Isolation and characterization of *Salmonella enterica* in day-old ducklings in Egypt

Kamelia M. Osman[1], Sherif H. Marouf[1], Tara R. Zolnikov[2], Nayerah AlAtfeehy[3]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt, [2]North Dakota State University, Developmental Science, Fargo, USA, [3]Department of Poultry Diseases, Animal Research Institute, Dokki, Egypt

Importing day-old ducklings (DOD) unknowingly infected with non-typhoid *Salmonella* (NTS) may be associated with disease risk. Domestic and international trade may enhance this risk. *Salmonella enterica* serovars, their virulence genes combinations and antibiotic resistance, garner attention for their potentiality to contribute to the adverse health effects on populations throughout the world. The aim of this study was to estimate the risk of imported versus domestic DOD as potential carriers of NTS. The results confirm the prevalence of salmonellosis in imported ducklings was 18.5% (25/135), whereas only 12% (9/75) of cases were determined in the domestic ducklings. Fourteen serovars (*Salmonella enteritidis*, *Salmonella kisii*, *Salmonella typhimurium*, *Salmonella gaillac*, *Salmonella uno*, *Salmonella eingedi*, *Salmonella shubra*, *Salmonella bardo*, *Salmonella inganda*, *Salmonella kentucky*, *Salmonella stanley*, *Salmonella virchow*, *Salmonella haifa*, and *Salmonella anatum*) were isolated from the imported ducklings, whereas only *S. enteritidis*, *S. typhimurium*, *S. virchow*, and *S. shubra* were isolated from the domestic ducklings. The isolated *Salmonella* serovars were 100% susceptible to only colistin sulphate and 100% resistant to lincomycin. The 14 *Salmonella* serovars were screened for 11 virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sil*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcf*C) by PCR. The *inv*A, *sop*B, and *bcf*C genes were detected in 100% of the *Salmonella* serovars; alternatively, the *gip*A gene was absent in all of the isolated *Salmonella* serovars. The 11 virulent genes were not detected in either of *S. stanley* or *S. haifa* serovars. The results confirm an association between antibiotic resistance and virulence of *Salmonella* in the DOD. This study confirms the need for a country adherence to strict public health and food safety regimes.

Keywords: Antibiotic resistance, Day-old ducklings, *Salmonella* serovars, Virulence genes

Introduction

Ducks are frequently used by human populations throughout the world for a variety of reasons; duck meat and duck eggs are consumed for protein-specific dietary purposes, raised as pets for children, used for natural décor on small ponds or lakes, hunted in game preserves or conservation areas, and finally, used for entertainment at country fairs.[1,2] In the United States of America (USA), 6.4 million households own and raise ducks in their backyards for meat, egg production, or as pets.[3,4] Unfortunately, disease risks are associated with contact with ducks and may contribute to adverse health effects in people. Aside from food-borne infections, a cluster of non-typhoid *Salmonella* (NTS) human infections has also been associated with day-old ducklings (DOD).[1–3,5–7] *Salmonella* outbreaks have intermittently occurred every few years since the 1950s when ducklings are purchased as pets during the Easter holiday season.[8–10] One case in the USA confirmed a single mail-order poultry hatchery was responsible for an 8-year *Salmonella* outbreak and thousands of infections in 43 states.[11] *Salmonella typhimurium* has been isolated from 40% of hatchlings and 1% of older ducklings in Taiwan, even though clear host species specific differences have also been detected.[12]

Salmonella has been previously isolated from imported DOD in Brazil and the USA.[11,13,14] Additionally, a significant amount of the infected duck products contained multi-drug-resistant (MDR) foodborne pathogens.[15] Because the prevalence of *Salmonella* in duck products poses a risk to human populations, an urgent need exists to investigate the prevalence, disease risk to human populations, and the global epidemiology of *Salmonella* serovars and specific clones. This information may be used to address *Salmonella* risk and promote evidence-based interventions in global public health.

A diversity of virulence factors have been previously described for *Salmonella*; these virulence factors are located on the bacterial chromosome,

Correspondence to: Kamelia M. Osman, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt. Email: kamelia-osman@hotmail.com

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frequently as part of pathogenicity islands, on plasmids and prophages.[16] Although all serovars of *Salmonella enterica* are considered pathogenic to man,[17] the distribution of virulence genes in serovars is not well understood.[18] An encompassing scientific literature review also confirms the negligible presence of research focussing on understanding virulence determinants associated with the isolated *Salmonella* serovars with special reference to DOD.

An accelerated dissemination of resistant pathogenic organisms from one geographic location to another has occurred as a result of globalization and international trade over the past decade.[19] Yet, there is a limited amount of data concerning the prevalence of foodborne pathogens and associated antimicrobial susceptibility phenotypes among imported poultry.[19] A very important issue that has been debated is whether the MDR strains are associated with virulence determinants[20] and thus have the propensity to be more virulent than their susceptible counterparts; therefore, this study attempted to address this outstanding issue on whether genetic determinants for both antibiotic resistance and virulence genes could be harboured by the same transferable element and further confirm the association between antibiotic resistance and virulence in DOD.

Consequently, the objectives of the current study were to determine and establish baseline data on the prevalence of *S. enterica* serovars, virulence genes, gene association and combinations, virulence genes encoding serotypes, and antibiotic-resistant *Salmonella* phenotypes in imported and domestic DOD in Egypt.

