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DETECTION OF SALMONELLA SEROVARS AND THEIR COMMON VIRULENCE GENES FROM DIFFERENT CHICKEN BREEDING SYSTEMS

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ABSTRACT

The present study investigated the occurrence, antibiotic sensitivity patterns, and virulence gene profiling of *Salmonella* serovars in chicken farms in Behaira governorate, Egypt. Three hundred samples including chicken's internal organs, cloacal swabs, litter samples and eggs were examined for the presence of *Salmonella* by standard microbiological techniques. All *Salmonella* isolates were tested for their sensitivity against 11 antibiotics and subjected to virulence genotyping by polymerase chain reaction (PCR). The overall isolation percentage of *Salmonella* was 13%. The prevalence of *Salmonella* was 6.8% in broilers, 42.3% in layers, 0% in breeders and cloacal swabs, 6.5% in litter and 15.6% in eggs. Four different serovars were found, with the predominant one being *Salmonella enteritidis*. *Salmonella* isolates were sensitive to most of the tested antibiotics, but they exhibited absolute resistance against ampicillin and cefotaxime. PCR investigation of *inv*A, *mgt*C, *sop*B and *bcf*C virulence genes in 8 *Salmonella* isolates revealed that *all* tested genes were expressed in all examined isolates. These results would greatly help in understanding the prevalence of virulence genes and antibiotic sensitivity among *Salmonella* serovars in chicken.

KEYWORDS: Chicken, PCR, *Salmonella*, Serotypes, Virulence Genes.

INTRODUCTION

Salmonella infection is one of the most serious problems that affect poultry industry causing high economic 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 (Gallegos-Robles et al., 2008).

Salmonella are members of the family *Enterobacteriaceae*. They are Gram-negative, facultative anaerobes and inhabit the intestinal tract of animals and can be isolated from many kinds of hosts, specially poultry and pigs, humans, foods and environment. Moreover, these bacteria may be pathogenic to domestic and wild animals, and humans (Holt et al., 1994).

Avian Salmonellosis is an important disease causing serious burden to the development of poultry industry especially in developing countries. Since no effective immuno-prophylactic measures are available for the disease to date, firm biosecurity is the only alternative to prevent the disease (Rajagopal et al., 2013).

Isolation and identification of *Salmonella* as well as testing for the best antimicrobial drug(s) for treatment of salmonellosis are very important to continually monitor the health of birds and its reflection on human health (Olivera et al., 2006).

Polymerase chain reaction (PCR) has been applied to rapidly and specifically detect *Salmonella* in feces and inner organs of infected birds and animals (Li et al., 2000). Furthermore, PCR is more accurate and confirmative for somatic sero-grouping with polyvalent antisera as sero-grouping is not possible when *Salmonella* isolates lack O-antigen (rough strain) or lack both O and H antigens (Roy et al., 2002).

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 are responsible for host cell invasion and intracellular pathogenesis. Other virulence factors of *Salmonella* include production of endotoxins and exotoxins and presence of fimbriae and flagella (Van Asten et van Dijk, 2005). The *inv*A gene of *Salmonella* contains sequences unique to this genus

and has been proven to be a suitable PCR target with potential diagnostic application (Jamshidi et al., 2009).

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

MATERIALS AND METHODS

Samples collection

A total number of 300 samples were collected as follows: 152 from broilers, 52 from layers, 5 from breeders, 32 cloacal swabs, 31 litter samples and 32 egg samples. Samples were obtained from different chicken farms, markets, backyards located in Behaira Governorate. Liver, cecum, heart, spleen, ovary, yolk sac and gall bladder from chicken were examined.

Isolation of *Salmonella*

According to ISO 6579 (2002), pre-enrichment of the collected samples was performed in Buffered Peptone Water as 1:10 dilution and then incubated aerobically at 37ºC for 18 hours. A volume of 0.1 ml was transferred to a tube containing 10 ml of the Rappaport Vassiliadis Soy broth and then incubated at 41.5ºC for 24 hours. One ml of the pre-enrichment culture was also transferred to a tube containing 10 ml of the Muller–Kauffmann tetrathionate/novobiocin broth and then incubated at 37ºC for 24 hours. From the enrichment culture, 10 µl were inoculated onto the surface of Xylose Lysine Deoxycholate (XLD), Hektoen Enteric, Brilliant Green, Salmonella-Shigella and MacConkey's agar plates then incubated at 37ºC for 24 hours. Typical colonies were picked and further tested by standard biochemical methods and serotyped using specific commercial sera.

