Salmonella enterica serotypes isolated from squabs reveal multidrug resistance and a distinct pathogenicity gene repertoire

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Summary

The consumption of squab (young unfledged pigeons) as part of the cuisine of many countries, together with the observation that squabs are vectors of zoonotic agents, may make them a public health risk. This study was designed to determine the serotypes, distribution of 11 virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, *bcf*C) and the antimicrobial resistance profiles of salmonellae recovered from squabs. Six isolates were identified from among 45 (13.3%) squabs sampled. Three serotypes were identified according to the Kauffman–White serotyping scheme: *Salmonella* Typhimurium (*S.* Typhimurium) (4/6; 66.7%), *S.* Braenderup (1/6; 16.7%) and *S.* Lomita (1/6; 16.7%). Polymerase chain reaction analyses revealed the presence of *inv*A, *sop*B and *bcf*C in all six isolates, whereas *sop*E1 and *gip*A were absent. All six isolates were resistant to lincomycin and streptomycin, but all were susceptible to ciprofloxacin, colistin sulphate and gentamicin. Among the *S.* Typhimurium isolates, seven resistance profiles were identified:

penicillins, aminoglycosides, fluoroquinolones, lincosamides, phenicols, tetracyclines and sulphonamides; four resistance profiles were identified in the isolates of *S.* Braenderup and *S.* Lomita: aminoglycosides, fluoroquinolones, lincosamides and polymyxin. Thus, the distribution of resistance to the antibiotics was largely dependent on serotype identity. The presence of *inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B and *bcf*C was associated with resistance to chloramphenicol; *inv*A, *sop*B and *bcf*C with resistance to streptomycin and lincosamide; and *inv*A and *sod*C1 with resistance to trimethoprim–sulfamethoxazole. The identification of serotypes *S.* Typhimurium, *S.* Braenderup and *S.* Lomita in the squab samples has important implications because these serotypes are significant causes of food poisoning and enteric fever in humans.

Keywords

Antibiotic resistance – Pigeon – *Salmonella* Braenderup – *Salmonella* Lomita – *Salmonella* Typhimurium – Squab – Virulence gene.

Introduction

The first historical mention of domesticated pigeons being used for food was in Egypt in 3,000 BC (1). Since their initial domestication, pigeons have been regarded as a cheap source of good meat, particularly the squab meat, which is valued highly. The Romans also kept pigeons for food and were familiar with the practice of force-feeding squabs in order to fatten the young birds more quickly. Wild pigeons were hunted as a cheap and readily available source of protein, although the meat from older and wild pigeons is tougher and requires a long period of stewing or roasting to tenderise. Usually considered a delicacy, squab is tender, moist and richer in taste than many commonly consumed poultry meats. Today, squab is part of the cuisine of many countries, including France, the United States (USA), Italy, Northern Africa, and several Asian countries (1, 2, 3). The Egyptians, Chinese and other Asians consider squab meat an aphrodisiac. In the USA, squab is 'increasingly a specialty item', as the larger and cheaper chicken has displaced it (4). Nevertheless, squab produced from specially raised utility pigeons continues to

grace the menus of American haute cuisine restaurants such as Le Cirque and the French Laundry (5, 6) and has enjoyed endorsements from some celebrity chefs (1). Accordingly, squab is often sold for much higher prices than other poultry, sometimes as high as eight US dollars (USD) per pound weight (1).

Human salmonellosis has been associated with contaminated poultry and with direct contact with food animals, highlighting the possibility of transmission of these organisms to humans through the food chain (7). Although there are few reports of disease transmission from pigeons to humans (8), their close interaction, together with the observation that these birds are vectors for zoonotic agents (9), may make them a public health risk. Squab meat is regarded as safer than some other poultry products, as it harbours fewer pathogens (2, 10), but there has not yet been a detailed study of the role of squab as a vector of *Salmonella* species.

To determine the extent to which squabs might harbour salmonellae and pose a risk to the human population in Egypt, squabs on sale in Cairo markets were screened for the presence of salmonellae relevant to public health. Isolates were examined for the presence of 11 virulence-associated genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *spv*C, *sop*E1, *bcf*C) and their antimicrobial resistance profiles were determined.

Methods

Sampling and isolation

The research was carried out in the Department of Poultry Diseases, Animal Research Institute, Dokki, Egypt (30° 3' North, 31° 15' East). The birds used in the study comprised 45 clinically healthy squabs randomly selected and purchased from local markets in Cairo and delivered alive to the laboratory. Sterile culturettes (Product No. 4360210, Becton Dickinson & Co., Cockeysville, MD, USA) were used to collect cloacal swabs for faecal culture. All swabs were immediately transported to the laboratory in ice-cold containers and

processed within 6 h of collection. Strains of salmonellae were isolated according to the standard ISO-6579 method (11).