Materials and Methods

Sampling and isolation

All imported bird samples were delivered to the Central Lab for Veterinary Quality Control on Poultry Production, Agriculture Research Center, Ministry of Agriculture. A total of 150 imported DOD were randomly collected from 50 boxes (25 birds per box; 3 birds/25 birds). Faecal samples were collected from the 150 imported DOD for culture and isolation of salmonellae; additionally, 150 faecal samples were taken from domestic DOD, which originated from both small-scale and commercial farms, whose production is primarily used for buying and selling DOD. The 300 faecal samples were analysed for *Salmonella* according to the ISO-6579-1993 standards.[21] Twenty five grams of faeces were mixed with 225 ml of buffered peptone water (Oxoid Ltd, Hampshire, England) for pre-enrichment. After incubation at 37°C for 24 hours, 0.1 ml were transferred to 10 ml of selective Rappaport-Vassiliadis broth (Oxoid Ltd) and were incubated for 24 hours at 42°C. A loopful of broth culture was streaked on Rambach agar (Merck, Darmstadt, Germany), xylose lysine desoxycholate agar (XLD, Oxoid Ltd), and Hektoen enteric agar (Oxoid Ltd); agar plates were then incubated at 37°C for 24–48 hours Presumptive *Salmonella* colonies were identified on the basis of a Gram stain, catalase reaction, oxidase reaction, and oxidation/fermentation of glucose. Gram negative bacilli, catalase positive, oxidase negative, and capability of oxidation and fermentation of glucose were inoculated onto microtubes of API 20E strips (bioMérieux, Marcy L'Étoile, France) in accordance with the manufacturers' instructions. The bacteria were identified using the database API LAB Plus version 3.2.2 (bioMérieux). On each positive sample, an individual *Salmonella* isolate was typed and subjected to further serotyping analysis. Serotyping was performed according to the Kauffmann–White typing scheme[22] using slide agglutination with standard antisera (Difco Laboratories, Detroit, MI, USA).

Phenotypic Virulence Assays

Invasiveness profiles

Congo red binding

The method of Qadir *et al.*[23] was used to test invasiveness profiling. Congo red (0.003%, w/v) was incorporated into nutrient agar before autoclaving. Plates streaked with test strains were incubated at 37°C for 18 hours Colonies were examined for the presence (red, crb +ve) or absence (white, crb −ve) of Congo red binding.

Serum survival

Bacterial survival in normal, uninfected duck and chicken sera was assayed through Congo red binding.[24] Counts were determined at times T = 0 hour and T = 2 hours following addition of the inoculum to 10% normal duck and chicken sera in single strength Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5.6 mM D-glucose, 0.02% phenol red, distilled water to 1000 ml; membrane filtre sterilized (pore size: 0.45 μm). Cultures of bacteria (*Salmonella enteritidis*, *S. typhimurium*, *Salmonella kisii*, *Salmonella gaillac*, *Salmonella uno*, *Salmonella eingedi*, *Salmonella shubra*, *Salmonella bardo*, *Salmonella inganda*, *Salmonella kentucky*, *Salmonella stanley*, *Salmonella haifa*, *Salmonella virchow*, and *Salmonella anatum* in addition to the two untypable isolates) in the log growth phase were prepared in 1 × phosphate buffered saline (PBS); 10 μl of each individual sample was added to 90 μl of freshly thawed, undiluted serum for a final bacterial concentration of 10^6 CFU/ml. The mixtures were incubated at 37°C with gentle rocking (20 rpm); sequentially, 10 μl of each sample was withdrawn at 45, 90, and 180 minutes. Serial dilutions of the samples were plated onto LB agar plates to determine

the viable bacterial counts. Survival, expressed as serum resistance factor, was determined at either CFU at $T = 2$ hours or CFU at $T = 0$ hour. Mean results were based on three independent determinations.

MDCK and Vero cell ingestion assay

The ingestion assay on Madin Darby Canine kidney (MDCK) and Vero cells was conducted according to Raja *et al.*[25] and were obtained from American Type Culture Collection, Rockville, Madison, USA. Cell free culture supernatants (CFCS) of *S. enteritidis*, *S. typhimurium*, *S. kisii*, *S. gaillac*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. stanley*, *S. haifa*, *S. virchow*, and *S. anatum*, in addition to the two untypable isolates, were prepared. Each bacterial strain was grown in brain heart infusion (BHI) broth for 24 hours at 37°C. After incubation, an inoculum containing 5×107 CFU/ml was taken from which 100 µl was added to the wells containing Vero and MDCK cells. Bacterial cells were allowed to infect the epithelial cells for 30 minutes–12 hours at 37°C. A control assay with uninfected cell lines was also maintained. Live cells were removed, and cytological changes were observed using an inverted microscope.

Chicken embryo lethality assay

Embryo lethality was performed according to Wooley *et al.*[26]. Overnight broth cultures of each isolate (*S. enteritidis, S. typhimurium, S. kisii, S. gaillac, S. uno, S. eingedi, S. shubra, S. bardo, S. inganda, S. kentucky, S. stanley, S. haifa, S. virchow, S. anatum*, and two untypable isolates) were washed twice in PBS, resuspended, diluted in PBS, and inoculated (total volume of 0.1 ml) into the allantoic cavity of six 12-day-old embryonated chicken eggs. Six PBS inoculated and uninoculated control embryonated eggs were also included in embryo lethality tests. Eggs were candled daily; deaths were recorded as they occurred until the embryos were 18 days of age. Allantoic fluid, liver, spleen, heart, and the brain of each embryo were collected immediately upon death and used for reisolation of the infected *Salmonella* serotypes. Isolates causing embryo death of > 29, 10–29, and $< 10\%$ were considered virulent, moderately virulent, and avirulent isolates, respectively.

Mannose sensitive and mannose resistance haemagglutination assay

The tests were performed in the presence or absence of D-mannose at 37 and 18°C for 24 hours.[27] *Salmonella enteritidis, S. typhimurium, S. kisii, S. gaillac, S. uno, S. eingedi, S. shubra, S. bardo, S. inganda, S. kentucky, S. stanley, S. haifa, S. virchow, S. anatum*, and the two untypable isolates were briefly grown on buffered glucose nutrient agar supplemented with 5% sheep blood. The bacteria were then suspended in PBS for a final concentration of approximately 10^{10} bacteria/ml; two-fold serial dilutions were created in 96-well round-bottom microtitre plates. An equal volume of 3% red blood cell (RBC) suspension (duck, chicken, guinea pig, and human type O) was added to each well of the bacterial suspensions. For mannose-resistant haemagglutination (MRHA) test, 20 µl of bacterial suspension was mixed with an equal volume of RBC suspension on a cavity glass slide and then, 20 µl of PBS was added to the mixture. Reactions were recorded as (+) for haemagglutination or (−) for no haemagglutination. A control, consisting of RBCs suspended in PBS, was included in the test.

