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Research Article

Salmonella Enteritidis in Broiler Chickens: Isolation, Antibiotic Resistance Phenotyping and Efficacy of Colistin on Control of Experimental Infection

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ABSTRACT

Out of 400 examined samples 45 suspected Salmonella isolates (11.25%) were obtained 19 (9.5%) out of apparently healthy and 26 (13%) from diseased chickens. Intestinal samples had more isolates (29, 14.5%) more than liver (16, 8%). Identified *S.Enteritidis* from suspected salmonella was 16/45 (35.6%) with a rate of 8% out of the examined 400 samples, 6 (3.0%) out of apparently healthy and 10 (5.0%) from diseased chickens. Intestinal samples had more isolates (11, 5.5%) than liver (5, 2.5%). The Antibiotics susceptibility profile of *S. Enteritidis* isolates revealed 100% resistance to trimethoprim/sulfamethoxazole, followed by oxacillin (62.5%), 56.3% for each of ampicillin, clindamycin, enrofloxacin and doxycycline, 50% for chloramphenicol, 43.8% for streptomycin, 37.5% to cephalosporins and 18.8% for colistin. Tested *S. Enteritidis* isolates are classified into 11 profiles and are resistant to two - nine antibiotic classes with resistant index 0.2- 0.9. Only two isolates are NDR (12.5%), most of isolates 10/16 (62.5%) are MDR and 25% are EDR to 8-9 antibiotics. Clinical signs in experimentally infected chickens appeared at 2nd dpi, mortality started at the 4th to reach 27.5% in infected nontreated and 5% in colistin treated. Signs and lesions were markedly severe in infected nontreated than treated. *S. Enteritidis was* re-isolated from dead infected birds. *S. Enteritidis* intestinal count in sacrificed infected nontreated was higher than treated. Colistin treated group showed higher FCR, EEF and CV% (1.52, 402.8 and 6.12%) than infected non-treated (1.73, 222.6 and 14.83%). It could be concluded that *S. Enteritidis* is prevalent in broiler chicken flocks. Most of the isolates are MDR. Experimental infection of broiler with *S. Enteritidis* field isolates resulted in high mortality and the addition of colistin sulphate in drinking water controlled the infection and restores the productivity of infected broiler chickens.

Key words: Broiler, *S. Enteritidis*, isolation, Multidrug resistance, Experimental infection, Colistin.

INTRODUCTION

Bacterial poultry diseases cause severe economic losses in poultry industry, one of these diseases is *Salmonella Enteritidis* (*S.Enteritidis)*, which is induced Salmonellosis in poultry and food poisoning in human (Akinyemi *et al.*, 2007). *S. Enteritidis* is one out of approximately 10-20 serovars of group 2 of genus Salmonella of the family Enterobacteriaceae. This group consists of non-host adapted and invasive serovars that are able to cause an invasive infection in poultry and may be capable of infecting humans (Hafez, 2001).

S. Enteritidis in poultry causes serious economic losses due to increase mortality rate (4-50%) and decrease in egg production worldwide (Shittu *et al.*, 2014). In broiler chickens *S. Enteritidis* causes variable mortality 20-96% (Barrow, 1991) especially in vertical transmitted chicks, characteristic pericarditis with necrotic foci and petechial hemorrhage on liver (O'Brien, 1988). During the last few years over 85% of broiler chickens infections was caused by *S. Enteritidis* from products of poultry origin (Altekruse *et al.*, 2006). Organism may cause systemic infection in chicks and laying hens accompanied by prolonged fecal shedding. Some variations in the mortality rates, clinical symptoms, fecal shedding and frequency of production of contaminated eggs were observed in the chicks and hens experimentally infected with S. *Enteritidis* isolates (Suzuki, 1994).

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Persistent environmental contamination of poultry houses is an important factor in the maintenance of *S. Enteritidis* and other salmonellas in poultry flocks (Baggesen *et al.,* 1992). Moreover, it was found that Salmonellas persist in dry livestock buildings for many months (Bale *et al.,* 1993). Soliman *et al.* (2018) reported that incidence rate of salmonella (20.24%) was highly reported on 5th week age out of them 56% was *S. Enteritidis*, and salmonella infection still prevalent in poultry farms in Egypt.

