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Some Molecular Studies on Salmonella on Chickens

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Abstract: ***The objective of these study to observe the distribution of three such genes, namely, Salmonella enterotoxin (stn)[14, 12,] (, Salmonella Enteritidis fimbrial (sef) and plasmid encoded fimbrial (pef) genes[15], among different serovars of Salmonella enterica isolated chicken. The work included: Isolation of salmonellae from broiler breeder chicken farms. Phenotypic characterization of Salmonella isolates using: (Morphological, cultural and biochemical identification. Serological identification.) Molecular identification of the isolates using polymerase chain reaction (PCR). Dead birds were collected from broiler chicken farms from 3 distinct governorates, Giza, Monofia and Qaluobia suspected to be salmonellae infected. Specimens for salmonellae isolation included liver, spleen, and intestine that were collected under aseptic condition. A total isolate belonging to S. Typhimurium, S. Enteritidis, S. Blockley, and S. Kentucky serovars were subjected to polymerase chain reaction (PCR) assay for the detection of stn, sef and pef genes using their specific primers and the PCR products were analyzed by 1 per cent agarose gel electrophoresis for the presence of the respective genes. Results: Varying distribution pattern of these genes was observed amongst the isolates. While, stn was found in all the strain we isolated, sef was found only among the S. Enteritidis isolates. The pef gene was found to be absent in some isolates. Conclusion: Findings indicated that the stn gene is widely distributed among Salmonella irrespective of the serovars and source of isolation. However, the sef gene appears to be serovar specific. Since the stn gene is found in all the isolates, it can be a viable target gene to explore the possibility of direct detection of Salmonella from samples from biological sources.***

Keywords: Salmonella, Pcr, Fimbrial Genes

1. Introduction

Salmonella bacteria are intracellular pathogens which causing localized or systemic infections, in addition to a chronic asymptomatic carrier state. Salmonella typhoid and paratyphoid caused by several species of Salmonellae infection which consider as important food borne human diseases [(8)].

Diarrhea, vomiting, abdominal pain, and nausea are the main symptoms of Salmonellosis and occurs both in outbreaks affecting a family or several hundreds or thousands of people in a population and sporadic cases[(1)] Salmonella is the genus name for a large number (over 2, 500) of types of bacteria. Each type is distinctly identifiable by its specific protein coating. The types are otherwise closely related. Salmonellae are short bacilli, 0.7-1.5 x 2.5 µm, Gram-negative, aerobic or facultative anaerobic, positive catalase, negative oxidase; they ferment sugars, produce H2S, are non porogenic, and are normally motile with peritricheal flagella, except for Salmonella Pullorum and Salmonella Gallinarum, which are nonmotile [(6)].

Salmonella were identified as a first time at the end of the 19th century. Isolate and makemorphological description to salmonella carried out in 1884[(7)].Pathogenic mechanisms of salmonella including production of enterotoxin [(2)]. This enterotoxin production is mediated by the *stn* gene [(3)].

Pathogenesis of Salmonellosis depends upon a large number of factors controlled by an array of genes that synergise into the actual virulence of Salmonella. Virulence genes encode products that assist the organisms in expressing its virulence in the host cells. Some genes are known to be involved in adhesion and invasion viz., sef, pef, spv or inv; others are associated with the survival in the host system- mgtC or in the actual manifestation of pathogenic processes viz., sop, stn, pip A, B, D. Nucleic acid based diagnostic techniques are being employed for the detection of various gene-encoded virulence factors viz., Salmonella enterotoxin (stn) [(8)] Salmonella Enteritidis fimbriae (sef) and plasmid encoded fimbriae (pef) genes.[(15)].The (sef) operon contains four structural genes (sefABCD) required for the translocation and biogenesis of SEF14 fimbriae: sefA encodes the major subunit, sefB and sefC encode the chaperone and usher, respectively, and sefD encodes the putative adhesion. Some evidence suggests that SEF14 fimbriae may play a role in pathogenesis. For example, immunization of mice with purified SEF14 subunits induces a strong T lymphocyte response, and the mice present a delayed-type hypersensitive response to whole S. enteritidis, demonstrating that these fimbriae are expressed in vivo and stimulate cell-mediated immunity [(10)]

2. Materials and Methods

Samples: Morbid and freshly dead birds were collected from 20 broiler chicken farms from 3 distinct governorates, Giza, Monofia and Qaluobia suspected to be salmonellae infected. The collected bird necropsied and examined for gross post mortem (PM) lesions. Specimens for salmonellae isolation included liver, spleen, and intestine that were collected under aseptic condition.