To demonstrate mannose sensitive haemagglutination (MSHA) of the isolates, a 1% (w/v) D (+) mannose solution was used to examine the inhibition of haemagglutination. The effect of carbohydrates was assessed by adding 20 µl of a sugar solution to the bacteria-RBC suspension. A positive control was also included in each test; this control comprised equal volumes of bacterial suspension. If positive haemagglutination turned negative, inhibition was recorded. The highest dilution reciprocal of bacterial suspension with complete haemagglutination of RBCs was considered the endpoint. Reactions with titres above 1:4 were considered positive, indicating the presence of specific hemagglutins. Each test was duplicated throughout the haemagglutination assay.

Antimicrobial susceptibility testing by disc diffusion

To determine antibiotic resistance of the isolated *Salmonella* serotypes, each isolate was inoculated onto Muller–Hinton agar (Oxoid) and incubated at 37°C for 24 hours These techniques were conducted according to Clinical and Laboratory Standards Institute (CLSI)[28] guidelines for disc diffusion techniques using commercial discs (Becton, Dickinson and Company, Sparks, Maryland, USA). After this procedure, the zones of inhibition were measured in order to assess resistance or susceptibility. The panel of antibiotic discs used in panel screens belonged to eight drug classifications. Selected antimicrobials were chosen based on their common use in treating or preventing *Salmonella* infection in humans. The antimicrobial agents and corresponding concentrations used in the study included ampicillin (10 mg), amoxicillin (20 mg), gentamicin (10 mg), neomycin (30 µg), streptomycin (10 mg), ciprofloxacin (5 µg), norfloxacin (5 µg), nalidixic acid (30 mg), lincomycin (30 µg), chloramphenicol (30 mg), colistin (10 mg), tetracycline (30 mg), trimethoprim (5 µg), and sulphamethoxazole + trimethoprim (23.75 + 1.75 mg).

Detection of virulence determinants

Detection of 11 virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcf*C) in the 14 typable serotypes and 2 untypable isolates

was conducted through PCR amplification. Table 1 outlines the primers, sequences, conditions, and predicted sizes of the amplified products. These virulence determinants represent either highly conserved (Salmonella pathogenicity islands, SPIs) or variable (prophages, plasmid) regions within the *Salmonella* genome. Bacterial strains of *S. enteritidis* (ATCC 13076) and *Escherichia coli* (ATCC 50034) were used as reference strains. Except *sop*E1, a positive control DNA from *S. enterica* serovar *S. typhimurium* strain LT2 was used for all targets.[32] For *sop*E1, DNA from *S. enterica* serovar Hadar strain 99-0601 was used as the positive control.

Statistical analysis

The distribution of single or multi-drug antibiotic resistance phenotypes among serotypes was determined by a contingency table analysis; this table analysis included labelled rows = serotypes and columns resistance versus susceptible. The statistical significance of homogeneity in antimicrobial resistance patterns among the groups was assessed using Pearson's chi^2 exact test using SAS version 9.2 (SAS, Cary, NJ, USA). Findings were considered statistically significant when $P < 0.05$. A contingency table allows researchers to test the proportion of resistance bacteria across different serotypes while using Pearson's

Table 1 Virulence factor targets and primers, including nucleotide sequences, PCR conditions, and references

Gene designation	Location on SP1/gene function	Oligonucleotide sequences (5′–3′)	PCR conditions[a]			Product size (bp)	References
			Denaturing	Annealing	Extension		
*inv*A	Type III secretion system apparatus SPI-1/invasion of macrophages	gtg aaa tta tcg cca cgt tcg ggc aa tca tcg cac cgt caa agg aac g	94°C for 60 seconds	64°C for 30 seconds	72°C for 30 seconds[b]	284	29
*avr*A	SPI-1/controls *Salmonella*-induced inflammation	cct gta ttg ttg agc gtc tgg aga aga gct tcg ttg aat gtc c	95°C for 30 seconds	58°C for 30 seconds	72°C for 30 seconds[b]	422	30
*ssa*Q	SPI-2/secretion system apparatus protein, component of second T3SS	gaa tag cga atg aag agc gtc gtc c cat cgt gtt atc ctc tgt cag c				455	
*mgt*C	SPI-4/Mg^{2+} uptake	tga cta tca atg ctc cag tga at att tac tgg ccg cta tgc tgt tg				677	
*sii*D (*Spi4*D)	Type I secretion/SPI-4	gaa tag aag aca aag cga tca tc gct ttg ttc acg cct ttc atc				655	31
*sop*B	SPI-5/inositol polyphosphate, phosphatase that promotes macropinocytosis, regulates SCV localization, and promotes fluid secretion	tca gaa gRc gtc taa cca ctc tac cgt cct cat gca cac tc				517	30
*gip*A	Gifsy-1 bacteriophage/Peyer's patch-specific virulence factor	acg act gag cag cgt gag ttg gaa atg gtg acg gta gac				518	
*sod*C1	Gifsy-2 bacteriophage/periplasmic Cu, Zn-superoxide dismutases	cgg gca gtg ttg aca aat aaag tgt tgg aat tgt gga gtc				424	
*sop*E1	Cryptic bacteriophage/promotes membrane ruffling and disrupts tight junctions	act cct tgc aca acc aaa tgc gga tgt ctt ctg cat ttc gcc acc				422	
*spv*C	pSLT/A phosphothreonine lyase required for complete virulence in murine models	acc aga gac att gcc ttc c ttc tga tcg ccg cta ttc g				467	
*bcf*C	Chromosome/bovine colonization factor, fimbrial usher	acc aga gac att gcc ttc c ttc tgc tcg ccg cta ttc g	95°C for 30 seconds	53°C for 30 seconds	72°C for 30 seconds[b]	467	

[a] PCR was done for 35 cycles.
[b] After 30 cycles, final extension step of 4 minutes at 72°C was performed.
[c] SCV, Salmonella-containing vacuole.

chi^2 test to assess the statistical significance between proportions. A high chi^2 value confirms resistant phenotypes are not proportionately distributed among serotypes; although, some serotypes may have a higher frequency of resistant phenotypes than others.