Reducing *S. Enteritidis* in chicken intestinal tract could reduce contamination of poultry products (Altekruse *et al.,* 1993), therefore, antibiotic treatment reduced the prevalence of infection S. Enteritidis but did not eliminate the organism (Seuna *et al.,* 1980), while antibiotic followed competitive exclusion shown to be effective in eliminating Salmonella from chickens (Reynolds *et al.,* 1997).

The goal of antimicrobial susceptibility testing of bacterial isolates is to detect possible drug resistance and to assure there susceptibility to drugs of choice for particular infections (Jorgensen and Ferraro, 2009). The isolates had multiple antibiotic resistance (MAR) index ≥ 0.3 indicated that these isolates were multidrug resistant (resistant to 3 or 4 class of antibiotics), expanded drug resistant (resistant to ≥5 antibiotics classes). The most resistance patterns observed among the MDR isolates suggesting that this resistant isolate originated from a high-risk source of contamination where antibiotics are often used or that a large proportion of the bacterial isolates have been pre-exposed to several antibiotics (Christopher *et al.***,** 2013).

Colistin sulphate is one of used antibiotics to control *S. Enteritidis* infection in broilers. It was reported that colistin decreases the rate of infection and contamination of carcasses together with improving live weight gain by 14% and the feed conversion rate by 8% (Fard, 2004). Unfortunately this antibiotic showed multi-drug resistance nowadays together with antimicrobial genetic elements that can be exchanged between intestinal bacteria (da Costa *et al*., 2013).

The objective of the present study was to isolate *S.Enteritidis* from apparent healthy and diseased broiler chickens at marketing age (35-42 days), detection of their antibiotic resistance phenotype, and study the ability of colistin sulphate in control of *S. Enteritidis* in experimentally infected broiler chickens.

MATERIALS AND METHODS

Samples

A total of 400 aseptically collected samples (1 gram from livers and 1 gm intestinal content close to the diverticulum) representing 100 apparent healthy and 100 diseased broiler chickens showing diarrhoea and respiratory distress at 35 to 42 day old age. Collected samples were transported to our laboratory under cooling for bacterial isolation.

Isolation of Salmonella

Both liver homogenate and intestinal content (1 ml) was aseptically added to 10 ml of Rappaport-Vassiliadis (RV) broth and incubated at 42°C for 24 hs. The broths were subcultured on xylose-lysine-desoxycholate (XLD) agar (Oxoid Limited, UK) and aerobic incubated at 35ºC for 24 h. Red to pink colonies with black center were picked and kept in 25% glycerol broth at -80ºC for further identification.

Identification of Isolates

The obtained isolates were identified and characterized on the basis their colonial morphological, Gram's stained for morphological and stain character and biochemical properties (Forbes *et al.*, 2002; Greenwood *et al.,* 2005). Biochemical characterization was done on the basis API identification kits (API System, France) were analyzed using Bergey's manual of systematic bacteriology (Sneath *et al*., 1986).

Serological identification

The serogroup characterization of isolates was performed via slide-agglutination test according to Quinn *et al.* (2002). Diagnostic poly and monovalent *Salmonella* O and H antisera were used for serological identification of the obtained *S. Enteritidis* isolates **(***Salmonella* diagnostic antisera, PRO-LAB, 3 Bassendale Road, Bromborough, Wirral, Merseyside, CH62 3QL, UK. Vision antisera are prepared for use in serological identification of organisms belonging to the genus Salmonella according to Kauffmann White classification (Kauffmann, 2001).

In vitro Antimicrobial susceptibility test

The antibiotics susceptibility phenotype or profile of the identified of *S. Enteritidis* isolates were determined using disc agar diffusion (DAD) technique (CLSI, 2016). Separate and similar colonies on solid media plate were emulsified in 2 ml of normal saline and the turbidity was adjusted to McFarland standard tube 0.5. All isolates were tested for resistance to 10 antimicrobials including: ampicillin 10 µg (AMP), calindamycin 2 µg (DA), cephalosporins 30 µg (CVN 30), colistin 10 µg (CT), chloramphenicol 30 µg (C30), doxycycline 5 µg (DO), enrofloxacin 5 µg (ENR), oxacillin 30 µg (OX), streptomycin 10 µg(S), trimethoprim/sulfamethoxazole 1:19 25µg (STX); representing ten antimicrobial classes. Antibiotic sensitivity disc were obtained from Oxoid Limited, UK. Sensitivity and resistance were determined according to CLSI (2016). *E. coli* laboratory isolate susceptible to all of the antibiotics, was used for control.

