 100 bp plus DNA ladder marker (Thermo scientific), Lane +ve: ST.LT2 reference positive control, Lane -ve: Negative control, Lane 1-12: *Salmonella typhimurium* isolates

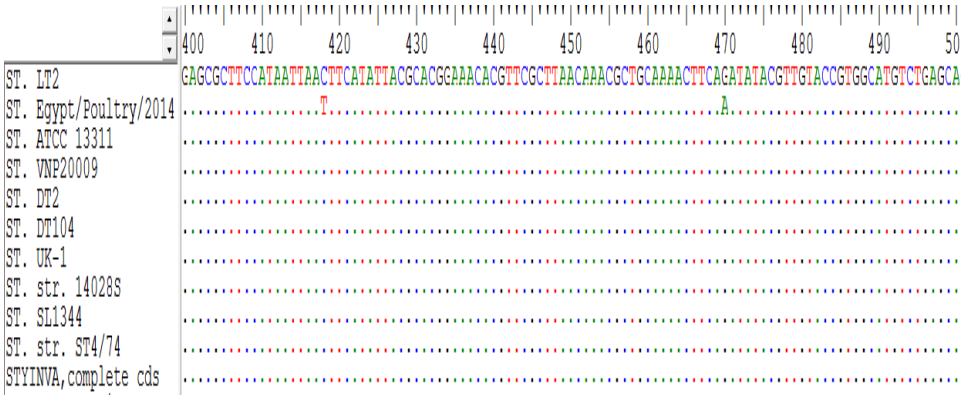

Fig. 2: Multiple alignment of nucleotide sequence of invA in Egyptian *Salmonella typhimurium* strain compared with other 10 invA in *Salmonella typhimurium* strains published sequences on GenBank

fragment of invA gene which contains unique sequences to this genus and has been proved as a specific PCR target[5,7]. Electrophoresis of the amplified products on 1.5% agarose gel revealed the presence of specific product at the correct size (2058 bp) according to the standard DNA ladder as shown in Fig. 1.

This finding agreed with Abd El-Tawwab *et al.*[25] who reported that the invA gene was detected in all isolates (100%) from chickens and ducks samples. While Sharma and Das[6] found that, the invA gene codes for protein in inner bacterial membrane, which is important for invasion of the host epithelial cells, they detected invA genes in 22 out of 40 *Salmonella* isolates from poultry meat samples (55%).

Due to invA that encodes a protein in the inner bacterial membrane which is responsible for invasion to the host epithelial cells, the ability of *Salmonella* specific primers to detect *Salmonella* species was rapid and accurate[9,26] and now has been proved as an international standard for identification of genus *Salmonella*[7].

The nucleotide sequence of 2058 bp PCR fragment representing the invA gene of *S. typhimurium* isolate was compared with other 10 *S. typhimurium* strains published sequences on GenBank as shown in Table 2 using Bio Edit software (ClastalW) representing 10 complete sequences of invA gene of different *S. typhimurium* strains. The different important part of this data is summarized in Fig. 2. After the nucleotide sequence analysis of the invA gene of *Salmonella typhimurium* local isolate ST. Egypt/Poultry/2014 (ST12) by using the 2058 bp InvA specific primers, the obtained sequence was submitted to GenBank by direct submission

