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Phenotypic Characterization and Genotypic Analysis of Salmonella Typhimurium Isolated from Egyptian Pigeons

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Abstract

Bacteriological findings of 102 examined cases from adult pigeons and squabs revealed that 4 out of 102 (3.9%) cloacal swab samples were positive for Salmonella species, while 16 drag swabs collected from pigeon houses and dovecotes were negative. Culture characters as well as the identical biochemical and serological tests identified Salmonella species. By using slide agglutination test, four Salmonella isolates from pigeons were serotyped using O and H poly, monovalent antisera, three isolates were serotyped as Salmonella Typhimurium, and one isolate was un-typed. By using agar gel diffusion method, the antimicrobial sensitivity test for Salmonella Typhimurium isolated from pigeons were sensitive to Amoxicillin, Ciprofloxacin, Doxycycline, Enrofloxacin, Gentamicin, Norfloxacin and Streptomycin, while they were resistant to Erythromycin. PCR technique was used to detect different virulent genes as invasion (invA) and flagellin (fliC) genes and the three isolates gave positive to all previous genes. Two fliC sequences were submitted to GenBank database and obtained accession numbers {HSE-1-2013 (KJ700871) and HSE-2-2013 (KJ671550)}and showed 99.8 % identity with each other and different identity percent with 22 randomly selected strains from GenBank. strain HSE-1-2013 consists of 158 amino acid showed 100% identity with the second strain HSE-2-2013 and the twenty two randomly selected strains from GenBank, while strain HSE-2-2013 consists of 159 amino acid showed 100% identity with Egyptian strains (Azhar1,2,3, and 4) and 99.5% identity. Other fifteen strains on the GenBank (there is protein changed at position159, the standard amino acid V Valine mutated to the amino acid E Glutamic acid). The restriction enzyme map was constructed for the two sequenced PCR products of Salmonella Typhimurium and the enzyme BbvI was found to cut five times at the positions 134,361,418,441 and 457.

Key words: Phenotypic, Genotypic, S. Typhimurium, PCR, Phylogenetic analysis, Antibiotic sensitivity.

Introduction

Salmonellosis is a disease caused by bacteria of thegenusSalmonellaofthefamily Enterobacteriaceae. Pigeons may acquire Salmonella through consumption of contaminated feed or water and by direct contact with contaminated feathers, dust or feces. Concerning the public health, pigeons play an important role in the transmission of diseases that affect humans and domestic animals, such as toxoplasmosis, Newcastle disease, aspergillosis and salmonellosis Sousa et al., (2010). The PCR test combined with Rappaport-Vassiliadis selective enrichment is more sensitive in detecting Salmonella at genus level than bacteriological methods. At serovar level, PCR-RV and (SMT) Standard microbiological techniques showed similar sensitivity. However, the PCR test combined with non-selective enrichment was not as effective as PCR-RV or SMT for detection and identification of Salmonella, indicating the need for selective enrichment prior to the PCR test. The PCR-RV protocol described also decreases the time needed to detect Salmonella and can easily be implemented in diagnostic and food analysis laboratories Oliveira et al., (2003). The ideal microbial typing technique should enable differentiation of epidemiological unrelated strains and group epidemiological related (outbreak) strains, and give information that will help to understand the evolutionary history of multiple strains within a clonal lineage, Foxman et al., (2005) and Parkhill and Wren (2011). Therefore, we applied PCR and sequencing to be able to make genotypic analysistotheisolatedstrains.

Material and Methods

Samples:-

One hundred and two cloacal swabs from pigeons (adult & squabs) and sixteen samples from pigeon houses and dovecotes were collected from Menoufia governorate, Egypt in order to examine the causative agents of paratyphoid in pigeons. All samples were collected under aseptic condition and laboratory safety precautions. Cloacal swabs were collected by using sterile cotton swabs, while the drag swabs were collected by using sterile gauze pads, which were dragged more than one time across the surface of the floor and dropping pit in the pigeon houses. Finally, the swabs were placed in sterile plastic bags and transported in icebox to the bacteriology laboratory as soon as possible.

Procedure for Isolation and Identification of Salmonella:-

Xylose lysine deoxycholate (XLD) agar medium and Hektoen Enteric (HE) agar medium. The plates were incubated at 37°C for 24h. Suspicious colonies morphologically similar to Salmonella were sub cultured in semisolid agar media tubes for further examination. Identification of the biochemical characteristics was performed according to **Quinn et al. (2002),** using triple sugar iron (TSI) medium, urease test, lysine-iron agar (LIA) medium, indole test, methyl red test, Voges- Proskauer test and Simmon's citrate medium.

Serotyping of Salmonella Species Isolated from Pigeons:-

All isolates presumptively identified as Salmonella, were serotyped according to **Kauffmann and Das-kauffmann (2001)** using Salmonella "O" and "H" poly and monovalent antisera based on slide agglutination tests to determine O and H antigens, respectively. S. Typhimurium isolates were identified according to their serotyping formula. To confirm the results of serotyping, all isolates tested by conventional PCR (cPCR).

