Characterization of Paratyphoid Salmonellae Isolated from Broiler Chickens at Sharkia Governorate, Egypt

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Abstract
Paratyphoid Salmonella has emerged as a global problem for humans and poultry. Therefore, in this study we investigated the occurrence, serological, antimicrobial and molecular characteristics of paratyphoid Salmonella isolated from chicken flocks at Sharkia Governorate during 2015-2016. The prevalence of paratyphoid Salmonella among the 150 suspected flocks was 32.6% (49/150), the highest rate (41/49; 80%) was among young ages (1-10 days old) and the lowest rate (8/49; 16%) among older ages (11-21 days old). The highest recovery was from liver (30.66%), followed by spleen (25.33%), caecum (20%) and yolk sac (15.7%). Serotyping of 49 Salmonella isolates revealed 11 different serogroups, with Salmonella Typhimurium being the most prevalent one (24.49%), followed by Salmonella Kentucky (18.36%) and Salmonella Enteritidis (14.28%). The most sensitive antibiotics were apramycin (82%) and ciprofloxacin (65%). Multidrug resistance (MDR) was significant to ampicillin, gentamycin and cefotriaxone in all Salmonella isolates. All phenotypically identified MDR Salmonella were found to possess invA, hilA, pefA (100%) and avrA (95%) genes by polymerase chain reaction (PCR), confirming that these virulence genes are important virulence markers for rapid diagnosis of Salmonella infection.

Keywords: Multidrug resistant Salmonella, Virulence genes, Serotyping, Chicks.

Introduction
Salmonellosis is a serious problem and a public health risk [1], causing high economic losses in poultry industry due to high mortality in young chicks and debilitating effect predisposing to other diseases [2]. Avian Salmonella infection occurs in the form of acute or chronic disease caused by genus Salmonella, of the family Enterobacteriaceae [3]. Paratyphoid Salmonellae include more than 2400 serovars [4], causing pasty diarrhea, inappetence, dehydration, growth retardation, blindness and lameness in one week old broiler chicks. The main gross lesions are hepatomegaly with necrotic foci, splenomegaly, pericarditis, panophthalmitis, persistent yolk sac and arthritis [5]. Paratyphoid infection affects chickens at any age and of any type. In young birds, high mortality rates may reach 80% or higher while older ages, over 3 weeks old, paratyphoid infection rarely causes mortality but the survivors become carriers and excrete the organisms in the environment [6].

The pathogenicity of Salmonella depends on a set of virulence associated factors harbored by the bacterium, the bird and its environment. Adhesion and penetration of the bacterium into intestinal mucosa is a prerequisite for systemic infection [7]. The infection usually starts by ingestion, followed by intestinal colonization and penetration of the mucosal epithelium which results in a systemic infection with colonization in the spleen and liver [8]. Salmonella pathogenicity islands (SPIs) are large clusters of chromosomal conserved virulence genes in pathogenic Salmonella spp. that are absent in nonpathogenic spp. [9]. Several pathogenicity
islands have been identified in Salmonella Typhimurium, S. Dublin and S. Enteritidis [10, 11]. SPI-1 encodes a type III secretion system (TTSS), which is required for the uptake of Salmonella by intestinal epithelial cells [12, 13]. The deletion of this island resulted in avirulent mutant by the oral route of infection but are virulent by the intravenous route, indicating that SPI-1 is required for intestinal invasion but not for systemic infection [14]. SPI-2 is essential for intramacrophage survival [15] and is required for the systemic phase of infection [16]. SPI-3 encodes the mgtCB operon that is required for both intramacrophage survival and growth in Mg±2-limiting conditions [17]. Salmonella of serotypes Dublin, Pullorum, Gallinarum, Choleraesuis, Abortusovis, and some strains of Typhimurium and Enteritidis harbor virulence plasmids that encode genes required for the ability to cause systemic disease [18, 19].

Salmonella infection can be detected via bacterial isolation, identification, serological tests [20] and polymerase chain reaction (PCR) which is more accurate especially in case of rough strain lacking O-antigen [21]. Efforts to reduce Salmonella include guidelines adoption for antibiotic misuse, continuous antimicrobial susceptibility surveillance to identify the changing pattern of Salmonella resistance [22] and alternative additives other than antibiotics as probiotics, prebiotics, synbiotics and organic acids [23]. Therefore, the aim of this study was to investigate the occurrence, serological, antimicrobial and molecular characteristics of paratyphoid Salmonella isolated from chicken flocks at Sharkia Governorate, Egypt.