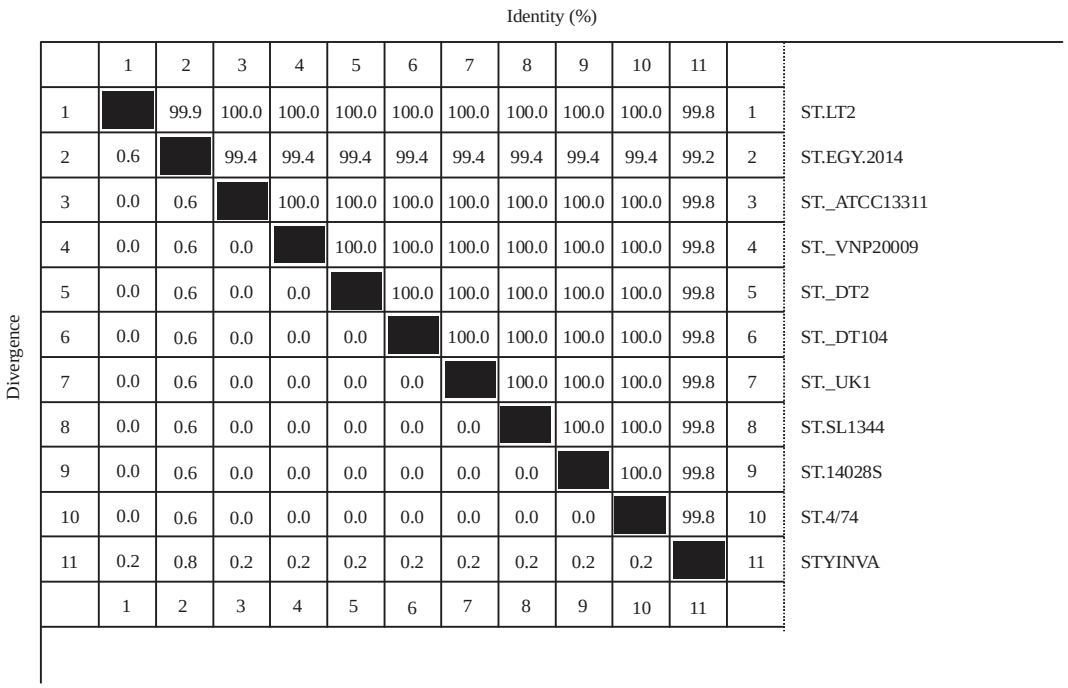

Fig. 3: Homology percentage of nucleotide sequence of InvA in Egyptian *Salmonella typhimurium* strain in comparison with other published *Salmonella typhimurium* strains

using Bankit submission tool under Accession No. (KR185982). The obtained sequences of local isolate covered around 2058 bp that encodes 685 deduced amino acid. Multiple alignment revealed nucleotide substitution at the base pair number 470 from the start codon which lead to amino acid substitution from CAG (Serine (Ser/S)) to CAA (Phenylalanine (Phe/F)) at residue number 530, this change is very characteristic and unique for the local isolate than the rest of isolates and strains. Also some different silent nucleotides substitution, non-significant differences, not affected on amino acid substitution occurred in the sequence at the base pairs number: A 208 G, T 418 C, T 589 C, T 1156 A, C 1165 T, A 1288 G, A 1381 G, G 1408 A, G 1654 A, G 1801 A and T 1813C.

The homology percentage of nucleotide sequence results (Fig. 3) showed high homology (99.4%) between ST/Egypt/Poultry/2014 strain and ST.LT2, ST.ATCC13311, ST.VNP20009, ST.DT2, ST.DT104, ST.UK1, ST.SL1344, ST.14028S and ST.4/74 but it gives (99.2%) with STYINVA strain. These results agree with Shi *et al.*[27] who found the high invA homology between *Salmonella* strains (72.9-97.2%). Also agree with Jarvik *et al.*[28] who found the 14028s and LT2 genomes have more than 98% similarity in sequence and more closely related than any pair of *Salmonella* strains examined in his studies and in UK-1 strain two unique genes were predicted which are highly homologous to the genes involved in the type III secretion system(as invA gene)[29].

The results in this study were parallel also with Edwards *et al.*[30] who described that all of the SPI loci (which contain invA genes) from different serotypes sharing nearly the same distribution of identity as a whole genomes (varying between 97.7 and 98.6% similarities).

The multiple alignment of deduced amino acid (685 amino acid) in Fig. 4 of Egyptian isolate with other published *S. typhimurium* strains on GenBank showed only one amino acid difference at (F 530 S) and this significant difference led to high identity percentage between *S. typhimurium* strains. Amino acid substitution from CAG (Serine (Ser/S)), which is considered as a polar amino acid, to CAA (Phenylalanine (Phe/F)), which is a hydrophobic amino acid, at residue number 530. This change is very characteristic and unique for the local isolate ST. Egypt/Poultry/2014 than the rest of published isolates.

The homology percentage of deduced amino acid results (Fig. 5) showed high homology (99.9%) between our Egyptian isolate of ST/Egypt/Poultry/2014 strain and ST.LT2, ST.ATCC13311, ST.VNP20009, ST.DT2, ST.DT104, ST.UK1, ST.SL1344, ST.14028S and ST.4/74 but it gives (99.6%) with STYINVA strain.