Determination of multiple antibiotic resistances index

Isolate MAR index was determined by using the formula MAR= The number of antibiotics to which the test isolate depicted resistance/Total number of antibiotics to which the test isolate has been evaluated for susceptibility (Paul *et al.,* 1997). For interpretation of antimicrobial susceptibility, narrow drug resistance (NDR) isolates have index <0.3, isolates have index ≥0.3-0.7 are multidrug resistant (resistant to 3 or 7 class of antibiotics) are MAR or extensively drug-resistant (XDR) bacterial isolates resistant to 8 or 9 antibiotics classes and pan drug resistant (PDR) isolates non susceptible to all agents in all antimicrobial categories (Christopher *et al.,* 2013).

Chickens

A total 130, 1-day-old Cobb broiler chicks as hatched; ten chicks were sacrificed and examined bacteriologically to prove their freedom from *S. Enteritidis* infection. The birds were kept in cleaned and disinfected separate cages and given feed and water ad libitum till the age of 21 days. All birds were vaccinated using live Hitchner B_1+ IB and La Sota vaccine strains via eye drop at 5 and 16 days of age, respectively. Infectious bursal disease live 228 E was given in drinking water at 14 days.

Ration

Commercial starter and grower broiler chicken ration were given till 21 and 32 days of age, respectively. The used commercial balanced ration based on yellow corn and soya bean according breed requirements.

Colistin sulphate

Colistin6M®: each gm contains 6000.000 IU Colistin sulphate. Lot No. 150415. Colistin6M®is produced by Jordan Vet and Agr Med. Ind. Co – Amman – Jordan. It was used in drinking water in dose of 0.5gm/lit for 5 successive days.

Experimental infection

The inoculum was prepared according to the method of Timms *et al.* (1990). At 21 days of age, each bird in the experimentally infected groups was inoculated orally with 0.5 ml/ containing 10^9 CFU/ml *S. Enteritidis* (Okamoto *et al.*, 2007).

Re-isolation of *S. Enteritidis*

a. Dead birds post challenge was collected and the liver, heart, spleen and caecum were used for *S. Enteritidis* re-isolation. Samples were inoculated into RV broth, incubated at 37°C for 24 hr, streaked onto XLD agar and incubated at 37°C for 24 hr. Suspected colonies were identified morphologically and biochemically.

b. At 3, 5 and 7 days after treatment 2 birds were sacrificed. Equal amount (about 1 gm) from duodenum, middle part close to the diverticulum and caecum were collected and mixed. One gram from each sample was subjected to total colony count on XLD agar plate containing streptomycin 10 mg/ ml.

Experimental design

A total number of 120, 21-day chicks divided into 3 equal groups, 40 chicks in each. Chicks of groups 1 and 2 were infected by gavage into the crop, each by 1.5 ml containing 10^9 CFU/ml *S. Enteritidis.* Group 3 was kept as negative non-infected non-treated. All chicken groups were daily observed for clinical signs, mortalities and gross lesions in dead birds. Colistin sulphate (0.5gm/lit) was given to group 1 start from 3nd day post infection (dpi); with the appearance of clinical signs and mortality; for 5 successive days. Samples from dead birds including liver, heart, spleen and cecum were aseptically collected from each group post infection for *S. Enteritidis* re-isolation. Intestinal content at 3, 5 and 7 day from medication were aseptically collected for recording total *S. Enteritidis* count. Total feed intake (TFI), and average body weight gain (BWG) were recorded for calculation of FCR with calculation of European efficacy factor (EEF) and production CV% at the 5th week of age.

Production parameters

FCR, livability, EEF and CV% were calculated for the same group birds during a given period (including weight gain of birds which died during the given period) according to Sainsbury (1984).