Antibiotic Sensitivity Test:-

The antibiotic sensitivity test of Salmonella Typhimurium isolates to eight antibiotics was determined using the disc diffusion technique according to Finegold and Martin (1982). Five well isolated colonies were selected with sterile loop and transferred to a tube containing Muller-Hinton broth and mixed well. The broth culture is incubated at 37°C until it achieves the turbidity of the 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 x108 CFU / ml for E.coli ATCC 25922. A sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. The dried surface of a Müeller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. Antimicrobial disks were disposed on the surface of inoculated agar media aseptically and incubated at 37°C for 18-20 h. The zone of on inhibition around each disk were measured and the results were interpreted based on comparison to standards, as described by **the** Clinical and Laboratory Standards Institute (CLSI 2013).

Genomic DNA Extraction:-

DNA extraction of all Salmonella Typhimurium isolates were performed from overnight culture in buffered peptone according to ABIO pure Genomic DNA extraction kit, (USA) instructions.

PCR (Polymerase Chain Reaction) for Detection of Salmonella Typhimurium:-

The purified DNA was used as a template for the PCR assay. For the cPCR, two primer pairs were used. The sequence of primers used in this study is shown in Table (1). The 139 and 141 primers are specific for the invA gene of Salmonella spp, Oliveira et al., (2003) and Fli15 and Tym primers are specific for the fliC gene of Salmonella Typhimurium, Soumet et al., (1999). Reactions with these primers were carried out in a 25µl amplification mixture (Table 2), and the cycling condition is shown in Tables (3) and (4). Amplified products were electrophoresed in 1.5% agarose gel, **Sambrook et al., (1989)** and a Gel Pilot 100 bp ladder, (Cat. No.239035) was used as a size reference. After staining with ethidium bromide the gel was photographed by a gel documentation system and the data were analyzed through computer software. Deionized distilled water was used as a template for negative control and S. Typhymurium (ATCC: 25923) was used as a positive control.

Table (1): Oligonucleotide primers sequences.

Primer	Target gene	Primer sequence(5'-3')	Length of amplified product	Reference
Fli15	fliC	CGGTGTTGCCCAGGTTGGTAAT	559 bp	Soumet et al. (1999)
Tym		ACTCTTGCTGGCGGTGCGACTT		
139	invA	GTGAAATTATCGCCACGTTCGGG CAA	284 bp	Oliveira et al. (2003)
		CATCGCACCGTCAAAGGAACC		

Table (2): Component of Emerald Amp®GT PCR master mix (Takara) Code No. RR310A

Component	Volume/reaction
Emerald Amp GT PCR mastermix (2x premix)	12.5 µl
PCR grade water	4.5 µl
Forward primer (20 pmol)	1 µl
Reverse primer (20 pmol)	1 µl
Template DNA	6 µl
Total	25 µl

Table (3): Cycling conditions of the invA primers during cPCR (Oliveira et al., 2003).

Step	Temperature	Time	No. of cycles
1. Primary denaturation and activation of Taq DNA polymerase.	94°C	6 min.	1 cycle
2. Cycling			35 cycles
A. Secondary denaturation	95°C	30 sec.	
B. Primer annealing	55°C	30 sec.	
C. Extension	72°C	30 sec.	
3. Final extension	72°C	7 min.	1 cycle

Table (4): Cycling conditions of the fliC primers during cPCR (Soumet et al., 1999).

Step	Temperature	Time	No. of cycles
1. Primary denaturation and activation of Taq DNA polymerase.	94°C	6 min.	1 cycle
2. Cycling			35 cycles
A. Secondary denaturation.	95°C	30 sec.	
B. Primer annealing	56°C	30 sec.	
C. Extension	72°C	30 sec.	
3. Final extension	72°C	7 min.	1 cycle

Nucleotide Sequencing and Sequence Analysis:

The amplified (559bp fliC gene) fragment (from pigeon) was purified using Gene Jet PCR purification kit; Fermentas (cat no.K0701). Sequencing was performed at Sigma Company (Germany). Identification of homologies between nucleotide and amino acid sequences of the Salmonella Typhimurium strains were compared with other strains published on GenBank using BLAST 2.0 and PSI- BLAST search programs, respectively. The obtained nucleotide sequences comparisons and their multiple alignments with reference S. Typhimurium strains as well as the detection of amino acid sequences were done using the BioEdit sequence alignment editor, CLUSTALX software for multiple sequence alignment.

Results

1-Incidence of Bacteria:-

Bacteriological findings of examined cases from diseased pigeons and squabs revealed that 4 out of 102 (3.9%) cloacal swab samples were positive for isolation of Salmonella species, while bacteriological findings of examined cases from pigeon houses and dovecotes revealed that all 16 drag swab samples were negative for isolation of Salmonella species as shown in Table (5).

Table (5): Incidence of Salmonella species isolated from pigeons and dovecotes % was calculated according to total number of examined samples.

Type of samples	of Examined Samples	Positive Salmonella Spp.		Positive Salmonella Typhimurium	
		Number	Percent	Number	Percent
Cloacal swabs	102	4	3.9%	3	2.94%
Drag swabs	16	-	-	-	-
Total	118	4	3.4%	3	2.54%

2- Identification of Salmonella Species Isolated from Pigeons:

Culture characters as well as the classical biochemical tests identified Salmonella species. Salmonella species colonies on XLD medium appeared pink with black center, while Salmonella species colonies on HE medium appeared green to blue – green color with black center.

Biochemical Tests used for Identification of Salmonella Species:

1. TSI test Slant-alkaline: red color, Butt-acidic: yellow color, H2S production: black color, Gas accumulation of gas beneath the butt.
2. Lysine iron test- Purple color in the slant and butt with blacking (H2S production) at the middle of the tube (+ve).
3. Urease test - Yellow color (-ve).