Materials and Methods

Bird examination and sample collection

A total of 150 broiler farms (Cobb, Ross, Arbor Acres, Sasso and Balady breeds) during 2015-2016 outbreaks were divided as 100 (1-10 days old) and 50 (11-21 days old) with a history of whitish diarrhea and mortality were examined by clinical and postmortem (PM) examination for Salmonella infection. Pooled samples including three of each organ; liver, spleen, caecum (150 for each) and unabsorbed yolk sac (70) were aseptically collected and transferred in ice box to the avian bacterial pathogen laboratory of Avian and Rabbit Medicine Department, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Bacterial isolation and identification

Samples were cultivated on buffered peptone water (Oxoid, UK) at 37 °C/18 h, from which 0.1 mL was transferred to a 10 mL Rappaport Vassiliadis broth (Oxoid, UK) at 41.5 °C/24 h. Ten microliter of broth culture were inoculated onto xylose lysine deoxycholate agar (XLD) and MacConkey’s agar (HiMediaTM Laboratories, India) at 37 °C/24 h. Pure colonies were microscopically examined, stabbed into semisolid agar for motility detection and biochemically identified by urea hydrolysis, triple sugar iron agar (TSI), lysine decarboxylase and indole production, methyl red test, Voges-Proskauer test, and citrate utilization test (IMVC) (Oxoid, UK). Isolates were preserved on tryptic soy agar (TSA) slants (Oxoid, UK) [24].

Serogrouping

Forty nine isolates were subjected to serotyping using polyvalent and monovalent somatic (O) and flagellar (H) Salmonella antisera via slide and tube agglutination test, respectively at the serology unit of the Faculty of Veterinary Medicine, Benha University, Egypt [25].

Antibiotic sensitivity test

The recovered isolates were tested for antibiotic susceptibility on Muller Hinton agar by disc diffusion method according to clinical and laboratory standard institute (CLSI) guide lines. Swabs from standardized suspension of colonies (match 0.5 McFarland standard) were streaked evenly on Mueller Hinton agar plate (Oxoid, UK). The antibiotic discs (thiamphenicol, 30 µg; florphenicol, 30 µg; ampicillin, 10 µg; erythromycin, 15 µg; apramycin, 15 µg; ceftriaxone, 30 µg; doxycycline, 30 µg; gentamicin, 10 µg; spectinomycin, 100 µg and ciprofloxacin, 5 µg, Oxoid, UK) were distributed evenly and firmly pressed on media. The plates were inverted and incubated for 18 h at 37° c. The diameter of inhibition zone was measured to the nearest mm with a ruler and interpreted according to CLSI breakpoints [26].
DNA Extraction and PCR assay for virulence genes

Twenty MDR isolates including (5 S. Typhimurium, 3 S. Enteritidis, 3 S. Kentucky, 2 S. Molade and one each of S. Infantis, S. Takoradi, S. Papuana, S. Labadi, S. Tsevie, S. Larochele and S. Angers) were tested using two biplex PCR one for invA and pefA and other for hilA and avrA. DNA was extracted using QIAamp kit (Qiagen, Germany). Test was done according to EmeraldAmp GT PCR mastermix (Takara, Europe) instructions. Thirty μl of each amplified samples, negative and positive control were gel electrophorized for 30 min and examined by UV-transilluminator [27]. The primer sequences, their thermal cycling condition for both PCRs and their amplicon sizes are shown in Table 1.

Table 1: Oligonucleotide primer sequences, amplicon sizes and cycling conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplicon size(bp)</th>
<th>Reference</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>PefA</td>
<td>plasmid encoded fimbriae</td>
<td>TGTTTCCGGGCTTGCTGTG</td>
<td>700</td>
<td>[5]</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>invA</td>
<td>responsible for invasion</td>
<td>GTGAAATTATCGCCACGTTCG</td>
<td>284</td>
<td>[17]</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>hilA</td>
<td>hyper invasive locus</td>
<td>CATGGCTGGTCAGTTGGAG</td>
<td>150</td>
<td>[18]</td>
<td>4°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>58°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>avrA</td>
<td>effector protein of TTSS</td>
<td>CCTGTATTGTTGAGCTGG</td>
<td>422</td>
<td>[19]</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>55°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>72°C 10 min.</td>
</tr>
</tbody>
</table>

Results

Clinical and PM findings of examined chickens

Diseased broiler chicks showed whitish diarrhea, progressive somnolence and mortality. Acute cases were septicemic, while chronic cases showed necrotic foci in the liver, enlarged gall bladder, spleen and liver, unabsorbed yolk sac, cheesy cecal cores and urates-filled ureters (Figure 1).

