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 Enteritidis* O₉ H₁g,m H₂- (2 isolates) and *Salmonella Kossein* O₂ H₁l,v H₂1,5 (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.
Agarose gel electrophoreses according to Sambrook et al., 1989 with modification.

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

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

**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**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primary denaturati on</th>
<th>Secondary denaturati on</th>
<th>Anneali ng</th>
<th>Extensio n</th>
<th>No. of cycle s</th>
<th>Final extensio n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>invA</em></td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>55˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td><em>avrA</em></td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>58˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td><em>sopB</em></td>
<td>94˚C 5 min.</td>
<td>94˚C 30 sec.</td>
<td>58˚C 30 sec.</td>
<td>72˚C 30 sec.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
<tr>
<td><em>Stn</em></td>
<td>94˚C 5 min.</td>
<td>94˚C 1 min.</td>
<td>59˚C 1 min..</td>
<td>72˚C 1 min.</td>
<td>35</td>
<td>72˚C 10 min.</td>
</tr>
</tbody>
</table>
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/H1:1,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.; 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.  
5- 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

<table>
<thead>
<tr>
<th>Salmonella gene factors</th>
<th>invA</th>
<th>Stn</th>
<th>sopB</th>
<th>avrA</th>
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<tr>
<td>Salmonella strains</td>
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<tr>
<td>S. Enteritidis</td>
<td>+</td>
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<tr>
<td>S. Enteritidis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>S. Koessen</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>S. Koessen</td>
<td>+</td>
<td>-</td>
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**Figure (1)** Agarose gel electrophoresis of amplified invA, avrA & sopB genes PCR products (284bp, 422bp & 517bp, respectively) in Salmonella serovars

<table>
<thead>
<tr>
<th>sopB</th>
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<td>Lane</td>
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<td>2</td>
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<td>4</td>
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<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>

**Lane 1:** Negative control for sopB gene.  
**Lane 2 & 5:** Negative strains for sopB gene.  
**Lane 3 & 4:** Positive strains for sopB gene (517 bp)  
**Lane 6:** Positive control for sopB gene.  
**Lane 7:** One step ladder (600bp).  
**Lane 8:** Negative control for avrA gene.  
**Lane 9, 10, 11, 12:** Negative strains for avrA gene.  
**Lane 13:** Positive control for avrA gene.  
**Lane 14:** One step ladder (10 bp)  
**Lane 15:** Positive control for invA gene.  
**Lane 16, 17, 18 & 19:** Positive strains for invA gene (284 bp)  
**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 7: 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 belonged to Salmonella Enteritidis O:9/H1:g,m/H2:- (2 isolates), (1.81%) of the examined poultry meat samples and Salmonella Koessen O:2,12/H1:I,v/H2:1,5 (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 invAgene 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 \textit{avrA} gene of \textit{Salmonella}, with a size of 422 base pairs. \textit{Zou et al (2011)} detected \textit{iacP}, \textit{avrA}, \textit{invH}, \textit{rhuM}, \textit{sirA}, \textit{sopB}, \textit{sopE} or \textit{sugR} genes in 40 to 80\% of \textit{Salmonella} strains, isolated from food and/or the food animal environment, using microarray platform assay. \textit{AvrA} was the effector responsible for the observed inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\kappa B) signaling (\textit{Collier-Hyams et al, 2002}). NF-\kappa B plays a key role in regulating the immune response to infection (\textit{Albensi & Mattson, 2000}).

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

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

It has been proposed that \textit{Salmonella} enterotoxin (\textit{Stn}) is a putative virulence factor and causative agent of diarrhea (\textit{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 (\textit{Nakano et al, 2012}).

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