Molecular Analysis of Some Virulence Genes of Salmonellae Isolated from Chicken at Sharkia Governorate

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Abstract

Poultry is one of the most reservoirs of Salmonella that can be transmitted to humans through the food chain causing high risk bacterial food poisoning. In the present study, bacteriological examination of 200 samples from broiler internal organs (liver, cecum, un absorbed yolk sac) from 1 day old chicks suffering from omphalitis and respiratory disorders after hatching, revealed 26 Salmonella isolates 13%. Serotyping of 26 Salmonella isolates by slide agglutination test using specific monovalent and polyvalent O and H Salmonella sera revealed eleven different Salmonella serotypes, with S. Enteritidis as the most prevalent serotype (38.5%) followed by S. Tamale (23.07%), S. Typhymurium (19.2%), S. Kentucky (15.4%) and S. Heldberg (3.8%). Most Salmonella isolates were sensitive to Ciprofloxacin, sulfamethoxazole – trimethoprim, Chloramphenicol and gentamycin. While they were resistant to Erythromycin, Rifamycin, Amoxicillin /clavulnic acid, colistin sulfate and cefotaxime. PCR detection of 6 virulent genes of 5 strains of Salmonella isolates which proved to play an important role in the virulence of Salmonella in chicken. In this study detection of (invA gene, hilA gene, stn gene, spvc gene, fmH gene and pefA gene) is with percentages of (100%, 80%, 100%, 0%, 100% and 40%) respectively. From examined 5 MDR Salmonella isolates, Integron class 1 were detected in all of them (100%) but no strain was found to harbor Integron class 2.

Key words: Salmonella, virulence genes, integrin, chicken.
Introduction

Salmonella isolates are considered as the most circulating and frequent bacterial agents causing disease in poultry and other avian species. It is associated with high mortality, morbidity, and impaired production (Sedeik et al., 2019). Salmonellosis has been associated with the infection of broiler flocks that has the ability of vertical transmission to progeny (Irshad et al., 2013). The high prevalence of multidrug resistant Salmonella to be commonly used antimicrobial in veterinary and public health sectors has emerged as a global problem that resulting in treatment failure (Piddock, 2002, Molla et al., 2003 and Yan et al., 2003 Khemtong and Chaunchuen, 2008).

The present work is planned out as an attempt to throw some spotlights on:

1. Isolation of Salmonella serovars from different poultry farms.
2. Biochemical and serological identification of Salmonella isolates.
3. Antimicrobial sensitivity test on Salmonella isolates.
4. Genotypic characterization of obtained Salmonella isolates to detect the most predominant virulence genes.
5. Detection of class1 and cass2 integrin in multi-drug resistant isolates by polymerase chain reaction.

Materials and Methods

1. Sampling

200 samples from broiler internal organs (liver, cec, unabsorbed yolk sac) from 1 day old chicks suffering from omphalitis and respiratory disorders after hatching. Samples were collected aseptically to prevent cross contamination by wearing disposable gloves and using sterile sampling materials (swabs, bags, and syringes). The samples were collected and transported in ice boxes with ice packs as early as possible to the laboratory for bacteriological examination and keeping on refrigerator.

2. Isolation of Salmonella, biochemical identification and serotyping

We follow ISO 6579 (2002) procedure. Suspected Salmonella colonies were confirmed serologically by Kauffman – White scheme (Kauffman, 1974) for the determination of (O) and (H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan). Biochemically by (TSI) test, Urea hydrolysis test, Lysine decarboxylation test, Indole production test and Simmon Citrate utilization test. The isolates were then serotyped by the Animal health research institute in Dokki -Giza. Only
confirmed Salmonella were tested for their susceptibility to different antibiotic and the presence of the virulant genes class1 and class2 integron.

3 Resistance to the antimicrobial agents

We detect susceptibility of isolates to antimicrobial agents following the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, CLSI, 2007) for the disk diffusion technique. The antimicrobials and concentrations tested were Amoxicillin/clavulanic acid, Norfloxacin, Gentamicin, Cefotaxin, Sulfamethoxazole/trimethoprim, Ciprofloxacin, Nalidixic acid, Doxycycline, Erythromycin, Chloramphenicol, Colistin sulphate, Rifamycin. The inhibition zones were scored as sensitive, intermediate susceptibility or resistant according to the CLSI.

4 Polymerase chain reaction for amplification of the most important virulent genes of Salmonella isolates (invA gene, hilA gene, stn gene, spvC gene, fmH gene and pefA gene)
a. Extraction of DNA according to QIAamp DNA mini kit instructions.
b. Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit.

