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Bacteriological and molecular studies on Salmonella isolated from duckling farms at Kaliobia, Egypt

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**ABSTRACT**

This study was conducted on 21 commercial duckling farms (1-20 days old) inspected to show Salmonella infection in different localities at Kaliobia Governorate. Samples were taken from diseased ducklings and freshly dead for bacteriological examination which resulted in, 94 samples were positive from 630 isolates, where 28 isolates from 33 diseased ducklings and 66 isolates from 72 freshly dead ducklings. Three serogroups of Salmonella were obtained by serological identification (Salmonella Typhimurium, Salmonella Enteritis and Salmonella Blegdam). The antibiotic sensitivity tests for the isolated strains showed multiple antibiotic resistances (oxytetracycline; amoxicillin; ampicillin; streptomycin; erythromycin and trimethoprim/sulphamethoxazol) but gentamycin, norfloxacin and ciprofloxacin are the most effective antibiotics on the isolated Salmonella and can be used for treatment of Salmonellosis in duck farms. PCR results appeared that, invA and stn genes were detected in all studied Salmonella isolates; pefA gene was detected in four out of five studied isolates but setC gene was detected in two isolates only. Finally, isolated Salmonellae are virulent pathogens responsible for disease in ducklings resulting in high mortality and morbidity, gentamycin, norfloxacin and ciprofloxacin are the most proper antibiotics used for treatment of Salmonellosis in duck farms.

1. INTRODUCTION

Salmonella infection is one of the most important duck diseases with a significant economic losses and public health importance as infected duck flocks are considered the most important reservoir of Salmonellae which can transmit it to human (Yang et al., 2019). They are Gram-negative, short plump shaped rods, non-spore forming, non-capsulated, aerobic and facultatively anaerobic organisms and classified under the family Enterobacteriaceae (Mondal et al., 2008a). One of the antigens classify Salmonella into serotypes is the “O” antigen determined based on oligosaccharides associated with lipopolysaccharide. Then the “H” antigen is determined based on flagellar proteins (H is short for the German Hauch meaning "breeze"). Since Salmonella typically exhibit phase variation between two motile phenotypes (Chiou et al., 2006), different “H” antigens may be expressed. Salmonella that can express only one “H” antigen phase consequently have motile and non-motile phenotypes and are termed monophasic. The emergence of antimicrobial resistance among Salmonella strains of poultry origin has important public health implications similar to food poisoning, such as diarrhea and acute gastroenteritis. Several studies resulted in Salmonella infections in human showing drug resistance were caused by strains from poultry (Mondal et al., 2008b; Husain, 2010).

It is caused chiefly by a bacterium *Salmonella Typhimurium* and to a less extent by other species of motile Salmonella. The virulence of Salmonella species is associated with a combination of chromosomal and plasmid factors (Oliveira et al., 2003). Salmonella produces both endotoxins and exotoxins (Zou et al., 2012). The endotoxin is lipid A of the outer membrane lipopolysaccharide (LPS) of Salmonella. The exotoxins are of two type viz., cytotoxins and enterotoxins. Differences in virulence among Salmonella serovars have been attributed to the variable acquisition and evolution of virulence genes (VanAsten and Van Dijk, 2005). Several Salmonella specific virulence genes which takes an important role in the pathogenicity have been identified, that are known to be

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involved in adhesion and invasion, like sefC; fimH; invA; pef (Murugkar et al., 2003; Singh et al., 2013; Akeem et al., 2017) and other genes associated with toxin production viz., stn (Marcus et al., 2000; Singh et al., 2013a). According the prevalence and characterization of Salmonella in ducks farms, Salmonellosis is one of the most important diseases facing duck industry in Egypt. Therefore, this study was conducted to throw light over the isolation, identification and characterization of Salmonella species in duckling farms at Kaliobia Governorate, which may provide beneficial information for the development of the duck industry and public health.