Figure 1: Congested spleen and urates filled ureters of 10 days old Hubbard chick (a); cheesy caecal core (b); swollen caecum and congested liver of 2 week old balady chick (c).
Salmonella isolation, identification and serotyping

The prevalence of paratyphoid Salmonella among the 150 suspected flocks was 32.6% (49/150), the highest rate was (41/49; 83.6%) among young ages (1-10 days) and the lowest rate was (8/49; 16.4%) among older ages (11-21 days). The highest recovery was from liver (30.66%), followed by spleen (25.33%), caecum (20%) and yolk sac (15.7%).

Salmonella spp. were Gram negative bacilli showed pale colonies on MacConkey's and reddish colonies with black center on XLD agar; urease negative; IMVC - + - + ; red/yellow (acid butt/alkaline slant) with black color due to H$_2$S production on TSI agar and violet slant/ violet butt with black color on lysine iron agar. Serogrouping of 49 Salmonella isolates by slide agglutination test using specific monovalent and polyvalent O and H Salmonella sera revealed 11 different Salmonella serotypes (Table 2).

### Table 2: Serotypes of Salmonella isolates, their prevalence and multidrug resistance

<table>
<thead>
<tr>
<th>Flock No.</th>
<th>Isolates</th>
<th>%</th>
<th>Group</th>
<th>Antigenic structure</th>
<th>Resistance to</th>
<th>No. of antibiotics (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>123</td>
<td>S. Angers</td>
<td>2.04</td>
<td>C3</td>
<td>8, 20</td>
<td>Z35: Z6</td>
<td>- 1</td>
</tr>
<tr>
<td>20, 35, 67, 90, 101, 107, 144</td>
<td>S. Enteritidis</td>
<td>14.2</td>
<td>D1</td>
<td>1, 9, 12</td>
<td>g, m: -</td>
<td>- - - - 7</td>
</tr>
<tr>
<td>23, 57, 83, 105</td>
<td>S. Infantis</td>
<td>8.1</td>
<td>C1</td>
<td>6, 7, 14</td>
<td>r: 1,5</td>
<td>- 1 2 1</td>
</tr>
<tr>
<td>3, 51, 69,73,85,100,118, 127, 146, 140</td>
<td>S. Kentucky</td>
<td>18.3</td>
<td>C3</td>
<td>8, 20</td>
<td>i: Z6</td>
<td>- 1 2 5 1</td>
</tr>
<tr>
<td>26, 93</td>
<td>S. Labadi</td>
<td>4.08</td>
<td>C3</td>
<td>8, 20</td>
<td>d: Z6</td>
<td>- - - 2</td>
</tr>
<tr>
<td>78</td>
<td>S. Larochelle</td>
<td>2.04</td>
<td>C1</td>
<td>6, 7</td>
<td>d: 1,6</td>
<td>1 - -</td>
</tr>
<tr>
<td>42, 64, 88, 110, 128</td>
<td>S. Molade</td>
<td>12.2</td>
<td>C2</td>
<td>8, 20</td>
<td>Z10: Z6</td>
<td>- - - 2 4</td>
</tr>
<tr>
<td>49, 72, 150</td>
<td>S. Papuana</td>
<td>6.1</td>
<td>C1</td>
<td>6, 7</td>
<td>r: e, n, Z15</td>
<td>- - 1 2</td>
</tr>
<tr>
<td>17, 114, 136</td>
<td>S. Takoradi</td>
<td>6.1</td>
<td>C2</td>
<td>8, 20</td>
<td>i: 1,5</td>
<td>- - - 2 1</td>
</tr>
<tr>
<td>53</td>
<td>S. Tsevie</td>
<td>2.04</td>
<td>B</td>
<td>4, 5</td>
<td>i: e, n, z15</td>
<td>- - 1 -</td>
</tr>
<tr>
<td>8, 12, 32, 59, 81, 97, 104, 119, S. 125, 131, 133, 149</td>
<td>Typhimurium</td>
<td>24.4</td>
<td>B</td>
<td>1, 4, 5, 12</td>
<td>i: 1,2</td>
<td>- 3 3 2 4</td>
</tr>
</tbody>
</table>

Total no (%): 1 5 8 11 17 7

### Antimicrobial resistance

Salmonella isolates were resistant to ampicillin, gentamycin and cefotaxime (100%), followed by doxycycline (96%), florfenicol (84%), thiamphenicol (76%), spectinomycin (73%) and erythromycin (63%). Sensitivity was highest to apramycin (82%) and ciprofloxacin (65%). Multidrug resistance was observed in all isolates at least to 4 antibiotics and approximately half (49%) of the isolates were resistant to 8-9 drugs (Table 2).

