Phenotypic and genotypic characterization of locally isolated *Salmonella* strains used in preparation of *Salmonella* antigens in Egypt

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**Abstract**

**Aim:** This work was conducted to study the phenotypic and genotypic characterization of locally isolated *Salmonella* strains (*Salmonella Pullorum*, *Salmonella Enteritidis*, and *Salmonella Typhimurium*) from poultry used in the preparation of *Salmonella* antigens in Egypt.

**Materials and Methods:** The phenotypic characterization of *Salmonella* strains was done using standard microbiological, biochemical, and serological techniques. Molecular identification was done using different sets of primers on different genes using different polymerase chain reaction (PCR) techniques.

**Results:** The phenotypic characterization of *Salmonella* strains was confirmed. Molecular identification revealed detection of 284 bp fragment of InvA gene in all studied *Salmonella* strains. Furthermore, multiplex PCR was used for more confirmation of being *Salmonella* spp., generally at 429 bp as well as genotyping of *Salmonella* Typhimurium and *Salmonella* Enteritidis at 559 and 312 bp, respectively, in one reaction.

**Conclusion:** The locally isolated field *Salmonella* strains were confirmed phenotypically and genotypically to be *Salmonella Enteritidis*, and *Salmonella Typhimurium* and could be used for the preparation of *Salmonella* antigens.

**Keywords:** characterization, duplex polymerase chain reaction, multiplex polymerase chain reaction, *Salmonella* spp.

**Introduction**

*Salmonella* organisms are responsible for a variety of acute and chronic diseases in poultry, animals, and humans [1]. *Salmonella* bacteria are facultative intracellular pathogens causing localized or systemic infections, in addition to a chronic asymptomatic carrier state. Many different serotypes of *Salmonella* have been isolated from poultry, most of them have a public health significance, but some include *Salmonella Typhimurium*, *Salmonella Enteritidis*, *Salmonella Pullorum*, and *Salmonella Gallinarum* can cause considerable losses in birds of less than a few weeks of age [2]. *Salmonella enterica* serovars *Typhimurium* and *Salmonella enterica* serovars *Enteritidis* are the most frequently isolated serovar from foodborne outbreaks throughout the world [3]. *Salmonella Pullorum* is a typical bacterial disease that has threaten the modern poultry industry over the past years. Chicken becomes the carrier in the spread of *Salmonella Pullorum* and may cause economic losses worldwide through mortality, morbidity, and reductions in egg production [4]. Establishing conventional methods was applied to detect and identify *Salmonella* include selective enrichment and plating followed by biochemical tests and serological identification [5]. In general, these techniques are time-consuming since they give only presumptive results after 3-4 days and definitive results after 5-6 days [6]. However, because of controversy in interpreting results, low sensitivity and specificity of these methods, 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 [7].

In *vitro* amplification of DNA by the polymerase chain reaction (PCR) method is a powerful tool in microbiological diagnostics [8]. Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and fecal samples. Virulence chromosomal genes - including invA, invE and himA, phoP - are target genes for PCR amplification of *Salmonella* species [9]. The invA gene of *Salmonella* contains sequences unique to this genus and has been proved as a suitable PCR target with potential diagnostic applications [10]. Multiplex PCR simultaneously detecting several pathogens in a single-tube reaction and has the potential of saving time and effort, lowering testing-related laboratory cost [11]. Typing of
**Salmonella** Enteritidis and Typhimurium using multiplex PCR reaction is depending on sefA gene which encodes for SEF14 fimbrial antigen characteristic for *Salmonella* Enteritidis while fliC gene variable region encoding for flagellin H1 was characteristic for *Salmonella* Typhimurium [12].

The objective of this work was to characterize the locally isolated *Salmonella* strains used in the preparation of *Salmonella* antigens in Egypt by both phenotypic and genotypic methods.

### Materials and Methods

#### Ethical approval

The approval from the Institutional Animal Ethics Committee to carry out this study was not required as no invasive technique was used.

#### Bacterial strains

Three local field *Salmonella* strains (*Salmonella* Pullorum, *Salmonella* Enteritidis, and *Salmonella* Typhimurium) isolated from chickens, kindly obtained from Bacterial Sera and Antigens Research Department, Veterinary Serum and Vaccine Research Institute, Abbassia, Egypt were used to study their phenotypic and genotypic characterization. All isolates were confirmed as *Salmonella* different types using both morphological and biochemical identification [13]. Serological typing was performed using reference *Salmonella* antisera [14].