2. MATERIAL AND METHODS

2.1. Samples
A total of 21 commercial duckling farms (1-20 days old) were inspected for Salmonella infection from different localities at Kaliobia Governorate. Samples were taken from internal organs of 105 ducklings from different breeds; 33 diseased and 72 freshly dead ones of different breeds after clinical and postmortem examination. Each organ was taken alone in sterile plastic bags, kept in icebox and transferred with minimum delay to the laboratory.

2.2. Bacteriological examination
The surface of the examined organs was seared by hot spatula, small pieces of them were taken under aseptic condition and putted in sterile Stomacher bag with 45 ml sterile buffered peptone water, then prepared for bacteriological examination following (APHA, 2001).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5'→3')</th>
<th>Amplified segment (bp.)</th>
<th>Primary Denaturation</th>
<th>Amplification (25; 35 cycles)</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>invA</td>
<td>GTGAAATTTCGCCACCTC6GGC6AA</td>
<td>284 bp.</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>72°C 7 min.</td>
<td></td>
<td>Olsthoorn et al. (2003)</td>
</tr>
<tr>
<td>Stn</td>
<td>TTG TGT CGC TAT CAC TGG CCA CC</td>
<td>617 bp.</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>59°C 40 sec.</td>
<td>72°C 45 sec.</td>
<td>72°C 10 min.</td>
<td></td>
<td>Murugkar et al. (2003)</td>
</tr>
<tr>
<td>pefA</td>
<td>GTG TTC CGC GCT TGT GCT</td>
<td>700 bp.</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>93°C 30 sec.</td>
<td>45°C 45 sec.</td>
<td>72°C 10 min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sefC</td>
<td>GCC ACC AAA ACT CGG ACT GTA</td>
<td>1103 bp.</td>
<td>94°C 5 min.</td>
<td>94°C 60 sec.</td>
<td>94°C 60 sec.</td>
<td>72°C 60 sec.</td>
<td>72°C 10 min.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. RESULTS

1. Isolation of Salmonella
The recovered results in Table (2) cleared that, 94 from 630 samples (14.9%) were positive for Salmonella isolation, where 28 isolates (14.1%) were from internal organs of 33 diseased ducklings and 66 isolates (15.3%) from 72 freshly dead ducklings. Moreover, Salmonella spp. isolates were isolated mostly from 71 intestine samples (75.5%) succeeded by 9 from heart blood samples (9.6%) then 7 from liver samples (7.4%); 4 from lung samples (4.3%); 2 from spleen samples (2.1%) and 1 from kidney samples (1.1%).

2. Identification of Salmonella isolates
Biochemically, all 94 isolates had characteristic biochemical features as that of Salmonella, where, they were positive for Methyl red test; citrate utilization test; H2S production test; lysine iron agar and nitrate reduction test. Meanwhile, they were negative for indole; Voges-Proskauer; oxidase and urease tests. Moreover, the serological examination of five random Salmonella isolates Table (3) appeared that, they were serotyped as Salmonella Typhimurium (2/5); Salmonella Enteritidis (2/5) and Salmonella Bledgam (1/5).

2.2.1. Isolation and identification of Salmonella strains following ISO 6579 (2002) and Markey et al. (2013):
The suspected colonies were sub-cultured into nutrient agar plate and incubated at 37°C for 24 hours. Then, the purified colonies were identified morphologically by Gram stain then biochemically and serologically using Salmonella antiserum. Typical Salmonella colonies grown on XLD agar medium had a pink color with black center; while on MacConkey’s agar the colonies appeared as pale, colorless smooth and transparent; grey- reddish / pink and slightly convex colonies on Brilliant Green agar plate and pale color colonies indicated non-lactose fermenting with or without black centers on Salmonella- Shigella agar (DENKA SEIKEN Co., Japan) according to Kaufmann (1973) and Markey et al. (2013).

