ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2017.303.309

Research Article
Virulence Associated Genes and Antibiotic Resistance Profiles in *Salmonella* Species Isolated from Chickens

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Abstract

Background and Objective: Infections due to *Salmonella* serovars represent a significant public health risk and are economically important for the poultry industry. Genes involved in pathogenesis of *Salmonella* serovar are clustered within *Salmonella* pathogenicity islands. Meanwhile, over use of antibiotics in poultry farms has led to an increase in antibiotic resistant *Salmonella* strains, which can be challenging to control. The present study was conducted to determine antibiotic resistance profiles and to detect the presence of five major pathogenicity islands among *Salmonella* serovars isolated from chickens in Egypt. **Materials and Methods:** Samples (n = 930) taken from chicken hearts, livers, caeca, yolk sacs, ovaries and cloacal swabs were collected and used for isolation and serotyping of *Salmonella* species. Antibiotic resistance was determined using the antibiogram method. The PCR was used for the molecular detection of *Salmonella* species using primers targeting *inv*A and the pathogenicity islands genes *inv*AE/A, *ssa*Q, *mgt*C, *spid*R and *sop*B, which are found in the most prevalent *Salmonella* serovars that present public health concerns. **Results:** The detection of 30 *Salmonella* isolates was confirmed by conventional and PCR methods and additional 5 *Salmonella* isolates were detected only by PCR. Among the isolates, *Salmonella Enteritidis*, *Salmonella Typhimurium*, *Salmonella Muenster*, *Salmonella Anatum* and *Salmonella Virchow* were the most prevalent serotypes at 36.7, 26.7, 20, 10 and 6.6%, respectively. The *Salmonella* serovars showed three antibiotic susceptibility patterns and all prevalent serovars carried the five virulence genes. **Conclusion:** *Salmonella* serovars that are pathogenic in chickens and that have public health relevance, including *Salmonella Enteritidis*, *Salmonella Typhimurium* and *Salmonella Muenster*, are circulating in chicken farms in Egypt.

Key words: *Salmonella* serovars, virulence genes, chickens, antibiotic resistance patterns, public health

Received: June 07, 2017 Accepted: July 04, 2017 Published: July 15, 2017

Citation: Nabila Osman and Dina Waheed, 2017. Virulence associated genes and antibiotic resistance profiles in *Salmonella* species isolated from chickens. Int. J. Poult. Sci., 16: 303-309.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Salmonella infection can cause severe economic losses for the poultry industry[1]. Chickens can be infected with many different serovars of paratyphoid *Salmonella*, including *Salmonella Typhimurium*, *Salmonella Enteritidis* and *Salmonella Heidelberg,* which are avian pathogens that exist worldwide[2].

Salmonella is the most common etiological agent of foodborne diarrheal illness[3-5]. Thus, detection of *Salmonella* in primary poultry production is an issue of interest since control of this zoonotic disease is mainly based on restricting pathogen distribution on chicken farms[6]. Furthermore, there is increasing concern about *Salmonella* pathogens due to increasing spread of antibiotic resistance and evolution of more pathogenic strains[7,8]. The inappropriate use of antibiotics on chicken farms in developing countries, including Egypt, is thought to be a main reason for the increasing the frequency of multidrug resistant *Salmonella*[9]. Multidrug resistant *Salmonella* serovars include *Salmonella Typhimurium* and *Salmonella Enteritidis*, which have been able to infect humans and cause systemic infection and death due to treatment failure[10].

The outer proteins of *Salmonella* spp. (SOPs) contribute to invasion by these bacteria through the compromise of membrane integrity[11] and cytoskeletal alterations in host cells[12]. Meanwhile, *Salmonella* spp. pathogenicity islands (SPIs) are of a critical importance for *Salmonella* virulence, as they encode a molecular apparatus called the type III secretion system (TTSS) that injects bacterial effector proteins through bacterial and host membranes to interact with host cells[13]. The ability of *Salmonella* to efficiently colonize host cells is attributed to gene clusters, including SPIs, which encode virulence factors that are distributed in the *Salmonella* genome[2]. Several major *Salmonella* pathogenicity islands have been reported for different serovars, SPI-1-5 is the predominant type in most serovars whereas, others are less widely distributed[2,13,14]. In general, SPI-1 is responsible for the invasion of host cells and induction of macrophage apoptosis, SPI-2 contributes to systemic infection and replication within macrophages, SPI-3 is required for bacterial survival in macrophages and for *Salmonella* growth in low magnesium environments, SPI-4 is essential for intra-macrophage survival and harbors genes that are important for toxin secretion and apoptosis, whereas, SP-5 encodes genes for multiple Type III secretion system effector proteins[2,13-19].