The phylogenetic analysis of nucleotide sequence (Fig. 6) showed two major clusters or branches, one representing the Egyptian isolate ST/Egypt/Poultry/2014 and the second divided in two sub-branches, the first for STYINVA strain and

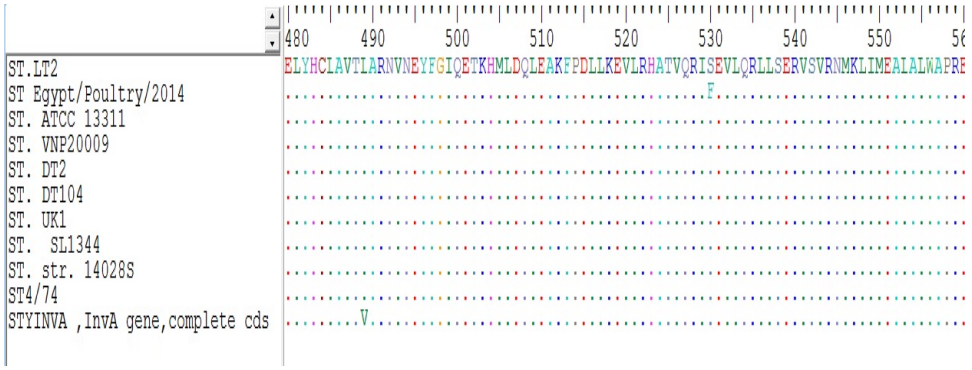

Fig. 4: Multiple alignment of deduced amino acid sequence of InvA in Egyptian *Salmonella typhimurium* strain compared with other 10 InvA in *Salmonella typhimurium* strains published sequences on GenBank

Identity (%)

	1	2	3	4	5	6	7	8	9	10	11		
1		99.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	1	ST.LT2
2	0.1		99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.6	2	ST.EGY.2014
3	0.0	0.1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.7	3	ST._ATCC13311
4	0.0	0.1	0.0		100.0	100.0	100.0	100.0	100.0	100.0	99.7	4	ST._VNP20009
5	0.0	0.1	0.0	0.0		100.0	100.0	100.0	100.0	100.0	99.7	5	ST._DT2
6	0.0	0.1	0.0	0.0	0.0		100.0	100.0	100.0	100.0	99.7	6	ST._DT104
7	0.0	0.1	0.0	0.0	0.0	0.0		100.0	100.0	100.0	99.7	7	ST._UK1
8	0.0	0.1	0.0	0.0	0.0	0.0	0.0		100.0	100.0	99.7	8	ST.SL1344
9	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0		100.0	99.7	9	ST.14028S
10	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0		99.7	10	ST.4/74
11	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3		11	STYINVA
	1	2	3	4	5	6	7	8	9	10	11		

Divergence

Fig. 5: Homology percentage of deduced amino acid sequence of InvA in Egyptian *Salmonella typhimurium* strain in comparison with other published *Salmonella typhimurium* strains

the second divided in two small subgroups, the first contains ST.4/74 and ST.SL1344 strains and the second contains ST.LT2, ST.ATCC13311, ST.VNP20009, ST.DT2, ST.DT104, ST.UK1 and ST.14028S strains. Therefore, ST. Egypt/Poultry/2014 is unique in their position with other *Salmonella typhimurium* strains.

The phylogenetic analysis of deduced amino acid (Fig. 7) showed two major clusters or branches on rooted tree, one representing the STYINVA (M90846.1) strain and the second one divided in two sub-branches, the first for ST/Egypt/Poultry/2014 Egyptian local strain (KR185982) and the second for remaining strains: ST.LT2 (AE006468.1), ST.ATCC13311 (CP009102.1), ST.VNP20009 (CP007804.2), ST.DT2 (HG326213.1), ST.DT104 (HF937208.1), ST.UK1 (CP002614.1), ST.SL1344 (CP001363.1), ST.14028S (FQ312003.1) and ST.4/74 (CP002487.1). Therefore, ST/Egypt/Poultry/2014 is unique in their position with other *Salmonella typhimurium* strains.