2.2.2. In-Vitro anti-microbial sensitivity test
In-Vitro sensitivity test was done on each isolated Salmonella isolates to study its antibiotic sensitivity using disc diffusion test according to the recommendation of the Clinical Laboratory and Standards Institute (CLSI, 2018).

2.2.3. Detection of virulence genes in Salmonella isolates by Polymerase Chain Reaction
Genotyping detection of invasion gene (invA); the heat-labile Salmonella enterotoxigenic (stn); plasmid encoded fimbiae (pefA) and S. Enteritidis fimbiae (sefC) genes, in five random Salmonella isolates using PCR, following QIAamp® DNA Mini Kit instructions (Qiagen, Germany, GmbH), Emerald Amp GT PCR mastermix (Takara, Japan) and 1.5% agarose gel electrophoreses (Sambrook et al., 1989) by using the Primers sequences, target genes, amplicons sizes and cycling conditions showed in Table (1).
intermediate sensitive to neomycin (68.1%) and doxycycline (67.0%). Moreover, they were highly resistant for oxytetracycline (87.2%) followed by amoxycillin (80.8%); ampicillin (79.8%); streptomycin (68.1%); erythromycin (61.7%) and trimethoprim/sulphamethoxazol (59.6%).

Table 2 Prevalence of isolated Salmonella from studied duckling samples

<table>
<thead>
<tr>
<th>Duckling cases</th>
<th>No. of ducklings</th>
<th>No. of samples</th>
<th>No. of Positive organ samples</th>
<th>Total Positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heart Blood</td>
<td>Lung</td>
</tr>
<tr>
<td>Diseased</td>
<td>33</td>
<td>198</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Freshly dead</td>
<td>72</td>
<td>432</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>TOTAL.</td>
<td>105</td>
<td>630</td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3 Serological typing of Salmonella isolates

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Serotyping Isolate</th>
<th>O Antigen</th>
<th>H Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Salmonella Typhimurium</td>
<td>B</td>
<td>1,4,12</td>
</tr>
<tr>
<td>2</td>
<td>Salmonella Enteritidis</td>
<td>D1</td>
<td>1,9,12</td>
</tr>
<tr>
<td>3</td>
<td>Salmonella Enteritidis</td>
<td>D1</td>
<td>1,9,12</td>
</tr>
<tr>
<td>4</td>
<td>Salmonella Blegdam</td>
<td>D1</td>
<td>9,12</td>
</tr>
<tr>
<td>5</td>
<td>Salmonella Typhimurium</td>
<td>B</td>
<td>1,4,12</td>
</tr>
</tbody>
</table>

Table 4 Anti-microbial Sensitivity test for isolated Salmonella in vitro

<table>
<thead>
<tr>
<th>Antimicrobial agents</th>
<th>Disk concentrations</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>25 µg</td>
<td>1</td>
<td>1.1</td>
<td>17</td>
<td>18.1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 µg</td>
<td>2</td>
<td>2.1</td>
<td>17</td>
<td>18.1</td>
</tr>
<tr>
<td>Ceftoxamine</td>
<td>30 µg</td>
<td>58</td>
<td>61.7</td>
<td>28</td>
<td>29.8</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5 µg</td>
<td>77</td>
<td>81.9</td>
<td>12</td>
<td>12.8</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>30 µg</td>
<td>17</td>
<td>18.1</td>
<td>63</td>
<td>67.0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>5 µg</td>
<td>72</td>
<td>76.6</td>
<td>16</td>
<td>17.0</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 µg</td>
<td>8</td>
<td>8.5</td>
<td>28</td>
<td>29.8</td>
</tr>
<tr>
<td>Flophencol</td>
<td>30 µg</td>
<td>72</td>
<td>76.6</td>
<td>14</td>
<td>14.9</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg</td>
<td>79</td>
<td>84.0</td>
<td>11</td>
<td>11.7</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30 µg</td>
<td>17</td>
<td>18.1</td>
<td>64</td>
<td>68.1</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>10 µg</td>
<td>79</td>
<td>84.0</td>
<td>14</td>
<td>14.9</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>30 µg</td>
<td>1</td>
<td>1.1</td>
<td>11</td>
<td>11.7</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S/10</td>
<td>3</td>
<td>3.2</td>
<td>27</td>
<td>28.7</td>
</tr>
<tr>
<td>Trimethoprim/ Sulphamethoxanol (1.25/23.75) mcg</td>
<td>20</td>
<td>21.3</td>
<td>18</td>
<td>19.1</td>
<td>56</td>
</tr>
</tbody>
</table>

