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Short communication

Antimicrobial resistance of non-typhoidal *Salmonella* isolates from egg layer flocks and egg shells

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ABSTRACT

This study was conducted to examine the antimicrobial resistance (AMR) of *Salmonella* spp. isolated from commercial caged layer flocks in New South Wales and South Australia. All *Salmonella* isolates (n = 145) were subjected to phenotypic and genotypic characterisation of AMR and carriage of integrons. The majority of *Salmonella* isolates (91.72%) were susceptible to all antimicrobials tested in this study. Limited resistance was observed to amoxicillin and ampicillin (5.51%), tetracycline (4.13%), cephalothin (2.06%) and trimethoprim (0.68%). None of the isolates were resistant to cefotaxime, ceftiofur, ciprofloxacin, chloramphenicol, gentamycin, neomycin or streptomycin. A low frequency of *Salmonella* isolates (4.83%) harboured antimicrobial resistance genes and a class 1 integron. The most commonly detected AMR genes among the *Salmonella* isolates were bla_{TEM} (2.07%), *tet* A (1.38%) and *dhfr*V (0.69%). Overall, *Salmonella enterica* isolates exhibited a low frequency of AMR and represent a minimal public health risk associated with the emergence of multidrug resistant *Salmonella* spp. from the Australian layer industry.

1. Introduction

Salmonella spp. is a major cause of foodborne illness worldwide. Consumption of contaminated food products such as pork, meat, egg and egg related products are among the most common sources of *Salmonella* infection (Hur et al., 2012). In Australia, outbreaks of human salmonellosis are associated with the consumption of contaminated food products containing chicken meat or egg products (OzFoodNet Working Group, 2012). In Australia, a total of 11,992 *Salmonella* cases were reported in 2010, representing 53.7 cases per 100,000 people which is higher compared to the previous 5 years with an average infection rate of 41.8 cases per 100,000 people (OzFoodNet Working Group, 2012).

Typically, infection with *Salmonella* is self-limiting producing mild gastroenteritis, however, severe infection occurs common in elderly and immunocompromised individuals (Parry and Threlfall, 2008). Severe, systemic salmonellosis may require treatment with antimicrobials such as fluoroquinolones and extended-spectrum cephalosporins (Parry and Threlfall, 2008). The use of antimicrobial agents in the prevention and treatment of many infectious diseases and as a growth promoter is well known both in veterinary and human medicine (Hur et al., 2012). Indiscriminate use of antibiotics in both animal and human populations has, however, led to an emergence of multidrug resistant *Salmonella* strains (Anjum et al., 2011). The emergence and dissemination of antibiotic resistance to *Salmonella* is of significant global concern for both animal and public health. Moreover, the transfer of multidrug resistant *Salmonella* spp. to humans through food producing animals can compromise the treatment options (Hur et al., 2012).

Compared with many other countries, Australia currently has a very conservative approach for antibiotic usage in commercial egg layer flocks. Antimicrobials, such as fluoroquinolones, are prohibited and ceftiofur is not approved for mass administration in food producing animals (Cheng et al., 2012; Obeng et al., 2012). To date, there is little information available characterising antimicrobial resistance (AMR) in *Salmonella* isolated from commercial Australian egg layer flocks. In this study, the phenotypic and genotypic AMR was characterised for multiple *Salmonella* isolates recovered from layer flocks and egg shells.

2. Materials and methods

2.1. Bacterial strains and serotyping

A total 145 *Salmonella* isolates were used in this study. Samples were isolated from 33 commercial caged layer flocks sourced from a total of 13 farms from New South Wales (10 farms) and South Australia (3 farms). All *Salmonella* isolates used in this study were previously isolated in our laboratory during epidemiological studies (Chousalkar and Roberts, 2012; Gole et al., 2014a, 2014b). Details of *Salmonella* isolates, sources and their distribution are presented in Table 1. All isolates were serotyped by the *Salmonella* Reference Laboratory, Institute of Medical and Veterinary Science, SA Pathology (Adelaide, South Australia).

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Table 1
Distribution and sources of *Salmonella* isolates.

Salmonella (*S.*) serovar	Source				Total
	Dust	Egg belt	Faeces	Shell wash	
S. Agona	4	2	0	0	6
S. Anatum	2	4	0	0	6
S. Infantis	0	2	3	11	16
S. Mbandaka	7	11	12	0	30
S. Oranienburg	8	8	8	6	30
S. Typhimurium	14	5	6	1	26
S. Worthington	7	8	14	2	31
Total	42	40	43	20	145

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of each *Salmonella* isolate to 12 antibiotics (Table 2) was determined using the broth microdilution method and the results were interpreted according to the established Clinical and Laboratory Standards Institute (CLSI), guidelines (CLSI, 2013). In cases where CLSI breakpoints were absent, results were evaluated according to the National Antimicrobial Resistance Monitoring guidelines (National Antimicrobial Resistance Monitoring System, 2012) or Swedish Veterinary Antimicrobial Resistance Monitoring guidelines (Swedish Veterinary Antimicrobial Resistance Monitoring, 2012). All tests were performed using *Escherichia coli* ATCC 25922 as control strain. *Salmonella* isolates showing resistance to more than three classes of antimicrobial agents were classified as multi-drug resistant (MDR).