Both phylogenetic trees (Fig. 6, 7) demonstrated that the Egyptian isolate (ST/Egypt/Poultry/2014) is closely related to the other *S. typhimurium* isolates but in a separate cluster because the presence of single, significant amino acid substitution at position (F 530 S), due to the change of

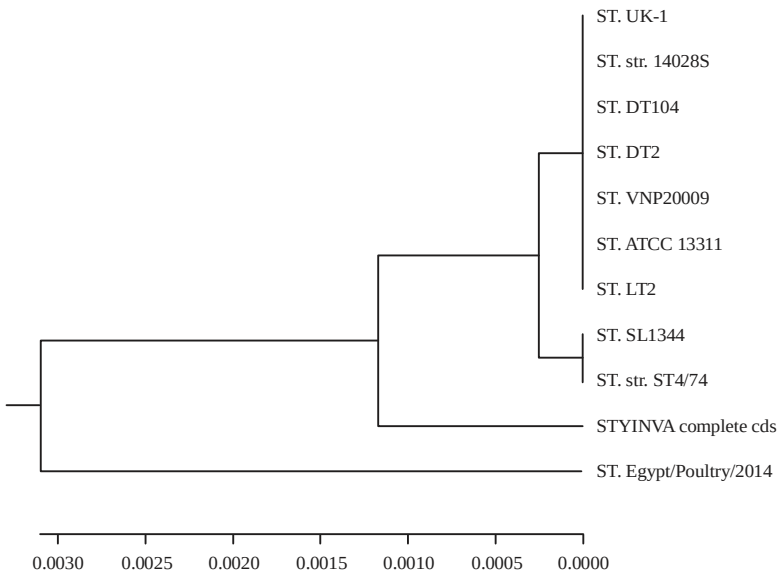

Fig. 6: Phylogenetic tree of nucleotide sequence of InvA gene of Egyptian *Salmonella typhimurium* strain with other published *Salmonella typhimurium* strains

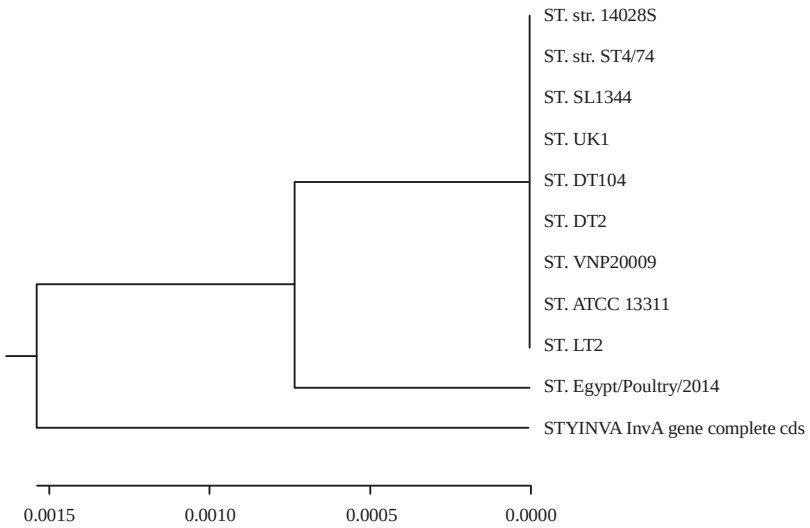

Fig. 7: Phylogenetic tree of deduced amino acid sequence of InvA gene of Egyptian *Salmonella typhimurium* with other published *Salmonella typhimurium* strains

nucleotide at position (A 470 G) and non-significant changes in another nucleotides. These results agree with Kingsley *et al.*[31] who found the isolates of DT2 formed a distinct phylogenetic cluster within *Salmonella typhimurium* serotypes. Comparative genomic analysis of DT2 94-213 and *S. typhimurium* SL1344, D23580 and DT104 identified few differences in genetic content with the exception of variations within prophages. Also, in this study found that the STYINVA strain was in a separate cluster in both phylogenetic trees due to some amino acid substitutions when comparing with other *Salmonella* strains, this differences may be due to long years lasts from isolation and sequencing of this strain science 1992[32], while the other strains (including Egyptian isolate) were isolated and sequenced from 2001 until 2014, which may explains the minor differences them.

All these results of invA alignment agree with Galan and Curtiss[33] and Shi *et al.*[27] who said that the invA is present and functional in most, if not all, virulent *Salmonella* strains and its conserved gene.

CONCLUSION

Regular monitoring of the Egyptian *S. typhimurium* sequence changes is an important discriminatory marker that could be used in epidemiological investigations as well as during vaccine preparation and evaluation.

ACKNOWLEDGMENT

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SIGNIFICANCE STATEMENT

This study discovered the changes in invA gene sequence between local Egyptian *S. typhimurium* strain and other published strains on GenBank. These changes should be taken in consideration during manufacturing and evaluation of new the *S. typhimurium* vaccine.

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