Results

Prevalence and serotyping of Salmonella

The 14 isolated serotypes were identified as *S. enteritidis, S. kisii, S. typhimurium, S. gaillac, S. uno, S. eingedi, S. shubra, S. bardo, S. inganda, S. kentucky, S. stanley, S. virchow, S. haifa*, and *S. anatum* from the imported ducklings and *S. enteritidis, S. typhimurium, S. virchow*, and *S. shubra* from the domestic ducklings. The prevalence of salmonellosis in the imported ducklings was $<$ 18.5% (25/135); serotyping of the *Salmonella*-isolated strains *S. enteritidis* and *S. kisii* confirmed a prevalence of 2.2% (3/135) in each strain. The prevalence decreased to 1.5% (2/135) in serotypes *S. typhimurium, S. gaillac, S. uno, S. eingedi, S. shubra, S. bardo, S. inganda, S. kentucky, S. stanley, S. virchow, S. haifa*, and *S. anatum*, while the one untypable isolate was $<$ 1% (1/135). The prevalence of salmonellosis in tested local ducklings was 12% (9/75) and serotyping of the *Salmonella*-isolated strains, *S. enteritidis, S. typhimurium, S. virchow*, and *S. shubra*, recorded a prevalence of 2.7% (2/75) and 1.3% (1/75) in the untypable isolate.

Phenotypic Virulence Assays

Congo red binding, serum survival, MDCK, chicken embryo lethality assay, Vero cell ingestion assay, and haemagglutination tests

Interestingly, *S. enteritidis*, *S. typhimurium*, *S. kisii*, *S. gaillac*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. virchow*, *S. anatum*, and the two untypable isolates displayed strong positive Congo red binding, were resistant to serum bactericidal activity, and survived for 3 hours in chicken and duck sera. Alternatively, *S. stanley* and *S. haifa* were weakly positive to Congo red binding (displayed by the orange colour) and were unable to survive over 1 hour in the chicken and duck sera; at the same time, the results confirmed 100% of susceptibility (after 12 hours of exposure to the *Salmonella* cytotoxin in cell free sonic lysate) and 100% death of the embryos during the periods of day-13 and day-17 in the two cell lines, Vero and MDCK.

The results of the tested serovars *S. kisii*, *S. gaillac*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. anatum*, *S. stanley*, *S. haifa*, and the two untypable isolates were 100% MRHA to duck erythrocytes, whereas *S. eingedi*, *S. stanley*, *S. bardo*, *S. kentucky*, and *S. haifa* were 100% MRHA to chicken erythrocytes. In all, 100% MRHA to guinea pig erythrocytes was recorded for the serotypes *S. kisii*, *S. uno*, *S. eingedi*, *S. stanley*, *S. inganda*, *S. kentucky*, and *S. haifa*. Serotypes *S. kisii*, *S. uno*, *S. eingedi*, *S. kentucky*, and *S. haifa* were 100% MRHA to human group O erythrocytes.

Distribution of resistance to individual antimicrobial agents

All *Salmonella* serovars were resistant to more than one tested antimicrobial; the serovars were susceptible to only 1 (colistin sulphate) of the 14 antimicrobials (Table 2), but were all 100% resistant to lincomycin. Resistance frequencies varied depending on the antibiotic selection; all 14 *Salmonella* serovars and untypables exhibited significant diversity in their resistance patterns. From the eight different classes, *S. typhimurium*, *S. shubra*, *S. eingedi*, *S. bardo*, *S. haifa*, *S. kentucky*, and *S. stanley* were resistant to seven of them with the exception of polymyxin (colistin sulphate). *Salmonella enteritidis*, *S. gaillac*, and *S. inganda* were resistant to six different antimicrobial resistance profiles (aminoglycosides, fluoroquinolones, lincosamides, phenicols, tetracyclines, and sulphonamides). A wide variety of resistant antimicrobials occurred in the untypable *Salmonella* isolates; the six antimicrobial-resistant profiles included penicillins, aminoglycosides, fluoroquinolones, lincosamides, phenicols, and sulphonamides. *Salmonella kisii* and *S. virchow* isolates were resistant to only five antimicrobial resistance profiles (aminoglycosides, fluoroquinolones, lincosamides, phenicols, and sulphonamides). The least four antimicrobial-resistant profiles for *S. uno* included aminoglycosides, fluoroquinolones, lincosamides, and phenicols, whereas for *S. anatum* the least four resistant profiles included aminoglycosides, lincosamides, phenicols, and sulphonamides.

Statistically significant correlations for resistance between the antimicrobials at the isolate level are presented in Tables 2 and 4. The distribution of resistance to the different antibiotics was largely dependent on the serovar identity. Significant resistance ($P < 0.001$) to amoxicillin (chi^2 = 13.0) was associated with serovars *S. eingedi*, *S. shubra*, *S. bardo*, *S. kentucky*, *S. stanley*, and *S. haifa*. Chloramphenicol (chi^2 = 13.0) confirmed significant resistance to serovars *S. virchow*, *S. shubra*, *S. eingedi*, *S. haifa*, *S. kentucky*, *S. stanley*, *S. anatum*, and *S. inganda*. Gentamicin (chi^2 = 15.1) was associated with serovars *S. kentucky*, *S. stanley*, and *S. gaillac*. Neomycin (chi^2 = 14.2) was associated with serovars *S. kentucky*, *S. stanley*, *S. haifa*, *S. gaillac*, *S. anatum*, and *S. inganda*. Ciprofloxacin (chi^2 = 34.0) was associated with serovars *S. shubra* and *S. kentucky*. Tetracycline (chi^2 = 26.8) was associated with serovars *S. shubra*, *S. eingedi*, *S. bardo*, *S. haifa*, *S. kentucky*, *S. stanley*, and *S. gaillac*. Finally, trimethoprim (chi^2 = 12.2) was found to be associated with *S. shubra*, *S. eingedi*,

Table 2 Distribution of resistance to individual antimicrobial agents among sources and *Salmonella enterica* serovars in day-old ducklings (DOD)