Serotyping of salmonellae

Purified isolates were serotyped using a slide agglutination method with O- and H-group antigen-specific antisera (Denka-Seiken, Tokyo, Japan). Once the antigenic formulae were obtained, the Kauffman–White serotyping scheme was used to identify the serotype.

Antimicrobial susceptibility testing using the disk diffusion method

The antimicrobial resistance profiles of the *S.* Typhimurium, *S.* Braenderup and *S.* Lomita isolates were determined using disks impregnated with a range of antibiotics at fixed concentrations (Becton, Dickinson and Company, USA). The following antimicrobials were chosen on the basis of their common use in treating and preventing *Salmonella* spp. infection in poultry and humans, and because they represent a range of antimicrobial classes: ampicillin (10 mg), amoxicillin (20 mg), gentamicin (10 mg), neomycin (30 µg), streptomycin (10 mg), lincomycin (30 µg), chloramphenicol (30 mg), colistin (10 mg), tetracycline (30 mg), trimethoprim (5 µg), sulfamethoxazole + trimethoprim (23.75 mg + 1.75 mg). In addition, resistance was determined against three broad-spectrum fluoroquinolones: ciprofloxacin (5 µg), norfloxacin (5 µg) and nalidixic acid (30 mg).

Each isolate was inoculated onto a Muller–Hinton agar (Oxoid) plate and antibiotic disks were placed on the surface of the agar (six disks per plate). The plates were incubated for 24 h to 48 h at 37°C. After incubation, the diameter of the halos was measured to assess resistance or susceptibility according to the interpretation criteria for *Escherichia coli* (*E. coli*) (ATCC No. 25922) established by the Clinical Laboratory Standards Institute (12). Multidrug resistance is defined as resistance to two or more antibiotics belonging to different antibiotic classes (9).

Detection of virulence determinants

A polymerase chain reaction (PCR) amplification assay (for primers and assay conditions, see Table I) was used to detect virulence genes in all the isolates. Briefly, isolates were grown overnight at 37°C in Luria–Bertani broth (Sigma, St. Louis, MO). Genomic DNA was extracted using a boiling method and used as the target for PCR assays. Lysates were chilled on ice and then spun for 5 min in a microcentrifuge at 14,000 *g* to pellet the debris. Portions (5 μl) of the supernatants were used as templates in the PCR. The isolates were screened for the presence of 11 virulence genes (*inv*A, *avr*A, *ssa*Q, *mgt*C, *sii*D, *sop*B, *gip*A, *sod*C1, *sop*E1, *spv*C, *bcf*C). The PCR conditions and primers used for the target genes have been previously published (13, 14, 15). The specificity of the oligonucleotide primers was confirmed using strains of *S.* Enteritidis (ATCC 13076) and *E. coli* (ATCC 50034) as reference samples.

Statistical analysis

The distribution of resistance phenotypes among the serotypes, either resistance to a single antibiotic or multidrug resistance, was tested using a contingency table analysis where rows = serotype and columns = resistance versus susceptible. The significance of homogeneity in antimicrobial resistance patterns among the groups was assessed using Pearson's χ^2 exact test and SAS version 9.2 software (SAS, Cary, NJ). Findings were considered significant where $p < 0.05$. A high χ^2 value indicates that the resistant phenotypes are not proportionately distributed among serotypes, some serotypes having a higher frequency of resistant phenotypes than others. To assess the significance of differences (D) between individual D values, 95% confidence intervals (95% CIs) were calculated. The normal approximation to the binominal distribution was used to calculate the 95% CIs for the proportion of isolates resistant to individual antimicrobial agents.

Results

A total of six (13.3%) isolates of salmonellae were identified from the 45 squabs. Serotyping of the isolates differentiated them into *S.* Typhimurium (4/6; 66.7%), *S.* Braenderup (1/6; 16.7%) and *S.* Lomita (1/6; 16.7%).

Virulotyping

All isolates were screened using PCR analysis for the presence or absence of 11 selected virulence genes (Table II). The genes *inv*A and *sop*B (carried by *Salmonella* pathogenicity islands [SPIs]) and *bcf*C (fimbria-related) were detected in all six isolates. In contrast, *sop*E1 (encoding a translocated effector protein) and *gip*A (encoding a Peyer's patch-specific virulence factor) were absent. The gene *spv*C, carried by the *Salmonella* virulence plasmid, was present in three of the four isolates of *S.* Typhimurium but was absent from both the Braenderup and Lomita isolates. The Braenderup and Lomita isolates carried *avr*A (located in SPI-1) and *mgt*C (carried by SPIs), both of which were absent from the Typhimurium isolates. In contrast, *sodC1* (located on a bacteriophage) was identified in the Typhimurium isolates only.

