ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Consequences to international trade of chicken hatchlings: *Salmonella enterica* and its public health implications

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Manuscript Info

Manuscript History:

Received: 12 March 2014
Final Accepted: 22 April 2014
Published Online: May 2014

Key words:
Chicken hatchlings; *Salmonella enterica*; virulence genes; antibiotic resistance

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Abstract

Chicken hatchlings (CH) contaminated with non-Typhoidal *Salmonella* involves a degree of disease risk to the importing country. The potential national and international trade impacts from *Salmonella enterica* serotypes and repertoire of virulence genes garners attention because of its distinctive multi-drug resistant characteristics and its international spread which poses a threat to the public health. Eighteen *Salmonella enterica* isolates were recovered from the CH (imported: 13/110, 11.8% and domestic: 5/80, 6.3%). The serotypes that were recovered from the imported CH were Enteritidis (3/13, 23%), Typhimurium (2/13, 15.4%), Dublin (2/13, 15.4%), Shagoua (2/13, 15.4%), Hindmarch (2/13, 15.4%) and Inganda (1/13, 15.4%) and one untypable (1/13, 15.4%). From the domestic CH the serotypes isolated were Enteritidis (1/5, 20.0%), Typhimurium (1/5, 20.0%), Dublin (1/5, 20.0%), and Infantis (2/5, 40.0%). These strains were screened for 11 potential 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) by polymerase chain reaction. All 18 isolates were resistant to at least one of 14 antibiotics used in this study. All isolates were primarily 100% resistant to lincomycin and 100% susceptible to ciprofloxacin and colistine sulphate. The high rate of resistance in *S*. Enteritidis strains, sometimes to multiple drugs, may complicate future options for treating human infections. The carriage of virulence-associated genes in these isolates suggests that they could cause serious disease and give rise to public health problems if they were to be dispersed in the general human population complicating future options for human treatment. The findings provide useful information for public health projects in Egypt and that the implementation of the Codex Committee on Food Import and Export Inspection and Certification Systems (CCFICS) to develop principles and guidelines in this area has become a must and that food control should cover both export and import.

Introduction

World-wide, birds are grown for meat or egg production, companionship, sports, scientific or educational purposes. In 2007, an estimated 17.9 billion chickens were farmed world-wide (Hoelzer et al., 2011). A number of people raise chickens in their backyards as pets (CDC, 2000; AVMA, 2012). Those mail-order chicks that wind up in children's Easter baskets and backyard farms have been linked to more than 300 cases of non-typhoidal *Salmonella* (NTS) in the U.S. — mostly in children — since 2004 (Anonymous, 1996; 2000; Hoelzer et al., 2011; CBC, 2012). Such outbreaks have been documented every few years since the 1950s (CDC, 2009). An estimated 50 million live poultry are sold through the mail each year in the United States in a business that has been booming because of the growing popularity of backyard chicken farming as a hobby among people who like the idea of raising their own food. Conditional analysis revealed CH as reservoirs for NTS and as an important risk factor to a farm (Coyle et al., 1988; Humphrey et al., 1988; Humphrey, 2000; Van Immerseel et al., 2004, 2005; Kim et al., 2007; Namata et al., 2009; Pui et al., 2011).

In the last decade, the international trade of poultry has increased resulting in imported food products containing multi-drug resistant foodborne pathogens (Aarestrup et al., 2007). Although relative to other infectious diseases, *Salmonella enterica* has few cases, the emergence of a drug-resistant bacteria and its rapid international spread over a couple of years have raised a sense of urgency to prevent a more threatening drug-resistant bacteria from emerging and unleashing a pandemic (Kwon, 2011). Due to the use of antibiotics for the promotion of growth and prevention of disease in food animals, there is an increase of human salmonellosis cases caused by foodborne multidrug-resistant (MDR) *Salmonella* (Yang et al., 2010). An inevitable side effect of antibiotic use is the emergence and dissemination of resistant bacteria, not only in pathogenic bacteria but also in the endogenous flora of man and animals. In a 2008 study of attributable medical costs for antibiotic resistant infections, it was estimated that infections in 188 patients from a single healthcare institution cost between $13.35 and $18.75 million dollars (Roberts et al., 2009).

The international trade in livestock and livestock products is a growing business, accounting for about one sixth, by value, of all agricultural trade (FAO, 2002. World Livestock Trade. Spotlight. Accessed online January, 2009 [http://www.fao.org/ag/magazine/0204sp1.htm]). To liberalize international trade, the General Agreement for Tariffs and Trade (GATT) was established in 1947. Recognizing that animal health and food safety standards can be nontariff barriers to international free trade, the World Trade Organization (WTO) also incepted Sanitary and Phytosanitary (SPS) measures. The Office International des Epizooties (OIE) was tasked to set appropriate global standards for animal health, while the Codex Alimentarius Commission sets standards for food safety (Aarestup E ed. 2006. Antimicrobial Resistance in Bacteria of Animal Origin. ASM Press, Washington DC. 442 pp.).