Molecular detection of Salmonella virulence genes by PCR

The 4 virulence markers; invA, hilA, avrA and pefA revealed bands of 284, 150, 422 and 700 bp., respectively (Figures 2and 3) and detected in all 20 MDR Salmonella serotypes (100%), except avrA (95%).
Figure 2: Agrose gel for amplicons generated in duplex PCR for detection of pefA and invA genes of Salmonella spp. lanes 1-11, 13-21, samples positive for pefA (700 bp) and invA gene (284 bp), lane 12, 1.5 kb DNA ladder, lane 22, positive control (S. Enteritidis a local lab strain), lane 23, negative control (E. coli DH5α).

Figure 3: Agrose gel for amplicons generated in duplex PCR for detection of avrA and hilA of Salmonella spp. lanes 1-10, 12-21, samples positive for avrA gene (422 bp) and hilA (150 bp) except sample in lane 13 that was negative for avrA gene, lane 11, 0.6 kb DNA ladder, lane 22, positive control, (S. Enteritidis a local lab strain), lane 23, negative control (E. coli DH5α).

Discussion

In the present study, 150 broiler chicken flocks revealed high isolation rate (32.6 %) of Salmonella, that was higher than those recorded by Ammar et al. [28] (17 %) and El-Zeedy et al. [29] (4.1 %) respectively. This may be explained by low biosecurity measures inside farms and possibility of disease transmission via different reservoirs and workers in farms [5].

A higher percentage (41 %) of Salmonella isolation was recorded at 1-10 days old, while lower percentage (16 %) was recorded at 11-21 days old. This could be attributed to acquisition of protective microflora in young birds that either competes with Salmonella for intestinal receptors or produces antagonistic factors [6, 7].

Forty nine Salmonella isolates were serologically identified into 11 serotypes by slide agglutination test using specific
monovalent and polyvalent O and H antisera. S. Typhimurium was the most prevalent one (24.49 %), followed by S. Kentucky (18.36 %), S. Enteritidis (14.28 %), S. Molade (12.24 %), S. Infantis (8.16 %), S. Takoradi, S. Papuana (6.12 %, for each), S. Labadi (4 %), while S. Tsevie, S. Larochelle and S. Angers were the least ones (2 %, for each).

Our results agreed with Abd El- Tawab et al. [31], who reported low percentage of S. Papuana, S. Takoradi, S. Labadi, and S. Angers, each as 2.3 %, while disagreed with Moussa et al. [32] who reported that S. Enteritidis was the most predominated serotype in Saudi Arabia (55.6 %), followed by S. Typhimurium (22.2 %). Our results were lower than those obtained by Abd- El-Ghany et al. [33] who reported that S. Enteritidis was the most prevalent one (37.25 %), followed by S. Typhimurium (29.41 %), S. Infantis (19.6 %), while S. Tsevie (3.92 %) and S. Kentucky (7.84 %) were the least ones. The high prevalence of multiple paratyphoid Salmonella serotypes emphasizes the need to develop appropriate preventive and control measures to minimize their presence in chicken flocks and its potential transmission to humans in Egypt.

MDR Salmonella isolates were detected in our study to at least 4 antibiotics while 49 % of isolates where resistant to 8-9 antibiotics and also by Shah and Korgo, [34] to 11 antibiotics and Zahraei et al. [35] to more than 8 antibiotics. A lower resistance was recorded by Kusumaningrum et al. [36] and Taddele et al. [37] to at least 2 antibiotics. All Salmonella isolates were resistant to ampicillin, gentamycin and cefotrixone (100 %). This is not surprising because these antibiotics are commonly used in humans and poultry. Another factor is the antibiotic misuse by poultry producers including subtherapeutic doses, unauthorized use without prescription and usage as preventive tool in poultry, leading to development of enteric flora resistance, from which pathogenic Salmonella may acquire and transfer resistance to human’s strains through food chain leading to emergence of multidrug resistance Salmonella [38].

Cefotrixone and ampicillin resistances were higher than those recorded by Learn et al. [39] in Malaysia (78%) and Parvej et al. [40] in Bangladesh (87%). On contrary, Habrun et al. [41] found that 99.3 % of Salmonella isolates were sensitive to gentamycin.