#### Total DNA extraction of *Salmonella* isolates

That was performed by boiling the overnight incubated culture broth for 10 min in dry bath and centrifuged at 5000 × *g* for 10 min. The supernatant was used for amplification by PCR using *Salmonella*-specific primers. The extract was divided into aliquots and kept at −20°C until use as PCR template [15].

#### Primers set

Primers used were supplied by Metabion (Germany) and summarized in Table-1. For diagnosis of *Salmonella* spp. generally, a primer set was used for amplification of 284 bp of InvA gene [10]. Another primer sets were used for general identification of *Salmonella* spp. as well as typing of *Salmonella* Typhimurium and *Salmonella* Enteritidis in a multiplex PCR reaction [12]. Typing of *Salmonella* Pullorum was done using a duplex PCR, to differentiate between *Salmonella* Gallinarum and *Salmonella* Pullorum depending on the presence of speC gene in both strains but glgC gene is unique for *Salmonella* Gallinarum only [16].

#### PCR amplification

Amplification was performed as following: 12.5 μl of ×2 Dream Taq Green PCR Master Mix (Fermentas), 100 pmol of upstream primer, 100 pmol of downstream primer, 4 μl of template DNA and nuclease-free water up to 25 μl using thermal cycler PerkinElmer Gene Amp PCR system 9700. Amplification conditions of 284 bp of InvA gene where the thermal cycler were adjusted to 1 cycle at 95°C for 1 min, then 35 cycles at 95°C for 1 min, 64°C for 30 s, 72°C for 30 s followed by 1 cycle at 94°C for 4 min [17]. For multiplex PCR, the amplification conditions were adjusted to 1 cycle at 94°C for 1 min, 35 cycles at 94°C for 30 s, 56°C for 1 min 30 s, 72°C for 30 s followed by 1 cycle at 72°C for 10 min [12]. Duplex PCR was performed [16,18], with a wide range of annealing temperatures, where PCR conditions were 1 cycle at 95°C for 5 min, 35 cycle of 95°C for 30 s, 55-65°C for 30 s, and 72°C for 30 s followed by a final extension step at 72°C for 10 min. Sterile DNase and RNase free water were used as negative PCR control.

#### Analysis of PCR products

All amplified products were analyzed by electrophoresis using 1-1.5% agarose gel (Applichem, Germany, GmbH) and visualized by ultraviolet transilluminator after gel staining with ethidium bromide stain (Fisher). The product size was measured using 100 bp DNA Ladder (Fermentas) that was used as a

### Table-1: Primer sets for *Salmonella* strains PCR.

| Primer set | *Salmonella* strain | Target gene | Primer sequence 5’-----3’ | Length | Amplicon fragment (bp) |
|------------|---------------------|-------------|---------------------------|--------|-----------------------|
| S139       | *Salmonella* spp.   | invA gene   | GTG AAA TTA TCG CCA CGT TCG GGC AA  | 26     | 284                   |
| S141       | *Salmonella* spp.   | invA gene   | TCA TCG CAC CGT CAA AGG AAC C  | 22     |                       |
| ST11       | *Salmonella* spp.   | Randomly cloned chromosomal fragment | AGGCAACCATGTCAATATGGGCA  | 25     | 429                   |
| ST15       | *Salmonella* Typhimurium | fliC | GGTGAAATCTCCAGCGGTTACTG | 24     |                       |
| Fli 15     | *Salmonella* Typhimurium | fliC | CGG TGT TCC CCA GTG TGG TAA T  | 22     | 559                   |
| Tym        | *Salmonella* Enteritidis | SefA gene | ACT CTG GCT GGC GGT GGC ACT T  | 22     |                       |
| Sef167     | *Salmonella* Enteritidis | SefA gene | AGG TTC AGG CAG CCG TTA CT  | 20     | 312                   |
| Sef478     | *Salmonella* Pullorum | glgC | GGC ACA TTT AGC TTT TCT TG  | 20     |                       |
| SG-L       | *Salmonella* Pullorum | glgC | GAT CTG CTG CCA GCT CAA  | 18     | 252                   |
| SG-R       | *Salmonella* Pullorum | glgC | GCG CCC TTT TCA AAA CAT A  | 19     |                       |
| SGP-L      | *Salmonella* Pullorum | glgC | CGG TGT ACT GCC CGC TAT  | 18     | 174                   |
| SGP-R      | *Salmonella* Pullorum | glgC | CTG GGC ATT GAC GCA AA  | 17     |                       |

PCR=Polymerase chain reaction
marker for PCR products. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra), and the data were analyzed through computer software.