Although some *Salmonella* serotypes have been subjected to virulotyping studies in chicken (Prager et al., 2003; Herrero et al., 2006; Soto et al., 2006; Dione et al., 2011; Hur et al., 2011) yet, the CH was not mentioned in any, with the exception of four studies (Oblapenko et al., 1991; Mulika and Yuwapanichsampan, 2008; Anyanwu et al., 2010; Osman et al., 2010) covering the serotypes encountered in the imported and domestic CH. It is noteworthy to indicate that, although the CH were linked to human outbreaks of salmonellosis (CDC 2011a,b,c; CDC 2012a,b,c,d; Gaffga et al., 2012, MMWR, 1997, 2007, 2009), yet MDR profiling and virulotyping were not studied in the CH. Therefore, it was essential to study the distribution of virulence determinants, antibiotic resistance and whether the MDR strains are associated with virulence determinants in the strains isolated from imported and domestic CH. They were screened for eleven potential 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) by polymerase chain reaction (virulo-PCR**).**

Materials and methods

Sampling and isolation

Samplings were performed following current governmental guidelines. All of the imported bird samples were sent to the Central Lab for Veterinary Quality Control on Poultry Production, Agriculture Research Center, Ministry of Agriculture. Chicken hatchlings, were collected per flock (maximum 50 boxes per flock; 25 birds per box). Live CH (n=110) were taken randomly from each flock. The domestic CH came from both small-scale and commercial farms which produced mainly CH for sale to small scale farms (n=80). These CH were euthanized and necropsied. Pools of faecal swabs were collected from each flock for culture, isolation and biochemical testing of salmonellae following the procedure outlined by the ISO 6579:2002 European International Standard Method (Osman et al., 2010). Briefly, samples for *Salmonella* isolation were pre-enriched in sterile buffered peptone water (Merck, Darmstadt, Germany) in a dilution of 1:10. The mixture was incubated at 37^{o}C for 18 h. One milliliter of incubated broth was transferred to 9mL of Muller–Kauffmann tetrathionate novobiocin broth (MKTTn broth; Oxoid, United Kingdom) and incubated at 378C for 18 h. In addition, a second transfer of incubated broth was transferred to 9mL of Rappaport–Vassiliadis broth (RV broth; Difco), which was prepared according to the instructions on the package. The RV broth was incubated for 24 h at 41.58C. One milliliter of the MKTTn broth and

0.1mL of the RV broth samples were streaked onto Xylose Lysine Desoxycholate Agar, Brilliant Green Agar, and Hektoen Enteric Agar (Oxoid) and incubated at 378C for 24 h. Black or red colonies (non-lactose fermenters) were inoculated onto Triple-Sugar Iron Agar, using a sterile inoculating needle, and were incubated for 24 h at 378C. Tubes with red slants and black or yellow butts were identified to be due to salmonellae. Further confirmation was done based on agglutination reaction with somatic (O), flagellar (F), and virulence (Vi) antisera.

***Salmonella* serotyping**

Typical *Salmonella* isolates were serotyped by using O and H-antigen-specific sera available from Difco Diagnostics (Berlin, Germany) antisera following the Kauffmann–White typing scheme.

Antimicrobial susceptibility testing by disk diffusion

Inhibitors of the cell wall synthesis (aminopenicillins, colistin), protein synthesis (gentamicin, streptomycin, tetracyclines, chloramphenicol, lincomycin and neomycin) and nucleic acid synthesis (norfloxacin**,** ciprofloxacin, trimethoprim-sulfamethoxazole, trimethoprim, nalidixic acid) were employed for inhibition tests. The panel of antibiotic disks (Becton, Dickinson and Company, Maryland, USA) used in panel screens belonged to 8 drug classes. Each isolate was inoculated onto Muller–Hinton agar (Oxoid) and incubated at 37^{o}C for 24 h according to National Committee for Clinical Laboratory Standards (CLSI, 2010) and the zones of inhibition were measured to assess resistance or susceptibility. The following antimicrobials were chosen given their common use in treating and preventing Salmonella infection in poultry and human and because they represented a variety of antimicrobial classes.

Virulo-PCR screening

Detection of the eleven 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 all isolates was undertaken by PCR amplification. The PCR conditions and primers used for the target genes have been previously published (Salehi et al., 2005; Huehn et al., 2010; Hauser et al., 2011) and Table 1outlines the sequences, conditions and predicted sizes of the amplified products. Six targets (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, and *sop*B) were located on the *Salmonella* pathogenicity islands (SPIs) 1–5, three targets (*gip*A, *sod*C1, and *sop*E1) on prophages, one (*spv*C) on the *Salmonella* virulence plasmid, and one (bcfC) on a fimbrial cluster.

Statistical analysis

The distribution of resistance phenotypes among serotypes, either resistance to a single antibiotic or multidrug-resistance, was tested using a contingency table analysis where rows = serotype and columns = resistance vs. susceptible. The statistical significance of homogeneity in antimicrobial resistance patterns among the groups was assessed using Pearson's chi-square exact test using SAS version 9.2 (SAS, Cary, NJ). Findings were considered significant where $P<0.05$. Contingency table allows to test if the proportion of resistance bacteria is the same across the different serotypes and uses the Pearson's χ^2 test to assess the statistical significance of the difference between the proportions. A high χ^2 value means that the resistant phenotypes are not proportionately distributed among serotypes, some serotypes having higher frequency of resistant phenotype than others. To assess the statistical significance of differences between individual D values, 95% confidence intervals (95% CIs) were calculated. Ninety-five percent confidence intervals for the proportion of isolates resistant to individual antimicrobial agents were calculated using the normal approximation to the binominal distribution.