Isolation and identification of Salmonella was done according to **ISO 6579 (2002)[(8)]** method. Buffered peptone water as pre enrichment non-selective liquid media was inoculated with the examined samples - incubated at 37ºC ± 1ºC for 18 h ± 2 h. 0.1 ml of the pre-enrichment culture was transferred to a tube containing **10** ml of Selenite-F-Broth, as enrichment selective liquid media then incubated at 41.5ºC ± 1ºC for 24 h ± 3 h. 10 µl of the previously inoculated and incubated selective enrichment culture were plated onto the surface of Xylose Lysine Deoxycholate, Salmonella-

Shigellaagar , MacConkey agar and then incubated at 37ºC ± 1ºC for 24 h ± 3 h.

Identification of Salmonella spp. according to Quinn et al. (2002) [13] by:

1) H_2S production using Triple sugar iron agar (TSI).It was used for detection of H2S production and fermentation of sugar triple sugar iron (TSI) agar tubes were inoculated with the suspected colonies and incubated at 37° C for 18- 24 hours. Isolates showed growth surface with an alkaline slant (red) acid butt gas with or without H2S production (black color between slant and butt) were considered positive for salmonella.
2) Citrate utilization test. Simmons citrate agar tubes were inoculated with the suspected isolates incubated at 37 ºC for 5 days. Tubes develop blue coloration considered as positive for salmonella.
3) Indole production test. The tube of SIM media was inoculated with the isolate and incubated for 24 hours at 37 ºC. Then 0.5 ml of Kovac´s reagent was gently dropped on the side of tube. Tubes showing rosy color were discarded, where negative reaction was considered positive for salmonella.
4) Methyl red test. Tubes of dextrose phosphate broth were inoculated with the suspected colonies and incubated at 37 ºC for 48 hours. Then 5 drops of methyl red reagent were added. Positive reaction (red coloration) due to high hydrogen ion concentration indicates presence of salmonella [5].
5) Urease test. The purpose of this test is to determine whether an organism can split urea into ammonia and carbon dioxide by the action of the enzyme urease. Christensen's urea medium without agar is used. The production of ammonia leads to alkalinity in the medium and will cause the medium to turn red (the indicator used is phenol red). Salmonella gave -ve reaction: The medium is more yellow than the inoculated medium due to acid produced during fermentation of glucose.

Table 1: Biochemical tests used for identification of Salmonella spp. the changes of the different biochemical media.

Biochemical tests	Results
TSI	(K / A / G / H2S)*
Urease test	Yellow color (-ve)
Indole	Colorless ring (-ve).
Methyl red test	Red color (+ve).
Voges Proskeaur test	Yellow color (-ve)
Simmon's citrate test	Blue color (+ve)

*K=alkaline: red *A=acid: yellow.
G= Gas production H2S= Blacking.

Serotyping of the isolated Salmonella spp.

Serological identification of the suspected Salmonella isolates was carried out according to [11] **Popoff.**