A higher sensitivity rate of Salmonella to ciprofloxacin (65 %) was recorded in this study that was lower than Munawwar et al. [42] (87.88 %). Molbak et al. [43] has reported an increased quinolone resistance in Salmonella. Controversy, no Salmonella resistance was observed from broiler carcasses in Barazil [44]. Florfenicol and doxycycline resistance was recorded in this study as 84 % and 96 %, respectively that partially matched with Ghoddusi et al. [45] as 72 % and 100 %, respectively. Molecular characterization of pathogenic organism is of most importance to diagnose, characterize and understand the pathogenesis of the disease in order to facilitate its control. Therefore, the present study aimed to investigate the PCR protocol as a rapid method for the identification of paratyphoid Salmonella. Paratyphoid Salmonella has several virulence genes include plasmid encoded fimbriae (pefA), hyper invasive locus (hilA), involved in adhesion and invasion [46]. In addition to, avrA an effector protein of type III secretion system (TTSS) complex, limiting the host’s inflammatory responses via induction of macrophage apoptosis [47] and invA, a conserved gene in all Salmonella spp. responsible for invasion [48] and located on SPI-I with hilA gene in all MDR Salmonella, indicating that Salmonella may exhibit several determinants to induce pathogenicity [49].

Our PCR result detected invA and hilA genes in all Salmonella serovars (100%) that matched with Lampel et al. [50], Shabnam and Kwai, [51], respectively, and Malorny et al. [52] for both genes, indicating the specificity of invA gene as Salmonella specific and virulence marker. The obtained results were matched with the traditional microbiological identification methods, confirming that invA and hilA PCR is rapid, sensitive and specific method for diagnosis of Salmonella infections in poultry.

The avrA gene was detected in 95 % of Salmonella serotypes by PCR, which was similar to Hopkins and Threlfall [53]. On the
other hand, Ben-Barak et al. [47] considered the high frequency of avrA gene was only in the most important Salmonella serovars. The obtained results found PefA gene in all Salmonella isolates (100 %), which was higher than Murugkar et al. [46](89 %).

**Conclusion**

The prevalence of paratyphoid Salmonella among the examined flocks at Sharkia Governorate was (32.6%) with the highest rate among 1-10 days old chicks. Serotyping revealed eleven different Salmonella serotypes with S. Typhimurium as the most prevalent serotypes. MDR was recorded in 49 % of the examined Salmonella isolates. The prevalence of invA, hilA and pefA genes were 100% and avrA (95%), indicating their importance as genus specific primers and thus can be used for rapid diagnosis of Salmonella infection in poultry.

**Conflict of interest**

The authors declared that they have no conflict of interest.

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المختص العربي

توصف سالمونيلا بارايتيفود المعزولة من دجاج التسمين بمحافظة الشرقية بمصر

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برزت سالمونيلا البارايتيفود كمشكلة عالمية للإنسان والدواجن. ولذلك قمنا في هذه الدراسة بالتحري عن تواجد والخصائص المصلية والجزئية ومقاومة المضادات الحيوية للسالمونيلا بارايتيفود المعزولة من قطعان الدجاج في محافظة الشرقية خلال الفترات 2015-2016. تم معدل الإصابة بسالمونيلا بارايتيفود بين 50 قطيعاً مشتبهاً به 32.6% و في الأعمار الصغرى كانت نسبة العزل أعلى بنسبة 84% (10-11 أيام) وأدنى معدل (12%) في الأعمار السابق سنًا (11-12 يوم). كان أعلى نسبة عزل من الكبد (34.77 %)، تلبي الحجل (33.72 %)، الأوعية (5.02 %) وكيس المطح (13.42%). كانت الأشجار المصيلة لـ 49 عزلة السالمونيلا عن 11 مجموعة مختلفة، وكان سالمونيلا تيفيموريوم هو الأكثر انتشاراً (44.18%)، سالمونيلا كتاكا (18.32%) و سالمونيلا أنتيردير (10.28%) وكانت المضادات الحيوية الأكثر حساسية هي الأقراص البنية (22 %) والسيبروفلوكاسين (86 %). كانت القدرات متعددة العقاير للأميسلين والختاميين و invA والسيروفلافونكسام في جميع عزلات السالمونيلا. جميع ميزولات السالمونيلا متعددة مقاومة العقاقير تحتوي على avrA بنسبة 100 % و pkfA و shiA بنسبة 95 % بواسطة تفاعل البلامرة المتسلسل، مما يؤكد أن هذه الجينات من جينات الصراوة المحددة والمهمة للتشخيص السريع لعذوى السالمونيل.