Serological and antibacterial characteristics of salmonella isolates from chickens in Assiut, Egypt

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ABSTRACT

Two hundred and twenty specimen from diseased and freshly dead chickens were gathered under complete aseptic conditions and transported to the laboratory of Faculty of Veterinary Medicine, Assiut University. Samples were processed for bacteriological and biochemical examinations. Suspected Salmonella isolates were serologically identified. Standard disc diffusion technique was used for antibacterial sensitivity testing of the isolated Salmonella. Minimum antibacterial inhibitory concentration (MIC) was also determined. 11.36% (25/220) out of the examined chicken samples were infected with Salmonella. The infection was more prevalent in two-weeks-old chickens (20%) than four-weeks-old (5%). Salmonella was frequent in liver and spleen (13.33%, 11.26%, respectively) than caecum and yolk sac (9.3%, 6.25%, respectively). The isolated Salmonellae were serotyped as S. Kentucky (27.77%), S. Enteritidis (22.22%), S. Typhimurium (16.66%), S. Molade (11.11%), while S. Inganda, S. Papuana, S. Wingrove and S. Larochelle encountered in the same percent (5.55%). These obtained isolates exhibited complete antibacterial resistance to amoxicillin and ampicillin (100%), but (88.88%) resistance to cefotaxime and oxytetracycline, (83.33%) to erythromycin, (72.22%) to doxycycline, (66.66%) to neomycin and (61.11%) resistance to amikacin. Somewhat, antibacterial sensitivity was noticed to colistin (55.55%), spectinomycin (44.44%) and norfloxacin (33.33%). Based on MIC, colistin and florfenicol were the most sensitive antibacterial at a lower MIC value (<2μg/ml).

1. INTRODUCTION

Over 125 years, salmonellosis has been known to cause great losses in poultry, animals, and human. Poultry are a primary Salmonella reservoir, as avian gut provides a diverse polymicrobial environment that could potentially provide selective pressure to alter the genetic composition of Salmonella in such a manner to better adapt to the poultry environment (Foley et al., 2013; CDC, 2017). Chicken salmonellosis either acute or chronic causes high financial losses in poultry industry due to high mortalities in young chicks and debilitating effect predisposing to other diseases (Crum et al., 2015). Salmonella belongs to the family Enterobacteriaceae and divided into 51 serogroups which collectively have 2600 serotypes (Gal-Mor, 2018). Chicken salmonellosis is manifested in 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 (Swayne, 2020). 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 (Gast and Beard, 1992). Flagella, capsule, plasmids, adhesion systems, toxins, and type 3 secretion systems (T3SS) play important role in Salmonella pathogenicity (Boko et al., 2013). Salmonella infection can be detected via bacterial isolation, identification, serological tests (Hendriksen, 2003) and polymerase chain reaction (PCR) which is more accurate especially in case of rough strain lacking O-antigen (Roy et al., 2002). Efforts to reduce Salmonella include guidelines adoption for antibiotic misuse, continuous antibacterial susceptibility surveillance to identify the changing pattern of Salmonella resistance (Kumar et al., 2012). Therefore, this study aimed at investigating the incidence of Salmonellae spp. in freshly dead and diseased broiler chickens, serological identification of Salmonella serotypes and studying the antimicrobial susceptibility of the prevalent Salmonella isolates to different antimicrobial drugs.

2. MATERIAL AND METHODS

2.1. Sampling:

220 specimens (liver, spleen, caecum, and yolk sac) from diseased and freshly dead chickens aging 1-40-days old were gathered under complete aseptic conditions from different diagnostic labs and farms in Assiut, Egypt, during December 2019 to May 2020, and transported to the laboratory of Faculty of Veterinary Medicine, Assiut University.
2.2. Bacteriological and biochemical examinations:

Under complete aseptic conditions, swabs from each organ were inoculated separately into Rappaport-Vassiliadis broth (Salmonella selective broth- HiMEDia, India) and incubated at 42°C for 24 hours. Then, a loopfull from the inoculated broth was streaked onto Xylose Lysine Deoxycholate (XLD) agar (Salmonella selective medium- HiMEDia/India) and incubated at 37°C for 24 hours. The Salmonella suspected colonies were identified morphologically and biochemically according to Quinn et al. (2002), colonies were sub-cultured on MacConkey’s agar plates to exclude any lactose fermenter coliforms and stained with gram’s stain for microscopical examination (Jawetz et al., 2007; WHO, 2010). The purified suspected Salmonella colonies were biochemically identified (urea hydrolysis, triple sugar iron agar, lactose fermentation, indole, catalase, and oxidase test) according to Barrow and Feltham, (1993) and MacFaddin, (2000).