3-Result of Antibiogram Sensitivity Test for Salmonella Typhimurium Isolated from Pigeons:

Salmonella Typhimurium isolated from pigeons were sensitive to Amoxicillin, Ciprofloxacin, Doxycycline, Enrofloxacin, Gentamicin, Norfloxacin and Streptomycin, while they were resistant to Erythromycin.

4-Results of PCR for Detection of Salmonella Typhimurium in Pigeons:

4-1 PCR for Amplification of Inva Gene from the DNA of Salmonella Typhimurium In Pigeons

Photo (1) illustrates the agarose gel electrophoresis with positive PCR amplification of 284 bp fragment of invA gene.

4-2 PCR for Amplification of Flic Gene (Flagellin Gene) from the DNA of Salmonella Typhimurium in Pigeons.

Photos (2) illustrated the agarose gel electrophoresis with positive PCR amplification of 559 bp fragment of filC gene from the DNA of Salmonella Typhimurium isolated from pigeons.

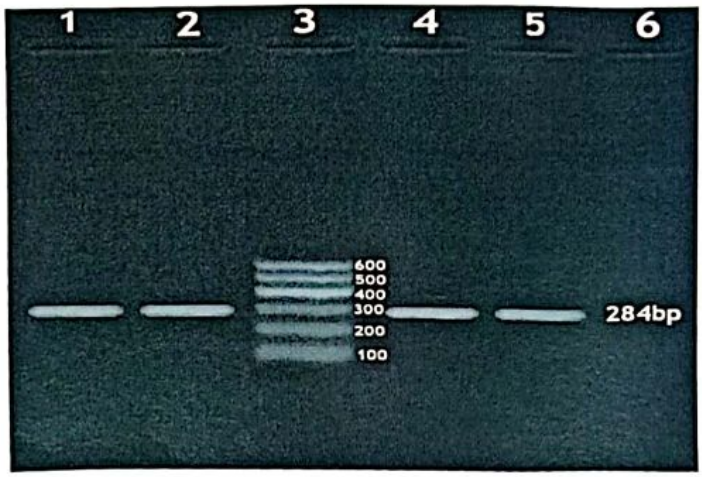


Photo (1) Agarose gel electrophoresis with positive PCR amplification of 284 bp fragment of invA gene from the DNA of Salmonella Typhimurium isolated from pigeons. (Lane, 3) Marker (100- 600 bp ladder) - (Lane, 1, 2, and 5) Positive sample from the pigeons (isolate one, isolate 2, and isolate 3). - (Lane, 4) Positive control (Lane, 6) Negative control

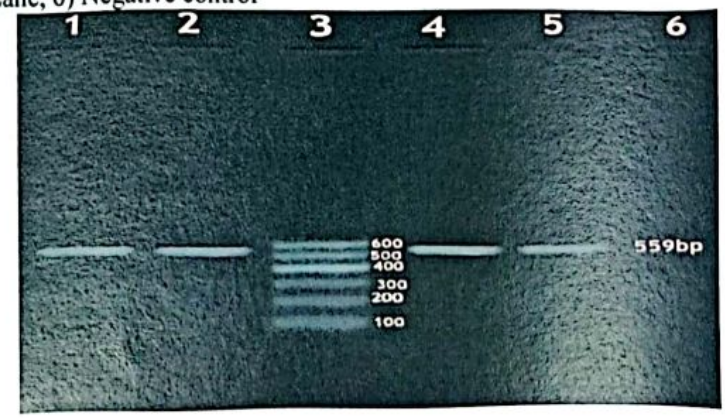


Photo (2) Agarose gel electrophoresis with positive PCR amplification of 559 bp fragment of fliC gene from the DNA of Salmonella Typhimurium isolated from pigeons. (Lane, 3) Marker (600 bp) - (Lane1, 2 & 5) Positive samples from the pigeons (isolate 1, isolate 2 and isolate 3) - (Lane, 4) Positive control (Lane, 6) Negative control.

Two fliC sequences were submitted to GenBank database and obtained accession numbers **(KJ700871** and **KJ671550).**

In the present study the nucleotide identity percent of the sequenced fliC gene of S. Typhimurium pigeon strains and twenty two randomly selected aligned strains from GenBank, revealed that the first Salmonella strain **(HSE-2-2013)** showed 99.8% identity with the first strain **HSE-1-2013** and the Salmonella Typhimurium Egyptian strains **(Azh1, 2,3&4).** In addition, it showed 99.6% identity with strain **(S. Typhimurium DT2 HG326213.1),** which is isolated from pigeons as shown in Table (6).

5-ucleotideSequencingandSequenceAnalysi:

In the present work, the nucleotide sequence of 559 bp PCR product representing the fliC gene of Salmonella Typhimurium from two strains isolated from pigeons had been sequenced by Sigma Company, Germany.