Table I. 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			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 sec	64 °C for 30sec	72 °C for 30sec [a]	284	Salehi et al. (2005)
*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 sec	53 °C for 30 sec	72 °C for 30 sec[b]	422	Huehn et al. (2010)
*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 (Spi4D)	Type I secretion/SPI-4	gaa tag aag aca aag cga tca tc gct ttg ttc acg cct ttc atc	95 °C for 30 sec	58 °C for 30 sec	72 °C for 30 sec[b]	655	Hauser et al. (2011)
*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	
*gip*A	Gifsy-1 bacteriophage/Peyer's patch-specific Virulence factor	acg act gag cag cgt gag ttg gaa atg gtg acg gta gac	95 °C for 30 sec	53 °C for 30 sec		518	Huehn et al. (2010)
*sod*C1	Gifsy-2 bacteriophage/Periplasmic Cu, Zn-superoxide dismutases	cgg gca gtg ttg aca aat aaag tgt tgg aat tgt gga gtc			72 °C for 30 sec[b]	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				467	

[a] PCR was done for 35 cycles.
[b]After 30 cycles, final extension step of 4 min at 72°C was performed

Results

Prevalence and serotyping of *Salmonella*

An overall total of eighteen *Salmonella enterica* isolates were recovered from the CH (imported: 13/110, 11.8% and domestic: 5/80, 6.3%). The serotypes that were recovered from the imported CH were Enteritidis (3/13, 23%), Typhimurium (2/13, 15.4%), Dublin (2/13, 15.4%), Shagoua (2/13, 15.4%), Hindmarch (2/13, 15.4%) and Inganda (1/13, 15.4%) and one untypable (1/13, 15.4%). From the domestic CH the serotypes isolated were Enteritidis (1/5, 20.0%), Typhimurium (1/5, 20.0%), Dublin (1/5, 20.0%), and Infantis (2/5, 40.0%).

Antimicrobial resistance

Table 2 shows the susceptibility of isolates to antimicrobials commonly used in Enterobacteriaceae. All strains were resistant to at least one antimicrobial tested in this study. All isolates were primarily 100% resistant to lincomycin (18/18) and 100% susceptible to ciprofloxacin and colistine sulphate (0/18). Among the 18 strains, 17

and 15 strains were resistant to chloramphenicol and streptomycin respectively. There was also resistance to nalidixic acid (9/18), neomycin (8/18), ampicillin, gentamicin, norfloxacin and tetracycline (6/18 each), amoxicillin and trimethoprim/sulfamethoxazole (5/18 each) and trimethoprim (4/18).

Statistically significant correlations for resistance among various antimicrobials at the isolate level are presented in Table 2. The distribution of resistance to the different antibiotics was largely dependent on the serotype identity. More precisely, highly significant resistance ($P<0.001$) to ampicillin ($\chi^2 = 11.7$) amoxicillin ($\chi^2 = 15.0$) was associated to serotypes Infantis and Hindmarch; to gentamycin ($\chi^2 = 15.6$) associated to serotypes Dublin and Hindmarch; to norfloxacin ($\chi^2 = 18.0$) associated to Enteritidis and Shagoua.

A significant resistance at $P<0.003$ was recorded to tetracycline ($\chi^2 = 9.0$) was associated to serotypes Enteritidis, Infantis, Hindmarch and Inganda. A $P<0.004$ value was seen to trimethoprim ($\chi^2 = 8.1$) when associated to serotypes Typhimurium, Infantis and Hindmarch.

A lower significant resistance ($P<0.01$) to streptomycin ($\chi^2 = 2.9$) associated to serotypes Shagoua, Infantis, Hindmarch, Inganda and the Untypable isolate; and also to nalidixic acid ($\chi^2 = 6.9$) which was associated to serotypes Infantis, Hindmarch and Inganda. Significance at $P<0.02$ to chloramphenicol ($\chi^2 = 5.3$) was observed to be associated with serotype Typhimurium and to sulphamethoxazole + trimethoprim ($\chi^2 = 5.6$) was associated to serotypes Enteritidis, Dublin, Infantis and Inganda.

In addition, significance at $P<0.03$ to neomycin ($\chi^2 = 4.5$) was associated to serotype Shagoua and the untypable strain.

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Table 2. Distribution of resistance to individual antimicrobial agents among sources and *Salmonella enterica* serotypes in chicken hatchlings