2.3. Serological examination:

Isolates preliminarily identified as Salmonella isolates were serologically identified according to Kauffman – White scheme (Kauffman, 1974) through determination of their Somatic (O) and flagellar (H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan).

2.4. Determining antibiotic sensitivity pattern of the isolated Salmonella:

Standard disc diffusion technique was used for in-vitro antibacterial sensitivity testing of the isolated Salmonella to 15 antibacterial agents according to Bauer et al. (1966) following Clinical Laboratory Standards Institute (NCCLS, 1999; CLSI, 2010; CLSI, 2018) Table 1, as the following:

2.4.1. Bacterial inoculum preparation:

Three to five well isolated colonies were suspended in 5 ml of brain heart infusion broth and incubated at 37°C for 24 hours.

2.4.2. Streaking of test plates:

1. About 2 ml of the broth culture were transferred into the plate and the plate rotated approximately 60° each time to ensure of well distribution of the inoculum.

2. All excess fluids were removed using sterile pipette and leave the plate to dry for 15-20 min. at room temperature before the application of the antimicrobial discs.

3. The antimicrobial discs were dispensed onto the surface of the inoculated agar plate and each disc has been pressed gently to ensure complete contact with the agar surface by using sterilized forceps.

4. The plates were inverted then placed in an incubator at 37°C for 24 hours.

5. The diameter of growth inhibition zones was measured by using the transparent ruler.

6. The sensitivity and the resistance were determined by the criteria of the Clinical and Laboratory Standard Institute (CLSI, 2010; CLSI, 2018).

2.5. Determining minimum antibacterial inhibitory concentration (MIC) to Salmonella isolates:

The antibacterial effect of Neomycin, Spectinomycin, Streptomycin, Erythromycin, Florfenicol, Colistin, Sulphaquinoxaline, and Chlortetracycline (Table 2) was checked in 96 wells microtiter plate using double fold micro-dilution method against all obtained Salmonella serovars in a density of 10^5 CFU (CLSI, 2018). The concentration of each antibacterial was 10μg/mL, 2.56μl of each antibacterial was added in 2 wells of the first row of plate then 100μl broth with bacteria was added to first row of plate (wells of antibacterial) and 50 μl broth with bacteria was added to the rest of wells. Two-fold serial dilution technique was made and discard the last 50μl. The bacterial inoculum broth was taken as a positive control and another broth without bacterial inoculum was considered as a negative control. The microtiter plates were incubated at 37°C for 24 hours and examined for the lowest concentration showing no detectable growth (MIC).

### Tables:

#### Table 1: Antimicrobial used in MIC.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>10</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>10</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>10</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>10</td>
</tr>
<tr>
<td>Colistin</td>
<td>10</td>
</tr>
<tr>
<td>Sulphaquinoxaline</td>
<td>10</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30</td>
</tr>
<tr>
<td>Colistin</td>
<td>30</td>
</tr>
<tr>
<td>Sulphaquinoxaline</td>
<td>30</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
</tr>
</tbody>
</table>

#### Table 2: Antimicrobial used in disc diffusion test.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Symbol</th>
<th>Conc. (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>AK</td>
<td>30</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GN</td>
<td>10</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>S</td>
<td>10</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>SH</td>
<td>100</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Neo</td>
<td>10</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>CTX</td>
<td>30</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CIP</td>
<td>5</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>NOR</td>
<td>10</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>E</td>
<td>15</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C</td>
<td>30</td>
</tr>
<tr>
<td>Colistin</td>
<td>CL</td>
<td>10</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>AMP</td>
<td>10</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>AM</td>
<td>10</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>DO</td>
<td>30</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>OT</td>
<td>30</td>
</tr>
</tbody>
</table>

### 3. RESULTS

The birds were examined for clinical signs and postmortem lesions. The common encountered signs in the checked birds were retarded growth, depression, lameness, ruffled feathers, huddling, respiratory troubles, whitish watery diarrhea, and dirty vent. Septicemia, bronzy discolored enlarged liver with necrotic foci, splenomegaly with necrotic foci, congested kidneys, enteritis and typhlitis with chalky cecal core, and unabsorbed yolk sac in young chicks were the main postmortem lesions. (Fig. 1,2)
22-28 days old, and 5% (2 / 40) in 29 days old till slaughtering age (Fig.3).
Regarding internal organs liver, spleen, cecum, and yolk sac showed 13.33%, 11.26%, 9.3%, and 6.25% Salmonella recovery rates, respectively (Fig. 4).