	Distribution of *Salmonella* resistance to antimicrobials														
	S. enteritidis 5/34	*S. kisii* 3/34	*S. typhimurium* 4/34	*S. virchow* 3/34	*S. shubra* 4/34	*S. eingedi* 2/34	*S. bardo* 3/34	*S. uno* 2/34	*S. haifa* 1/34	*S. kentucky* 1/34	*S. stanley* 1/34	*S. gaillac* 2/34	*S. anatum* 1/34	*S. inganda* 1/34	Untypable 2/34
Antimicrobials	No. of resistant isolates														
Penicillins															
Ampicillin	0/5	0/3	2/4	0/3	3/3	2/2	3/3	0/2	1/1	1/1	1/1	0/2	0/1	0/1	1/2
Amoxicillin	0/5	0/3	3/4	0/3	3/3	2/2	3/3	0/2	1/1	1/1	1/1	0/2	0/1	0/1	0/2
Aminoglycosides															
Gentamicin	0/5	0/3	0/4	0/3	2/3	1/2	1/3	0/2	0/1	1/1	1/1	2/2	0/1	0/1	0/2
Neomycin	1/5	1/3	0/4	0/3	1/3	1/2	1/3	0/2	1/1	1/1	1/1	2/2	1/1	1/1	1/2
Streptomycin	2/5	2/3	3/4	2/3	3/3	2/2	3/3	2/2	1/1	1/1	1/1	2/2	1/1	1/1	2/2
Fluoroquinolones															
Ciprofloxacin	0/5	0/3	0/4	0/3	3/3	0/2	0/3	0/2	0/1	1/1	0/1	0/2	0/1	0/1	0/2
Nalidixic acid	2/5	2/3	3/4	1/3	2/3	2/2	1/3	1/2	1/1	1/1	1/1	2/2	0/1	1/1	1/2
Norfloxacin	0/5	0/3	0/4	0/3	1/3	1/2	0/3	0/2	0/1	1/1	1/1	2/2	0/1	0/1	1/2
Lincosamides															
Lincomycin	5/5	3/3	4/4	3/3	3/3	2/2	3/3	2/2	1/1	1/1	1/1	2/2	1/1	1/1	2/2
Phenicols															
Chloramphenicol	3/5	2/3	3/4	3/3	3/3	2/2	2/3	1/2	1/1	1/1	1/1	1/2	1/1	1/1	1/2
Polymyxin															
Colistin sulphate	0/5	0/3	0/4	0/3	0/3	0/2	0/3	0/2	0/1	0/1	0/1	0/2	0/1	0/1	0/2
Tetracyclines															
Tetracycline	1/5	0/3	1/4	0/3	3/3	2/2	3/3	0/2	1/1	1/1	1/1	2/2	0/1	1/1	0/2
Sulphonamides															
Trimethoprim	1/5	1/3	2/4	0/3	3/3	2/2	2/3	0/2	1/1	1/1	1/1	1/2	1/1	0/1	1/2
Trimethoprim–sulfamethoxazole	2/5	2/3	3/4	1/3	3/3	1/2	2/3	0/2	1/1	1/1	1/1	0/2	0/1	1/1	1/2

S. haifa, *S. kentucky*, *S. stanley*, and *S. anatum*. Significant resistance (with a value of $P < 0.003$) was also recorded for ampicillin ($chi^2 = 8.5$) that was associated with serovars *S. shubra*, *S. eingedi*, *S. bardo*, *S. haifa*, *S. kentucky*, and *S. stanley*. Streptomycin ($chi^2 = 8.8$) was also observed to be associated with serovars *S. shubra*, *S. eingedi*, *S. bardo*, *S. uno*, *S. haifa*, *S. kentucky*, *S. stanley*, *S. gaillac*, *S. anatum*, *S. inganda*, and the untypable *Salmonella* isolate. Low significant resistance of sulphamethoxazole+trimethoprim ($chi^2 = 7.0$; $P < 0.008$) was associated with serovars *S. shubra*, *S. haifa*, *S. kentucky*, *S. stanley*, and *S. bardo*; low resistance also occurred in nalidixic acid ($chi^2 = 6.5$; $P < 0.01$), which was associated with serovars *S. eingedi*, *S. haifa*, *S. kentucky*, *S. stanley*, *S. gaillac*, and *S. inganda*.

Virulence genes

All isolates were screened by PCR analysis for the presence or absence of 11 selected virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcf*C) (Table 3). The PCR screening analysis detected the *inv*A, *sop*B, and *bcf*C genes that were confirmed in *S. enteritidis*, *S. typhimurium*, *S. kissii*, *S. virchow*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. gaillac*, and *S. anatum* isolates and the two untypable isolates; on the contrary, the *gip*A gene was absent from all of the isolated *Salmonella* serovars (*S. enteritidis*, *S. typhimurium*, *S. kissii*, *S. virchow*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. gaillac*, and *S. anatum*), whereas the *sop*E1 gene (encoding a translocated effector protein) was detected in the *S. inganda* isolate, but remained undetected in the remainder of the *Salmonella* serovars. None of the 11 virulent genes were detected encoding either of the *S. stanley* and *S. haifa* isolates. The *spv*C gene, carried by the *Salmonella* virulence plasmid, was expressed in 10 isolates of the isolated salmonellae, 9 of which were on the *S. enteritidis* and *S. typhimurium* isolated from the imported and local ducklings. The *sod*C1 gene, located on a bacteriophage, in nine isolates of the tested salmonellae, was isolated from the imported and local ducklings; eight of these isolates were *S. enteritidis* and *S. typhimurium*.