Microscopic Identification of *Salmonella* Isolates

Films from suspected purified colonies were prepared, fixed and stained with Gram's stain (Cruickshank *et al*., 1975).

Biochemical Identification of *Salmonella* Isolates

According to **ISO 6579 (2002),** purified isolates were examined by different biochemical reactions as oxidase, urea hydrolysis, H_2S production on TSI, lysine decarboxylation, indole, methyl red test, Voges-Proskauer, and citrate utilization tests.

Serological Identification of Salmonella Isolates

Salmonella isolates were subjected to serological identification according to Kauffman-White Scheme (Kauffmann, 1973) for determination of somatic (O) and flagellar (H) antigens.

Antibiotic susceptibility testing

Determination of the susceptibility of the isolated *Salmonellae* (n= 39) to different antimicrobials was adopted using the disc diffusion technique according to Clinical and Laboratory Standards Institute instructions (Clinical and Laboratory Standards Institute, 2015). The discs used were: ampicillin (10 µg), amoxiclav (30 µg), cefotaxime (30 µg), gentamycin (10 µg), streptomycin (10 µg), tetracycline (30 µg), doxycycline hydrochloride (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), sulphamethoxazole-trimethoprim (25 µg) and chloramphenicol (30 µg).

Detection of Virulence Genes using Polymerase chain reaction (PCR)

DNA was extracted using QIAamp DNA Mini Kit according to manufacturer's instructions. Primer sequences and PCR conditions used for the study are listed in Table (1). Preparation of PCR Master Mix was done according to Emerald Amp GT PCR master mix kit (Takara; Code No. RR310A). Temperature and time conditions of the PCR were according to Huehn et al., (2010), Olivera et al., (2003). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose in Tris–acetate–EDTA (TAE) buffer.

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

Primer	Sequence	Amplified product	Reference
invA	GTGAAATTATCGCCACGTTCGGGCAA	284 bp	**Olivera et al., (2003)**
	TCATCGCACCGTCAAAGGAACC		
sopB	TCA GAA GRC GTC TAA CCA CTC	517 bp	**Huehn et al., (2010)**
	TAC CGT CCT CAT GCA CAC TC		
mgtC	TGA CTA TCA ATG CTC CAG TGA AT	677 bp	
	ATT TAC TGG CCG CTA TGC TGT TG		
bcfC	ACC AGA GAC ATT GCC TTC C	467 bp	
	TTC TGC TCG CCG CTA TTC G		

RESULTS

Prevalence of *Salmonellae* in different samples

Salmonella was recovered from 39 samples with an incidence rate of 13% (39 out of 300) (Table 2).

Table (2): Prevalence of Salmonella in samples from chicken internal organs, cloacal swabs, litter and egg samples.

Samples	Chicken Type	No. of examined samples	No. of positive samples	%
Chicken (internal organs)	Broiler	148	10	6.8
	Layer	52	22	42.3
	Breeder	5	0	0
	Total	205	32	15.6
Cloacal Swabs	Broiler	16	0	0
	Layer	16	0	0
	Total	32	0	0
Litter	Broiler	15	0	0
	Layer	16	2	12.5
	Total	31	2	6.5
Eggs		32	5	15.6
Total		300	39	13

Incidence of *Salmonellae* in different internal organs of different chicken types

Salmonella was isolated from liver and cecum of broiler chickens with an incidence of 7.4%, 5.4%, respectively. The percentages of *Salmonella* isolation from layer internal organs (liver, cecum, spleen, heart, ovary and bile) were 28.8%, 10%, 16.7%, 0%**,** 40%, 0%, respectively. All internal organs of breeders were negative for *Salmonella* isolation as in Table (3).

Table (3): Incidence of *Salmonellae* in different internal organs of different chicken types.

