Molecular studies on *Salmonella* species isolated from food products of animal origin

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Abstract

Foodstuffs of animal origin may present intrinsic hazards, due to microbiological contamination. This study was conducted to determine *Salmonella* spp.in some types of animal source foods. Bacteriological examination of total 246 raw food samples of animal origin, (raw milk, (105); beef meat (31) and poultry meat (110), collected from different localities in Ismailia City, showed isolation of 4 isolates of *Salmonella* spp., at percentage of 1.62% of the examined samples. Serological studies of the isolated *Salmonella* revealed identification of *Salmonella Entritidis*
O_9 H_1g,m H_2- (2 isolates) and *Salmonella Kossein* O_2 H_1l,v $H_2$1,5 **(**2 isolates). The isolated *Salmonella* (100%) showed sensitivity to Polymyxin B (PB) 300 units, (Polypeptides). On the other hand, 50% of the total tested *Salmonella* strains were resistant against 4 antimicrobial groups, (B-Lactams, B-Lactam/B-lactamase inhibitor combinations, Aminoglycosides and Tetracyclines), so considered multi drug resistant. Isolated *Salmonella* strains were submitted to molecular studies for detection of the specific DNA bands for *invA* (*Salmonella* invasion protein), *stn* (enterotoxin), *avrA* (**recombinant protein**) and *SopB* (effector protein) gene factors by using PCR technique**.**

Introduction

Salmonella species are Gram-negative, flagellated, facultative anaerobic bacilli, most are motile, ferment glucose with the production of acid and gas or acid only. Some *Salmonella* produce three main types of disease in humans: enteric fever (typhoid fever), bacteraemia and enterocolitis, but mixed forms are frequent **(*Jawetz & Adelberg, 2004)***. Although more than 2500 serovars of *Salmonella enterica* had been identified, most human *Salmonella* infections were caused by a limited number of serovars. *S. Enteric, S. Typhimurium* and *S. Enteritidis* were the most common of *Salmonella* serovars worldwide ***(Xia et al, 2009).*** The genomic reservoir of *Salmonella* species contains horizontally transferred

genetic elements, including some virulence genes that may play roles in pathogenicity and disease development ***(Malorny et al, 2007).*** The target genes most frequently utilized for the specific detection of *Salmonella* are associated with virulence, including, for example, *invA* (*Salmonella* invasion protein gene) ***(Rahn et al, 1992; Ferretti et al, 2001 & Malorny et al, 2003a)*** and *stn* (enterotoxin gene) ***(Dinjus et al, 1997).*** Some of these genes have been reported to be absent in a few *Salmonella* serovars, whereas others yielded false positive results when detection methods based upon them were tested against non-*Salmonella* strains ***(Rahn et al, 1992; Malorny et al, 2003a and Moore & Feist, 2007).***

Material & methods:

1- Isolation and identification of *Salmonella* species:

Salmonella were isolated from a total number of (105) raw milk samples, (31) beef meat samples and (110) poultry meat samples, by cultivation into 1% peptone water as pre-enrichment media, Rappaport vassiliadis (RV) broth as enrichment media then on *Salmonella-Shigella* agar plates and were identified by biochemical tests, then serologically identified by using slide agglutination test and submitted to molecular studies for detection of the specific DNA bands for *invA* (*Salmonella* invasion protein), *stn* (enterotoxin), *avrA* (**recombinant protein**) and *SopB* (effector protein) gene factors, by using PCR technique. Antibiotic susceptibility of the isolated *Salmonella* was tested using standard plate technique as recommended by **Clinical laboratory Standard Institute (CLSI)**.

2-Molecular studies on *Salmonella* species:

2-1-Extraction of DNA, according to QIAamp DNA mini kit instructions

2-2-Oligonucleotide primers sequences of *Salmonella* genes:

2-3- Cycling conditions of the different primers during PCR of the tested *Salmonella* genes

2-4- DNA Molecular weight marker

The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

The used ladders were:

-Gel Pilot 100 bp ladder

(cat. no. 239035) supplied from **QIAGEN (USA).**

Number of bands: 6 Size range: 100-600 bp.

Used for detection of ***Salmonella*** genes (*invA; sopB* & *avrA*).

-Gene ruler 100 bp plus DNA ladder

(cat. no. SM0323) supplied from Fermentas**.**

Number of bands: 14 Size range: 100-3000 bp.

Used for detection of *stn* gene of ***Salmonella***.