Table 3 Distribution of the virulence genes among the *Salmonella* serovars isolated from day-old ducklings (DOD)

	Virulence genes										
***Salmonella* serotypes**	***inv*A**	***avr*A**	***ssa*Q**	***mgt*C**	***sii*D**	***sop*B**	***gip*A**	***sod*C1**	***sop*E1**	***spv*C**	***bcf*C**
From imported ducklings											
S. enteritidis	+	+	ND	+	ND	+	ND	+	ND	+	+
S. enteritidis	+	+	ND	+	ND	+	ND	+	ND	+	+
S. enteritidis	+	ND	ND	ND	ND	+	ND	+	ND	+	+
S. typhimurium	+	ND	ND	ND	ND	+	ND	+	ND	+	+
S. typhimurium	+	ND	+	+	ND	+	ND	+	ND	+	+
S. kisii	+	ND	+	ND	ND	+	ND	ND	ND	ND	+
S. kisii	+	+	+	+	ND	+	ND	ND	ND	ND	+
S. kisii	+	+	+	+	ND	+	ND	ND	ND	ND	+
S. virchow	+	+	+	+	ND	+	ND	ND	ND	ND	+
S. uno	+	+	+	+	ND	+	ND	ND	ND	ND	+
S. uno	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+
S. eingedi	+	+	+	+	+	+	ND	+	ND	+	+
S. eingedi	+	+	+	+	+	+	ND	ND	ND	ND	+
S. shubra	+	ND	+	ND	+	+	ND	ND	ND	ND	+
S. shubra	+	ND	+	+	+	+	ND	ND	ND	ND	+
S. bardo	+	+	+	+	+	+	ND	ND	ND	ND	+
S. bardo	+	+	+	+	+	+	ND	ND	ND	ND	+
S. inganda	+	+	+	+	+	+	ND	ND	+	ND	+
S. kentucky	+	+	+	+	+	+	ND	ND	ND	ND	+
S. stanley	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. haifa	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
S. gaillac	+	+	+	+	+	+	ND	ND	ND	ND	+
S. gaillac	+	+	+	+	+	+	ND	ND	ND	ND	+
S. anatum	+	ND	+	+	+	+	ND	ND	ND	ND	+
Untypable	+	ND	+	+	+	+	ND	ND	ND	ND	+
From domestic ducklings											
S. enteritidis	+	+	+	+	ND	+	ND	+	ND	+	+
S. enteritidis	+	+	+	+	ND	+	ND	+	ND	+	+
S. typhimurium	+	ND	ND	ND	ND	+	ND	+	ND	+	+
S. typhimurium	+	ND	ND	ND	ND	+	ND	ND	ND	+	+
S. virchow	+	+	+	+	ND	+	ND	ND	ND	ND	+
S. virchow	+	ND	+	ND	ND	+	ND	ND	ND	ND	+
S. shubra	+	ND	+	ND	+	+	ND	ND	ND	ND	+
S. shubra	+	ND	+	+	+	+	ND	ND	ND	ND	+
Untypable	+	+	+	+	+	+	ND	ND	ND	ND	+

ND: not detected.

Virulence genes combinations

The 14 serovar collections provided significant diversity in the virulence genes combinations. The 11 genes were absent from the isolated *S. stanley* and *S. haifa*, which were isolated from the imported ducklings providing the first virulent gene combination. A second combination occurred in the *S. eingedi* (2/2), *S. bardo* (2/2), and *S. kentucky* (1/1). A third combination occurred in *S. kissii* (2/3), *S. virchow* (1/1), and *S. uno* serotypes (1/2). A fourth combination was recorded with *S. enteritidis* (2/3). A fifth combination occurred in *S. enteritidis* (1/3) and *S. typhimurium* (1/2). The two *S. gaillac* isolates had a common virulence gene combination, but varied from the other 34 isolates. Lastly, virulence gene repertoires occurred in *S. anatum* and the untypable isolate.

Association of antimicrobial resistance phenotype with virulence-associated genes

The presence of *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcfC* genes in *S. enteritidis, S. kissii, S. typhimurium, S. gaillac, S. uno, S. eingedi, S. shubra, S. bardo, S. inganda, S. kentucky, S. stanley, S. virchow, S. haifa, S. anatum*, and the one untypable isolate in the imported ducklings, alongside the Salmonella isolated strains (*S. enteritidis, S. typhimurium, S. virchow* and *S. shubra*, and the untypable isolate) from the domestic ducklings, displayed various antimicrobial resistance patterns (Table 4). A detailed analysis displayed associations of resistance and susceptibility phenotypes with potential virulence genes. The study confirmed 14 tested antimicrobials were not associated with any of the 11 virulent genes, which were not detected in either of the

Table 4 Distribution of virulence genes combinations in the different *Salmonella* serovars and antibiotic resistance phenotypes isolated from day-old ducklings (DOD)