The produced colonies on XLD agar were typical reddish with black center, white to white grey, small, smooth, rounded on Nutrient agar. Fig. 5a, b The gram-stained films from these colonies showed gram-negative bacilli.

Biochemically the isolated suspected Salmonella were catalase positive, urease, indole, and oxidase negative, and produced red butt yellow slant and H₂S on TSI agar. (Table 3).

The biochemically identified Salmonella isolates were serotyped according to Kauffmann – White scheme as S. Kentucky (27.77%), S. Enteritidis (22.22%), S. Typhimurium (16.66%), S. Molade (11.11%) while S. Inganda, S. Papuana, S. Wingrove and S. Larochelle encountered in the same percent (5.55%) as shown in Fig. 6 and Table 4.

In-vitro antibacterial susceptibility of the isolated Salmonellae:
All the obtained Salmonella serotypes were resistant to most tested antibacterial. Complete resistance (100%) to amoxicillin and ampicillin was observed. The resistance rates were (88.88%) to cefotaxime and oxytetracycline, (83.33%) to erythromycin, (72.22%) to doxycycline, (66.66%) to neomycin and (61.11%) to amikacin. Sensitivity rates (55.55%, 44.44%, and 33.33%) to colistin, spectinomycin, and norfl oxacin, respectively, as presented in Fig. 7 and Table 5.

All the obtained isolates showed multidrug resistance (MDR) as they exhibited resistance to at least 5 antibacterial while 83.3% of isolates were resistant to 8 spectinomycin, and norfloxacin, respectively. as presented in Fig. 7 and Table 5.

Minimum antibacterial inhibitory concentration:
Basing on MIC test for the tested antibacterial, 14 Sulpha quinoxaline and 7 neomycin resistant isolates showed very high (MIC) value (>256μg/ml), 12 spectinomycin, 10 florfenicol, and 7 colistin resistant isolates showed high MIC values (>128 μg/ml), 16 streptomycin resistant isolates showed 4–8fold increase in MIC compared to the CLSI resistance breakpoint of 16 μg/ml. All erythromycin resistant isolates showed 4–10 fold increase in MIC compared to CLSI resistance breakpoint (>
8μg/ml). 15 chlorotetracycline resistant isolates showed 2–8 fold increase in MIC compared to CLSI resistance breakpoint (> 16μg/ml). 9 and 3 isolates showed sensitivity to colistin and florfenicol respectively at a lower MIC value (<2μg/ml) as illustrated in Table 7.

4. DISCUSSION

Despite being old problem, salmonellosis still very interesting and worth pursuing. Salmonella particularly paratyphoid causes worldwide significant economic and public health losses particularly in developing countries due to its relevant implications in poultry industry and human. The extensive irrational use of antibiotics and chemotherapeutics to prevent and control bacterial diseases led to development of drug-resistant bacteria that became increasingly difficult to be controlled and eradicated. So, this study investigated chickens Salmonella infection in Assiut, Egypt, and determining the serological and antibacterial characteristics of isolated Salmonellae. Depending on conventional bacteriological examination and biochemical identification, from 220 broilers chickens’ samples, 25 (11.36%) were infected with Salmonella. These results indicate existence of Salmonella in commercial chickens although the extensive use of antibiotics particularly at the first days of chicken’s life and need to be ascertained in large scale studies. Similarly, Kaoud et al. (2018) observed average salmonellosis prevalence 11.33 % in open broiler houses in Egypt. Conversely, lower infection rates were obtained by Ibrahim et al. (2018) 6.8% in broilers in Beheira Province, Egypt and Sedek et al. (2019) 7.5% in newly hatched chicks in five different governorates in Egypt (El-Gharbia, Kafr Elshiekh, El-Beheira, Alexandria and Matrouh).