Combinations of virulence genes

Over all, four different combinations of virulence genes were detected among the serotypes (Table III). The four Typhimurium isolates possessed three combinations of virulence genes, two of them being identical (*sod*C1, *sop*B, *spv*C and *bcf*C).

Distribution of resistance to individual antimicrobial agents

Frequent resistance to the antimicrobials tested was evident in all six isolates of salmonellae (Table IV). In particular, all isolates were resistant to lincomycin and streptomycin but all were susceptible to ciprofloxacin and gentamicin; resistance patterns to the other antimicrobials exhibited great diversity. The four Typhimurium isolates showed seven different resistance profiles (penicillin, aminoglycosides, fluoroquinolones, lincosamides, phenicols,

tetracyclines, sulphonamides). Interestingly, four of the resistance profiles of Braenderup and Lomita isolates were the same as for Typhimurium (aminoglycosides, fluoroquinolones, lincosamides, polymyxin). Significant correlations among various antimicrobials at isolate level are shown in Tables III and IV. The distribution of resistance to the antibiotics was largely dependent on serotype identity. Resistance to colistin sulphate ($\chi^2 = 6.0$; $p < 0.014$) was associated with serotypes Braenderup and Lomita.

Association of antimicrobial resistance phenotype with virulence-associated genes

Analysis of the presence of the 11 virulence-associated genes in the tested isolates is shown in Table III. Detailed analysis revealed associations of resistance/susceptibility phenotypes with potential virulence genes. The association of genes *inv*A, *sop*B and *bcf*C with particular antimicrobial resistance phenotypes (streptomycin and lincosamide) has not been recorded previously. The presence of *inv*A and *sod*C1 was associated with resistance to trimethoprim-sulfamethoxazole.

Discussion

Although many bacteria in poultry and poultry-related samples have been identified, to the authors' knowledge and from a thorough scrutiny of the literature, no studies have yet reported on virulence genes associated with different *Salmonella* serotypes or/and antimicrobial resistance in salmonellae recovered from squabs.

Notably, serotypes *S*. Braenderup and *S*. Lomita have never been detected in squabs until now. The age of the bird does not influence the *Salmonella* infection rate in pigeons and squabs (10); however, having a parent that tests positive for salmonellae greatly increases the odds of testing positive as a squab, which may imply transovarian transmission or transmission through intimate contact, either by the faecal-oral route or possibly from the crop of the parent to the oral cavity of the squab (10). The successful isolation of serotypes Typhimurium, Braenderup and Lomita reported here has

important implications, because they are a significant cause of food poisoning and enteric fever in humans (16), which makes the spread of Braenderup and Lomita among pigeons a concern for human health. Both these serotypes have been isolated from cases of food poisoning in England and Wales (17), Germany (18), Switzerland (19), the USA (20) and Japan (21, 22). With regard to isolates derived from squabs, rodents acting as reservoirs for the bacteria help to maintain and spread the infection in the pigeon population; for example, by contaminating food sources.

Many of the virulence factors in salmonellae are present as part of pathogenicity islands on the chromosome, although some virulence factors are encoded on plasmids or bacteriophages (23). In the present study, the *Salmonella* invasion gene *inv*A, which is essential for entry of the bacteria into epithelial cells, is a putative inner membrane component of the SPI-1-dependent Type III secretion system (TTSS-1) virulence apparatus (24) and was present in all six isolates. The virulotyping results for isolates carrying the *Salmonella* genomic island-1 (SGI-1) indicated that little or no variation occurred for most genes incorporated in SPIs and for the fimbrial marker. Thus, the genes *sop*B and *bcf*C were also present throughout all serotypes. It was observed that some virulence patterns were serotype specific; for example, the isolates of Braenderup and Lomita shared a common virulotype that was characterised by the presence of seven genes and the absence of *sod*C1 and *spv*C. The association of a certain serotype with a virulence-associated gene panel was also confirmed by the microarray results reported by Beutlich *et al*. (25) and a previous study by Huehn *et al*. (14) of European isolates belonging to different serotypes of salmonellae.