- The suspected colonies were cultured on nutrient agar and incubated at 37 °C for 24 hours.
- One drop of the saline solution was placed on a cleaned glass slide and dispersed in the drop, by means of a loop, part of the colony to be tested. In order to obtain a homogenous and turbid suspensions.
- The slide gently was rocked for 30-60 seconds and observed the result against a dark background, preferably with the aid of a magnifying glass.
- **Examination for O- antigens:** one non-auto agglutinating pure colony was added to one drop of the anti-O serum instead of saline solution, if agglutination occurs, the reaction considered positive. Then poly-and monovalent sera were used one after the other.
- **Examination for H- antigens:** A semi-solid nutrient agar was inoculated with a pure non- agglutinable colony and incubated at 37°C ± 1°C for 24 h. ± 3 h. The culture was used for examination for the H-antigens, then proceeded as before.
- **Detection of stn, sef and pef genes by polymerase chain reaction (PCR):** For the detection of stn, sef and pef genes by PCR analysis, bacterial cells from the overnight cultures were suspended in 350 µl distilled water and boiled at 100°C for 10 min. After boiling, the cell suspensions were cooled on ice and were immediately tested for the presence of stn, sef and pef genes by PCR analysis [14, 15]. Primers used for stn gene were: Stn P1 5 - TTG TGT CGC TAT CAC TGG CAA CC - 3 (upper primer) and Stn M13- 5 - ATT CGT AAC CCG CTC TCG TCC - 3 (lower primer) [14] (GENSET, Singapore), which flank a 617 bp segment in the stn gene sequence. The PCR mixture (25 µl) included 12.5 µl master mix (QIAGEN, USA) containing 2.5 U Taq DNA polymerase, 20µM each of dATP, dCTP, dTTP and dGTP and PCR buffer, 5 µl (1µM) each of upper and lower primers and 2.5µl of template DNA (bacterial cell suspension). PCR incubation was performed in a thermocycler (Perkin-Elmer, USA) in 25 cycles of denaturation (94°C for 1 min), primer annealing (59°C for 1 min) and primer extension (72°C for 1 min) followed by incubation at 72°C for 10 min. For sef gene, the primers used were, sef C 5 - GCG AAA ACC AAT GCG ACT GTA - 3 (upper primer) and sef C 5 - CCC ACC AGA AAC ATT CAT CCC - 3 (lower primer) [15]that flank a 1103 bp segment in the sef gene sequence. PCR incubation was performed in 25 cycles of denaturation (94°C for 1 min), primer annealing (55°C for 1 min) and primer extension (72°C for 1 min) followed by incubation at 72°C for 10 min. For pef gene the primers used were, pef A1 5 TGT TTC CGG GCT TGT GCT - 3 (upper primer) and pef A2 5 - CAG GGC ATT TGC TGA TTC TTC C - 3 (lower primer) [14].These primers flank a 700 bp segment in the pef gene sequence. The PCR was run for 25 cycles of denaturation (94oC for 55 sec), primer annealing (55 °C for 55 sec) and primer extension (72°C for 55 sec) followed by incubation at 72°C for 10 min. A 15 µl aliquot of each PCR product was electrophoretically separated on agarose gel (1%, containing 0.5 µl/ml ethidium bromide, Pharmacia, Sweden), analyzed under UV light (300 nm) and photographed.

Table 2: Oligonucleotide primers sequences encoding for stn, sef and pef genes:

Primer	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)
StnP1	Stn	TTG TGT CGC TAT CAC TGG CAA CC	617 bp
StnM13		ATT CGT AAC CCG CTC TCG TCC	
SefC-F	**Sef**	GCG AAA ACC AAT GCG ACT GTA	1103 bp
SefC-R		CCC ACC AGA AAC ATT CAT CCC	
PefA1	**Pef**	TGT TTC CGG GCT TGT GCT	700 bp
PefA2		CAG GGC ATT TGC TGA TTC TTC C	

3. Results

- Out of 20 samples subjected for Salmonella isolation, 7 were suspected positive samples for the presence of Salmonella colonies were obtained. Selected colonies in the positive samples were picked up and further cultured on selenite-f-broth then the purified colonies were cultured on MacConcky agar, S.S agar and XLD for further identification. The isolates showed red black center due to H_2S production on XLD, colorless black center due H_2S production on S.S agar and colorless on MacConkey agar. All isolates were serotyped using O and H poly and monovalent Salmonella antisera. The isolates were serotyped as, (1) Salmonella Enteritidis, (2) Salmonella Typhimurium, (2) Salmonella Kentucky and (1) Salmonella Blockley.
- Table (3) shows the antigenic formula of the isolated Salmonella.

Table 3: Antigenic structure of Salmonella isolates

Salmonella serotype	Number of sample	Antigenic formula
Kentucky	Sample NO (1, 2, 3)	O : 8, 20 H_1 :i H_2 :Z_6
Enteritidis	Sample NO (4)	O : 1, 9, 12 H_1 : g, m H_2 :-
Blockley	Sample NO (5)	O : 6, 8 H_1 : K H_2 :1, 5
Typhimurium	Sample NO (6, 7)	O : 1, 4, (5), 12 H_1:i H_2 : 1, 2

- PCR assay carried out for the detection of three such genes, namely, Salmonella enterotoxin (stn), Salmonella Enteritidis fimbrial (sef) and plasmid encoded fimbrial (pef) genes, in Salmonella isolates from chicken sources has revealed that the (stn) gene was present in all the isolates that was demonstrated by presence of a 617 bp PCR product (Fig.1). These results are in agreement with many reports. The (stn) genes are widely distributed among the Salmonella strains and absent in S. bongori strains [(12)] and also the other members of Enteriobacteriaceae which have enterotoxigenic potential [(9)]. (stn) gene contained sequence unique to Salmonella strains present in all the Salmonella serotypes. So, use PCR in detection (stn) gene is a suitable way for detection of Salmonella strains in field. However, Salmonella Enteritidis fimbriae 14 (SEF 14), one of the major fimbriae of Salmonella, consists of 4 major protein subunits SefA (14 kd), SefB (28 kd), SefC (90kd), and SefD (10 kd), which are arranged sequentially in the fimbrial structure and are encoded by fimbrial operons sefA, sefB and sefC and sefD, respectively. SefC forms the largest component of the fimbriae and its presence indicates the presence of the SEF adhesion[(4)]. The sefC gene, detected by the presence of a 1103 bp product was found to be present in only isolates belonged to S. Enteritidis serovar. So sefC consider as serotype specific presence and can use in detection and identification of salmonella Enteritidis. On the other hand, there was no serotype specific presence or absence of the pef gene.