Results & Discussion

Results revealed 26 Salmonella isolates from 200 samples with percentage (13%). Serotyping of 26 Salmonella isolates by slide agglutination test using using specific monovalent and polyvalent O and H Salmonella sera detected eleven different Salmonella serotypes, with S. Enteritidis as the most prevalent serotype (38.5%) followed by S. Tamale (23.07%), S. Typhymurium (19.2%), S. Kentucky (15.4%) and S. Heldberg (3.8%). Most Salmonella isolates were sensitive to Ciprofloxacin, Sulfamethoxazole – trimethoprim, Chloramphenicol and gentamycin, while they were resistant to Erythromycin, Rifamycin, Amoxycillin/clavulanic acid, Colistin sulfate, and Cefotaxime.

Examination of samples collected from diseased and died sasso chicks from different hatcheries in Sharkia using SMT, 26 Salmonella isolates was isolated in an over prevalence of 13% (26/200). The results of serological identification in present study detected 5 different serovars from 26 Salmonella isolates, S. Enteritidis predominated with higher percentage (38.5%) while the remained isolates were serotyped as S. Tamale, S. Typhymurium, S. Kentucky, S. Heldberg, with percentages (23.07%),
Genotypic characterization of some *Salmonella* serotypes: *S. Enteritides* (105), *S. Tamale* (13), *S. Typhimurium* (17), *S. Kentucky* and *S. Heldberg* (97) were subjected to PCR genotyping for detection of some virulent genes (*invA*, *fimH*, *hilA*, *pef*, and *spvC*) and Integron (Int1 and Int2).

It was evident that the oligonucleotide primer pairs targeting the genes under study successfully amplified the DNA extracted from tested *Salmonella* isolates for each primer. As expected, PCR confirmation of bacteriology positive strains was documented by appearance of amplified DNA fragment of 284bp for the *invA* gene, a target for *Salmonella* genus in all examined serotypes (100%)

**Detection of class 1 integron and class 2 integron among multidrug resistant isolates:**

From examined 5MDR *Salmonella* isolates, Integron class 1 were detected in all of them (100%) but no strain was found to harbor Integron class 2 (0%) as shown in figures 7&8.

Association between phenotypic antimicrobial results and genotypic detection of some virulence genes of different *Salmonella* species could be effective in providing a more accurate profile for understanding the dangerous spread of virulence genotypes and antibiotics resistance in *Salmonella* serovars.

From the above mentioned results, it is important to note that amplification of *invA* gene as *Salmonella* species specific and virulance factor in this study produced a PCR product of approximately 284bp in all *Salmonella* species tested (100%). All 5 *Salmonella* serotypes which Identified phenotypically were found to possess *invA* gene indicating that PCR result from *invA* gene detection in agreement with these of conventional methods.

Several authors proposed a rapid, sensitive and specific PCR method using primers for *invA* gene for the detection of *Salmonella* serotypes in many clinical samples within a maximum of 12hr, thus confirming its affiliation to *Salmonella* species (*Lampel et al., 2000; Ferretti et al., 2001; Schneder et al., 2002; and Ammar et al., 2014*).

In addition, *Oliveria et al.* (2002) and *Malorny et al.* (2003) and *Lin et al.* (2007) reported that *invA* gene abled to identify all examined *Salmonella* serovar by PCR technique.

In the last decade, there has been a wide interest in the use of PCR technique. PCR have been
applied to detect different species of several microorganisms, to differentiate closely related species Settani and Corsetti, (2007). The primary advantages of PCR tests are increased sensitivity and less time required to process samples in the laboratory when compared to standard culture method Lampel et al. (2000).

In the present work, PCR approaches have been applied to detect different virulent genes that are (invA, hilA, pefA, stn, spvc and fimH) and also detect integron class 1 and integron class 2 that is responsible for antibiotic resistance. Finally, amplification of invA and hilA genes now has been recognized as an international standard for detection of genus Salmonella Maloeny et al. (2003).

It is alarming that Salmonella induced diarrhea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin which is mediated by stn gene. Therefore, a uniplex PCR assay was carried out for detection of this gene in the representative Salmonella isolates. The results revealed the presence of stn gene in all tested Salmonella isolates with a PCR product at 619bp. This finding is in a greement with earlier reports of Parger et al. (1995) and Morogkar et al. (2003) who detected stn gene among different Discussion 78 Serovars of Salmonella, indicating that stn gene is widely distributed among Salmonella irrespective of their Serovars and source of isolation. Moreover, fimbriae play an important role in the pathogenicity of bacteria which is a key factor for bacteria invasion and survival inside the host cells Finaly and Falkow (1989). PefA gene is encoded by pef operon located in a plasmid Friedrich et al. (1993).