Salmonella serotype	Virulence genes combinations[a]	Antibiotic resistance[b]
Isolated from imported ducklings		
S. enteritidis	**inv**A, *avr*A, *mgt*C, *sod*C1, **sop**B, *spv*C, **bcf**C	Chl, Lin, Neo, Str, Tet, Sxt
S. enteritidis	**inv**A, *avr*A, *mgt*C, *sod*C1, **sop**B, *spv*C, **bcf**C	Chl, Lin, Neo
S. enteritidis	**inv**A, *sod*C1, **sop**B, *spv*C, **bcf**C	Lin
S. typhimurium	**inv**A, **sop**B, *sod*C1, *spv*C, **bcf**C	Lin, Str
S. typhimurium	**inv**A, *mgt*C, *ssa*Q, *sod*C1, **sop**B, *spv*C, **bcf**C	Amo, Amp, Chl, Lin, Na, Str, Tri, Sxt
S. kissii	**inv**A, *ssa*Q, **sop**B, **bcf**C	Chl, Lin, Na, Str, Tri, Sxt
S. kissii	**inv**A, *avr*A, *ssa*Q, *mgt*C, **sop**B, **bcf**C	Lin, Na, Neo, Sxt
S. kissii	**inv**A, *avr*A, ssaQ, *mgt*C, **sop**B, **bcf**C	Chl, Lin, Str
S. virchow	**inv**A, *avr*A, *ssa*Q, *mgt*C, **sop**B, **bcf**C	Chl, Lin, Nor
S. uno	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Chl, Lin, Neo, Str
S. uno	**inv**A, **sop**B, **bcf**C	Lin, Str
S. eingedi	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, *spv*C, **bcf**C	Amo, Amp, Chl, Lin, Na, Neo, Nor, Str, Tet, Tri, Sxt
S. eingedi	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Amp, Chl, Gen, Lin, Str, Tet, Tri
S. shubra	**inv**A, *ssa*Q, *sii*D, **sop**B, **bcf**C	Amo, Amp, Chl, Gen, Lin, Na, Str, Tet, Tri, Sxt
S. shubra	**inv**A, *ssa*Q, *mgt*C, *sii*D, *sod*C1, **sop**B, **bcf**C	Amo, Amp, Chl, Gen, Lin, Na, Neo, Nor, Str, Tet, Tri, Sxt
S. bardo	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Amp, Lin, Str, Tet, Tri, Sxt
S. bardo	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Amp, Chl, Gen, Lin, Str, Tet
S. inganda	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*E1, **sop**B, **bcf**C	Chl, Lin, Na, Neo, Str, Tet, Sxt
S. kentucky	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sod*C1, **sop**B, **bcf**C	Amo, Amp, Chl, Cip, Gen, Lin, Na, Neo, Nor, Str, Tet, Tri, Sxt
S. stanley	The virulence genes *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1,	Amo, Amp, Chl, Gen, Lin, Na, Neo, Nor, Str, Tet, Tri, Sxt
S. haifa	*spv*C, and *bcf*C were not detected	Amo, Amp, Chl, Lin, Na, Neo, Str, Tet, Tri, Sxt
S. gaillac	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Chl, Gen, Lin, Na, Neo, Nor, Str, Tet
S. gaillac	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Gen, Lin, Na, Neo, Nor, Str, Tet, Tri
S. anatum	**inv**A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Chl, Lin, Neo, Str, Tri
Untypable	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Lin, Neo, Str
Isolated from domestic ducklings		
S. enteritidis	**inv**A, *avr*A, *mgt*C, *ssa*Q, *sod*C1, **sop**B, *spv*C, **bcf**C	Chl, Lin, Neo, Str, Tri, Sxt
S. enteritidis	**inv**A, *avr*A, *mgt*C, *ssa*Q, *sod*C1, **sop**B, *spv*C, **bcf**C	Lin
S. typhimurium	**inv**A, *sod*C1, **sop**B, *spv*C, **bcf**C	Amo, Amp, Chl, Lin, Na, Str, Tri, Sxt
S. typhimurium	**inv**A, **sop**B, *spv*C, **bcf**C	Amo, Chl, Lin, Na, Tet, Sxt
S. virchow	**inv**A, *avr*A, *ssa*Q, *mgt*C, **sop**B, **bcf**C	Chl, Lin, Na, Str
S. virchow	**inv**A, *ssa*Q, **sop**B, **bcf**C	Chl, Lin, Sxt
S. shubra	**inv**A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Amp, Chl, Gen, Lin, Str, Tet, Tri, Sxt
S. shubra	**inv**A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Amo, Amp, Neo, Chl, Na, Lin, Str, Tet, Tri, Sxt
Untypable	**inv**A, *avr*A, *ssa*Q, *mgt*C, *sii*D, **sop**B, **bcf**C	Chl, Lin, Na, Nor, Str, Tri, Sxt

[a] Bold indicates where the frequency of the virulence genes is consistent.
[b] Amo: amoxicillin; Amp: ampicillin; Col: colistin sulphate; Tet: tetracycline; Cip: ciprofloxacin; Str: streptomycin; Nor: norfloxacin; Gen: gentamicin; Chl: chloramphenicol; Neo: neomycin; Lin: lincomycin; Na: nalidixic acid; Tri: trimethoprim; Sxt: trimethoprim+sulfamethoxazole.

S. stanley and *S. haifa* isolated from the imported ducklings; moreover, with the exception of *S. stanley* and *S. haifa*, the virulence-associated genes *inv*A, *sop*B, and *bcf*C were found to be 100% associated with one antimicrobial resistance phenotype (lincosamide), which was not recorded previously. Association was also found between chloramphenicol and the virulence genes *inv*A (94.1%; 32/34), *sop*B (71.8%; 23/32), and *ssa*Q (62.5%; 20/32).

Discussion

In spite of the long history of *Salmonella* and ducklings, literature on DOD is scarce.[14,21,33–35] The high frequency of *Salmonella* recovery from imported DOD causes great public health concern due to the zoonotic potential of this pathogen and its economical importance to commercial poultry breeding.[14] Our study provided evidence of *Salmonella* infection rates in imported and domestic DOD lower than those reported in previous studies[14,34–36] and higher than those in a previous study in Egypt by Osman *et al.*[21] and in Vietnam by Lam *et al.*[34] In 2005, Tsai and Pi-Hung[37] demonstrated that ducklings younger than 2 weeks of age had a significantly higher *Salmonella* prevalence rate than other age groups. Transovarian transmission is thought to be an important route of transmission to young birds; rodents and other vectors are also thought to play an important epidemiologic role in the transmission of *Salmonella* to the birds.[38,39–41] Horizontal transmission at hatcheries during feeding, handling, and transportation is also known to contribute to the spread of *Salmonella*;[10,38,42,43] in fact, Salmonella prevalence in hatcheries has been estimated between 20 and 60% for ducks.[10,44] A study by Henry[38] successfully confirmed the importance of hatchery contamination and *Salmonella* dissemination.

The 14 *Salmonella* serovars isolated from the imported DOD were substantially different from previous scientific literature.[1–3,5–7,11,13,14,34,45] The isolated serovars of DOD in the current investigation have not been previously reported in Egypt;[21] moreover, because contracting *Salmonella* causes adverse health effects and even mortality in humans,[46] this study may provide additional evidence that may be used to address this impending global public health issue in the future.[47,48] The high prevalence (5/34) of detected *S. enteritidis* is evident by the fact that serovar *S. enteritidis* may have contributed to filling the gap of an ecologic niche, apparent by the considerable increase in *S. enteritidis* prevalence after the eradication of biovar Gallinarum in the 1960s.[49]