Typhimurium pigeon strain**(HSE-1-2013)** showed 100% identity with the Salmonella Typhimurium Egyptian strains **(Azh1, 2,3&4)** that were isolated from wild birds. In addition, it showed 99.8% identity with strain **(S. Typhimurium DT2 HG326213.1),** which is isolated from pigeons. The second Salmonella Typhimurium pigeon

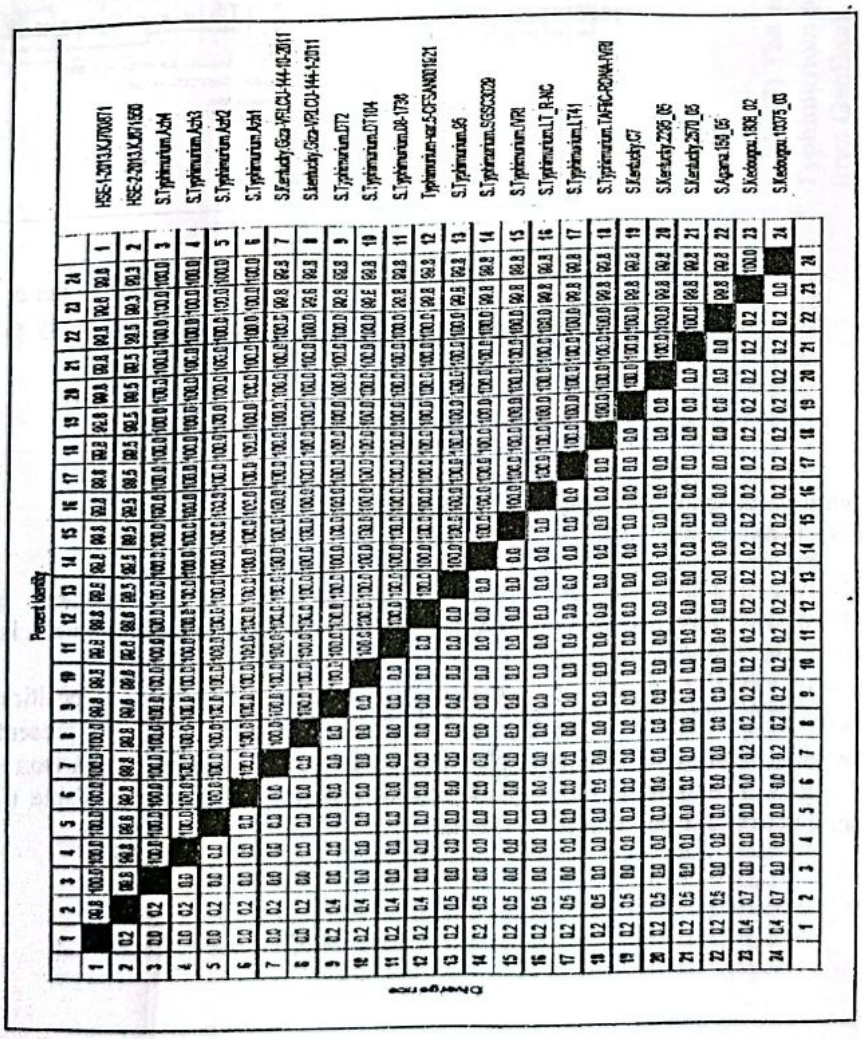


Table (6) The identity % of the sequenced *fliC* gene of *S.* Typhimurium pigeon strains and 22 randomly selected aligned strains from GenBank.

The phylogenetic tree was constructed, the tree having two groups A and B originated from the same base, group B contains Salmonella strains HSE-1-2013, HSE-2-2013, Salmonella Typhimurium Egyptian strains (Azh1, 2,3&4) and S. Typhimurium DT2 (HG326213.1), which isolated from pigeons. In addition, group B contains fourteen Salmonella strains isolated from worldwide as shown in Fig (1).

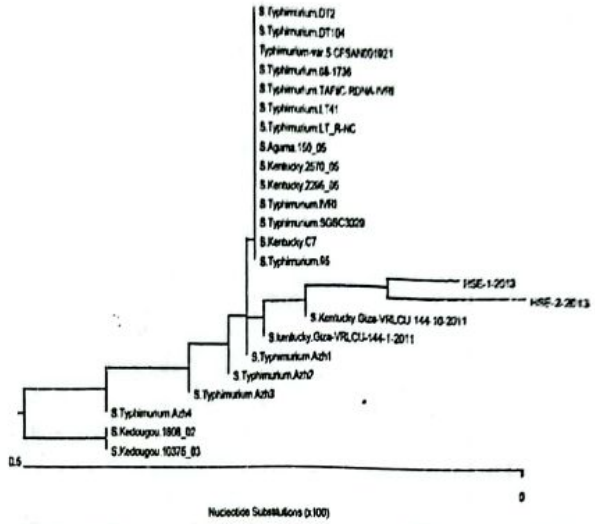
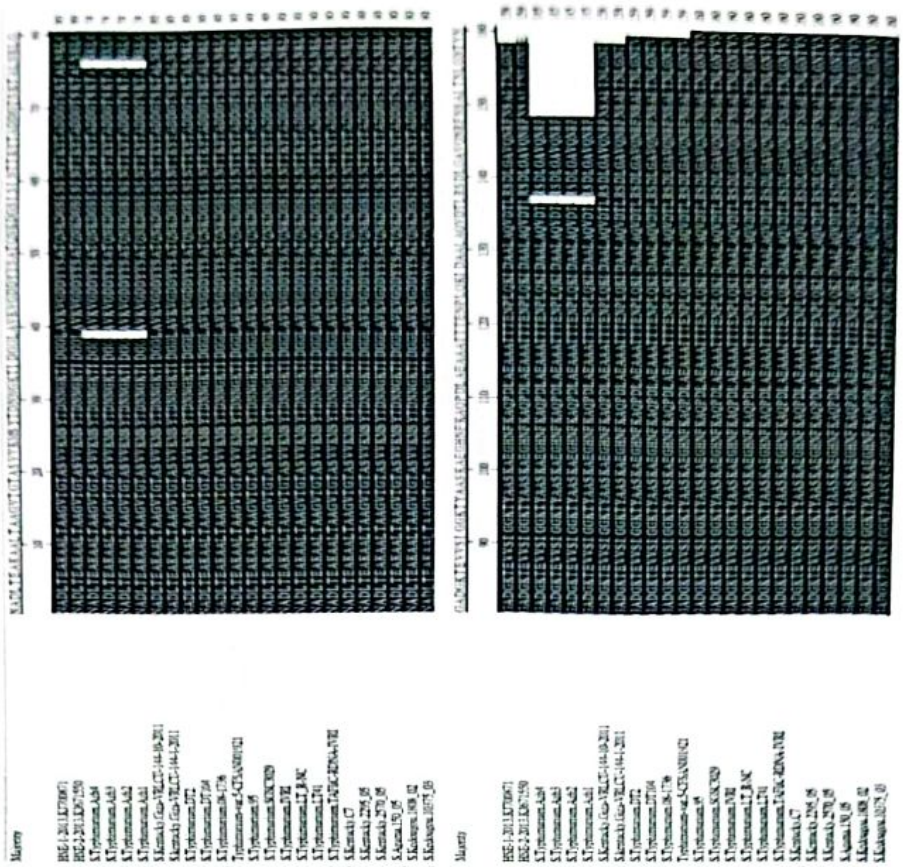