2-5- Agarose gel electrophoreses according to Sambrook *et al*., 1989 with modification

Table (1): *Oligonucleotide primers sequences of Salmonella genes:*

Primer	Sequence	Amplified product	References
invA	Forward/GTGAAATTATCGC CACGTTCGGGCAA	284 bp	**Oliveira *et al*., 2003**
	Reverse TCATCGCACCGTCAAAGGA ACC		
avrA	Forward/ cct gta ttg ttg agc gtc tgg	422 bp	**Huehn *et al*. 2010**
	Reverse/ aga aga gct tcg ttg aat gtc c		
sopB	Forward/ tca gaa gRc gtc taa cca ctc	517 bp	
	Reverse/ tac cgt cct cat gca cac tc		
Stn	Forward/ TTG TGT CGC TAT CAC TGG CAA CC	617 bp	**Murugkar *et al*., 2003**
	Reverse/ ATT CGT AAC CCG CTC TCG TCC		

Table (2): *Cycling conditions of the different primers during PCR of the tested Salmonella genes according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit*

Genes	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
invA	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.
avrA	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.
sopB	94˚C 5 min.	94˚C 30 sec.	58˚C 30 sec.	72˚C 30 sec.	35	72˚C 10 min.
Stn	94˚C 5 min.	94˚C 1 min.	59˚C 1 min..	72˚C 1 min.	35	72˚C 10 min.

Results

1-The trials applied for isolation of Salmonella in this study failed to detect those organisms from the examined raw milk or raw beef meat samples.

2- Four Salmonella species were isolated and biochemically identified at percentage of 3.63% of the examined raw poultry meat samples.

3- Serological studies on Salmonella isolates, obtained from a total 110 poultry meat samples revealed identification of two isolates of S. enterica serovar Enteritidis O:9/H1:g,m/H2:--, (1.81%) and two isolates of S. enterica serovar Koessen O:2,12/H1:I,v/H2:1,5, (1.81%). 4- The two strains of S. Enteritidis (100%) showed sensitivity to Gentamicin (CN) 10ug. and Polymyxin B (PB) 300 units. On the other hand, the two strains (100%) showed resistance against Amoxicillin/Clavulanic acid (AMC) 30ug; Cephazolin (KZ) 30ug; Cefuroxime sodium (CXM) 30ug.; Piperacillin/Tazobactam (TZP) 110ug; Aztreonam (ATM) 10ug. and Meropenem (MEM) 10ug. One strain (50%) showed sensitivity to Tetracycline (TE) 30ug. and the other one (50%) was resistant against it. One strain (50%) showed intermediate sensitivity to Colistin sulphate (CT) 10 ug. and the other strain (50%) was resistant against it.

5- The two strains of *S. Koessen* (100%) showed sensitivity to Polymyxin B (PB) 300 units. On the other hand, the two strains (100%) showed resistance against Amoxicillin/Clavulanic acid (AMC) 30ug.; Cephazolin (KZ) 30ug.; Cefuroxime sodium (CXM) 30ug.; Piperacillin/Tazobactam (TZP) 110ug.; Gentamicin (CN) 10ug.; Tetracycline (TE) 30ug.; Aztreonam (ATM) 10ug. and Meropenem (MEM) 10ug. One strain (50%) showed sensitivity to Colistin sulphate (CT) 10 ug. and the other strain (50%) was resistant against it.

6- As regarded to the molecular studies:

a- All (100%) of the isolated *Salmonella* strains had the specific DNA band for the *Salmonella* invasion protein (*invA*) gene of *Salmonella* with a size of 284 base pairs.