The inv*A* target gene is unique and present in all invasive strains of *Salmonella*,[50] although generally absent from related genera such as *Escherichia*.[51] Although the *inv*A gene was absent in our isolated strains of *S. stanley* and *S. haifa*, it was available in the rest of the isolated serovars.[52–54] Interestingly, the isolated *S. stanley* and *S. haifa* in our study lacked the 11 virulence genes. Therefore, in the absence of these 11 virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcf*C), we were interested in proving the virulence of *S. stanley* and *S. haifa* serovars and asked whether these serovars will present a comparable virulence phenotype as *S.* enteritidis, *S. kissii*, *S. typhimurium*, *S. gaillac*, *S. uno*, *S. eingedi*, *S. shubra*, *S. bardo*, *S. inganda*, *S. kentucky*, *S. virchow*, and *S. anatum* to Congo red binding, serum survival, MDCK and Vero cell ingestion, chicken embryo lethality, and MSHA and MRHA assays. The Vero and MDCK cell culture model was carried out in order to compare the abilities of clinical isolates of *S. stanley* and *S. haifa* that produce a disease status comparable to *in vivo* virulence and the results of the *in vitro* assay, which are often used as correlates of bacterial virulence phenotypes.[25] The results of the pathogenicity assays and the association between phenotypic virulence assays and the invasiveness level of *S. stanley* and *S. haifa* serovars concluded their virulence that encouraged us to hypothetically speculate that these serovars may be using alternative invasive mechanisms.[52,55,56]

In the decades following the licencing of fluoroquinolones, an increased prevalence of quinolone-resistant salmonellae has been observed in clinical[57,58] and poultry isolates worldwide;[59] however, these same classes of antimicrobial agents are also administered in animals consumed by human populations, which leads to the inevitable development of resistant bacteria.[60–62] The emergence of resistance to fluoroquinolones among NTS is of particular concern, because this class of antimicrobial agents constitutes the 'drug of choice' for treating potentially life-threatening *Salmonella* infections caused by multiple antibiotic-resistant strains.[63,64] *Salmonella typhimurium* is among the serovars with the lowest average antimicrobial resistance in the present study, which is a positive indicator considering the nature of *S. typhimurium*, an exposure to cause severe human health effects; however, it is known that *S. enteritidis* is less prone to developing resistance than other serovars.[65–67] The absence of resistance to ciprofloxacin and norfloxacin among serovars *S. enteritidis*, *S. kissii*, *S. typhimurium*, *S. uno*, *S. eingedi*, *S. bardo*, *S. inganda*, *S. virchow*, *S. haifa*, and *S. anatum* is of significant value because these two antimicrobials are the primary agents used against cases of invasive salmonellosis in humans.[20] It was previously indicated that salmonellae exhibiting nalidixic acid-resistance is usually displayed with decreased susceptibility to

ciprofloxacin.[68–70] This was not evident in our investigation. Six and eight serovars were resistant to ampicillin and chloramphenicol, respectively; however, it should be noted that these antimicrobials are frequently used on Egyptian and European poultry farms[71,72] and may be the cause of the resistance observed to the tested drugs in this study. The increase in resistance to streptomycin observed in the present study is not unexpected as this drug has been among the most frequently used antibiotics on poultry farms.[73] None of the serovars identified were resistant to colistin sulphate, whereas ciprofloxacin was only resistant to serovars *S. shubra* and *S. kentucky*. This suggests a potentially effective treatment option for *Salmonella* infections. The direct association of virulence and resistance is determined in this study. In studies of the relationship between antibiotic resistance and pathogenicity, the direct comparison of sensitive and resistant *Salmonella* strains in virulence assays has been a favoured approach. Virulence and resistance are not independent properties but rather are closely related. In fact, this relationship can be seen in two ways: as genetic elements carrying genes associated with both virulence/resistance, and when the acquisition of resistance affects the virulence of the bacteria.[74] Virulence and resistance might be contained in the same mobile genetic element.[74] The fact that genetic determinants for both antibiotic resistance and virulence genes could be harboured by the same transferable element implies that there is a significant association between the presence of some virulence genes and antibiotic resistance.[75,76] The majority of the molecular pathogenicity determinants are located on the chromosome or large virulence-associated plasmids,[77,78] whereas antibiotic resistance genes are also often located on extrachromosomal genetic elements or in segments inserted within the chromosome that originate from other genomes.[75] It has been reported that resistance plasmids carry genes encoding virulence factors.[79] Also, antibiotic resistance and virulence genes can be linked in the same replicon, or eventually a single determinant can be involved in both virulence and resistance.[79]

Conclusion

It is difficult to compare prevalence estimates obtained from different studies regarding *Salmonella* across geographic regions; several factors must be considered when making such comparisons. While they may not reveal true differences in the distribution of *Salmonella*, one suggestion could include obtaining and perusing information obtained from management systems,[10,80] raising practices, seasonal patterns, processing procedures, and varying techniques currently used to determine *Salmonella* prevalence.[36]

The emergence and development of antibiotic-resistant Salmonella in the DOD may be due to a variety of reasons. One such reason may be the practice of dipping hatching eggs in solutions containing antimicrobial agents or/and routine inoculation with antibiotics.[39] Also, the occurrence and proliferation of antibiotic-resistant Salmonella in the ducklings have been linked to the common casual usage of antimicrobials in veterinary practice.[73,81] In the present study, all the Salmonella serovars exhibited full resistance to lincomycin and total susceptibility to colistin sulphate; these antimicrobials are widely used in animal production environments for the treatment and prevention of disease as well as growth promotion and have been listed under the OIE list of antimicrobials of veterinary importance (Resolution No. XXVIII)[82] as critically important antimicrobials used in human medicine.[60,82]

This study confirms the need for improved import control and testing[83,84] in order to decrease the human health hazard risk associated with DOD.[2–7,39]

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83 FAO/WHO. APPENDIX IX - Working papers of the Second Global Forum of Food Safety Regulators Second FAO/WHO Global Forum of Food Safety Regulators-Proceedings of the Forum. Food export control and certification Agenda Item 4.5 GF 02/8a Food and Agriculture Organization of the United Nations World Health Organization Rome, 2005. Second FAO/WHO Global Forum of Food Safety Regulators 12–14 October 2004, Bangkok, Thailand. Building effective food safety systems Proceedings of the Forum) WHO 2005. Drug-resistant *Salmonella* Fact sheet N°139 Revised April 2005. Food Safety Department WHO, Geneva.

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