Antimicrobials	Distribution of resistance to antimicrobials														
	***S*. Enteritidis (n=4/18)**			***S*. Typhimurium (n=3/18)**			***S*. Dublin (n=3/18)**			***S*. Chagoua (n=2/18)**			***S*. Infantis (n=2/18)**		
	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI
Penicillins															
Ampicillin (10 mg)	1/4	25	1.32-78.06	1/3	33	1.77-87.47	0/3	0	3.18-69.00	0/2	0	4.89-80.21	2/2	100	19.79-95.11
Amoxicillin (20 mg)	0/4	0	2.35-60.42	0/3	0	3.18-69.00	1/3	33	1.77-87.47	0/2	0	4.89-80.21	2/2	100	19.79-95.11
Aminoglycosides															
Gentamicin (10 mg)	0/4	0	2.35-60.42	0/3	0	3.18-69.00	3/3	100	31.00-96.82	0/2	0	4.89-80.21	1/2	50	2.67-97.33
Neomycin (30 µg)	3/4	75	21.94-98.68	0/3	0	3.18-69.00	2/3	67	12.53-98.23	2/2	100	19.79-95.11	0/2	0	4.89-80.21
Streptomycin (10 mg)	3/4	75	21.94-98.68	2/3	67		2/3	67	12.53-98.23	2/2	100	19.79-95.11	2/2	100	19.79-95.11
Fluoroquinolones															
Ciprofloxacin (5 µg)	0/4	0	2.35-60.42	0/3	0	3.18-69.00	0/3	0	3.18-69.00	0/2	0	4.89-80.21	0/2	0	4.89-80.21
Nalidixic acid (30 µg)	3/4	75	21.94-98.68	0/3	0	3.18-69.00	0/3	0	3.18-69.00	1/2	50	2.67-97.33	2/2	100	19.79-95.11
Norfloxacin (5 µg)	4/4	100	39.58-97.65	0/3	0	3.18-69.00	0/3	0	3.18-69.00	2/2	100	19.79-95.11	0/2	0	4.89-80.21
lincosamides															
Lincomycin (30 µg)	4/4	100	39.58-97.65	3/3	100	31.00-96.82	3/3	100	31.00-96.82	2/2	100	19.79-95.11	2/2	100	19.79-95.11
Phenicols															
Chloramphenicol (30 µg)	4/4	100	39.58-97.65	2/3	67	12.53-98.23	3/3	100	31.00-96.82	2/2	100	19.79-95.11	2/2	100	19.79-95.11
Polymyxin															
Colistin sulphate (10 mg)	0/4	0	2.35-60.42	0/3	0	3.18-69.00	0/3	0	3.18-69.00	0/2	0	4.89-80.21	0/2	0	4.89-80.21

Tetracyclines														
Tetracycline (30 µg)	3/4	75	21.94-98.68	0/3	0	3.18-69.00	0/3	0	3.18-69.00	0/2	0	4.89-80.21	1/2	50	2.67-97.33
Sulphonamides														
Trimethoprim (5 µg)	0/4	0	2.35-60.42	1/3	33	1.77-87.47	0/3	0	3.18-69.00	0/2	0	4.89-80.21	2/2	100	19.79-95.11
Trimethoprim–sulfamethoxazole (23.75 + 1.75 mg)	1/4	25	1.32-78.06	0/3	0	3.18-69.00	1/3	33	1.77-87.47	0/2	0	4.89-80.21	2/2	100	19.79-95.11

Table 2 (continued). Distribution of resistance to individual antimicrobial agents among sources and *Salmonella enterica* serotypes in chicken hatchlings

Antimicrobials	**Distribution of resistance to antimicrobials**								
	***S*. Hindmarch (n=2/18)**			***S*. Inganda (n=1/18)**			***S*. untypable (n=1/18)**		
	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI	n=of resistant isolates	% of isolates	95% CI
Penicillins									
Ampicillin (10 mg)	2/2	100	19.79-95.11	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Amoxicillin (20 mg)	2/2	100	19.79-95.11	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Aminoglycosides									
Gentamicin (10 mg)	2/2	100	19.79-95.11	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Neomycin (30 µg)	0/2	0	4.89-80.21	0/1	0	10.78-94.54	1/1	100	5.46-89.22
Streptomycin (10 mg)	2/2	100	19.79-95.11	1/1	100	5.46-89.22	1/1	100	5.46-89.22
Fluoroquinolones									
Ciprofloxacin (5 µg)	0/2	0	4.89-80.21	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Nalidixic acid (30 µg)	2/2	100	19.79-95.11	1/1	100	5.46-89.22	0/1	0	10.78-94.54
Norfloxacin (5 µg)	0/2	0	4.89-80.21	0/1	0	10.78-94.54	0/1	0	10.78-94.54

lincosamides									
Lincomycin (30 µg)	2/2	100	19.79-95.11	1/1	100	5.46-89.22	1/1	100	5.46-89.22
Phenicols									
Chloramphenicol (30 µg)	2/2	100	19.79-95.11	1/1	100	5.46-89.22	1/1	100	5.46-89.22
Polymyxin									
Colistin sulphate (10 mg)	0/2	0	4.89-80.21	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Tetracyclines									
Tetracycline (30 µg)	1/2	50	2.67-97.33	1/1	100	5.46-89.22	0/1	0	10.78-94.54
Sulphonamides									
Trimethoprim (5 µg)	1/2	50	2.67-97.33	0/1	0	10.78-94.54	0/1	0	10.78-94.54
Trimethoprim–sulfamethoxazole (23.75 + 1.75 mg)	0/2	0	4.89-80.21	1/1	100	5.46-89.22	0/1	0	10.78-94.54

Number of isolates showing resistance to the specific antimicrobial drug out of 18 isolates.
Percentage of isolates resistant to the specific antimicrobial drug.
The 95% binominal confidence interval (95% CI) based on normal approximation.

Virulo-PCR screening

All isolates were screened by PCR analysis for the presence or absence of 11 selected virulence genes (Table 3). Altogether, in this *Salmonella* serotypes collection, 13 different combinations of virulence genes were detected. The serotypes isolated from the imported and domestic CH (18/18) carried a combination of the *inv*A and *sop*B (carried by *Salmonella* pathogenicity islands [SPIs]) genes in all the isolates tested. Also, except for one of the isolated *S.* Enteritidis strains (1/3), all isolated serotypes (17/18) carried the *bcf*C (fimbria-related).

