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Detection of virulence genes in Salmonella serovars isolated from broilers

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Abstract: This study was conducted to determine the prevalence of *Salmonellae* in broilers farms in Dakahlia Governorate, Egypt. A total of 1000 samples were collected from 200 broiler chickens (40 apparently healthy, 80 diseased chickens and 80 freshly dead broiler chickens).The samples were liver, caecum, heart blood, spleen & kidney. The colonial morphology, microscopical and biochemical identifications of the isolates revealed the presence of 37 out of 200 chickens (18.5%) *salmonella* species isolates, representing: 3 from apparently healthy chicken (7.5%), 21 from diseased chickens (26.25%) and 13 from freshly dead broiler chickens (16.25%).The rate of recovery of *Salmonellae* from the different internal organs showed that high recovery rate was from liver, caecum, spleen, heart then kidney as the follow (9.5%), (5.5%), (4.5%), (3%) and (2%), respectively. The serotyping of the isolated *salmonellae* from chickens were eight *S. enteritidis*, one *S. maccles Field*, two *S. wingrove*, one *S. eingedi*, three *S. rissen*,two *S. derby*, two *S. vejle*, one *S. magherafelt*, two *S. berta*, two *S. enterica sub.spp salamae*, one *S. gueuletapee*, one *S. blegdam*, five *S. kentucky*, two *S. newport*, two *S. agona* and two *S. virchow* were isolated from broilers. PCR assay was carried out for six serovars (*S. enteritidis, S. maccles Field, S. rissen, S. derby, S. magherafelt* and *S. enterica sub.spp salamae*) to detect the presence of *invA*, *sopB* and *stn* gene, All serovars had the three genes. Gentamycin, ciprofloxacin, colistin sulphate and enrofloxacin were found to be the most effective antimicrobials against the tested isolates; while a high resistance to erythromycin and flumequine were shown. High prevalence of *Salmonella* in broilers and multidrug resistance, constituting a major concern for public health. Further surveillance programs and research are a necessity to understand their epidemiology and to limit the spread of multidrug-resistant Salmonella spp.

Keywords: Salmonella, Broiler, Serotypes, Virulence Genes

1. Introduction

Salmonella infection is one of the most serious problems that affect poultry industry causing high economical losses not only due to high mortality in young chickens but also for the debilitating effect which predisposes for many other diseases. Salmonellosis is an important health problem and a major challenge worldwide. *Salmonella spp.* are recognized as the most causative agents of food poisoning. These organisms are Gram negative and rod shape which have been divided into over 2700 serotypes based on somatic, flagellar and capsular antigens [1] . *Salmonellae* are short bacilli, 0.7-1.5 x 2.5 µm, Gram-negative, aerobic or facultative anaerobic, positive catalase, negative oxidase; they ferment sugars with gas production, produce H2S, are non sporogenic, and are normally motile with peritricheal flagella, except for *Salmonella Pullorum* and *Salmonella Gallinarum*, which are nonmotile [2]

The genus *Salmonella* is divided into two species *Salmonella enterica* and *Salmonella bongori*; *Salmonella enterica* itself is comprised of 6 subspecies. They are *S. enterica subsp. enterica, S. enterica subsp. arizonae, S. enterica subsp. diarizonae, S. enterica subsp. indica, S. enterica subsp. houtenae or I, II, IIIa, IIIb, IV and VI*, respectively [3].

Salmonella enterica serovar typhimurium and S. enterica serovar enteritidis are the most frequent isolated serovars worldwide [4]. In Egypt *S. enteritidis* were isolated from broiler chicken, chicken meat and food poisoning patient. The clinical illness characterized by fever, nausea and diarrhea, vomition and abdominal pain after an incubation period of 12 to 72 hrs [5].

Many of the virulence genes of *S. enterica* are chromosomal genes located on pathogenicity islands referred to as Salmonella Pathogenicity Islands (SPI). These genes are believed to have been acquired by Salmonella from other bacterial species through horizontal gene transfer. They responsible for host cell invasion and intracellular pathogenesis. Other virulence factors of *Salmonella* include production of endotoxins and exotoxins, and presence of fimbrie and flagella [6]

This study was planned to identify biochemically and serologically the prevalent *Salmonella* species in broilers farms in Dakahlia Governorate, Egypt, to detect common virulence genes of Salmonella serovars using Polymerase Chain Reaction, and to detect its susceptibility to various antibiotics.

2. Material and Methods

2.1. Sample Collection

A total of 200 samples from broilers farms were collected for Salmonella isolation and these samples include liver, caecum, spleen, heart and kidney. All samples were put in sterile plastic bags in ice box and transported directly to Mansoura laboratory (Animal Health Research Institute).