All the isolates of Typhimurium, Braenderup and Lomita that were tested showed multidrug resistance. Nevertheless, all isolates were sensitive to gentamicin and ciprofloxacin, and sensitivity to ampicillin, amoxicillin, neomycin, norfloxacin, chloramphenicol, tetracycline, trimethoprim and trimethoprim-sulfamethoxazole was also frequent. Ciprofloxacin is the drug of choice for treating potentially life-threatening infections caused by multidrug-resistant

strains of salmonellae in adult humans (26). However, recent studies have documented the emergence of ciprofloxacin resistance in salmonellae isolated from foods (27, 28), with nalidixic acid resistance indicating the first step in the development of resistance to ciprofloxacin (29). Thus, nalidixic-acid-resistant serotypes of Typhimurium, Braenderup and Lomita usually show decreased sensitivity to ciprofloxacin (29, 30). In the present study, however, although the isolates were resistant to nalidixic acid they remained sensitive to ciprofloxacin. For comparison, it is noteworthy that isolates from pigeon eggs were sensitive to ciprofloxacin, norfloxacin, gentamicin and chloramphenicol (100%), but resistant to tetracycline (50%) (31). Realistically, antibiotic resistance can be considered a virulence factor (32). Resistance mechanisms are pandemic and create an enormous clinical and financial burden on healthcare systems worldwide (32). The almost complete absence of resistance to ciprofloxacin and norfloxacin among the isolates tested is important, because these two antimicrobials are the primary agents used against invasive salmonellosis cases in humans (33).

In Senegal and Gambia, significant associations have been found between the presence of some virulence genes and resistance to commonly used antimicrobials (34). The isolates of Typhimurium, Braenderup and Lomita in the present investigation have a core pattern of resistance to the four antimicrobials lincomycin, streptomycin, nalidixic acid and colistin sulphate, referred to as quattuor-resistance type LSNaCs. The five genes for this resistance type, *inv*A, *ssa*Q, *sii*D, *sop*B, and *bcf*C, are clustered within the genome on the genomic island SGI-1, which also contains horizontally transferable genetic elements such as phage- and plasmid-related genes (35, 36).

An inevitable side effect of antimicrobial use is the emergence and dissemination of resistance in pathogenic bacteria, with the possibility of resistant zoonotic bacteria reaching the intestinal tract of humans (37, 38). The emergence and development of resistance to lincomycin, streptomycin, nalidixic acid and colistin sulphate has been linked to the wide use of antimicrobials in veterinary practice (39, 40, 41, 42,

43) for the treatment and prevention of disease and for growth promotion.

Conclusion

Understanding the associations between human cases of salmonellosis, animal sources and the environment is epidemiologically important in attempts to control the spread of these infections within communities (7, 44). The isolation of *S. enterica* serotypes Typhimurium, Braenderup and Lomita from squabs has important implications, because they are significant causes of food poisoning and enteric fever in humans (16, 17, 18, 19, 20, 21, 22). Carriage of the virulence-associated genes *inv*A, *sop*B, and *bcf*C among multidrug-resistant strains may increase the propensity of such strains to be of major clinical relevance, particularly if specific virulence factors are also carried (33).

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1 **Table I**

2 **Virulence factor targets and primers, including nucleotide sequences, polymerase chain reaction conditions and references**

Gene designation	Location on SPI and gene function	Oligonucleotide sequences (5'-3')	PCR conditions [a]			Product size (bp)	Reference
			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	72°C for 30 s [b]	64°C for 30 s	94°C for 60 s	284	13
*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 s	58°C for 30 s	72°C for 30 s [b]	422	14
*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	14
*mgt*C	SPI-4: Mg^{2+} uptake	tga cta tca atg ctc cag tga at / att tac tgg ccg cta tgc tgt tg	"	"	"	677	14
*sii*D (Spi4D)	Type I secretion: SPI-4	gaa tag aag aca aag cga tca tc / gct ttg ttc acg cct ttc atc	"	"	"	655	15
*sop*B	SPI-5: Inositol polyphosphate phosphatase that promotes macropinocytosis, regulates SCV localisation and promotes fluid secretion	tca gaa gRc gtc taa cca ctc / tac cgt cct cat gca cac tc	"	"	"	517	14
*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	14
*sod*C1	Gifsy-2 bacteriophage: Periplasmic Cu-, Zn-superoxide dismutases	cgg gca gtg ttg aca aat aaag / tgt tgg aat tgt gga gtc	"	"	"	424	14
*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	14
*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	14
*bcf*C	Chromosome: Bovine colonisation factor, fimbrial usher	acc aga gac att gcc ttc c / ttc tgc tcg ccg cta ttc g	72°C for 30 s [b]	53°C for 30 s	95°C for 30 s	467	14