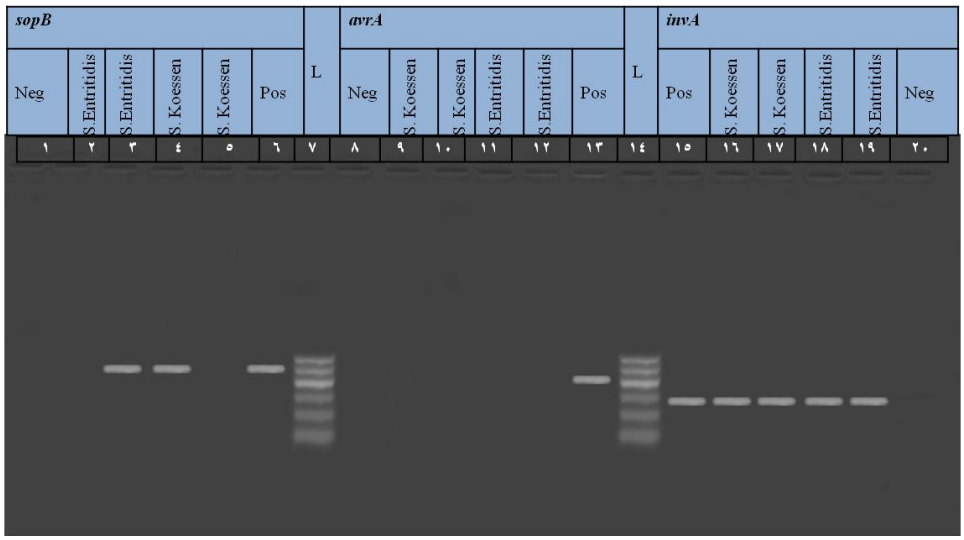
b- None (0%) of the isolated *Salmonella* strains, had the specific DNA band for the Recombinant Avirulence protein A **(***avrA*) gene of *Salmonella*, with a size of 422 base pairs.

c- Two strains (50%) of the isolated *Salmonella* serovars , *S. Enteritidis* (one isolate) & ***S***. ***Koessen*** (one isolate), had the specific DNA band for the Effector protein (*sopB*) gene of *Salmonella* with a size of 517 base pairs.

d- One strain (25%) of the isolated *Salmonella* strains (S. *Enteritidis*), had the specific DNA band for the enterotoxin (*stn*) gene of *Salmonella*, with a size of 617 base pairs, while the other three strains did not.

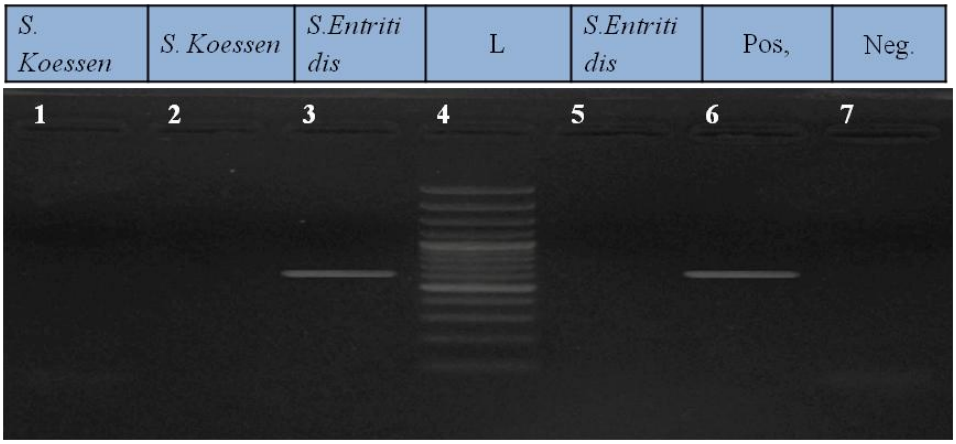
Table (3) *Detection of invA, avrA, sopB & stn genes in the isolated Salmonella serovars, using PCR technique*

***Salmonella* gene factors** / ***Salmonella* strains**	***invA***	***Stn***	***sopB***	***avrA***
S. Enteritidis	+	-	-	-
S. Enteritidis	+	+	+	-
S. Koessen	+	-	+	-
S. Koessen	+	-	-	-



Figure(1) *Agarose gel electrophoresis of amplified invA, avrA & sopB genes PCR products (284bp, 422bp & 517bp,respectively) in Salmonella serovars*

Lane 1: Negative control for *sopB* gene.	**Lane 7:** One step ladder (600bp).	**Lane 14:** One step ladder (10
Lane 2 & 5: Negative strains for *sopB* gene.	**Lane 8:** Negative control for *avrA* gene.	**Lane 15:** Positive control for *invA* gene.
Lane 3 & 4: Positive strains for *sopB* gene (517 bp)	**Lane 9, 10, 11, 12:** Negative strains for *avrA* gene.	**Lane 16, 17, 18&19 :** Positive strains for *invA* gene (284 bp).
Lane 6: Positive control for *sopB* gene.	**Lane 13:** Positive control for *avrA* gene.	**Lane 20:** Negative control for *invA* gene.