RESULTS AND DISCUSSION

In the present study, out of 400 examined samples 45 suspected isolates (11.25%) were obtained 19 (9.5%) out of apparent healthy and 26 (13%) from diseased chicken (Table 1). similar isolation rate of salmonella from chicken was 14% (Rabie *et al.*, 2012). Intestinal samples had more isolates (29, 14.5%) more than liver (16, 8%) (Table 2). The highest incidence of *Salmonella* isolation was from the intestine than liver agree with those previously reported by Menghistu *et al.* (2011). Also, Gong *et al.* (2014) reported that prevalence of *Salmonella* sp. in rectal swab samples were 9.8% in chicken examined farms**.** Fasure *et al.* (2012) reported isolation of salmonella (12.5%) from human and broilers. *Salmonella* strains were isolated 38% of the screened 5-week-old broiler flocks (El-Sharkawy *et al.,* 2017). Similar to our incidence of salmonella from liver was 12% (Mohamed (1998), while, Putturu *et al.* (2012) reported higher (50%) rate of salmonella isolation from liver.

Regarding the identified *S. Enteritidis* out of the examined samples (Table 1) 16 isolates (8.0%) were obtained 6 (3.0%) out of apparent healthy and 10 (5.0%) from diseased (Table 1). Rate of identified *S. Enteritidis* from salmonella isolates were 16/45 (35.6%). Our results are close to those reported by Fasure *et al.* (2012) 6.25% and lower than previously reported the incidence of *S. Enteritidis* out the examined samples as 40% (Abdallah, 1995), 81.5% (Carli *et al.,* (2001), 52.9% (Marin *et al.,* 2011), 37.25% (Abd El-Ghany *et al.,* 2012a), and 58.33% (Rabie *et al.,* 2012). Apparent healthy and diseased intestinal samples had *S. Enteritidis* isolates (11, 5.5%) more than liver (5, 2.5%) (Table 2). Similar results were obtained by El-Sharkawy *et al.* (2017) who reported that *S. Enteritidis* was 2 (0.98%) from liver and 1 (0.49%) from intestine.

Antibiotics susceptibility profile (Table 3) of *S. Enteritidis* isolates revealed 100% resistance to Trimethoprim/Sulfamethoxazole, followed by Oxacillin (62.5%), 56.3% for each of ampicillin, Clindamycin, Enrofloxacin and Doxycycline, 50% for Chloramphenicol, 43.8% for Streptomycin, 37.5% to Cephalosporins and lowest percentagefor Colistin(18.8%). Previous worker reported similar resistant results, 31.25%to ciprofloxacin and 87.5% to amoxicillin (Aditya, 2015); 100% to ampicillin and 90.6% to tetracycline (Fasure *et al.,* 2012); 82.2% to ampicillin, 80% to tetracycline and 54.2% to chloramphenicol (Asif *et al.*, 2017). Also, 65.6% of clinical isolates were resistant to ampicillin and tetracycline (Mezal *et al.,* 2014). In contrast El-Sharkawy *et al* (2017) reported that S. enterica serovar Enteritidis isolates were susceptible to all tested antimicrobials.

Table 1: Isolation (suspected) and identification of *S. Enteritidis* from intestine and livers of healthy and diseased broiler chickens (n=100).

Chickens	Organs	No. of Suspected isolates	No. of Identified isolates	% of *S. Enteritidis*
Apparent	Intestine	12 (12%)	4 (4%)	33.3
healthy	Liver	7 (7%)	2(2%)	28.6
Diseased	Intestine	17 (17%)	7 (7%)	41.2
	Liver	9 (9%)	3 (3%)	33.3
Total	400	45 (11.25%)	16 (8.0%)	35.6

Table 2: Isolation (suspected) and identification of *S. Enteritidis* from Intestine and livers of broiler chickens (n=200).

Organs	No. and % Suspected isolates	No. and % of Identified isolates
Intestine	29 (14.5%)	11 (5.5%)
Liver	16 (8%)	5 (2.5%)

Table 3: Antibiotics profile of *S. Enteritidis* isolated from broiler chickens.