In this study, it is reported that the isolation, identification, serotyping and antibiotic resistance patterns of *Salmonella* spp. are isolated from chickens in Upper Egypt. In addition, the frequency of virulence-associated genes in isolates was assessed, particularly for those genes that have zoonotic importance.

MATERIALS AND METHODS

Sample collection: A total of 240 diseased and freshly slaughtered chickens (155 baby chicks, 70 broilers and 15 layers) were subjected to post-mortem examination were used in this trial. A total of 930 samples from livers, hearts, caeca and yolk sacs (baby chicks only) as well as ovaries and cloacal swabs (layer flocks only) were collected from the birds under completely aseptic conditions.

Isolation and identification of *Salmonella* isolates: The *Salmonella* isolation and identification processes were carried out according to ISO 6579[20]. In brief, the samples were directly inoculated in buffer peptone water (Oxide) and incubated at 37°C for 18 h as a pre-enrichment step. Then, 100 µL of culture was transferred into a tube containing selenite cysteine broth (Oxide) and incubated at 37°C for 24 h before a loopful of bacterial from selective enriched media was streaked onto MacConkey's agar (Oxide), *Salmonella* Shigella agar (Oxide), Bismuth sulfate agar (Oxide) and Xylose-lysine deoxycholate (XLD) agar (Oxide) and incubated at 37°C for 24 h. The suspected *Salmonella* colonies were identified by Gram staining and motility tests. Biochemical assays included triple sugar iron (TSI) agar (Oxide), urease agar (Oxide) and citrate agar (Oxide) according to ISO 6579[20].

Serotyping of *Salmonella* isolates: Isolates that were biochemically identified as *Salmonella* spp. were serotyped using O and H antisera (Difco, Detroit, USA) by a slide agglutination test, according to the Kauffmann-White classification scheme[21].

Determination of the antibiotic susceptibility profile of *Salmonella* serovars: Strains were tested for antibiotic resistance by the plate disc diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[22]. The following discs were included in the test: Ciprofloxacin (CIP), 5 mg (Bioanalyses), levofloxacin (LEV), 5 mg (Bioanalyses), norfloxacin (NOR), 10 mg (Oxide), chloramphenicol (C), 30 mg (Bioanalyses), amoxicillin/clavulanic acid (AMC), 30 mg (Bioanalyses), streptomycin (S), 10 mg (Bioanalyses), trimethoprim (TMP), 5 mg (Bioanalyses), doxycycline (DO), 30 mg (Oxide), cephradine (CE), 30 mg (Oxide), rifampicin (RD), 5 mg (Oxide)

Table 1: Oligonucleotide sequences of primers used to detect *Salmonella* species and virulence associated genes in *Salmonella* isolates

Primer name	Nucleotide sequence (5'-3')	Target region	Product size (bp)
S139F	Gtgaaattatcgccacgttcgggcaa	*inv*A (*Salmonella* ssp)	284
S141R	Tcatcgcaccgtcaaaggaacc		
InvE/A-F	Tgccttacaagcatgaaatgg	SPI-1 (*inva*E/A)	450
InvE/A-R	Aaactggaccacggtgacaa		
SsaQ-F	Gaatagcgaatgaagagcgtcc	SPI-2 (*ssa*Q)	677
SsaQ-R	Catcgtgttatcctctgtcagc		
MgtC-F	Tgactatcaatgctccagtgaat	SPI-3 (*mgt*C)	655
MgtC-R	Atttactggccgctatgctgttg		
SpidR-F	Gatatttatcagtctataacagc	SPI-4 (*spid*R)	1269
SpidR-R	Attctcatccagatttgatgttg		
SopB-F	Gatgtgattaatgaagaaatgcc	SPI-5 (*sop*B)	1170
SopB-R	Gcaaaccataaaaactacactca		

and lincomycin (L), 2 mg (Oxide). The results were interpreted as recommended by the CLSI[22] to determine if the strain was resistant, intermediate or susceptible to the tested antibiotics.