Figure(2) *Agarose. gel electrophoresis of amplified stn gene PCR products (617bp) in Salmonella serovars*
Lane 1, 2 & 5: Negative strains for stn gene factor.
Lane 3: Positive strain for stn gene factor (617 bp).
Lane 4: One stip ladder (100-3000 bp).
Lane 6: Positive control for stn gene factor.
Lane ٧: Negative strain for stn gene factor.

Discussion

The trials applied for isolation of *Salmonella* in this study failed to detect those organisms from all the examined milk samples. The same results were showed by ***Stephan & Buehler (2002)*** and ***EL-Jendy (2004).*** Different result was reported by ***Kaushik et al (2014),*** who found that, 7.7% (11/142) of market milk samples were positive for *Salmonella* based on biochemical reactions.

Salmonella failed to be isolated from the investigated beef meat samples in the present study. Similar results were reported by ***Ibrahim (1997).*** Opposite result was recorded by ***Yang et al (2010)*** who reported that 17% (13) of the determined beef samples were positive for *Salmonella.*

In the present study**,** *Salmonella* was recorded at percentage of 3.63% of the examined raw poultry meat samples. This result was agreed with that recorded by ***Mona (2002),*** **(**4%) of the examined frozen poultry carcasses. Higher values were reported by ***Kaushik et al (2014),*** who found that 23.7% (54/228) chicken meat samples were positive for *Salmonella* based on biochemical reactions.

Salmonella is of an increasing public health concern because they are the most incriminated pathogenic microorganisms of bacterial food poisoning especially present in poultry meat, with infection being through the handling of raw poultry carcasses and products, together with the

consumption of undercooked poultry meat ***(Panisello et al, 2000).*** Serological identification of the (4) *Salmonella* strains, isolated from a total of 110 poultry meat samples, revealed that they were belonged to *Salmonella Enteritidis* O:9/H_1:g,m/H_2:- **(**2 isolates), (1.81%) of the examined poultry meat samples and *Salmonella Koessen* O:2,12/H_1:I,v/H_2:1,5 **(**2 isolates), (1.81%) of the examined poultry meat samples, table (27). ***Kaushik et al (2014)*** reported that serotyping of *Salmonella* strains, isolated from raw chicken meat showed an incidence of 6.1% of *Salmonella Typhimurium*, 2.6% of *S. Newport*, 1.7% of *S. Gallinarum* and 0.4% each of *S. Enteritidis*, *S. Infantis* and *S.Worthington*.

Antibiogram study revealed that 50% of the total tested *Salmonella* strains were resistant against 4 antimicrobial groups, (B-Lactams, B-Lactam/B-lactamase inhibitor combinations, Aminoglycosides and Tetracyclines). These results appeared multi resistance for several antibiotics and appear sensitivity for others. The cause of this multi resistance is trance from resistance bacteria to sensitive that alive in same environments by genes on conjugate plasmid ***(Hoge et al, 1996).*** Multidrug resistant (MDR) *Salmonella* serovars were recorded by different authors as ***Rad et al (2012)*** who subjected a total of 83 epidemiologically unrelated clinical isolates of *Salmonella enterica* serovars to antimicrobial susceptibility testing. Eleven isolates (13.1%) which were resistant to at least 4 groups of antimicrobial agents considered as multidrug resistant (MDR) *Salmonella* serovars.

The using of antimicrobial drugs in poultry production, whether for prophylactic, therapeutic or performance enhancing purposes, contributes to the development of resistance in pathogens and has serious consequences for the treatment of human illness from these organisms ***(Threlfall et al, 2003).*** Results of PCR confirmation showed that all (100%) of the isolated *Salmonella* strains had the specific DNA band for the *invA* gene of *Salmonella* with a size of 284 base pairs. This result was confirmed with the finding of ***Das et al (2012),*** who recorded invasive gene (*invA*; 244bp) in 100% of *Salmonella* ***enterica*** strains, isolated from commercial food stuffs by polymerase chain reaction (PCR). Different results were obtained by ***Kaushik et al (2014),*** who recorded positive presence of *Salmonella* in 18.42% of examined chicken meat and 5.6% of examined market milk samples, by using polymerase chain reaction targeting *invA* gene. Amplification of *invA*gene of *Salmonella* has been reported as a suitable target for PCR amplification, with potential diagnostic applications ***(Malorny et al, 2003 b).*** None (0%) of the investigated *Salmonella* strains,

reacted positively with the *avrA* gene of *Salmonella*, with a size of 422 base pairs. ***Zou et al (2011)*** detected *iacP*, *avrA*, *invH*, *rhuM*, *sirA*, *sopB*, *sopE* or *sugR* genes in 40 to 80% of *Salmonella* strains, isolated from food and/or the food animal environment, using microarray platform assay.