2.2. Isolation of Salmonella

According to ISO 6579 (2002) method [7]: Each sample was inoculated separately in selenite F broth and incubated at 37°C for not more than 18 hours or Rappaport-Vassiliadis Soya broth (RVS) and incubated at 42°C for 24 hours. Then a loopful from selective enriched media was streaked onto plates of MacConkey's, Salmonella–Shigella (S.S) and xylose lysine deoxycholate and incubated overnight at 37 °C. Typical colonies were picked and further tested by standard biochemical methods and serotyped using specific commercial sera.

2.3. Identification of Salmonella Isolates

2.3.1. Microscopic Examination

Films from suspected purified colonies were prepared, fixed and stained with Gram's.

2.3.2. Biochemical Identification

According to ISO 6579 (2002) method: Purified isolates were examined by different biochemical reactions either by oxidase, urea hydrolysis, H_2S production on TSI, lysine decarboxylation, indole, methyl red test, Voges-Proskauer, citrate utilization and Analytical profile index 20 E (API 20 E).

2.3.3. Serological Identification

Salmonella isolates were serotyped by the slide agglutination method using O and H antisera (Difco, Detroit, USA), according to the manufacturer's instructions.

2.4. Detection of Virulence Genes

Table (1). *PCR primers for amplification of virulence genes*

Primer	Sequence	PCR product	Reference
invA	Gtgaaattatcgccacgttcgggcaa Tcatcgcaccgtcaaaggaacc	284bp	[15]
sopB	Tcagaagcgtctaaccactc Taccgtcctcatgcacactc	517 bp	[8]
stn	Ttg tgt cgc tat cac tgg caacc Att cgt aac ccg ctc tcg tcc	617 bp	[19]

DNA was extracted using QIAamp DNA Mini Kit according to the instructions of the manufacturer. Detection of virulence genes was performed by PCR. Primer sequences and PCR conditions used for the study listed in Table (1). PCR performed in T3 Thermal cycler (Biometra). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose in Tris–acetate–EDTA (TAE) buffer at 100 V. And Gel Pilot 100 bp ladder (QIAGEN, USA) was included in each agarose run, accordingly the amplified product.

2.5. Antibiotic Sensitivity Testing

According to ISO 6579 (2002) method: Determination of the susceptibility of the isolated strains to antibiotic discs was adopted using the disc diffusion technique according to Clinical and Laboratory Standards Institute (CLSI) instructions (CLSI, 2006) [9].

3. Results

3.1. The Prevalence of Salmonella Spp

Table (2). *The prevalence of Salmonella spp. in examined chickens*

Examined chicken	Number of examined chicken	Number of positive	%
Apparently healthy chicken	40	3	7.5
Diseased chicken	80	21	26.3
Freshly dead chicken	80	13	16.3
Total	200	37	18.5

Salmonella suspected isolates showed smooth red colored colonies with black center on XLD, while on Hektone enteric it appeared as deep blue colonies but on MacConkey's agar appeared as pale, colorless smooth, transparent and raised colonies and on Salmonella Shigella (S-S) agar, Salmonella produce colorless colonies with black centers due to H2S production. The staining characters appeared as Gram negative, non-spore forming &short rod shaped. Biochemically, all *Salmonella* suspected isolates were non-lactose fermenting colonies and negative oxidase, urea hydrolysis, indole and Voges-Proskauer tests. Meanwhile, most isolates produced H_2S and positive methyl red, citrate

utilization and lysine decarboxylation.

The prevalence of *Salmonella spp.* in examined chickens was depicted in table (2), Salmonella was recovered in 37 samples with an incidence rate 18.5% (37 out of 200).

3.2. Recovery rate of Salmonella from Internal Organs

Table (3). *recovery rate of Salmonella from internal organs.*

Examined organs	No. of positive	Percentage
Liver	19	9.5
Caecum	11	5.5
Spleen	9	4.5
Heart	6	3
Kidney	4	2
Total	49	24.5

The rate of recovery of *Salmonella* from internal organs was depicted in table (3), the highest recovery rate of Salmonella isolates was found in liver (9.5%) followed by caecum (5.5%); spleen (4.5%); heart (3%) and kidney (2%).

3.3. Serotyping

The results of serotyping of isolated Salmonella species were listed in Table (4). Salmonella isolates (37) were serotyped using "O" and "H" antisera, serotypes recovered were eight S. enteritidis, one S. macclesfield, two S. wingrove, one S. eingedi, three S. rissen,twoS. derby, two S. vejle, one S. magherafelt, two S. berta, two S. enterica sub.spp salamae, one S. gueuletapee, oneS. blegdam, five S. kentucky, two S. newport, two S. agona, two S. virchow were isolated from broilers with percentage of (21.62%), (2.7%), (5.4%), (2.7%), (8.1%), (5.4%), (5.4%), (2.7%), (5.4%), (5.4%), (2.7%), (2.7%), (13.5%), (5.4%), (5.4%) and (5.4%) respectively.