Samples	Chicken type	No. of samples	Positive	
			No.	%
Liver	Broiler	95	7	7.4
	Layer	52	15	28.8
	Breeder	5	0	0
	Total	152	22	14.5
Cecum	Broiler	56	3	5.4
	Layer	30	3	10
	Breeder	5	0	0
	Total	91	6	6.6
Spleen	Broiler	10	0	0
	Layer	6	1	16.7
	Breeder	3	0	0
	Total	19	1	4.8
Heart	Broiler	6	0	0
	Layer	16	0	0
	Breeder	5	0	0
	Total	27	0	0
Ovary	Broiler	0	0	0
	Layer	10	4	40
	Breeder	5	0	0
	Total	15	4	26.7
Yolk sac	Broiler	3	0	0
	Layer	0	0	0
	Breeder	0	0	0
	Total	3	0	0
Bile	Broiler	4	0	0
	Layer	13	0	0
	Breeder	0	0	0
	Total	17	0	0

Serotyping of the isolated *Salmonella*

Four serotypes were detected from all samples. Serotypes were 29 *S. enteritidis* (74.4%), 5 *S. kentucky* (12.8%), 4 *S. bardo* (10.6%) and 1 *S. atakpame* (2.7%).

Seven *S. enteritidis* and 3 *S. kentucky* were isolated from broiler chickens with percentages of 70% and 30%, respectively. Also, 16 *S. enteritidis*, 4 *S. bardo* and 2 *S. Kentucky* were isolated from layer chickens with percentages of 72.7%, 18.2% and 9.1%, respectively. In total chicken internal organs, *23 S. enteritidis*, *5 S. kentucky* and *4 S. bardo* were isolated with percentages of 71.9%, 15.6%, and 12.5%, respectively. *S. enteritidis* was the only serotype isolated from eggs with a percentage of 100%. From litter samples, 1 *S. enteritidis* and 1 *S. atakpame* were isolated with percentages of 50% and 50% as in table (4).

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

Samples		No. and percentage of isolated serotypes				
		S. enteritidis	***S. kentucky***	***S. bardo***	***S. atakpame***	**Total**
Chicken internal organs	**Broiler**	7 (70%)	3 (30%)	-	-	10 (100%)
	Layer	16 (72.7%)	2 (9.1%)	4 (18.2%)	-	22 (100%)
	Total	23 (71.9%)	5 (15.6%)	4 (12.5%)	-	32 (100%)
Egg samples		5 (100%)	-	-	-	5 (100%)
Layer litter samples		1 (50%)	-	-	1 (50%)	2 (100%)
Total		29 (74.4%)	5 (12.8%)	4 (10.6%)	1 (2.7%)	39 (100%)

Antimicrobial sensitivity of isolated *Salmonella*

Sensitivity of isolated *Salmonella* to different antimicrobials is listed in Table 5. Thirty-nine isolates were tested. No isolates were sensitive to ampicillin and cefotaxime. The highest sensitivity detected was for streptomycin (97.4%).

Table (5): Numbers and percentages of sensitive *Salmonella* isolates.

Antimicrobials	Sensitive isolates	
	No.	**%**
Ampicillin	0/39	0%
Amoxyclav	14/39	35.9%
Cefotaxime	0/39	0%
Gentamycin	37/39	94.9%
Streptomycin	38/39	97.4%
Tetracycline	32/39	82.1%
Doxycycline Hydrochloride	27/39	69.2%
Ciprofloxacin	35/39	89.7%
Norfloxacin	35/39	89.7%
Sulphamethoxazole-. Trimethoprim	29/39	74.4%
Chloramphenicol	34/39	87.2%

Detection of Virulence genes using polymerase chain reaction (PCR)

Eight *Salmonella* isolates (three *S. enteritidis*, two *S. kentucky*, two *S. bardo* and one *S. atakpame*) were examined for detection of virulence genes as *invA, mgtC, sopB* and *bcfC* by conventional PCR.

All examined *Salmonella* serovars in this study showed positive amplification of 284 bp, 677 bp, 517 bp and 467 bp fragments specific for the *inv*A (common gene), *mgt*C, *sop*B and *bcf*C genes, respectively as shown in Fig.1.