AvrA was the effector responsible for the observed inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling ***(Collier-Hyams et al, 2002).*** NF-κB plays a key role in regulating the immune response to infection *(Albensi & Mattson, 2000).*

Concerning to *sopB* gene factor, two strains (50%) of the isolated ***Salmonella*** serovars, *S. Enteritidis* (one isolate) & *S. Koessen* (one isolate), had the specific DNA band for the *sopB* gene of *Salmonella* with a size of 517 base pairs. Barman ***et al (2013)*** screened the presence of *sop*B from 41 *Salmonella* isolates from diarrheic as well as apparently healthy animals and birds and found that 87.80% of the strains were detected positivity to *sop*B.

SPI-5 is a small locus of 7.6 kb encodes at least six genes, *pipD*, *sigD/sopB*, *sigE*, *pipB*, *pipC* and *pipA* all of which contribute to enteropathogenesis as assessed in a calf model of infection ***(Wood et al, 1998).*** SPI (SPI-2, SPI-4 and SPI-5)-associated genes encode effector proteins that facilitate intracellular survival of *Salmonella* in the host cells, type one secreation system (T1SS) toxins and survival of these bacteria in macrophages ***(Schmidt & Hensel 2004, Hu et al, 2008 and Hansen-Wester & Hensel, 2001***Only one strain (25%) of the ***isolated*** *Salmonella* serovars (*S. Enteritidis*) had the specific DNA band for the *stn* gene of *Salmonella* with a size of 617 base pairs, while the other 3 isolates did not have it. This observation disagreed with the observation of *Das et al (2012)*, who detected *Salmonella* enterotoxin gene (*stn*; 617bp), in 100% of *Salmonella enterica* strains, isolated from commercial food stuffs by polymerase chain reaction (PCR).

It has been proposed that *Salmonella* enterotoxin (*Stn*) is a putative virulence factor and causative agent of diarrhea ***(Chopra et al, 1994; Chopra et al, 1999)*** and via regulation of outer membrane proteinA (ompA) membrane localization, functioned in the maintenance of membrane composition and integrity ***(Nakano et al, 2012)***.

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دراسات جزيئية عن ميكروب السالمونيللا المعزول من المنتجات الغذائية من أصل حيوانى

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***طبيبة بيطرية بكلية الطب البيطرى.

تمثل الأغذية ذات الأصل الحيوانى خطورة على الصحة العامة نتيجة ما قد تحمله من ملوثات بكتيرية وتعتبر الإجرءات الميكروبيولوجية وسيلة للحكم على مدى أمان وجودة هذه الأغذية. الفحص البكتيرى لعدد ٢٤٦ عينة غذاء من أصل حيوانى (١٠٥) عينة ألبان خام، (٣١) عينة لحوم عجالى و(١١٠)عينة لحوم دواجن ، أسفر عن عزل وتصنيف (بيوكيميائياً) عدد ٤ سالمونيللا ، بنسبة ١,٦٢% من مجموع العدد الكلى للعينات التى تم فحصها. كما تم تصنيف عترات السالمونيللا المعزولة من العينات المختبرة تصنيفاً مصلياً إلى سالمونيللا إنتريتيديس (عترتان) وسالمونيللا كوسين (عترتان). اختبار الحساسية لعترات السالمونيللا أظهر حساسة ١٠٠% لبولى ميكسين بى ٣٠٠ وحدة ومقاومة ١٠٠% ضد معظم مضادات الدراسة. أجريت اختبارات جزيئية على عترات السالمونيللا للبحث عن بعض عومل الضراوة المسئولة عن خطورتها كمسببات للأمراض ،حيث وجد جين سالمونيللا إنفاجين بروتين فى جميع العترات المعزولة من سالمونيللا إنتريتيديس وسالمونيللا كوسين بينما لم تحتوى أى منها على جين ريكومبينانت بروتين ،أما جين إفيكتور بروتين فقد وجد فى عترة واحدة من سالمونيللا إنتريتيديس وفى عترة واحدة من سالمونيللا كوسين ،ووجد جين إنتيروتوكسين فى عترة واحدة من سالمونيللا إنتريتيديس ولم يوجد فى أى من سالمونيللا كوسين.