Fig. (1) The phylogenic tree between sequenced fliC gene of S. Typhimurium isolated from pigeons and twenty two randomly selected aligned strains from GenBank

The amino acids identity percent between sequenced fliC gene of S. Typhimurium isolated from pigeons and twenty two randomly selected aligned strains from GenBank was constructed, the first strain (**HSE-1-2013**) is similar to the worldwide. The second strain (**HSE-2-2013**) was slightly different from them, with 99.5% to 100% similarity, because of changing in protein position 159 (The standard amino acid V Valine mutated to the amino acid E Glutamic acid), and this due to the substitution of the nucleotide at positions 560 and 563. The amino acid V Valine is present in all the Salmonella species in our comparison except the Egyptian strains, as shown in Table (7) and Fig.(2)

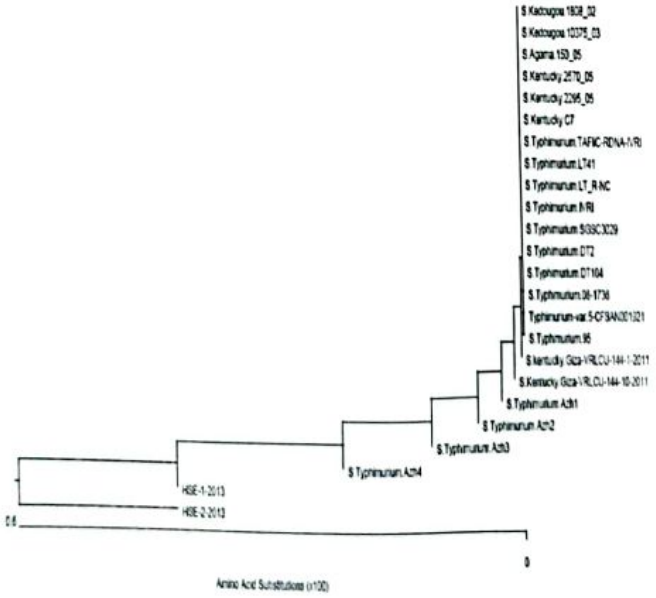
Salmonella Typhimurium Egyptian strains (**Azh1,2,3&4**) and to the other Salmonella isolated

Table (7) The amino acids identity % of the sequenced fliC gene of S. Typhimurium pigeon strains and 22 randomly selected aligned strains from GenBank.



and a leaflet that giving cluster 5, which contains S. Typhimurium Azh1, and a leaflet that giving cluster 6, which contains S. Kentucky Giza-VRLCU-144-10-2011 and a leaflet that giving cluster 7, which contains S. Kentucky Giza-VRLCU-144-1-2011 and another sixteen S. Typhimurium strains distributed world wide as shown in Fig. (3)

The phylogenetic tree of the amino acids was constructed. It consists of seven cluster consist of two main groups A and B. Group A contains the second S. Typhimurium (HSE-2-2013), group B contains (HSE-1-2013), and a leaflet that giving cluster 2, which contains S .Typhimurium Azh4, and a leaflet that gives cluster 3, which contains S. Typhimurium Azh3, and a leaflet that giving cluster 4, which contains S. Typhimurium Azh2,



pigeons and twenty two randomly selected aligned strains from GenBank.

Fig. (3) The amino acids tree between sequenced fliC gene of S. Typhimurium isolated from

Typhimurium strain HSE-1-2013 and strain HSE-2-2013 Using BioEdit version 7.2.5 Hall (1999).