Reactions		AMP	DA	CVN	CT	C	DO	ENR	OX	S	STX
R	No	9	9	6	3	8	9	9	10	7	16
	%	56.3%	56.3%	37.5%	18.8%	50%	56.3%	56.3%	62.5 %	43.8%	100%

R: Resistant. AMP: Ampicillin. DA: Calindamycin. CVN: Cephalosporins. CT: Colistin. C: Chloramphenicol. DO: Doxycycline. ENR: Enrofloxacin. OX: Oxacillin. S: Streptomycin .STX: Trimethoprim/Sulfamethoxazole.

Table 4: Distribution of antibiotic resistance rates, index and class of *S.Enteritidis* isolates.

Antibiotic pattern profile	Antibiotic	No of isolates	Resistant			
			No of Antibiotic	Index	Percent	Class
1	DA, STX	2	2	0.2	12.5%	NDR
2	AMP, OX, STX	1	3	0.3-0.7	62.5%	MDR
3	DA, CVN, DO, STX	2	4			
4	C, DO, ENR, OX	1				
5	AMP, CVN, C, ENR, STX	2	5			
6	AMP, DA, DO, OX, S, STX	2	6			
7	AMP, DA, CVN, ENR, OX, STX	1				
8	DA, C, DO, ENR, OX, S, STX	1	7			
9	AMP, CT, C, DO, ENR, OX, S, STX	3	8	0.8-0.9	25%	XDR
10	AMP, DA, C, DO, ENR, OX, S, STX	1	9			

AMP: Ampicillin. DA: Calindamycin. CVN: Cephalosporins. CT: Colistin: C: Chloramphenicol. DO: Doxycycline. ENR: Enrofloxacin. OX: Oxacillin. S: Streptomycin. STX: Trimethoprim/Sulfamethoxazole. NDR: Narrow drug-resistant, index < 0.3. MDR: Multidrug-resistant, index, 0.3-0.7. XDR: Extensively drug-resistant, index 0.8- 0.9.

Table 5: Mortality rate in *S. Enteritidis* infected treated and control chicken group (n=40).

Group	Infection	Treatment	No of dead birds after infection	Mortality rate
1	+	Colistin	2	5%
2	-	- ve	0	0
3	+	Non-treated	11	27.5%

Table 6: Performance of treated and control *S. Enteritidis* infected broiler chicken groups at the 5th week of age.

Gr N0.	Infection	Treatment	ABW/gm Mean ± SD	AFI/gm	FCR	Livability %	EEF*	CV%**
1	+	Colistin	2250.0±137.8	3420	1.52	95	401.8	6.12
2	-	- ve	2143.5±183.7	3280	1.53	100	400.1	8.57
3	+	Non-treated	1858.7±275.6	3210.5	1.73	72.5	222.6	14.83

* EEF: European Efficacy Factor. **CV%: Uniformity

Tested *S. Enteritidis* isolates are classified to 11 profiles. Regarding result of MDR, it was recorded that all tested isolates are resistant to two - nine antibiotics with resistant index 0.2- 0.9 (Table 4). Only two isolates are NDR (12.5%), most of isolates 10/16 (62.5%) are MDR and 4/16 (25%) are EDR having resistance to 8-9 antibiotics (Table 3). Our results are similar to result of *Elkenany et al.* (2019) where MDR was determined in 76.7% of the isolates with resistance index of 0.2- 0.6. Isolates of resistant index higher than 0.2 are of high risk in control (Miranda *et al.*, 2008). MDR was reported in 54.8% of *S. enteritidis* isolates and 20% were EDR (Aditya, 2015). Also, it was found that S. *Enteritidis* isolates were 63.9% (Medeiros *et al*., 2011) and 90% (Rizi *et al.*, 2015) were MDR.

Infected groups at the 2nd dpi showed clinical signs including ruffled feathers, off food, depression, closed eyes, wet dropping to diarrhea, pasty vent that lasted for the 7th day in control non-treated group that showed marked loss of weight (Abd El-Ghany *et al.*, 2012b). The mortality stared at the 4rd till the 9th dpi in non-treated. Lesion including congested liver and spleen and sever enteritis. The late mortality and sacrificed birds in non-treated birds show focal areas of necrosis on liver, spleen, intestinal mucosa and caecal cores. Clinical signs were subside in the 2nd day from treatment with improve in feed

intake and drooping. Sacrificed birds at 5 and 7 days from the start of treatment showed mild or non-obvious lesions.