Indeed, among the isolates analysed in this study pefA gene was present in 40% of these isolates. This result is go hand in hand with Mona 2014 that detect pefA with percentage 41.1%. On the contrary this result is markedly lower than that presented by Wood Ward et al. (1996) who found that 97.9% of S. Enteriditis isolates was positive for the presence of this gene and Morugkar et al. (2003) who found pefa in 89% of different Salmonella serovars in India.

Furthermore, Salmonella is plasmid virulence (spvc) is also related to survival and growth of the bacterium in host cells Swamy et al. (1996).

In this study, no isolate of Salmonella was positive for the presence of spvc gene that is differ from than that observed by Amini et al. (2010) who detecyed spv in 30% of
S. enteritidis strains isolated from poultry and Ihab et al. (2013) who found that the incidence of spvc genes was 31.5% in S. Enteritidis and 30% in S. Typhymurium isolates from poultry. Salmonella enterica serovar Enteritidis is presently the major causative agent of food born gastro enteritis in human worldwide and an important source of bacterial infection on poultry farms resulting in public health problems and economic loss. Infection by S. Enteritidis is initiated by the attachment and colonization of gut mucosa, which seem to be essential stage in the pathogenesis of salmonellosis. Increasing evidence suggests that bacterial fimbriae play an important role in these processes (Dagmara 2006).

S. Enteritidis genome contains many putative fimbrial operons: afg, bcf, fim and pef (Edward et al. 2002). Studies on S. Typhymurium revealed that FimH adhesion is responsible for bacteria binding to intestinal epithelial cells (Thankavel et al. 1999). The fim H gene produced an amplified PCR product at 164bp in all Salmonella tested. S. enterica serovar Typhymurium express type 1 fimbriae that enable the bacteria to bind to eukaryotic cell and have been implicated in mediation.

We have previously shown that fimH mutants of Salmonella are unable to adhere to eukaryotic cell, demonstration that FimH is critical for the ability of type 1 fimbriae to specifically bind to host cells (Brett et al. 2011). Association between phynotypic antimicrobial results and genotypic detection of some virulence genes of different Salmonella species could be effective in providing a more accurate profile for understanding the danderous spread of virulence genotypes and antibiotics resistance in Salmonella serovars. Overall, multidrug resistant Salmonella serotypes were also cabable for exhibiting several virulence determinants which are very important to induce Salmonella pathogenicity. This result corresponds closely to a previous report published by Amminin et al. (2011) who found the virulence gene invA, hila located on SPI-1 and other virulence genes not present on it such as stn and spvc were detectable in all multidrug resistant Salmonella isolates.

It is important to note that Salmonella can easily acquire multiple drug resistance to most antimicrobial and transform them to human through food chain.
Recently, the generation and the transformation mechanism of the drug resistance gene have become a hot research topic in order to control the spread of multi drug resistance bacteria. Integron, a novel DNA element, mediating the integration of antibiotic resistance gene through site specific recombination has great impact on human health. The backbone structure of an integron is probably the most prevalent type of integrin harboured by such isolates Sunde.2005 and Ren et al. (2013).

In the present study, class1 integron and class 2 integron, was screened among 5 multidrug resistance Salmonella strains using specific primer for integrase (IntI1) and (IntI2) gene. The result revealed the presence of class1 integrin in all tested serotypes and absence of class 2 integrin in all tested serotypes. Other reports have also revealed the prevalence of class 1 integron in gram negative isolates from food samples as 41.3% in united states Khaista et al. (2008), 45% in china Li et al. (2011), 49 % in Australia White et al. (2001) 99% In Bortugal Antunes et al. (2004) indicating that class 1 integron are wide spread among gm - ve isolates.

![Agarose gel electrophoresis showing the result of PCR for detection of invA gene from 5 Salmonella strains](image)

**Fig (1):** Agarose gel electrophoresis showing the result of PCR for detection of invA gene from 5 Salmonella strains

**Lanes 1,2,3,4,5:** Salmonella species
**Lane Pos:** positive invA control.
**Lanes 1,2,3,4,5:** positive amplification of 284bp for invA gene of different Salmonella species.
**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).
**Lane Neg:** negative invA control. (control negative).
Fig. (2): Agarose gel electrophoresis showing the result of PCR for detection of fimH gene from 5 Salmonella strains

**Lanes 1,2,3,4,5:** Salmonella species

**Lane Pos:** positive fimH control.

**Lanes 1,2,3,4 and 5:** positive amplification of 164bp for fimH gene of different Salmonella species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).