6-Restriction Enzyme Mapping

Restriction Enzyme map was constructed for Salmonella enterica subsp. enterica serovar

Table (8) Enzymes that cut five or fewer times:

Enzyme	Recognition	frequency	Positions
AanI	CACCTGCnnnn'nnnn_	1	116
AflIII	A'CryG_T	1	489
AlwNI	CAG_nnn'CTG	2	56 , 146
AseI	AT'TA_AT	1	264
BbvI	GCAGCnnnnnnnn'nnnn_	5	,361,418,441,457
BceAI	ACGGCnnnnnnnnnnnn'nn_	1	349
BmrI	ACTGGGnnnn_n'	1	329
BsaJI	C'CnnG_G	2	505, 547
BseMII	CTCAGnnnnnnnn_nn'	1	48
BsgI	GTGCAGnnnnnnnnnnnnnn_nn'	2	291, 531
BslI	CCnn_nnn'nnGG	1	32
BspCNI	CTCAGnnnnnnn_nn'	1	49
BspMI	ACCTGCnnnn'nnnn_	2	52,116
BsrI	ACTG_Gn'	1	324
BsrFI	r'CCGG_y	1	134
BsrGI	T'GTAC_A	1	73
BstF5I	GGATG_nn'	2	67,296
BtsI	GCAGTG_nn'	1	279
Cac8I	GCn'nGC	1	-10
DraI	TTT'AAA	1	403
EciI	GGCGGAnnnnnnnnn_nn'	1	437
FauI	CCCGCnnnn'nn_	2	33,459
FokI	GGATGnnnnnnnnn'nnnn_	2	74,283
Hin4I	GAynnnnnvTCnnnnnnnn_nnnnn'	2	281,313
HincII	GTy'rAC	1	487
Hpy8I	GTn'nAC	2	38,487
MnlI	CCTCnnnnnn_n'	2	52,94
MspA1I	CmG'CkG	1	454
NlaIV	GGn'nCC	1	253
PstI		2	286, 459
SfaNI		2	152,457
SfcI		2	
TaqII	C_TGCA'G	2	282,455
TatI	GCATCnnnnn'nnnn_	2	311,497
TspRI	C'TryA_G	1	73,514
XmnI	CACCCAnnnnnnnnn_nn'	1	286
	w'GTAC_w		524
	_nnCAsTGnn'		
	GAAnn'nnTTC		

EcoICRI, Eco57MI, EcoNI EcoO109I, EcoRI, EcoRV, FalI, FseI, FspI, FspAI, HaeII, HgaI, HindIII, HpaI, HphI Hpy188III, KasI, KpnI, MboII, MfeI, MluI, MlyI, MmeI, MscI, MslI, NaeI, NarI, NcoI NdeI, NgoMIV, NheI, NotI, NruI, NsiI, NspI, PacI, PciI, PflMI, PleI, PmeI, PmlI ,PpiI, PpiI, PpuMI, PshAI, PsiI, PspOMI, PsrI, PsrI, PvuI, PvuII, RsrII, SacI SacII, SalI, SanDI, SapI, SbfI, ScaI, SexAI, SfiI, SfoI, SgrAI, SmaI, SmlI, SnaBI ,SpeI, SphI, SrfI, SspI, StuI, StyI, SwaI, TaqII, TspDTI, TspGWI, Tth111I, XbaI ,XcmI, XhoI, XmaI, ZraI

Enzymes that do not cut

AatII, AccI, Acc65I, AclI, AfeI, AflII, AgeI, AhdI, AleI, AloI, AloI, AlwI, ApaI ,ApaLI, ApoI, AscI, AsiSI, AvaI, AvrII, BaeI, BaeI, BamHI, BanI, BanII, BbeI, BbsI ,BbvCI, BcgI, BcgI, BciVI, BclI, BfrBI, BglI, BglII, BlpI, Bme1580I, BmgBI, BmtI ,BplI, BpmI, Bpu10I, BpuEI, BsaI, BsaAI, BsaBI, BsaHI, BsaWI, BsaXI, BsaXI, BseRI BseYI, BsiEI, BsiHKAI, BsiWI, BsmI, BsmAI, BsmBI, BsmFI, Bsp1286I, BspEI, BspHI ,BsrBI, BsrDI, BssHII, BssSI, BstAPI, BstBI, BstEII, BstXI, BstYI, BstZ17I, Bsu36I ,BtgI, ClaI, DraIII, DrdI, EaeI, EagI, EarI, Eco57I,

Discussion

The present study tries to throw the light on the problem of Salmonella Typhimurium in pigeons in Menoufia governorate, Egypt. Pigeons are potential reservoirs for several pathogenic microorganisms, including Chlamydia spp., Salmonella spp. and Cryptococcus spp. In Japan, S. Typhimurium, C. psittaci and Mycobacterium spp. have been isolated from feral pigeons and the frequency of Salmonella spp. and Chlamydia spp. is particularly high. The presence of pigeon feces in public parks and railroad stations has contributed to the spread of infectious agents in the environment **Tanaka et al., (2005).**

In the present work, the incidence of Salmonella isolated from pigeons in Menoufia governorate, Egypt, was 2.94%. Isolation, identification and serotyping of the causative agents of paratyphoid infection in pigeon were detected. The antimicrobial susceptibility testing was done by the agar disk diffusion method as described by **CLSI (2013).**

Different virulent genes were examined using cPCR, the invA gene of Salmonella contains sequences unique to this genus and has been proved as a suitable PCR target, while the flagellin gene (fliC) encodes the major component of the flagellum in Salmonella enterica serovar Typhimurium.