Table (4). *Serotyping of isolated Salmonella species*

Serotype	Antigenic formula	No. of positive	%
S. enteritidis	O: 1,9,12.H 1 g, m, H2 -	8	21.62
S. macclesfield	O: 9,46.H1 g, m, S, H2 1,2,7.	1	2.7
S.Wingrove	O: 6,8. H1 C , H2 1,2	2	5.4
S. eingedi	O: 6,7. H1 F,g,t, H2 1,2,7	1	2.7
S.rissen	O: 6,7,14. H1 f,g. H2 -	3	8.1
S. derby	O: 1,4,[5],12 .H1 F, g. H2[1,2]	2	5.4
S.Vejle	O: 3,[10],[15].H1 e, h, H2 1,2	2	5.4
S. magherafelt	O: 8,20. H1 I, H2 1,w	1	2.7
S.berta	O: 1,9,12.H1 [F],g, [t] H2 -	2	5.4
S.enterica sub.spp salamae	O: 1,4,[5],12.H1 F,g,t. H2 Z6	2	5.4
S. gueuletapee	O:9,12, H1 g,m,s, H2 __	1	2.7
S.blegdam	O:9,12, H1 g,m,q, H2 __	1	2.7
S. kentucky	O: 8,20. H1: i, H2: Z6	5	13.5
S.newport	O :6,8,20. H1 :e,h , H2 :1,2	2	5.4
S. agona	O:1,4(5),12.H1:f,g,s, H2: (1,2)	2	5.4
S.virchow	O:6,7,14. H1: r, H2: 1,2	2	5.4

3.4. Virulence Genes

Six salmonella serotypes (S. enteritidis, S. macclesfield, S. rissen, S. derby, S. Magherafelt and S. enterica sub.spp salamae) examined for detection of virulence genes as invA, stn and sopB by conventional PCR.

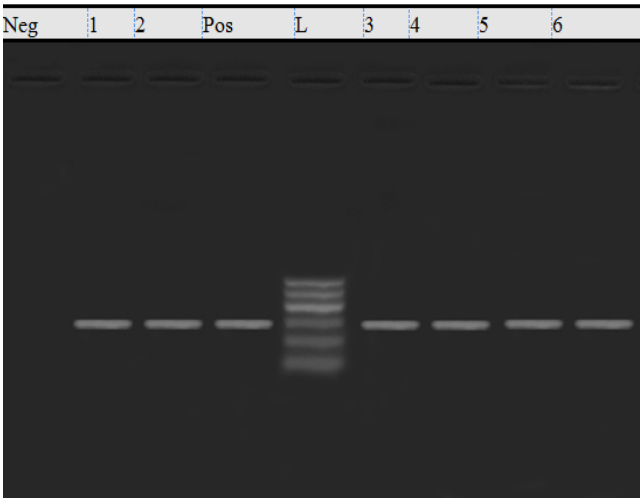

Photo (1). *Agarose gel electrophoresis of ampilified invA PCR product (284 bp). Lane L: 100-600pb DNA ladder;Pos.: Positive control; Neg.: Negative control; Lane 1, 2,3,4,5&6 examined Salmonella.*

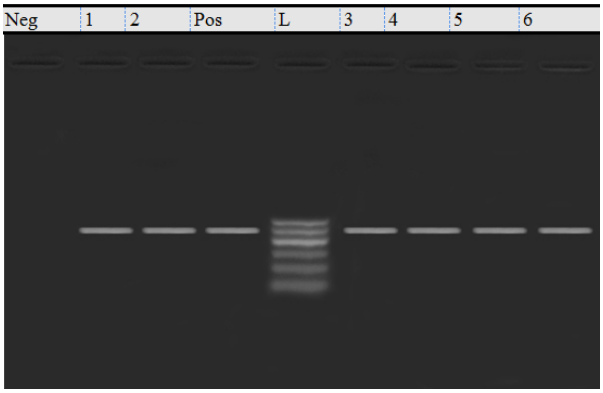

Photo (2). *Agarose gel electrophoresis showing Salmonella specific PCR of Salmonella isolates using primer set for the sopB gene(517 bp). Lane L: 100-600pb DNA ladder; Pos.: Positive control; Neg.: Negative control; Lane 12,3,4,5 &6 examined Salmonella.*