The serotypes isolated from the imported CH indicated that, gene bcfC was detected in 12/13 of the isolated strains (92.3%). Genes *avr*A located in SPI-1 (encoding a protein that inhibits activation of $NF\text{-}_kB$) and *mgt*C (encoding the intramacrophage survival protein for SPI-3) were found in 76.9% (10/13 each) of the isolates. Likewise, the *ssa*Q gene and the *sii*D were found in 69.2% (9/13) of the isolates. With lower percentages, the *sod*C1 gene (located on a bacteriophage) and the *spv*C gene (plasmid-encoded) were found in 30.8% of the isolates (4/13 each). The lowest percentage of presence was detected in the *sop*E1 gene (encoding a translocated effector protein) in only 7.7% of the isolates. The *gip*A gene (encoding a Peyer's patch-specific virulence factor) was absent. The *Salmonella* serotypes Hindmarch (two isolates), untypable (one isolate) and one of the Shagoua isolates, had identical virulence gene repertoires (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B and *bcf*C).

The five serotypes isolated from the domestic CH revealed that in addition to the *inv*A, *sop*B the *bcf*C genes were detected in all of the serotypes (100%) isolated. Genes *ssa*Q and *sii*D were detected in 80% of the isolates (4/5). With a lesser degree of presence, the genes *avr*A and *mgt*C were present in 60.0% of the isolates (3/5). The lowest degree of presence was for the genes *sod*C1 and *sop*E1 (40%, 2/5 each) and *spv*C (20.%, 1/5).

The association of the *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, and *bcf*C genes in the Enteritidis, Typhimurium, Dublin, Shagoua, Hindmarch and Inganda in addition to one untypable isolate from the imported CH in addition to the *Salmonella* isolated strains from the domestic CH (Enteritidis, Typhimurium, Dublin and Infantis) with various antimicrobial resistance patterns was recorded as shown in Table 4. A detailed analysis displayed associations of resistance/susceptibility phenotypes with potential virulence genes. The virulence associated genes *inv*A, *sop*B and *bcf*C genes were 100% associated with one antimicrobial resistance phenotype (lincosamide) which was not recorded previously.

Table 3. Virulence genes combinations among the *Salmonella* serotypes isolated from chicken hatchlings

***Salmonella* serotypes**	**Virulence genes**										
	***inv*A**	***avr*A**	***ssa*Q**	***mgt*C**	***sii*D**	***sop*B**	***gip*A**	***sod*C1**	***sop*E1**	***spv*C**	***bcf*C**
Imported chicken											
Enteritidis	+	+	ND	+	ND	+	ND	ND	ND	+	+
Enteritidis	+	+	ND	+	ND	+	ND	ND	ND	+	ND
Enteritidis	+	+	ND	+	ND	+	ND	ND	ND	ND	+
Typhimurium	+	ND	+	ND	+	+	ND	+	ND	+	+
Typhimurium	+	ND	+	ND	+	+	ND	+	ND	ND	+
Dublin	+	+	+	+	+	+	ND	+	ND	+	+
Dublin	+	+	+	+	+	+	ND	+	ND	ND	+
Chagoua	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+
Chagoua	+	+	+	+	+	+	ND	ND	ND	ND	+
Hindmarch	+	+	+	+	+	+	ND	ND	ND	ND	+
Hindmarch	+	+	+	+	+	+	ND	ND	ND	ND	+
Inganda	+	+	+	+	+	+	ND	ND	+	ND	+
Untypable	+	+	+	+	+	+	ND	ND	ND	ND	+
Domestic chicken											
Enteritidis	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+
Typhimurium	+	ND	+	ND	+	+	ND	+	ND	ND	+
Infantis	+	+	+	+	+	+	ND	ND	+	ND	+
Infantis	+	+	+	+	+	+	ND	ND	+	ND	+
Dublin	+	+	+	+	+	+	ND	+	ND	+	+

ND= Not Detected

Table 4. Distribution of virulence genes combinations in the different *Salmonella* serotypes isolated from chicken hatchlings and their antibiotic resistance phenotypes

***Salmonella* serotypes**	**Origin**	**Virulence genes**	**Antibiotic resistance [a]**
Enteritidis	**Imported**	*inv*A, *avr*A, *mgt*C, *spv*C, *sop*B, *bcf*C	Chl-Lin-Na-Str-Tet
Enteritidis	**Imported**	*inv*A, *avr*A, *mgt*C, *sop*B, *spv*C	Chl-Lin-Na-Neo-Nor-Str-Sxt
Enteritidis	**Imported**	*inv*A, *avr*A, *mgt*C, *sop*B, *bcf*C	Chl-Lin-Neo-Na-Nor-Str-Tet
Typhimurium	**Imported**	*inv*A, *ssa*Q, *sii*D, *sod*C1, *sop*B, *spv*C, *bcf*C	Amp-Lin-Tri
Typhimurium	**Imported**	*inv*A, *ssa*Q, *sii*D, *sod*C1, *sop*B, *bcf*C	Chl-Lin-Str
Dublin	**imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sod*C1, *sop*B, *spv*C, *bcf*C	Chl-Gen-Lin
Dublin	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sod*C1, *sop*B, *bcf*C	Amo-Chl-Gen-Lin-Neo-Str
Chagoua	**Imported**	*inv*A, *sop*B, *bcf*C	Chl-Lin-Na-Neo-Nor-Str
Chagoua	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *bcf*C	Chl-Lin-Neo-Nor-Str
Hindmarch	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B. *bcf*C	Amo-Amp-Chl-Gen-Lin-Na-Str-Tri
Hindmarch	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *bcf*C	Chl-Lin-Na-Str-Sxt
Inganda	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*E1, *sop*B, *bcf*C	Chl-Lin-Na-Str-Tet-Sxt
Untypable	**Imported**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *bcf*C	Amo-Lin-Neo-Str
Enteritidis	**Domestic**	*inv*A, *sop*B, *bcf*C	Amp-Chl-Lin-Nor-Tet
Typhimurium	**Domestic**	*inv*A, *ssa*Q, *sii*D, *sod*C1, *sop*B, *bcf*C	Chl-Lin-Str
Infantis	**Domestic**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*E1, *sop*B, *bcf*C	Amp-Amo-Chl-Lin-Na-Str-Tri-Sxt
Infantis	**Domestic**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*E1, *sop*B, *bcf*C	Amp-Amo-Chl-Gen-Lin-Na-Str-Tet
Dublin	**Domestic**	*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sod*C1, *sop*B, *spv*C, *bcf*C	Chl-Gen-Lin-Neo-Str-Sxt