Nucleotide sequences of fliC gene were done, and protein identification in comparison with Salmonella Typhimurium DT2 was 100%.

Investigation of sensitivity of the isolated Salmonella Typhimurium to antibiotics was carried out and it was found that all strain were sensitive to Amoxicillin, Ciprofloxacin, Gentamicin, Enrofloxacin, Doxycycline,Norfloxacinand Streptomycin while it was resistant to Erythromycin. There was a great variation in the sensitivity of the isolated Salmonella Typhimurium from pigeons to different antibiotics as also reported by **Ravaei et al.(2013)**.InthepresentstudySalmonellaTyphimurium strain was sensitive to Gentamicin ,and Streptomycin, which differ from **Frech et al. (2003)** who reported that all isolated Salmonella Typhimurium variant Copenhagen were resistance to Ampicillin, Streptomycin, Sulfamethoxazole and Tetracycline. Additional resistances to Chloramphenicol, Florfenicol, Kanamycin, Gentamicin and Trimethoprim were seen in the majority of the isolates while **Hosain et al. (2012)** reported that 80% of the Salmonella isolates from pigeons were sensitive to CiprofloxacinfollowedbySulphamethoxazole(70%),Chloramphenicol(60%), Kanamycin (60%), Gentamicin (60%) and Nalidixic acid (60%). On the other hand 90% of the Salmonella isolates were found resistant to Amoxicillin (90%), followed by Ampicillin (80%), Erythromycin (80%) andTetracycline(60%).Salmonellaisolationby conventional culture methods, are based on non-selective pre-enrichment followed by selective enrichment and plating on selective and differential agars. Suspected colonies were then confirmed by biochemical and serological methods, **Van Kessel et al., (2003).** Generally, these techniques take longer time, since they give only presumptive results after 3-4 days and definitive results after 5-6 days, **Malorny et al., (2003b).** Rapid detection methods, such as DNA or RNA probing, immuno-detection methods and nucleic acid hybridization have been developed, but they do not have enough sensitivity and specificity, **Zhu et al., (1996).** Amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics, **Malorny et al., (2003b).** Several genes have been used to detect Salmonella in natural environmental samples as well as food and feces samples.

The invA gene of Salmonella contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications, **Rhan et al., (1992).** Amplification of this gene now has been recognized as an international standard for detection of Salmonella genus, **Malorny et al., (2003a).** This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host, **Darwin and Miller (1999).** On the other hand, Salmonella Typhimurium possessed two non-allelic structural genes, fliC and fliB for flagglin, **Kutsukake et al., (2005).** The flagellin gene fliC encodes the major component of the flagellum in Salmonella enterica serovar Typhimurium, **Aldridge et al., (2006).** Due to high variability of its central region the fliC gene has also been

used for molecular typing studies on Salmonella, **Dauga et al., (1998).** This structural gene encodes the phase 1 flagellar protein (H1 antigen), and is expressed alternately with the fljB gene which encodes the phase 2 flagellar protein (H2 antigen).

Flagellin gene was detected in only three examined strains of Salmonella Typhimurium, and it is considered as the very important structural gene encoded the phase 1 flageller protein, **Macnab (1996).**

In the present work the nucleotide sequence of 559 bp PCR product representing the fliC gene of Salmonella Typhimurium from two strains isolated from pigeon had been sequenced by Sigma Company (Germany).

The nucleotide identity percent revealed that the first Salmonella Typhimurium pigeon strain HSE-1-2013 (KJ700871) showed 100% identity with the Salmonella Typhimurium Egyptian strains (Azh1,2,3,&4) isolated from wild birds and S. Kentucky Giza-VRLCU-144-10-2011 and S. Kentucky Giza-VRLCU-144-1-2011 isolated from Chickens). In addition, it showed 99.8% identity with strain (S. Typhimurim DT2 HG326213.1) which is isolated from pigeon. The second Salmonella Typhimurium pigeon strain HSE -2-2013 showed 99.8% identity with the first strain HSE-1-2013 and the Salmonella Typhimurium Egyptian strains(Azh1,2,3,&4). It also showed 99.6% identity with strain S. Typhimurium DT2, which is isolated from pigeon and with other three strains (S. Typhimurium DT104, S. Typhimurium 08-1736, and S. Typhimurium-var.5-CFSAN001921).

The phylogenetic tree was constructed to calculate and examine the evolutionary relationship of the sequences, in which the length of the horizontal line connecting one sequence to another was proportional to the estimated genetic distance between the sequences. The tree having two groups A and B originated from the same base, group B contains the two Salmonella strains HSE -1-2013 and HSE -2-2013 and also contains all Salmonella Egyptain strains (Azh1,2,3,&4) and S. Typhimurium DT2 HG326213.1 which is isolated from pigeon and also contains thirteen Salmonella strains isolated from worldwide

Sequencing and characterization of fliC was performed in the development of a molecular serotyping, **Mortimer et al., (2004).**

The amino acids tree between sequenced fliC gene of S. Typhimurium isolated from pigeons and twenty two randomly selected aligned strains from GenBank was constructed. The first strain HSE-1-2013 is similar to the Egyptian strains (Azh1, 2, 3, &4) and to the other Salmonella isolated worldwide, while the second strain HSE-2-2013 was slightly different from them with 99.5% similarity, there is protein changed at position 159 (The standard amino acid V Valine mutated to the amino acid E Glutamic acid) because of the substitution of the nucleotide at positions 560 - 563. In the present study the nucleotide phylogenetic tree and the amino acid phylogenetic tree were nearly similar and the identity between the strains were nearly homologous, these results agree with **McQuiston et al., (2004)** who mentioned that, alleles encoding the same flagellar antigen were homologous, while substantial sequence heterogeneity existed between alleles encoding different flagellar antigen and therefor suggesting that, flagellin genes may be useful targets for the molecular determination of flagellar antigen type.