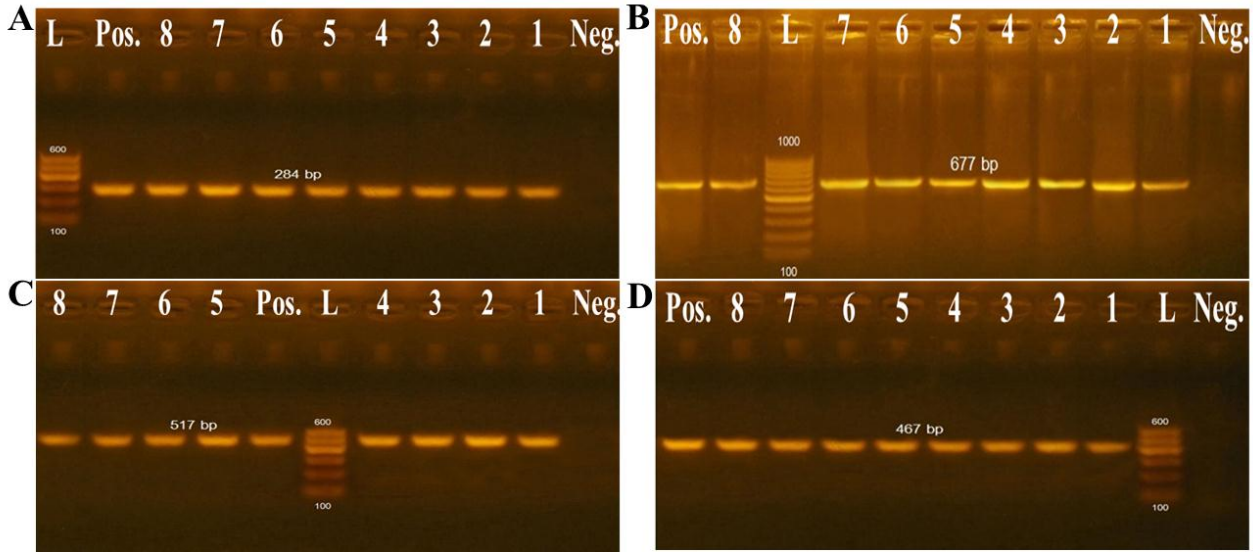

Figure 1: Agarose gel electrophoresis of amplified DNA showing the specificity of the single reactions for the detection of the (A) *inv*A (284 bp) gene, (B) *mgt*C gene (677 bp), (C) *sopB* gene (517 bp) and (D) *bcfC* gene (467 bp). Pos; positive control, Neg; negative control, L; 100 bp DNA ladder. 1-8; *Salmonella* isolates.

DISCUSSION

Salmonella infection is one of the most important bacterial diseases in poultry causing heavy economic losses through mortality and reduced production. In the present study, *Salmonella* was isolated from 10 samples of broilers (internal organs) with a percentage of 6.8%, which agreed with Schluter et al., (1994) who isolated *Salmonella* from chicken broiler flocks with an incidence of 6.2% and Abd El-Ghany et al., (2012) who isolated *Salmonella* from diseased broilers with an incidence of 7.03% and from dead birds with an incidence of 4.69%. On the contrary, Lamas et al., (2006) isolated *Salmonella* from poultry houses of broilers in Northwestern Spain with an incidence of 1.02%, while Kudaka *et al.*, (2006) found that 18% of broilers were positive for *Salmonella*.

Salmonella was isolated from 22 samples of layer chickens (internal organs) with a percentage of 42.3%و which agreed with Kudaka et al., (2006) who isolated *Salmonella* from layer hens in Japan with a percentage of 39.1%, while Hulaj et al., (2016) isolated *Salmonella* from layer samples with an incidence of 10.4% also, Temelli et al., (2010) found the incidence of *Salmonella* in layer hens by rPCR and culture to be 61.0% and 55.6%, respectively.

Salmonella was not isolated from breeders' samples unlike Sivaramalingam *et al.*, (2013) [20] who revealed that the prevalence of *Salmonella* was 47.4% in broiler-breeders.

Salmonella was not isolated from litter of broilers but Pieskus *et al.*, (2008) isolated *Salmonella* from broilers' feces with a percentage of 32.9% in Lithuania.