**DNA sequence and analysis**

PCR fragment of speC gene of Salmonella Pullorum was purified with agarose gel extraction kit Qiagen, Germany). Sequence analysis of this fragment was performed using the same PCR primers (Macrogen Inc., Seoul, Korea).

**Results**

In this study, three locally fields isolated Salmonella strains used in preparation of Salmonella antigens in Egypt were tested and confirmed to be Salmonella species phenotypically by culturing and biochemical testing. Furthermore, these strains were confirmed serologically to be Salmonella Pullorum, Salmonella Enteritidis, and Salmonella Typhimurium (Table-2). On the other hand, strains were confirmed to be related to Salmonella spp. by invA specific PCR methods as all isolates showed positive bands at 284 bp (Figure-1).

Genotype identification was done using multiplex PCR assay with simultaneous characterization of Salmonella spp. generally. The results obtained showed that the three used strains were positive by PCR primers set (ST11, ST15) showing specific bands at 429 bp (Figure-2) for all Salmonella spp. Multiplex PCR could differentiate between Salmonella Enteritidis and Salmonella Typhimurium that showing sharp specific bands at 312 and 559 bp, respectively (Figure-2).

Duplex PCR for identification of Salmonella Pullorum results as shown in Figure-3 revealed a band at 220 bp at 60°C annealing temperature only. None other bands were obtained by repeating the test with different PCR conditions. Furthermore, this test showed negative results or no product when tested with Salmonella Typhimurium and Salmonella Enteritidis. This band was purified for sequence analysis. The data obtained from sequence analysis of this fragment (data not shown) showed that this PCR fragment is not related to speC or glgC genes at all but it was related to yejBEF gene which is common among many other Salmonella spp.

**Discussion**

Salmonella contamination of eggs has been identified as a public health concern worldwide. Globally, Salmonella is one of the most prevalent causes of foodborne illness [19]. Culture techniques are universally recognized as standard methods for detection of bacterial pathogens, such as Salmonella in food-stuffs [20]. These techniques generally take longer time [8] and are less sensitive compared to PCR-based methods [21]. InvA gene specific PCR method is the most used in diagnostic and research laboratories, and Salmonella identification by molecular techniques is the simplest and less expensive method [10].

### Table-2: Results of serotyping of Salmonella strains.

| Salmonella groups and types          | Antigenic formula |
|--------------------------------------|-------------------|
|                                      | O     | H     |
| Salmonella Pullorum                  | 1, 9, 12 |
| Salmonella Typhimurium               | 1, 4, 5, 12 | 1, 1, 2 |
| Salmonella Enteritidis               | 1, 9, 12 | g, m (1, 7) |

( )=May be absent

In this study, all the three locally field isolated strains used in the preparation of Salmonella antigens in Egypt were tested and confirmed phenotypically to be Salmonella Pullorum, Salmonella Enteritidis and Salmonella Typhimurium by culturing, biochemical testing and serological characterization. These strains were confirmed to be related to Salmonella spp. by invA specific PCR methods as all isolates showed positive bands at 284 bp (Figure-1) which agree with the previously reported results [10,17]. Genotype identification was done using multiplex PCR assay with simultaneous characterization of Salmonella spp.
it was found that glgC gene was a pseudogene in Salmonella Gallinarum while speC was a pseudogene in both biovars. In bacterial genomes, pseudogenes are continually created from ongoing mutational processes and are subject to degradation and removal by further accumulation of mutations. Their retention time seems to be extremely short and, even in very closely related bacteria, they tend to be deleted at a relatively rapid rate [26]. All these findings decreased the sensitivity and reliability of this duplex PCR. More investigations are required for rapid and easy identification of Salmonella Pullorum, as the previously reported conventional DNA-based methods are not feasible due to a high level of sequence similarities among Salmonella serovars as well as the limitation in resolution between biovars Gallinarum and Pullorum [27,28]. Furthermore, post-PCR steps as RFLP is laborious and time-consuming [29,30].

**Conclusion**

The locally isolated field Salmonella strains were confirmed phenotypically and genotypically to be Salmonella Enteritidis and Salmonella Typhimurium and could be used for the preparation of Salmonella antigens. Further studies are required to develop and establish rapid and accurate protocols for genotyping of Salmonella Pullorum.

**Authors’ Contributions**

HMI and HAA designed the work. HMI, DAMA and HAA conducted the research work. Data analysis and manuscript were written by HMI, DAMA and HAA under the guidance of MIE. All the authors have read and approved the final manuscript.

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**Competing Interests**

The authors declare that they have no competing interests.

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