Smith and Selander (1990) and **Li et al. (1994)** applied the Salmonella fliC sequences and found that these Salmonellae were conserved at their terminal and variable in the central region between serotypes.

A restriction enzyme is an enzyme that cuts DNA at specific recognition nucleotide sequences known as restriction sites, **Kessler and Manta (1990).** Restriction enzymes are used widely in genetic engineering to cut a molecule of DNA at specific points, in order to insert or remove a piece of DNA. There are many different restriction enzymes; each cuts the DNA at a specific sequence of bases, allowing great precision in genetic engineering, **Roberts et al., (2007).** In addition, it will give idea about a map of the restriction enzymes of choice for using it in future for molecular analysis to this gene.

In the present study restriction Mapping was constructed from which 39 restriction enzymes cut sites and their frequency and positions on

the partial sequence of the fliC was determined.

The in silico detection of the cut sites of restriction enzymes of the fliC sequence of the two Salmonella Typhimurium isolates help us to know the restriction enzymes of choice and their cut sites also will help to know the enzymes that do not cut in our sequences to avoid using it in further typing. **El –Jakee et al. (2010)** used restriction enzyme Hind III to genotype S. Typhimurium isolates from different poultry origin and geographical areas in Egypt. In the present study Hind III enzyme does not cut at any site, so the in silico analysis could help us to avoid using the misleading restriction enzyme by knowing the restriction enzymes that does not cut in our sequences. Cocolin **et al. (1998)** used different restriction enzyme for typing Salmonella Typhimurium isolates as (ApaI, AluI, HinfI, HindIII, DpnI, RsaI, NotI, SfiI ,and SamaI). Some of these enzymes do not cut in our sequences as: ApaI, HindIII, NotI and SamaI

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الملخص العربى

تم جمع 112 عينه وفحصها بكتريولوجيا لمسببات البراتيفيود في الحمام البالغ وفراخة . تم عزل ميكروب السالمونيلا من 4 عينات بنسبة اجمالية 3.9٪ . كانت السالمونيلا إيجابية من مسحات المذارقية 102/4 بنسبة 3.9 ٪ في حين تم جمع 16 مسحات مجرورة من زرق الحمام المتواجد في منازل وابراج الحمام وفحصها بكتريولوجيا ولم يتم العثور على ميكروب السالمونيلا في جميع العينات.

تم عمل اختبار التلازن على الشريحة لعدد 4 من السالمونيلا المعزولة من الحمام باستخدام O و H عديد و احادى الانتى سيرم ووجد ثلاثه من المعزولات سالمونيلا التيفيموريوم و معزولة واحدة غير مصنفه .

عمل اختبار الحساسية لمضادات الميكروبات لسلالات السالمونيلا التيفيموريوم المعزولة من الحمام وكانت ذات حساسية للأموكسيسيلين ، سيبروفلوكساسين ، دوكسي سيكلين ، انروفلوكساسين ، جنتاميسين ، نورفلوكساسين و ستربتوميسين ، في حين أنها كانت مقاومة للاريثروميسين .

تم استخدام اختبار تفاعل البلمرة المتسلسل لفحص جينات الضراوة مثل (invA و fliC) وقد أعطت المعزولات نتيجة إيجابية لجميع الجينات السابقة. وقد تم ارسال اثنين من متواليات fliC إلى قاعدة بيانات بنك الجينات وحصلت على أرقام الانضمام (HSE-1-2013 "KJ700871") و("KJ671550" HSE-2-2013) وأظهرا تماثلا بنسبة 99.8 ٪ مع بعضهم البعض ونسب مختلفة مع 22 سلاله مختارة عشوائيا من بنك الجينات.

السلاله الاولى (KJ700871) HSE- 1-2013 تتكون من 158 حمض أميني أظهرت تماثلا بنسبة 100 ٪ مع السلالة الثانية (HSE- 2-2013 KJ671550) و اثنين وعشرين سلالات مختارة عشوائيا من بنك الجينات ؛ بينما سلالة KJ671550) HSE-2-2013 تتكون من 159 حمض أميني وأظهرت تماثلا بنسبة 100 ٪ مع المعزولات المصرية سلالات (Azhar،1و 2و3 و 4) وتتماثل بنسبة 99.5 ٪ مع خمسة عشر سلاله أخرى ببنك الجينات كما وجد تغيير بروتيني في الموقع 159 و تحور الحمض الأميني فالين V للحمض الجلوتاميك E).

تم عمل خريطة انزيمات التقطيع لاثنين من سلالات السالمونيلا ووجد انزيم BbvI ليقطع خمس مرات في مواقع 134و361و418و441 و 457.