Molecular detection of *Salmonella* species and five major virulence associated genes by PCR: The virulence genes *inva*E/A, *ssa*Q, *mgt*C, *spid*R and *sop*B were detected as described by Soto *et al.*[23] and Sanchez-Jimenez *et al.*[24]. The DNA was extracted from the cultures using a QIAamp DNA mini Kit (Qiagen, USA) according to the manufacturer's instructions. The PCR was performed in volume of 25 µL containing: 12.5 µL GoTaq® Hot Start Green Master mix (Promega, USA), 1 µL 20 µM each forward and reverse primer (Table 1), 3 µL DNA template and 7.5 µL nuclease-free H_2O. The PCR was carried out using a gradient thermal cycler (A200, gradient thermal cycler, Japan) under the following conditions: Initial denaturation at 95°C/2 min followed by 30 cycles of denaturation at 95°C/1 min, annealing at 51°C/1 min (*inva*E/A and *spid*R), 53°C/1 min (*sop*B), 54°C/1 min (*mgt*C) or 58°C/1 min (*ssa*Q) and extension at 72°C/1 min before a final extension at 72°C/5 min. A total of 15 µL of PCR product in 3 µL loading buffer was loaded on a 1.5% agarose gel and electrophoresed at 100 volts/35 min before staining with 0.5 µg ethidium bromide/1 mL of TAE running buffer for 30 min and visualization under a UV illuminator.

RESULTS

Incidence of *Salmonella* serovars isolated from chicken flocks: Out of the 240 chickens in the sample, 30 were positive for *Salmonella* (incidence of 12.5%) as indicated by conventional bacteriological methods using MacConkey's agar, Bismuth sulfite agar, *Salmonella* Shigella agar and XLD media (Table 2). All suspected *Salmonella* isolates were Gram negative, straight, non-spore forming rods by Gram staining of colonies. Furthermore, Gram staining showed Gram negative bacilli and a motility test indicated that the suspected isolates were highly motile. Biochemically, all suspected isolates produced alkaline (red) slant and acid (yellow) butt with or without H_2S production on TSI and were urease negative and citrate positive (Table 2).

Serotyping of *Salmonella* isolates: The 30 *Salmonella* isolates were serotyped using O and H antisera (Table 2). The recovered serotypes were 11 (36.7%) *Salmonella Enteritidis*, 8 (26.7%) *Salmonella Typhimurium*, 6 (20%) *Salmonella Muenster,* 3 (10%) *Salmonella Anatum* and 2 (6.6%) *Salmonella Virchow*.

Antibiotic susceptibility profile of isolated *Salmonella* serovars: An antibiogram of the isolated *Salmonella* serovars was compiled using 12 antibiotic discs to determine the most suitable antibiotic to control *Salmonella* infections (Table 3). The antibiogram showed three antibiotic susceptibility patterns with multidrug resistance: (i) Type I multidrug resistant to cephradine, rifampicin and lincomycin, including *Salmonella Enteritidis* and *Salmonella Virchow* isolates, (ii) Type II multidrug resistant to doxycycline, cephradine, rifampicin and lincomycin, including *Salmonella Typhimurium* and *Salmonella Muenster* isolates and (iii) Type III multidrug resistant to streptomycin, trimethoprim, doxycycline, cephradine, rifampicin and lincomycin, seen for *Salmonella Anatum* (Table 3). All identified *Salmonella* serotypes were 100% sensitive to ciprofloxacin, levofloxacin, norfloxacin, amoxicillin/clavulanic acid and chloramphenicol, 80% sensitive to streptomycin and trimethoprim, 100% resistant to cephradine, lincomycin and rifampicin and 60% to doxycycline.

Molecular identification and characterization of pathogenicity islands in *Salmonella* serovars: Among the 240 chickens tested with PCR and primers targeting a 284 bp region of *inv*A as a *Salmonella* specific gene, the *Salmonella*