Higher infection rates were documented by Abdeen et al. (2018) 14.3% in morbid Fayoumi chickens in Menoufia Governorate, Elkenany et al. (2019) 28.6% in live and dead broiler chickens in Sharkia Governorate, Egypt and El-Sharkawy (2018) 16% in Baladi and broiler chickens. The variation in the percentage of Salmonella detection among chicken species could be explained by different factors including management, biosecurity and prophylactic antibacterial used in each circumstance, different samples type, and different diagnostic techniques. Low biosecurity measures inside farms allow possibility of disease transmission via different reservoirs and workers in farms (El-Sharkawy et al., 2017).
In this study the Salmonella recovery rate was relatively higher in 2-weeks old chickens (20%) than those >4-weeks old (5%). This can be explained by that Salmonella intestinal colonization, invasion to internal organs, and persistence in colonized tissues are all higher in newly hatched chicks than in older birds due to their acquisition of protective microflora that either competes with Salmonella for intestinal receptors or produces antagonistic factors (Al-baqir et al., 2019; Swayne, 2020). These findings are in accordance with those of Al-baqir et al. (2019).

Focusing on body organs, the present study depended on investigation of Salmonella in more than one organ (liver, spleen, cecum, and yolk sacs). The liver showed the highest Salmonella recovery rate (13.33%), followed by spleen (11.26%) and cecum (9.3%) while the yolk sac revealed the lowest infection rate (6.25%). This denotes Salmonella ability to survive and multiply in internal organs with various frequency, especially liver and spleen as these areas provide sites where bacterial multiplication can occur without exposure to host defense mechanisms (Gast, 2003).

Coming along with our results Al-baqir et al. (2019) recorded the highest Salmonella recovery percent in liver (30.66%) followed by spleen (25.33%) cecum (20%) and yolk sac (15.7%).

The isolated Salmonellae were serotyped into 8 serotypes. S. Kentucky and S. Enteritidis were the most encountered serotypes, followed by S. Typhimurium and S. Molade. S. Inganda, S. Papuana, S. Wingrove and S. Larocheille were the least frequent and encountered in the same percent (Figure 6). This high variation in the prevalent salmonella serotypes can denote substantial burden on poultry industry particularly the high frequency of the S. Kentucky, S. Enteritidis, and S. Typhimurium that represent high public health significance.

These results were closely agreed with Awad et al. (2020) who reported the predominance of S. Kentucky (22.6%), S. Enteritidis (22.6%) and S. Typhimurium (19.4%) serovars in Egypt. Other previous studies recorded different results, Im et al., (2015) documented S. Bareilly as the most prevalent Salmonella serovars in the flocks (41.2%), followed by S. Mbandaka (32.4%) and S. Rissen (17.6%). Abd El-Tawab et al. (2015) demonstrated low S. Papuana, S. Takoradi, S. Labadi, and S. Angers incidence (2.3% each) in different Governorates in Egypt. Sedeik et al. (2019) found the most prevalent serovar detected in Egypt was S. Enteritidis 2.4% followed by S. Virchow 1.4%, S. Typhimurium 1.2%, and S. Kentucky 0.8%.

Generally, the frequencies of serovar isolation varied from location to another due to different management and hygienic regimes as well as environmental and individual differences as mentioned by Kirn et al. (1991).

It is evident from this work that all isolated Salmonella serotypes belong to serogroup (C3) and (D1) with large percent then (B), (C1) and (C2) of Kauffman white scheme. These results are like Al-Nakhli et al. (1999) who mentioned that 90% of Salmonella serotypes infecting both poultry and human are belonged to serogroup B,C,D and E. To antibacterial susceptibility, the dissemination of multidrug resistance MDR in Salmonella spp. became a major concern in poultry production. Certain Salmonella serovars were found to have a greater ability to acquire MDR than others.

The antibacterial susceptibility pattern of the isolated Salmonella serovars in this study was determined against the commonly used antibacterial in Egyptian poultry farms. All isolates (100%) exhibited resistance to at least 5 antibacterial, while 83.3 % of them showed resistance to 8-14 antibacterial. This is not surprising because these antibacterial are commonly used in humans and poultry. Moreover, the antibacterial 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 humans strains through food chain leading to emergence of multidrug resistance Salmonella (Cox et al., 2003).