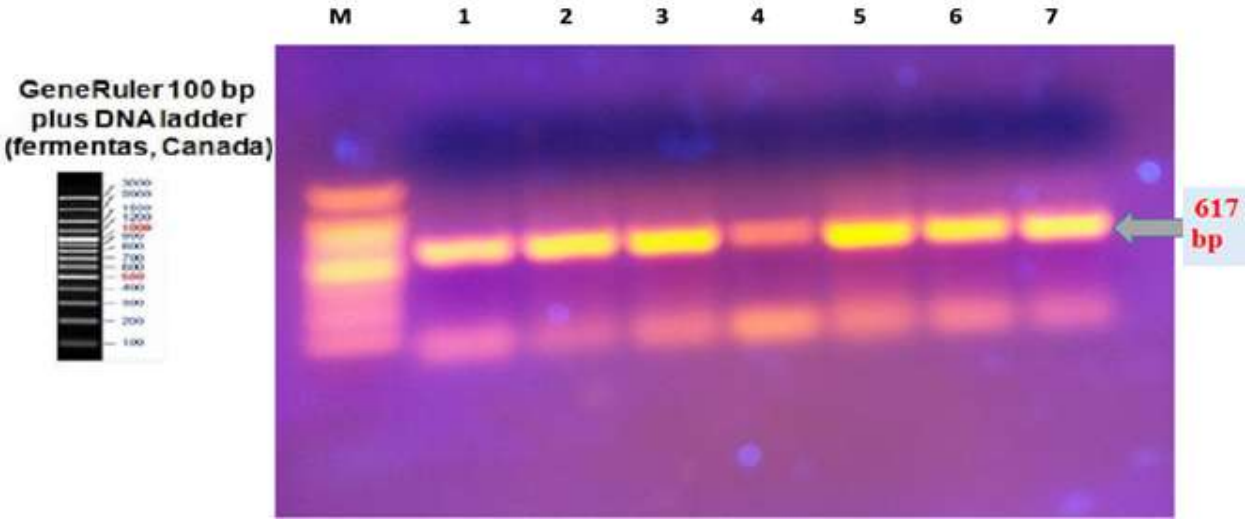

Figure 1: Detection of stn gene by polymerase chain reaction (PCR).