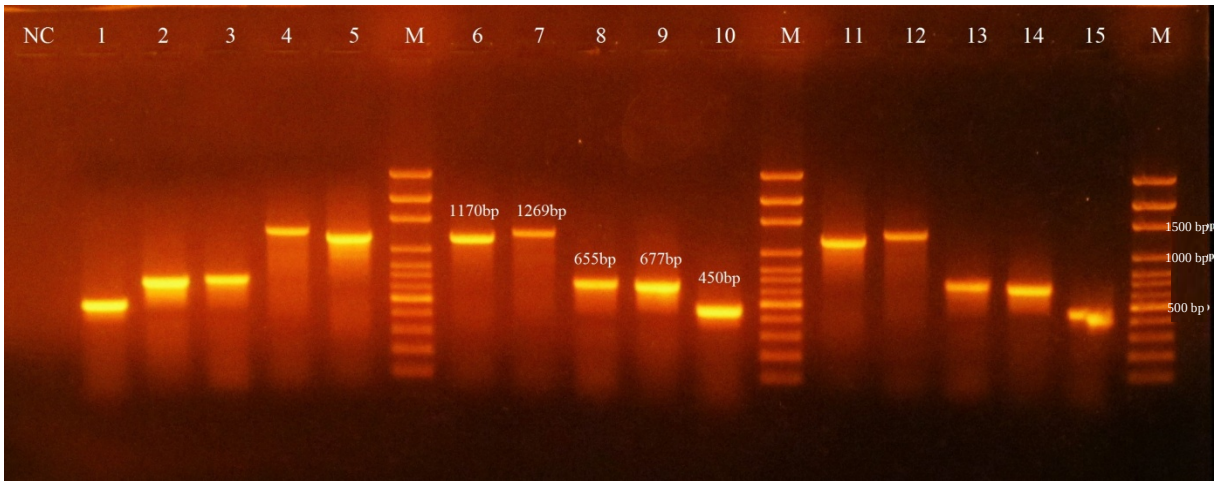

Fig. 1: PCR products of five pathogenicity island genes (SP-1 (450 bp), SP-2 (677 bp), SP-3 (655 bp), SP-4 (1269 bp) and SP-5 (1170 bp)) from the most prevalent *Salmonella* spp. that are a public health concern. The PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide (0.5 μg mL^{-1}) after electrophoresis and photographed using a PhotoDoc-It-Imaging gel documentation system (Ultra-Violet Products Ltd., UK) equipped with a Canon digital camera

Lane M: 100 bp DNA ladder (Solis BioDyne, Estonia), Lanes (1-5): Pathogenicity islands of *Salmonella Enteritidis*, Lanes (6-10): Pathogenicity islands of *Salmonella Typhimurium*, Lanes (11-15): Pathogenicity islands of *Salmonella Muenster*, NC: Negative control (PCR master mix without template DNA)

Table 2: Serotyping of *Salmonella* spp. isolated from chickens

		Antigenic structure				
			Flagellar			
No. of isolates	Groups	Somatic (O)	Phase 1	Phase 2	Serotype	Percentage
11	D1	1, 9, 12	g, m	1,7	*S. Enteritidis*	36.7
8	B	1, 4, 5, 12	i	1,2	*S. Typhimurium*	26.7
6	E1	3, 10, 15, 34	e, h	1,5	*S. Muenster*	20.0
3	E1	3, 10, 15, 34	e, h	1, 6	*S. Anatum*	10.0
2	C 1,2	6, 7, 14	r	1, 2	*S. Virchow*	6.6

Table 3: Antibiotic susceptibility of *Salmonella* serovars isolated from chickens

		Antibiotic susceptibility patterns		
Salmonella serovars	No. of isolates	Sensitive patterns	Resistant patterns	Profile types
S. Enteritidis	11	CIP, LEV, NOR, C, AMC, S, TMP, DO	CE, RD, L	I
S. Typhimurium	8	CIP, LEV, NOR, C, AMC, S, TMP	DO, CE, RD, L	II
S. Muenster	6	CIP, LEV, NOR, C, AMC, S, TMP	DO, CE, RD, L	II
S. Anatum	3	CIP, LEV, NOR, C, AMC	S, TMP, DO, CE, RD, L	III
S. Virchow	2	CIP, LEV, NOR, C, AMC, S, TMP, DO	CE, RD, L	I

AMC: Amoxicillin/clavulanic acid, C: Chloramphenicol, CE: Cephradine, CIP: Ciprofloxacin (CIP), DO: Doxycycline, L: Lincomycin, LEV: Levofloxacin, NOR: Norfloxacin, RD: Rifampicin, S: Streptomycin, TMP: Trimethoprim

prevalence was 14.6% (35 out of 240). All 30 positive isolates were identified biochemically and serologically as *Salmonella* spp., in addition to the 5 suspected samples that were atypical *Salmonellae* according to biochemical and serological methods.

The three highly prevalent *Salmonella* serotypes (*Salmonella Enteritidis*, *Salmonella Typhimurium* and *Salmonella Muenster*) were investigated for the presence of the five major SPIs, SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5 by conventional PCR using gene sequence-specific primers. The results indicated that the five pathogenicity islands encoding *inv*E/A, *ssa*Q, *mgt*C, *spid*R and *sop*B were found in the three predominant *Salmonella* serovars (Fig. 1).