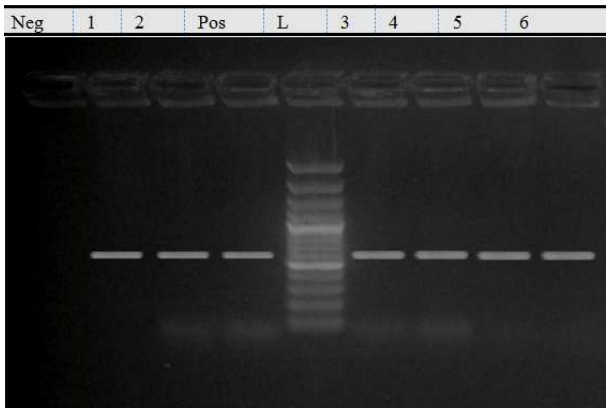

Photo (3). *Agarose gel electrophoresis showing Salmonella specific PCR of Salmonella isolates using primer set for the stn (617 bp) gene. Lane L: 100-3000pb DNA ladder; Pos.: Positive control; Neg.: Negative control; Lane 1, 2, 3, 4, 5&6 examined Salmonella.*

4. Discussion

Salmonella infection is one of the most important bacterial diseases in poultry causing heavy economic loss through

mortality and reduced production. In the present study, the incidence of *Salmonella* in broilers was 18.5% (37out of 200 chickens). This is in agreement with the results obtained by [10]who found that18% of broilers were positive for *salmonella*. However, [11] reported that *Salmonella* present in (62.5%) in examined chickens with higher incidence. The difference in the prevalence rates may be due to socio-economic factors.

Recovery of *Salmonella* species from internal organs of the examined chickens were higher from liver followed by caecum, spleen, heart and kidney 9.5%, 5.5%, 4.5%, 3% and 2% respectively. It was clear from these results, showed higher isolation rate of *Salmonella* species from liver. However, [12] isolated *Salmonella* from tissue samples 22% (22/100).

Serological identification of isolated *Salmonella* species revealed higher incidence of *S. enteritidis* (21.62%) followed by *S. kentucky*(13.5%), *S. rissen* (8.1%), 5.4% for each *S. wingrove, S. derby, S. vejle, S. berta, S. enterica sub.spp salamae, S. newport, S. agona & S. virchow* and 2.7% for each *S. maccles field, S. eingedi, S. magherafelt, S. gueuletapee& S.blegdam.*These results agree with that reported by [13]. They recorded that the predominant serotypes of *Salmonella* was *S. enteritidis*. [14] isolated *S. Kentucky* and *Salmonella enteritidis* with percentage of 21.64% and 5.15%, respectively.

[15] revealed that PCR method has high specificity and sensitivity and more importantly a less time consuming procedure than standard microbiological techniques for detection and identification of Salmonella. PCR assay using the *invA* primers specific for Salmonella spp. considerably decreases the number of false-negative results which commonly occur in diagnostic laboratories. Amplification of *invA* is now recognized as an international standard procedure for detection of *Salmonella* genus. In this study, PCR assay was carried out for the detection of the *inv*A gene from six isolated strains (*S. enteritidis, S. macclesfield, S. rissen, S. derby, S. magherafelt and S. enterica sub.spp salamae*) has revealed that the gene was present in all of the isolates (100%) that was demonstrated by the presence of a 284 bp PCR amplified fragment. The results obtained in the present study were in corroboration with [16]. PCR assay was carried out for the detection of the sopB gene from isolated strains has revealed that the gene was present in all of the isolates (100%) which was demonstrated by the presence of a 517 bp PCR product. The results obtained in the present study were in corroboration with [17] and [18]. Also, PCR assay carried out for the detection of the *stn* gene in *Salmonella* isolates has revealed that the gene was present in all the isolates (100%) that were demonstrated by the presence of a 617 bp PCR product. These findings are in agreement with [19]; [20]and [21]. Observations from the present study indicated that the stn gene is widely distributed among the *Salmonella* serovars.

In this study all *Salmonella* strains were sensitive to gentamycin, ciprofloxacin, colistin sulphate and enrofloxacin and this agree with [22]who reported that all the strains were sensitive to at least four antibiotics as gentamicin, chloramphenicol, ceftriaxone and ciprofloxacin. And [23] reported that salmonella isolates were 100% were resistant to each of erythromycin, penicillin, and amoxicillin, while 98·8%, 96·4%, 95·2%, and 91·6% were resistant to nalidixic acid, sulphamethoxazole, oxytetracycline, and ampicillin, respectively.

5. Conclusion

It could be concluded that there are high level of *Salmonella* isolation in broilers evaluated in this study may be attributed to horizontal and/or vertical transmission of *Salmonella* to the chicks. Also, the high rates of antibiotics resistance found in the present study can be explained by the spread of use of antibiotics agents given to poultry in Egypt as prophylaxis, growth promoters or treatment. The multiple resistances observed were to those antimicrobials frequently employed in veterinary practices. We recommend more restrictions on the irrational use of antibiotics and public awareness activities should be undertaken to alert the public to the risks of the unnecessary use of antibiotics. Also, the study recommends that PCR should be used for rapid and sensitive detection of Salmonella.

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