[a] Amo, amoxicillin; Amp, ampicillin; Col, colistin sulphate; Tet, tetracyclin; Cip, ciprofloxacin; Str, streptomycin**;** Nor, norfloxacin; Gen, gentamycin; Chl, chloramphenicol; Neo, neomycin; Lin, lincomycin; Na, nalidixic acid; Tri, trimethoprim; Sxt, trimethoprim-sulfamethoxazole

Discussion

Trade in poultry occurs on a global scale. While this makes the best breeding and commercial stock available to producers and offers consumers an increasingly greater choice, it has potential drawbacks in terms of spread of poultry diseases between trading partners. The international trade in CH, like that in poultry meat, may present an opportunity for the global spread of disease. *Salmonella enterica* is commonly acquired from contaminated poultry and is an important cause of illness worldwide (Hendriksen et al., 2011). Subtyping *Salmonella* by serotyping is useful for targeting interventions needed to control *Salmonella* (Hendriksen et al., 2011).

The comparison of *Salmonella* surveillance data in the literature and our results varied vastly. Inspite of the fact that, *S.* Enteriditis and Typhimurium are the two most important serotypes for salmonellosis transmitted from animals to humans (Foley and Lynne, 2008; Deng et al., 2012) yet, all serotypes can cause disease in humans (WHO, 2005). *Salmonella* serotypes and prevalence and pathogenic potential may vary considerably between localities, districts, regions, countries and seemed to be culturally linked (Poppe, 2000; Foley et al., 2008; WHO, 2010; Hendriksen et al., 2010) but lesser differences between countries within the same region (Poppe, 2000; Foley et al., 2008; Hendriksen et al., 2011; Hoelzer et al., 2011). In an epidemiological study of NTS in Israel serotypes Enteritidis, Typhimurium and Infantis were isolated among chicken breeding flocks (Gal-Mor et al., 2010; Bassal et al., 2012). The four *Salmonella* serotypes, Enteritidis, Typhimurium, Dublin and Infantis isolated in the present investigation, are consistently in the top 10 most frequently reported serotypes in Europe (Huehn et al., 2010). Together serotypes Enteritidis and Typhimurium are the most important ones causing approximately 82.8% of all human cases of salmonellosis (Hendriksen et al., 2010; Bugarel et al., 2011).

The *Salmonella* serotypes isolated from the imported CH were greatly diversable from the recorded CH in Russia (Oblapenko et al., 1991), Thailand (Mulika and Yuwapanichsampan, 2008), Nigeria (Anyanwu et al., 2010) and Egypt (Osman et al., 2010). Crucially, the isolated serotypes can cause disease in humans (WHO, 2005) making them a global concern for public health (Poppe, 2000; Hendriksen et al., 2010; Global Foodborne Infections Network (GFN), 2012). The CH can be infected by vertical transmission through infected parents or by horizontal transmission through hatcheries, sexing in contaminated hatcheries, cloacal infection, transportation equipment, and feed (Poppe, 2000). Environmental factors such as air, litter and unclean facilities, and vectors, such as insects, humans, and rodents, coula also participate in responsiblity for Salmonella contamination in poultry farms (Poppe, 2000). There has been a gradual and documented increase (30-50%) in CH mortality in many farms in parts of north central Nigeria. This was linked most often to hatchery originated infections (Anyanwu et al., 2010). Salmonella prevalence in hatcheries is estimated between 0 and 17% for chickens, (Hoelzer et al., 2011). A common serotype isolated from both imported and local CH was *S*. Enteritidis and in most of the cited literature makes it conceivable that serotype Enteritidis filled the ecologic niche left by the eradication of serotype Gallinarum biovar Gallinarum, since a considerable increase in Enteritidis prevalence coincided with the eradication of biovar Gallinarum in the 1960s (Foley et al., 2008).

For most bacterial pathogens, virulence requires multiple factors (Groisman and Ochman, 1997). The ability of NTS to cause invasive disease is attributed to arrays of virulence genes defined in the *Salmonella* pathogenicity islands (SPIs) (Blum et al., 1994). There are at least 60 genes associated with SPIs (Groisman and Ochman, 1997) and the majority of these determinants are located on the chromosome or on large virulence-associated plasmids (Groisman and Ochman, 1996; Hacker et al., 1997).