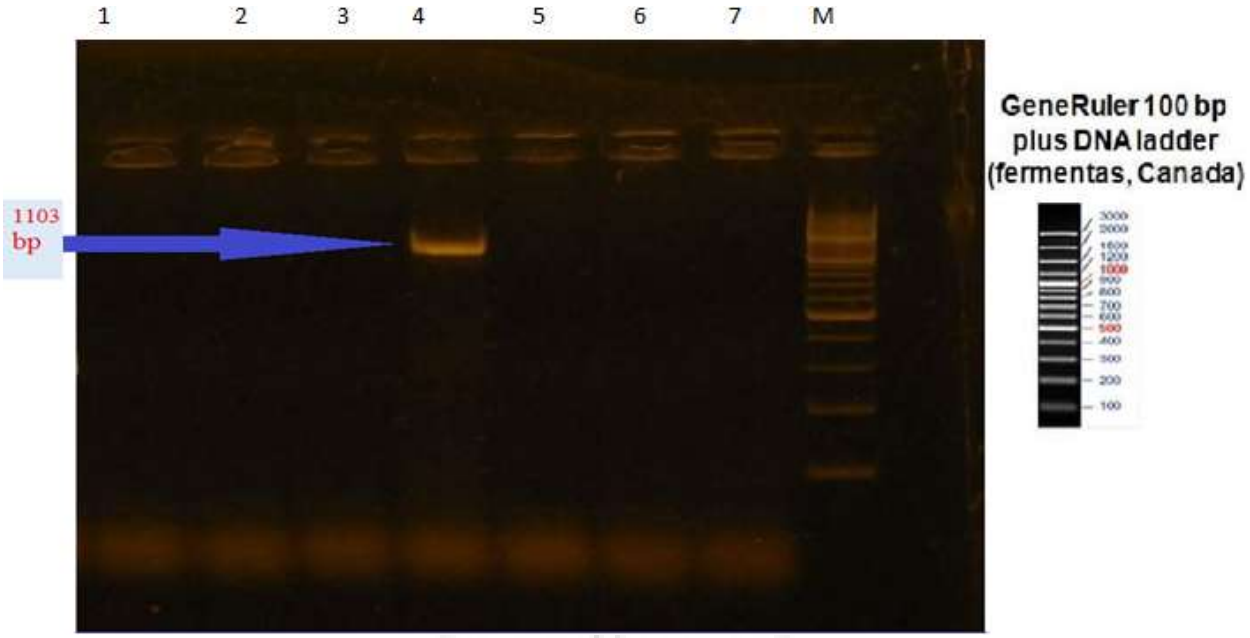

Figure 2: Detection of sef gene by polymerase chain reaction (PCR).

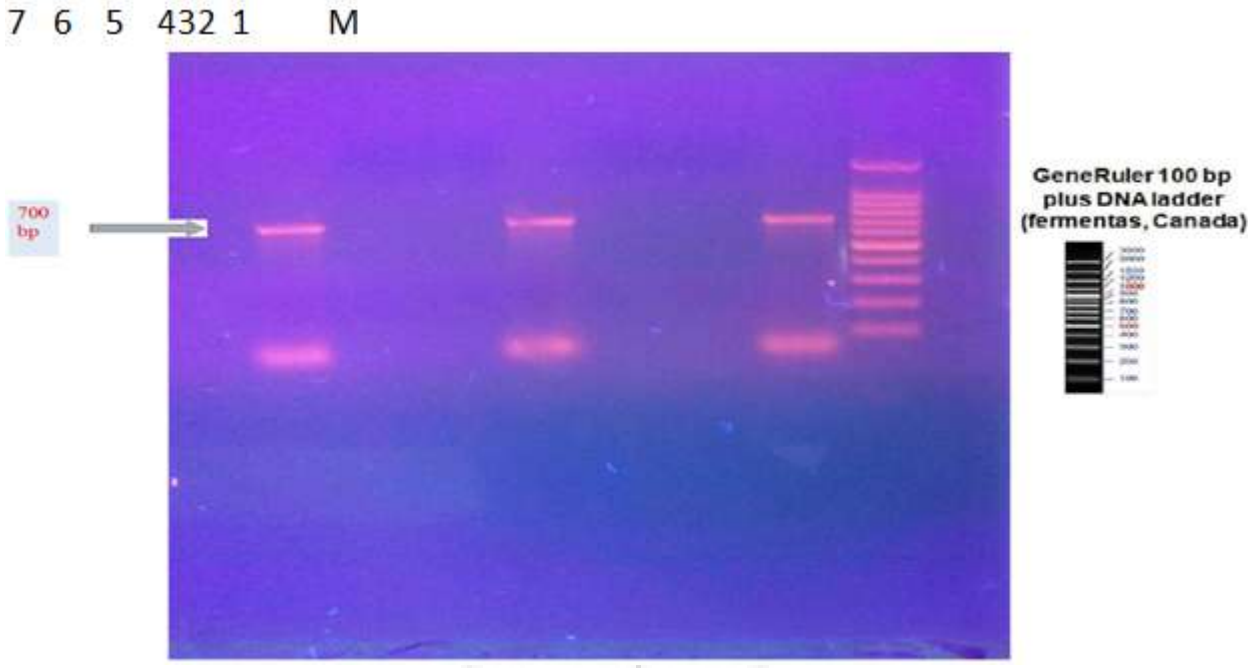

Figure 3: Detection of pef gene by polymerase chain reaction (PCR)

4. Discussion

The findings from the present study show that the stn gene is present in all the Salmonella serotypes and reports that the stn gene contained sequence unique to Salmonella strains, makes this gene a suitable PCR target for detection of Salmonella strains in field samples. The S. enteritidis fimbriae (SEF14) is restricted to S. enteritidis and other closely [(4)]. Therefore, an analysis of SEF14 fimbrial function may provide insight into the unique aspects of virulence that distinguish this group of Salmonellae, the primer for this gene therefore, was considered for detection of sef gene among the isolates in the present study. The sefC gene, detected by the presence of a 1103 bp product (Fig. 2), was found to be present in only all of which belonged to S. Enteritidis serovar (Table 3). In the present study, presence of pef gene was evaluated with the help of primer for pef gene, which encodes for the major portion of the pef operon. The pef gene, detected by the presence of a 700 bp PCR product (Fig. 3). There was no serotype specific presence or absence of this gene.

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