Mortality rate are shown in (Table 5), the highest mortality rate was 27.5% in infected non-treated group 3 (Abd El-Ghany *et al.*, 2012b) and 5% in treated (Group 1) while control negative (Group 2) showed 0% mortalities. Lesions in dead birds were congested muscles, hemorrhages on body and heart fat, congested internal organs and inflamed intestinal mucosa with watery to mucoid contents. The lesions were markedly severe in infected non treated broilers than treated ones. Similar lesions caused by *S. Enteritidis* in broiler chickens (Abd El-Ghany *et al.*, 2012b).

Collected samples from dead infected birds including liver, heart, spleen and cecum were positive for re-isolation of *S. Enteritidis* indicates the microorganism was the cause of mortality. Highest mortality rate in was in *S. Enteritidis* experimentally infected group3is matched with result of Abd El-Ghany *et al.* (2012b) and Morsy (2012) who reported that *S. Enteirtidis* is one of the bacterialcauses for high mortalities in broiler chickens. Mild signs and low mortality in treated group proved effect of colistin on *S. Enteritidis.* It was found that addition of colistin in broiler diet decrease the rate of infection of flocks and contamination of broiler carcasses due to *S. Enteritidis* (Fard, 2004), recently, Osman *et al.* (2014) noticed that *S. enteric spp.*, that isolated from imparted duckling showing 100% sensitivity to colistin.

S. Enteritidis intestinal count in sacrificed infected non-treated (group 3) was higher (3×10^6, 2.5×10^7 and 2.5×10^5at 3, 5 and 7 from medication, respectively) than in treated group 1 (2.6×10^4, 1.2×10^3 and 0.5×10^3 at 3, 5 and 7 from medication, respectively). This result proved that the used Colistin was effective in reducing clinical signs; lesion and intestinal content in *S. Enteritidis* infected treated broiler chickens (Fard, 2004). Colistin was effective against broiler infections caused by MDR *P. aeruginosa, A. baumannii* and Enterobacteriaceae (Rasool *et al.*, 2018). Colistin sulfate have specific and selective activity against intestinal bacteria as it is not absorbed and act local in the gastrointestinal tract (Collell and Segura, 2013).

Regarding production performance (Table 6) infected non-treated group showed the lowest ABW (1858.7±275.6gm) at the end of the 5th week followed by control negative group (2143.5±183.7gm) and the highest was in infected colistin treated which was 2250.0±137.8gm (Table 2). This result was matched with who noticed that chickens infected with *S. Enteritidis* organism had lower body weight compared to the controls.

FCR infected non-treated was the lowest (1.73), followed by colistin sulphate (1.52) and control negative (1.51). It is clearly noticed that treated group showed FCR close to control negative one. Liveability was 95, 100 and 72.5% in treated, non-treated and negative group; respectively (Table 6). Higher EEF in treated (402.8) followed by control group (400.1), while the nontreated infected showed the lowest factor (222.6). CV% in treated is the highest (6.12) followed by control group (8. 57) and that of nontreated infected showed the lowest (14.83). Our results agree with those of (Bozorgmehri, 2004) where colistin in feed reduces *S. Enteritidis* in broiler farm and increases live BWG (14 %) and FCR (8 %).

Treated group showed good uniformity (CV %) 6.12% than control negative 8.57% and infected nontreated group 14.83%.

Conclusions

It could be concluded that *S. Enteritidis* is prevalent in broiler chicken flocks. Most of isolates are MDR. Experimental infection of broiler with *S. Enteritidis* resulted in high mortality and addition of colistin sulphate in drinking water controlled the infection and restores the productivity of infected broiler chickens.

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Author's contributions

AMA and MMA designed and planned the study. EMH and AMG collect samples. All authors share laboratory tests, experimental work, and writing, drafted, revised the manuscript and approved the final manuscript.

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