Salmonella was isolated from litter of layers with a percentage of 12.5%, which agreed with Abdel Rahman *et al.*, (2014) who isolated *Salmonella* from layers drag swabs with a percentage of 11.4%, while Im *et al.*, (2015) isolated *Salmonella* from layers feces with a percentage of 41.8%.

Salmonella was isolated from table eggs with a percentage of 15.6%, which agreed with Ebrahim, (2014) who isolated *Salmonella* from chicken eggs with a percentage of 15 % but Saha *et al.*, (2012) isolated *Salmonella* from inner content of laid eggs with a percentage of 44.4% and Abdel Rahman *et al.*, (2014) isolated *Salmonella* from table eggs with a percentage of 1.5%.

Salmonella isolates were serotyped using poly and monovalent "O" and "H" antisera and the results revealed that: 23 *S. enteritidis*, 5 *S. kentucky* and 4 *S. bardo* were isolated from chickens' internal organs with a percentage of 71.9%, 15.6% and 12.5%, respectively. In chicken eggs, only five *S. enteritidis* were isolated with a percentage of 100%, and in litter samples, *S. enteritidis*, *S. atakpame* were both isolated with percentages of 50%. This agreed with Kanashiro *et al.*, (2005) who showed a high incidence of *S. enteritidis* in broilers flocks (84.0%) and Abdel Rahman *et al.*, (2014) who showed that the most common serovar identified in layer chickens in Egypt was *S. enteritidis*.

Sensitivity of isolated *Salmonella* to different antimicrobials was higher to gentamycin (94.9%), streptomycin (97.4%), tetracycline (82.1%), ciprofloxacin (89.7%), sulphamethoxazole-trimethoprim (74.4%) and chloramphenicol (87.2%), which agreed with Boris *et al.*, (2012) who found that all tested isolates were highly sensitive to chloramphenicol and streptomycin (99.3%), gentamicin (97.5%), tetracycline (85.4%), ciprofloxacin (81%) and sulfamethoxazole (95.5%), while Yah *et al.*, (2007) reported that the isolates were highly resistant to chloramphenicol,

gentamycin, trimethoprim, tetracycline, and sulfamethoxazole and to lesser extent resistant to ciprofloxacin, enrofloxacin, ofloxacin, ceftriaxone and cefuroxime. All *Salmonella* were resistant to ampicillin, which agreed with Abd-Elghany *et al.*, (2015) who showed that 91.6% of isolates were resistant to ampicillin but differ from Hulaj *et al.*, (2016) who reported that all isolates were sensitive to ampicillin. All isolates were resistant to cefotaxime, which differed from Lamas *et al.*, (2016) who reported that all *Salmonella* spp. were susceptible to cefotaxime.

The PCR technique has been developed in the last few years to rapidly and specifically detect *Salmonella* in feces and inner organs of infected birds and animals (Li *et al.*, 2000). In this study, eight *Salmonella* isolates (3 *S. enteritidis*, 2 *S. kentucky*, *2 S. bardo* and one *S. atakpame*) were examined for detection of virulence genes as *invA*, *mgtC*, *sopB* and *bcfC* by conventional PCR. The *inv*A gene was detected in all of the tested isolates, which agreed with the previous studies by Hong *et al.*, (2003), Herich *et al.*, (2004) and Salehi *et al.*, (2005). The *mgt*C gene was present in all of the tested isolates that was different from AL Atfehy, (2012) who detected *mgt*C in 54.3% of his isolates and Ebrahim, (2014) who detected *mgt*C in 50% of examined isolates. The *sopB* gene detection agreed with Ebrahim, (2014) and AL Atfehy, (2012) who detected *sopB* gene in almost all isolates (97.1%). The *bcfC* gene was present in all of the isolates, which agreed with AL Atfehy, (2012) who detected it in almost all isolates (95.7%) and Ebrahim, (2014) who detected it in all isolates.

CONCLUSION

It could be concluded that there is a high level of *Salmonella* isolation in chickens examined in this study. This may be due to vertical and/or horizontal transmission of *Salmonella*. Also, the high rates of resistance to some antibiotics found in the present study can be explained by the massive use of antibiotics in the field of poultry in Egypt for treatment or as prophylaxis and growth promoters. We recommend more restrictions on the illogical use of antibiotics and public realization activities should be undertaken to warn 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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