DISCUSSION

Salmonella infections not only adversely affect public health and the poultry industry but surveillance, treatment and prevention of infections by these bacteria can be costly and result in negative economic effects.

This study detected 30 (12.5%) *Salmonella* isolates in 240 chickens of different ages. This result is consistent with that reported by Ibrahim *et al*.[25] and El-Fakar and Rabie[26]. Although a study by Antunes *et al*.[27] showed a higher *Salmonella* incidence in chickens, it concerned poultry farms in countries that have different surveillance rates and biosecurity levels for poultry farms.

The isolates in this study were categorized antigenically and serologically into 5 serogroups with characteristic antigenic properties based on O and H antigens. *Salmonella Enteritidis* (11), *Salmonella Typhimurium* (8) and *Salmonella Muenster* (6) were the most prevalent serotypes followed by *Salmonella Anatum* (3) and *Salmonella Virchow* (2) (Table 2). These results supported those obtained by Fashae *et al*.[28], Muhammad *et al*.[29], Suresh *et al*.[30], Abd El-Ghany *et al*.[31] and Ahmed and Shimamoto[32].

The molecular detection of *Salmonella* species using PCR showed that there were 5 additional isolates that were typical for *Salmonella* by conventional (culture, biochemical and serological) methods. This result suggests that the PCR technique would be a sensitive, rapid and specific diagnostic tool for *Salmonella* detection on poultry farms[33].

The dissemination of antibiotic resistance genes and emergence of antibiotic resistant *Salmonella* serovars reflects the worldwide interest and public health concerns about these pathogens, especially in Africa and Asia[34,35]. Multidrug resistance, particularly in *Enterobacteriaceae,* represents a significant public health concern in both developing and developed countries[36]. Hence, the assessment of antibiotic resistance of *Salmonella* species isolated from chickens has become an important integrated process in pathogen control methods. Antibiotic susceptibility results of the current study revealed three antibiotic profiles among the *Salmonella* isolates (Table 3). All *Salmonella* isolates were phenotypically susceptible to ciprofloxacin and chloramphenicol as was reported by Murugkar *et al*.[37], Salehi *et al*.[38] and Begum *et al*.[39]. In contrast, Agbaje *et al*.[40] and Muthu *et al*.[41] showed that the majority of isolated *Salmonella* serovars were resistant to ciprofloxacin and chloramphenicol. This difference may be due to the continuous use of antibiotics in a given locality that leads to the emergence of resistant strains of the same pathogens.

Pathogenicity islands include large clusters of genes that facilitate *Salmonella* colonization, invasiveness and establishment of systemic infection in the host. They also facilitate acquisition of a single island that can convert a non-pathogenic microorganism to a pathogenic one[2,13-19]. In the current study the virulence genes for five major SPIs from the three major predominate *Salmonella* serovars: *S. Enteritidis, S. Typhimurium* and *S. Muenster* were examined and found that the five major pathogenicity islands (SP-1-SP-5) genes were present in all of them (Fig. 1), which is concurrent with studies by Soto *et al*.[23] and Sanchez-Jimenez *et al*.[24]. The results also revealed that all three predominate serovars, but particularly *S. Enteritidis* and *S. Typhimurium,* were present in our samples. These two serovars are pathogenic to both humans and chickens and cause 80% of *Salmonella* infections in humans.

CONCLUSION

There are several different *Salmonella* serotypes, including *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella muenster*, *Salmonella anatum* and *Salmonella virchow* circulating in chicken farms in Egypt. Among these, *Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes. These isolates represent a public health risk and also exhibit several antibiotic resistant patterns as well as the most common pathogenicity islands.

SIGNIFICANCE STATEMENTS

This study identified prevalent multidrug resistant *Salmonella* species among chickens in poultry farms in Egypt. These serovars carry five major virulence associated genes and represent potential public health hazards. As such, measures to control the prevalence of *Salmonella* in poultry farms are urgently needed. This study examined in greater detail the pathogenicity of *Salmonella* species in an agricultural setting and revealed critical areas where *Salmonella* control measures have been unsuccessful. These results may provide insight into antibiotic susceptibility and virulence-associated factors of *Salmonella* in poultry farms and form a basis for developing more successful control measures.

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