No.: Number of isolates. AA: Antibiogram activity.

4. Detection of virulence genes

Five random Salmonella isolates were screened by PCR for the identification of virulence-associated genes (invA, stn, pefA and sefC) and all were positive for at least two of the screened genes. The recovered PCR results in Fig. (1) indicated that, invA gene was amplified in all Salmonella isolates giving product of 284 bp. and also, the stn gene was amplified in all five studied Salmonella isolates giving product of 617 bp. (Fig., 2).

Meanwhile, Fig. (2) showed that, the pefA gene was amplified in four out of five studied Salmonella isolates giving product of 700 bp. Moreover, the sefC gene was amplified in two out of five studied Salmonella isolates only giving product of 1103 bp. (Fig. 4).

4. DISCUSSION

The recovered results in Table (2) were nearly similar to those recorded by Ismail, Rehab(2013); Badr, Heba and Nasef, Soad (2016) and Rahman et al. (2016). Meanwhile, they disagree with those of Osman; Kamelia et al. (2013); Lebdah et al. (2017) and Enany et al. (2018) who isolated Salmonella species from internal organs of duck and duckling with lower incidence.
The obtained results indicated the acute nature of the disease and the predominant role of Salmonella in causing enteritis and death of ducklings. Moreover, the variation of isolation rates in different localities may be due to the prophylactic and therapeutic use of antibiotics, vaccination against viruses and immune status of ducklings or variation in degree of hygiene and overloading in the farms. Regarding to the phenotypic characters of isolated Salmonella, the colonial appearance and the biochemical profile were similar to those previously reported such as the fermentation of certain sugars or enzymatic reaction that might be characteristic of highly virulent strains associated with Salmonellosis (Markey et al., 2013; Rahman et al., 2016; Ahmed et al., 2019). Moreover, the serogroups obtained Table (3) came in consistent with those of Adzitey et al. (2012); Ismail, Rehab (2013); Osman, Kamelia et al. (2013); Lebdah et al. (2017), and Enany et al. (2018), who recorded the same serotypes from ducklings affected with Salmonellosis. The results of in-vitro antimicrobial sensitivity tests Table (4) were nearly similar to that reported by Adzitey et al. (2012); Ismail, Rehab (2013); Badr, Heba and Nasef, Soad (2016); Rahman et al. (2016) and Enany et al. (2018). High resistance of Salmonella isolates to oxytetracycline, amoxicillin, ampicillin, streptomycin and erythromycin in this finding, indicated that, they are multidrug-resistant strains and of clinical serious concern as these drugs are still considered the most recommended for the treatment of bacterial infections in duck farms in Egypt.