2.3. DNA extraction

DNA extraction from all *Salmonella* isolates was performed using 6% Chelex® (Bio-Rad, Sydney, NSW, Australia) prepared in TE (10 mM Tris and 1 mM EDTA). Briefly, single colonies of fresh *Salmonella* isolates were grown in LB broth at 37 °C for approximately 16 h with shaking. 100 μL of overnight bacterial culture was mixed to 1 ml of sterile water and centrifuged at 14,000 g for 2 min. The supernatant in the tube was decanted and bacterial pellet was re-suspended in 200 μL of 6% Chelex solution. The tubes were incubated at 56 °C for 20 min. Following vortexing, tubes were incubated at 100 °C for 8 min. Samples were kept on ice for 5 min and centrifuged for centrifuged at 14,000 g for 10 min. Supernatants were recovered from each isolate and used as DNA template for polymerase chain reactions (PCR).

2.4. Analysis of antimicrobial resistance genes (ARG) and integrons

All 145 *Salmonella* isolates were screened for total of 20 antimicrobial resistance genes (ARG) by either multiplex or uniplex PCR (Van et al., 2008). Presence of class 1, 2 and 3 integrons in *Salmonella* isolates were investigated by multiplex PCR assay (Dillon et al., 2005). For each PCR reaction, both negative and positive controls were included. A set of five positive control *Salmonella* strains known to contain the targeted antimicrobial resistance genes were selected from isolates included in an earlier study (Abraham et al., 2014). PCR reactions for multiplex pool were performed in total volume of 25 μL containing 5 ng of DNA template and final concentrations of 10 μM of each dNTP (Bioline, Australia), 4 mM $MgCl_2$, 25 *p*M each primer pool and 1 U of Hotstart *Taq* polymerase (Qiagen, Australia). Uniplex PCR reactions were also performed in a total reaction volume of 25 μL including 2 μL DNA template. Each uniplex PCR reaction mixture consisted of final concentration of 1.5 mM $MgCl_2$, 2.5 μM of each dNTP (Bioline, Australia), 25 *p*M of each primer, and 1 U of *Taq* polymerase (Bioline, Australia). DNA amplification was carried out in T100 thermal cycler (Bio-Rad, Australia) using the following conditions: 15 min initial denaturation at 95 °C, following 30 cycles of denaturation at 94 °C for 30 s, annealing at various temperatures for 30 s (Table S1) and extension at 72 °C for 60 s and final amplification cycle at 72 °C for 10 min. PCR products were electrophoresed at 80 V for 2.5 h on 2% agarose gel. The size of PCR products was determined by comparing with standard 100 bp ladders (Thermo Fisher, Australia). The details of antimicrobial resistance genes, integrons and primer sequences are described in Supplementary Table S1.

3. Results

3.1. Antimicrobial susceptibility screening of Salmonella isolates

The *Salmonella* isolates selected for this study displayed a low but wide spectrum of antibiotic resistance (Table 2). A total of 91.72% (133/145) of the *Salmonella* isolates were susceptible to all tested antimicrobials. Overall, resistance was observed to amoxicillin and ampicillin (5.51%), tetracycline (4.13%), cephalothin (2.06%) and trimethoprim (0.68%) (Table 2). Resistance to cefotaxime, ceftiofur, ciprofloxacin, chloramphenicol, gentamycin, neomycin, or streptomycin was not observed for any isolate. *S.* Mbandaka, *S.* Typhimurium and *S.* Worthington showed resistance to amoxicillin, ampicillin and tetracycline whereas *S.* Agona, *S.* Anatum, *S.* Infantis and *S.* Oranienburg were susceptible to all tested antimicrobials.

No multidrug resistant phenotypes were identified from any *Salmonella* isolate included in this study. Results of resistance patterns of all *Salmonella* isolates are described in Table 3.

3.2. Detection of resistance genes and integrons

All 145 *Salmonella* isolates belonging to seven different serovars recovered from layer flocks were investigated for antimicrobial resistance genes and integrons by multiplex and uniplex PCR. A total of 4.83% (7/145) *Salmonella* isolates harboured antimicrobial resistance genes and class 1 integron (Table 4). The resistance genes most commonly detected were bla_{TEM} (2.07%), *tet* A (1.38%), *dhfr*V and class 1 integron (0.69%). One isolate, *S.* Mbandaka was positive for class 1 integron and contained the *dhfr*V gene conferring resistance to

Table 2
Percentage (%) of antimicrobial resistance among different serovars of *Salmonella* (n).

	Antimicrobials												
Serovar	No. of isolates tested	AMC	AMP	CTX	CEF	CPL	CIP	CHL	GEN	NEO	STR	TET	TRM
S. Agona	6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. Anatum	6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. Infantis	16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. Mbandaka	30	6.66 (2)	6.66 (2)	0.0	0.0	3.33 (1)	0.0	0.0	0.0	0.0	0.0	16.66 (5)	3.33 (1)
S. Oranienburg	30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. Typhimurium	26	3.84 (1)	3.84 (1)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.84 (1)	0.0
S. Worthington	31	16.12 (5)	16.12 (5)	0.0	0.0	6.45 (2)	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total	145	5.51 (8/145)	5.51 (8/145)	0.0	0.0	2.06 (3/145)	0.0	0.0	0.0	0.0	0.0	4.13 (6/145)	0.68 (1/145)

Abbreviations: AMC — Amoxycillin; AMP — Ampicillin; CTX — Cefotaxime; CEF — Ceftiofur; CPL — Cephalothin; CIP — Ciprofloxacin; CHL — Chloramphenicol; GEN — Gentamycin; NEO — Neomycin; STR — Streptomycin; TET — Tetracyline; TRM — Trimethorpim.