The evidence that the *Salmonella* serotypes isolated in this study often had two or more toxin genes, although in different combinations, highlights their potential pathogenicity. Virulotyping has recently been used as a molecular typing tool for the epidemiological study of pathogenic bacteria, such as *Salmonella* spp. Therefore, in this study, an attempt to establish the 11 virulence genes profiles for *S*. Enteritidis, Typhimurium, Dublin, Shagoua, Hindmarch, Inganda and Infantis for invasion, enterotoxin production and pathogenesis in the host collected in Egypt from CH were investigated. Eleven *Salmonella* virulence genes, including *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D , *sop*B, *gip*A, *sod*C1, *sop*E1, and *bcf*C, as well as *Salmonella* plasmid virulence gene *spv*C, were assayed. Our results showed that for the *Salmonella* serotypes collected in Egypt, their virulotypes are obviously different from those reported for those collected in Taiwan, Europe, Africa and the USA.

The majority of the isolates from CH harbored between seven to eight virulence genes, except for the *gip*A gene, indicating that these virulence genes are widespread in the *Salmonella* isolates. The *spv*C gene (carried by the *Salmonella* virulence plasmid) was found in only 27.8% of strains (5/18). These findings are in contrast with previous studies (Castilla et al., 2006; Skyberg et al., 2006; Kwag et al., 2008; Hur et al., 2011; Huehn et al., 2010; Mir et al., 2010; Bolton et al., 2012), which have reported the high prevalence of *spv*C gene in *Salmonella* isolates. Although it is not possible to predict whether a particular serovar of *Salmonella* will cause the disease merely by the presence or absence of a few virulence genes, the high prevalence of multiple virulence genes from the clinical isolates could explain the increased potential of these serotypes in causing severe infections in humans. All *Salmonella* serotypes isolates were examined for the presence of SPI associated genes and the *spv* operon. During the course of infection, *S. enterica* serotypes utilise many virulence factors, among which SPIs play a major role. The *Salmonella* invasion gene *inv*A, an essential regulatory gene of T3SS genes and the SPI-1 effectors, including *Sop*B and *Sop*E1, are associated with human and animal intestinal epithelial cell invasion and enterocolitis, and are known to act as major virulence factors for *Salmonella enterica* (Hu et al., 2008; Li et al., 2009). Yet, in this study, all isolates harboured the *inv*A gene (100%) and the SopB (100%) but on the contrary, the SPI-1-related gene *Sop*E1 was present in one isolate only (Inganda) from the imported CH (1/12; 8.3%) and in serotype Infantis (2/5; 40.0%) from the domestic CH.

Salmonella antibiotic resistance is a global concern that includes multidrug resistant strains (Hendriksen, 2010). Traditional first-line antibiotic medications include ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole. Resistance to these first-line antibiotics defines multidrug resistance in *S. enterica* (Crump and Mintz, 2010). The occurrence and proliferation of antibiotic-resistant *Salmonella* in the chicken may be due to the practice of dipping hatching eggs in solutions containing antimicrobial agents or/and routine inoculation with antibiotics (Kabir, 2010) and on-the farm usage of antibiotics as prophylactics, growth promoters or as therapeutics (Carraminana et al. 2004). *Salmonella* strains of avian origin are often resistant to variety of antimicrobials approved for poultry including tetracycline, oxytetracycline, penicillin, aminoglycosides and fluoroquinolones (Kabir, 2010). The large number of antibiotics to which the strains were resistant in the present study is consistent with the findings

of other authors in chicken (Logue et al., 2003; Su et al., 2004; Parveen et al., 2007; Hur et al., 2010; Pan et al., 2010; Yang et al., 2010).

An earlier study observed that most *S*. Enteritidis clinical isolates are drug-susceptible (Su et al., 2002), with antimicrobial resistant and MDR *S*. Enteritidis isolates being rarely reported and less prone to developing resistances than other serotypes (Dogru et al., 2009; Zou et al., 2012). All *S*. Enteritidis isolates examined in this study were resistant to at least one antibiotic. There was a high resistance rate to particular antimicrobials, notably norfloxacin, chloramphenicol, neomycin, streptomycin, nalidixic acid, and tetracycline. Fluoroquinolones were approved in numerous European countries from the 1980s onwards and these same classes of antimicrobial agents are also administered to food animals, which leads to the inevitable development of resistant bacteria (FAO/OIE/WHO, 2003; DANMAP, 2011; FDA, 2012). In the decades following the licensing of these drugs, there has been an increasing prevalence of quinolone-resistant salmonellae, observed in clinical and poultry isolates worldwide (Sáenz et al., 2001; Schroeter et al., 2004; Wilson, 2004; Dias de Oliveira et al., 2005; Ellerbroek et al., 2010; Shrestha et al., 2010). This fact is extremely worrisome, because, as indicated above, fluoroquinolones have long been considered a drug of choice to treat salmonellosis in human adults when necessary (Gunell, 2010). Fortunately, we found 25% of the isolates in this study were resistant to ampicillin but not to amoxicillin. The low rate of resistance to ampicillin could be attributed to its non-use in animal production. The high rates of resistance and MDR *S*. Enteritidis strains identified in this study suggest they may give rise to public health problems. Future options for treating *S*. Enteritidis infections may be complicated if these types of strains are distributed in the human population. Luckily no isolates were identified that were resistant to amoxicillin, gentamicin, ciprofloxacin, colistin sulphate and trimethoprim suggesting these as potentially effective treatments for *S*. Enteritidis infections. As in the present study, resistance to streptomycin, neomycin and tetracycline have also been frequently reported in a number of other studies on poultry products (Hur et al., 2010; Yildirim et al., 2011). The global increasing resistance to tetracycline and streptomycin has been observed in *Salmonella* of animal origin (Abdellah et al., 2009). This trend is not surprising, as tetracycline and streptomycin are among the most commonly used antibiotics for food animal production worldwide (Pezzella et al. 2004). Both drugs have also been approved for use in chicken production in the United States (FDA Approved Animal Drug products, 2012). Luckily, no isolates were identified that were resistant to gentamicin and ciprofloxacin, suggesting that these are potentially effective treatments for *Salmonella* infections. As in this current piece of work, no resistance was observed to these antimicrobials by Yildirim et al. (2011). The fact that *S*. Typhimurium is among the serotypes showing the lowest average antimicrobial resistances in the present study is a result in contrast with most surveys (Hernández et al., 2005; Abdellah et al., 2009; Berrang et al., 2009). This is a soothing information because, as previously indicated, *S*. Typhimurium causes more serious consequences on human health than most *Salmonella* serotypes. It should be pointed out that, cross-resistance or coresistance mechanisms (Capita and Alonso-Calleja, 2013) could be the cause of the resistance observed to chloramphenicol in the present report and other researches (van Duijkeren et al., 2003; Yildirim et al., 2011). The reemergence of chloramphenicol-sensitive strains in prior resistant organisms points towards the concept of antibiotic recycling (Harish and Menezes, 2011). In the present study, all the *Salmonella* serotypes exhibited total resistance to lincomycin and total susceptibility to ciprofloxacin and colistin sulphate, which are widely used in other animal production environments for the treatment and prevention of disease and growth promotion and have been Listed as the OIE List of Antimicrobials of Veterinary Importance at its 75th General Session in May 2007 (Resolution No. XXVIII) (FAO/WHO/OIE. 2008) and listed and categorized as critically important antimicrobials used in human medicine (WHO, 2009).