**Lane Neg:** negative fimH control. (control negative).

Fig. (3): Agarose gel electrophoresis showing the result of PCR for detection of hilA gene from 5 Salmonella strains

**Lanes 1,2,3,4,5:** Salmonella species

**Lane Pos:** positive hilA control.

**Lanes 2,3,4 and 5:** positive amplification of 150bp for hilA gene of different Salmonella species.

**Lane 1:** negative amplification of 150bp for hilA gene of different Salmonella species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).

**Lane Neg:** negative hilA control. (control negative).
Fig. (4): Agarose gel electrophoresis showing the result of PCR for detection of pef gene from *Salmonella* strains

**Lanes 1,2,3,4,5:** *Salmonella* species

**Lane Pos:** positive *Pef* control.

**Lanes 2 and 4:** positive amplification of 700bp for *Pef* gene of different *Salmonella* species.

**Lanes 1,3 and 5:** negative amplification of 700bp for *Pef* gene of different *Salmonella* species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder)

Fig. (5): Agarose gel electrophoresis showing the result of PCR for detection of spvC gene from *Salmonella* strains

**Lanes 1,2,3,4,5 *Salmonella* species**

**Lane Pos:** positive *spvC* control (reference strain).

**Lanes 1,2,3,4,5:** negative amplification of 467bp for *spvC* gene of different *Salmonella* species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).

**Lane Neg:** negative *spvC* control. (control negative)
Fig. (6): Agarose gel electrophoresis showing the result of PCR for detection of stn gene from 5 *Salmonella* strains.

**Lanes 1,2,3,4,5:** *Salmonella* species

**Lane Pos:** positive *stn* control (reference strain)

**Lanes 1,2,3,4,5:** positive amplification of 617bp for *qnrS* gene of different *Salmonella* species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).

**Lane Neg:** negative *stn* control. (control negative).

Fig. (7): Agarose gel electrophoresis showing the result of PCR for detection of *Int1* from 5 *Salmonella* strains.

**Lanes 1,2,3,4,5:** *Salmonella* species

**Lane Pos:** positive *Int1* (reference strain)

**Lanes 1,2,3,4,5:** positive amplification of 280bp for *Int1* of different *Salmonella* species.

**Lane L:** the DNA molecular weight marker (Gelpilot 100bp ladder).

**Lane Neg:** negative *Int1* control. (control negative).
Fig. (8): Agarose gel electrophoresis showing the result of PCR for detection of Int2 from 5 Salmonella strains

Lanes 1,2,3,4,5 Salmonella species
Lane Pos: positive Int2 control (reference strain).
Lanes 1,2,3,4,5: negative amplification of 250bp Int2 of different Salmonella species.
Lane L: the DNA molecular weight marker (Gelpilot 100bp ladder).
Lane Neg: negative Int2 control (control negative).

Based on the phenotypic and genotypic characterization, the isolates were categorized in five different well defined profiles as shown in table (1):

Table (1): Relationship between drug resistance and presence of virulent genes, Int 1 and Int 2 among Salmonella isolates:

<table>
<thead>
<tr>
<th>Code No.</th>
<th>Sample origin</th>
<th>Antimicrobial sensitivity (R&amp;I)</th>
<th>Virulent genes</th>
<th>Int 1</th>
<th>Int 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>105 S.E</td>
<td>Liver</td>
<td>AMC-S-NA-CTX- SXT- E-C-CT-RF.</td>
<td>InvA-stn-fimH.</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13 S.Ta</td>
<td>Liver</td>
<td>CTX-E-CT-RF</td>
<td>invA-hilA-stn</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>17 S.Ty</td>
<td>Yalk Sac</td>
<td>S-CN-NA- CTX- DO-E-C-CT-RF.</td>
<td>invA-hilA-stn</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>33 S.K</td>
<td>Liver</td>
<td>AMC-NA-CTX- SXT-DO-E-C-CT-RF.</td>
<td>invA-hilA-stn</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>97 S.H</td>
<td>2ceci</td>
<td>AMC-CN-NA-CTX- E-CT-RF.</td>
<td>invA-hilA-stn</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

References


**Dagmara Kisiela, Anna Laskowska, Anna Sapeta, Maciej Kuczkowski, Alina Wieliczko and Maciej Ugorski(2006):** Functional characterization of the FimH adhesion from *Salmonella* enterica serovar Enteritidis , Microbiology , 152, 1337–1346.


**Ihab M M , Yosra S A, Abdullah A A, Ashgan M H Azza S G \ and Refai M K.(2013):** Molecular characterization of *Salmonella* virulence genes isolated from different sources relevant to