The present results closely coincided with Abdeen et al. (2018) who revealed that all tested Salmonella isolates (100%) were MDR to five classes of antibacterial and higher than Elkenany et al. (2019) who revealed that MDR against three or more antibacterial in 76.7% of isolates. Sharma et al. (2019) showed that all isolates were MDR and 92.86% of these isolates showing resistance to 5 or more classes of antibacterial on disk diffusion.

All the examined Salmonella serovars in the current study were completely resistant (100%) to ampicillin and amoxicillin indicating the limited therapeutic value of these antibacterial to poultry, this may be due to their wrong and frequent use in the field. Higher rates of resistance were observed to cefotaxime, erythromycin, oxytetracycline, doxycycline, aminoglycosides, chloramphenicol, neomycin, ciprofloxacin and streptomycin. While rates of intermediate sensitivity were observed to colistin, spectinomycin and norfloxacin.

Interestingly S. Papuana was resistant to (14) antibacterial, S. Larocheille was resistant to (12) antibacterial. All isolates showed sensitivity to colistin except one S. Kentucky isolate was resistant.

These results were in a partial accordance with Tarabees et al. (2017) who stated that S. Typhimurium isolates were resistant to ampicillin, amoxicillin, penicillin, neomycin, ofloxacin, doxycycline, and chloramphenicol and higher than Moawad et al. (2017) who showed that most of the S.enterica isolates showed resistance to ampicillin (87.0%) and cefotaxime (80.0%) and Awad et al. (2020) who reported that the isolates collected from chicken carcasses in Mansoura, Dakahlia Province show resistance to Erythromycin 96.78%, Doxycycline 93.55%, Streptomycin 80.65% and Amoxicillin 67.8%.

On the other hand, our data showed contrast to the data obtained by Helal et al. (2019) who revealed that the examined Salmonella serovars were highly sensitive to Doxycycline, Chloramphenicol, followed by Amoxicillin, Ampicillin, Gentamycin and Sulphamethoxazole+ Trimethoprim. Elkenany et al. (2019) Who reported that Salmonella serovars were highly susceptible to Ciprofloxacin, Ceftaxime, Chloramphenicol and Ezzat et al. (2019) who mentioned that all strains were sensitive to amikacin and norfloxacin (100%) which was the most effective chemotherapeutic agent against Salmonella infection.

Moreover, 14 sulphaquinoxaline and 7 neomycin resistant isolates showed very high MIC values (> 256μg/ml). 12 spectinomycin, 10 florfenicol and 7 colistin resistant isolates showed high MIC values (>128μg/ml). All erythromycin resistant isolates showed 6–10 folds increase in MICs compared to CLSI resistance breakpoint (> 8μg/ml). 16 streptomycin resistant isolates showed 4–8 folds increase in MIC compared to the CLSI resistance breakpoints of 16μg/ml. 15 chloramphenicol resistant isolates showed 2–8 folds increase in MICs compared to CLSI resistance breakpoint (> 16μg/ml). While 9 and 3 isolates showed sensitivity to colistin and florfenicol, respectively at a lower MIC value (<2μg/ml). This may be due to colistin competitively displaces divalent cations.
(Ca²⁺ and Mg²⁺) from the phosphate groups of membrane lipids, which leads to disruption of the outer cell membrane, leakage of intracellular contents and bacterial death.

5. CONCLUSION

In conclusion, the data of the present study revealed the variation of Salmonella serovars recovered from chicken. These serovars expressed variable degrees of resistance to the available antibacterial used for the treatment of invasive Salmonellosis alarming the importance of the situation. This requires regular monitoring of the isolated Salmonella for their antibacterial susceptibility especially that of zoonotic importance and finding strict national plan for complete elimination of this disease. Therapeutic agents must not be given before microbial sensitivity testing and it’s advisable not to use the therapeutic antibacterial as a preventive feed supplement. Further investigations are important to track the emergence of new Salmonella serovars and the frequencies and patterns of its antibacterial resistance.

The higher MDR value observed in this study might be attributed to the widespread use of antibacterial in the locality of our study in Egypt. So, regular testing of the prevalent Salmonella isolates in poultry for their antibacterial susceptibility is required to avoid transmission of these serovars to human food chains and possibility of increasing in their pathologic potential to poultry. The current findings alert for seeking alternative safe ways like herbal extract, probiotic, and prebiotic to combat Salmonellosis instead of the traditional insensitive therapeutics.

6. REFERENCES


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