3 a) Polymerase chain reaction, 35 cycles

4 b) After 30 cycles, final extension step of 4 min at 72°C

bp: base pairs

PCR: polymerase chain reaction

SPI: *Salmonella* pathogenicity islands

SCV: *Salmonella*-containing vacuole

TTSS: Type III secretion system

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Table II

Distribution of virulence genes among three *Salmonella* serotypes

Salmonella serotypes	Virulence genes										
	*inv*A	*avr*A	*ssa*Q	*mgt*C	*si*D	*sop*B	*gip*A	*sod*C1	*sop*E1	*spv*C	*bcf*C
Typhimurium	+	ND	ND	ND	ND	+	ND	+	ND	ND	+
Typhimurium	+	ND	+	ND	+	+	ND	+	ND	+	+
Typhimurium	+	ND	ND	ND	ND	+	ND	+	ND	+	+
Typhimurium	+	ND	ND	ND	ND	+	ND	+	ND	+	+
Braenderup	+	+	+	+	+	+	ND	ND	ND	ND	+
Lomita	+	+	+	+	+	+	ND	ND	ND	ND	+

ND: not detected

Table III

Distribution of virulence gene combinations and antibiotic resistance phenotypes in three *Salmonella* serotypes

Serotype	Virulence gene combination [a]	Resistance phenotype
Typhimurium	*inv*A, *sod*C1, **sop**B, **bcf**C	Amp, Amo, Chl, Lin, Str, Na, Neo, Nor, Tet, Tri, Sxt
	*inv*A, *ssa*Q, *si*D, *sod*C1, **sop**B, *spv*C, **bcf**C	Lin, Str, Tet, Sxt
	*inv*A, *sod*C1, **sop**B, *spv*C, **bcf**C	Amo, Neo, Chl, Lin, Str, Na, Tet, Sxt
	*inv*A, *sod*C1, **sop**B, *spv*C, **bcf**C	Chl, Lin, Str, Na, Tet, Sxt
Braenderup	*inv*A, *avr*A, *ssa*Q, *mgt*C, *si*D, **sop**B, **bcf**C	Chl, Lin, Str, Na, Tet
Lomita	*inv*A, *avr*A, *ssa*Q, *mgt*C, *si*D, **sop**B, **bcf**C	Chl, Lin, Str, Na

a) Bold indicates consistent frequency of virulence genes

Amo: amoxicillin

Amp: ampicillin

Chl: chloramphenicol

Lin: lincomycin

Na: nalidixic acid

Neo: neomycin

Nor: norfloxacin

Str: streptomycin

Tet: tetracycline

Tri: trimethoprim

Sxt: trimethoprim-sulfamethoxazole

Table IV
Distribution of resistance to individual antimicrobial agents among three *Salmonella enterica* serotypes isolated from squabs

Antimicrobials	Distribution of resistance to antimicrobials								
	S. Typhimurium (*n* = 4/6)			*S.* Braenderup (*n* = 1/6)			*S.* Lomita (*n* = 1/6)		
	No. of resistant isolates	% of isolates	95% CI	No. of resistant isolates	% of isolates	95% CI	No. of resistant isolates	% of isolates	95% CI
Penicillins									
Ampicillin	2/4	50	9.18, 90.81	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Amoxicillin	2/4	50	9.18, 90.81	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Aminoglycosides									
Gentamicin	0/4	0	2.35, 60.42	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Neomycin	1/4	25	1.32, 78.06	0/1	0	10.78, 94.54	0/1	0	
Streptomycin	4/4	100	39.58, 97.65	1/1	100	5.46, 89.22	1/1	100	5.46, 89.22
Fluoroquinolones									
Ciprofloxacin	0/4	0	2.35, 60.42	0/1	0	10.78, 94.54	0/1	0	
Nalidixic acid	3/4	75	21.94, 98.68	1/1	100	5.46, 89.22	1/1	100	5.46, 89.22
Norfloxacin	1/4	25	1.32, 78.06	0/1	0	10.78, 94.54	0/1	0	
Lincosamides									
Lincomycin	4/4	100	39.58, 97.65	1/1	100	5.46, 89.22	1/1	100	5.46, 89.22
Phenicols									
Chloramphenicol	3/4	75	21.94, 98.68	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Polymyxin									
Colistin sulphate	0/4	0	2.35, 60.42	1/1	100	5.46, 89.22	1/1	100	5.46, 89.22
Tetracyclines									
Tetracycline	3/4	75	21.94, 98.68	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Sulphonamides									
Trimethoprim	2/4	50	9.18, 90.81	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54
Trimethoprim–sulfamethoxazole	3/4	75	21.94, 98.68	0/1	0	10.78, 94.54	0/1	0	10.78, 94.54

CI: 95% binominal confidence interval (95% CI) based on normal approximation