Polymerase chain reaction (PCR) is capable of identifying the pathogenic Salmonella isolates in duckling and duck farms (Gong et al., 2014; Lebdah et al., 2017; Yang et al., 2019). The invasin gene (invA) encodes a protein in the inner membrane of bacteria, which is necessary for invasion of the intestinal mucosa of the host (Singh et al., 2013a) and a common unique marker gene in all strains of Salmonella spp. (Liu et al., 2012). The results of invA gene amplification in Fig. (1) clarified that, they were Salmonella strains and PCR confirmed the conventional tests performed. Similar detection was recorded by Osman, Kamelia et al. (2014); Elghory, Amany et al. (2017) and Yang et al. (2019) who detected invA genes in all Salmonella serovars isolated from duckling and duck farms. Meanwhile, for the heat-labile Salmonella enterotoxin gene (stn) serve as effector proteins, which are involved in the pathogenesis of Salmonellosis and diarrhea (Murugkar et al., 2003; Singh et al., 2013a), the obtained results came in parallel with those of Ismail, Rehab (2013); Elghory, Amany et al. (2017); Lebdah et al. (2017) and Enany et al. (2018) who detected stn genes in all Salmonella serovars isolated from duckling and duck farms. Fimbriae play an important role in the pathogenicity of bacteria, because they promote their attachment to intestinal epithelial cells and encoded by the pef operon located in plasmid (Castilla et al., 2006). Similar findings of plasmid encoded fimbriae (pefA) gene (Fig. 3) in Salmonella strains isolated from duckling and duck farms were recorded by Gong et al. (2014) and Elghory, Amany et al. (2017). The fimbriae 14 (SEF14) of Salmonella Enteritidis is encoded by the sef operon, which contains sefC gene. It contains four major protein subunits SefA, SefB, SefC, and SefD. SEF14 plays important role in the ability of Salmonella to colonize Peyer’s patches and in the invasion and adhesion of epithelial cells of the host intestine (Castilla et al., 2006). The obtained results for PCR amplification of S. Enteritidis fimbriae (sefC) gene in Salmonella isolates (Fig., 11) appeared that, it was amplified in two Salmonella isolates only and as this gene is considered to be specific for serovar Salmonella Enteritidis (Murugkar et al., 2003), so, the two positive isolates were Salmonella Enteritidis strains. These results were similar to those obtained by Murugkar et al. (2003); Castilla et al. (2006); Das et al. (2012); Ammar et al. (2016) and Akeem et al., 2017), who detected sefC gene.

The obtained results indicated the acute nature of the disease and the predominant role of Salmonella in causing enteritis and death of ducklings. Moreover, the variation of isolation rates in different localities may be due to the prophylactic and therapeutic use of antibiotics, vaccination against viruses and immune status of ducklings or variation in degree of hygiene and overloading in the farms. Regarding to the phenotypic characters of isolated Salmonella, the colonial appearance and the biochemical profile were similar to those previously reported such as the fermentation of certain sugars or enzymatic reaction that might be characteristic of highly virulent strains associated with Salmonellosis (Markey et al., 2013; Rahman et al., 2016; Ahmed et al., 2019). Moreover, the serogroups obtained Table (3) came in consistent with those of Adzitey et al. (2012); Ismail, Rehab (2013); Osman, Kamelia et al. (2013); Lebdah et al. (2017), and Enany et al. (2018), who recorded the same serotypes from ducklings affected with Salmonellosis. The results of in-vitro antimicrobial sensitivity tests Table (4) were nearly similar to that reported by Adzitey et al. (2012); Ismail, Rehab (2013); Badr, Heba and Nasef, Soad (2016); Rahman et al. (2016) and Enany et al. (2018). High resistance of Salmonella isolates to oxytetracycline, amoxicillin, ampicillin, streptomycin and erythromycin in this finding, indicated that, they are multidrug-resistant strains and of clinical serious concern as these drugs are still considered the most recommended for the treatment of bacterial infections in duck farms in Egypt.

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in Salmonella Enteritidis strains isolated from different sources.

5. CONCLUSION

Finally, the present work concluded that Salmonella is a serious bacterial pathogen responsible for disease in ducklings resulting in high mortality and morbidity, as they are isolated with high percentages; multiple antibiotic resistances are widely spread among them, but gentamicin, norfloxacin and ciprofloxacin are the most effective against the isolated Salmonella in vitro and for treatment of Salmonellosis in duck farms. Moreover, all studied Salmonella by PCR were pathogenic as they had two virulence genes at least that play a role in pathogenicity and virulence of the Salmonella.

6. REFERENCES