Physical linkages and statistical associations between resistance and virulence genes have been reported in avian *Salmonella* strains (Prager et al., 2000; Rahman, 2006; Foley and Lynne, 2008; Borsoi et al., 2009; Dione et al., 2011). Dione et al. (2011) found that the *sop*E gene was common in serovars isolated from chicken in The Gambia. Previous studies have shown that the *sop*E gene was present in strains of *S*. Typhimurium associated with epidemic disease in both humans and animals (Mirold et al., 1999) and therefore the *sop*E gene if expressed may be implicated in diseases in both children and animals. Huehn et al. (2010) reported that their data indicated that the virulotype did not vary significantly with host source or geographical location which is contrary to our findings. One important virulence factor previously shown to be common among predominant NTS serotypes and associated with MDR is the *spv* operon (Gebreyes et al., 2009). Our *spv*C findings supports the hypothesis that the occurrence of virulence factor *spv*C within a strain exhibiting specific MDR phenotypes may make strains clinically more relevant (Gebreyes et al., 2009). The carriage of *spv*C among MDR strains may increase the propensity of such strains to be of major veterinary relevance. The fact that genetic determinants for both antibiotic resistance and virulence genes could be harboured by the same transferable element (Carattoli, 2003; Bugarel et al., 2011) implies that there is an association between antibiotic resistance and virulence. Such association could have an impact on the spread of

resistance clones of *Salmonella*. In this study, we observed that some virulence-associated genes significantly present in avian *Salmonella* strains susceptible to penicillins, streptomycin and chloramphenicol which are partly in agreement with previous reports that certain virulence-associated genes among human *Salmonella* isolates were significantly enriched in strains susceptible to certain antibiotics, such as chloramphenicol, tetracycline and others (Foley and Lynne, 2008; Borsoi et al., 2009; Dione et al., 2011). This suggests that certain virulence genes may significantly exist in susceptible poultry as well as human strains. This indicates that the relationship of resistance with virulence-associated genes may vary according to particular host. The difference in such associations may be related to virulence-associated genes, geographical origin and antimicrobial use of the strains under investigation.

Conclusion

The potential trade impacts from *S. enterica* serotypes and repertoire of virulence genes garners attention because of its distinctive multi-drug resistant characteristics and its international spread which poses a threat to the public health. The isolation of multi-drug-resistant *Salmonella* in this study suggests that CH may act as a reservoir for multi-drug-resistant *Salmonella* strains, which can be transferred to humans. Our results and those previously recorded, highlight the complexity of the global epidemiology of *Salmonella* and the need and importance for improving monitoring data of those serotypes of highest epidemiologic importance and global co-operation in controlling salmonellosis as emphasised at an early stage by the World Health Organization (WHO) (Negri, 2009). The high rate of resistance and multiple drug-resistant *S*. Enteritidis strains found here may complicate future options for treating human *S*. Enteritidis infections. Most isolates harboured the SPI-1 and SPI-2-associated genes and the *spv* operon, which are known to be associated with human infections. Although relative to other infectious diseases, *S. enterica* has few cases, the emergence of a drug-resistant bacteria and its rapid international spread over a couple of years have raised a sense of urgency to prevent a more threatening drug-resistant bacteria from emerging and unleashing a pandemic. Not only nationally, but internationally, countries must cooperate to prevent the relative spread of *Salmonella* to both neighboring and distant countries (Kwon, 2012). Our findings also provide useful information for public health projects in Egypt and that the implementation of the Codex Committee on Food Import and Export Inspection and Certification Systems (CCFICS) and the OIE Handbook on Import Risk Analysis for Animals and Animal Products publication, provide guidelines useful to bilateral parties to develop principles and guidelines in this area, has become a must and that food control should cover both export and import.

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