Antimicrobial resistance surveillance in *Salmonella* isolates from environments on Australian commercial egg farms

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Foreword

This project was conducted to identify the most appropriate mechanisms for obtaining and performing antimicrobial susceptibility testing on *Salmonella* isolates obtained from Australian layer shed environments.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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About the Authors

The author of this report is Professor Darren J. Trott, Director of the Australian Centre for Antimicrobial Resistance Ecology, School of Animal and Veterinary Sciences, the University of Adelaide.

Professor Trott completed his veterinary degree at Murdoch University and worked in small animal practice for eight years. Following an honours project on *Listeria monocytogenes* he became interested in the ecology of infectious diseases and completed a PhD with Prof David Hampson on intestinal spirochaetes (awarded in 1998). After completing a three-year post-doc at the National Animal Disease Center in Ames, Iowa, USA, he accepted a lectureship at the University of Queensland (UQ) School of Veterinary Science in 2000. He taught veterinary microbiology and antimicrobial chemotherapy to veterinary undergraduates at UQ for 10 years and conducted research on gastrointestinal diseases, microbial ecology and virulence/antimicrobial resistance in companion animal, livestock and human bacterial pathogens.

He joined the University of Adelaide School of Animal and Veterinary Sciences in January 2010 and his research areas have expanded to also include antimicrobial resistance ecology, repurposing existing drug classes for development as new anti-infectives, including pre-clinical assessment in murine bioluminescent models of infection, and the effect of antimicrobials on the gut microbiome.

Professor Trott recently established and is the inaugural Director of a new Research Centre at the University of Adelaide, the Australian Centre for Antimicrobial Resistance Ecology. He works closely with Australia's major animal industries (both livestock and companion animal) in the areas of prudent antimicrobial use, antimicrobial stewardship and confirming/ensuring that rates of resistance in animal pathogens, zoonotic and commensal bacteria remain low by international standards.

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Abbreviations

AMR	Antimicrobial resistance
ACARE	Australian Centre for Antimicrobial Resistance Ecology
AST	Antimicrobial susceptibility testing
ASTAG	Australian Strategic and Technical Advisory Group
AVA	Australian Veterinary Association
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CLSI	Clinical and Laboratory Standards Institute
DPI	Department of Primary Industries
ECDC	European Centre for Disease Prevention and Control
ECOFF	European Committee for Antimicrobial Susceptibility Testing epidemiological cut-off value
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee for Antimicrobial Susceptibility Testing
EFSA	European Food Safety Authority
HBA	Horse blood agar
MALDI-TOF	Matrix assisted laser desorption/ionisation-time of flight (mass spectrometry)
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
NARMS	National Antimicrobial Resistance Monitoring System (USA)
STs	Sequence types

Executive Summary

This proof-of-concept Antimicrobial resistance (AMR) surveillance study was undertaken to identify the most appropriate mechanisms for obtaining and performing antimicrobial susceptibility testing on *Salmonella* isolates obtained from Australian layer shed environments.

A total of 307 *Salmonella* spp. isolates from the years 2015-2018, proportionally representative of the number of layer flocks in each Australian state, were obtained from reference, research and State Department of Health laboratories as well as directly from drag swab samples of Tasmanian shed environments. Antimicrobial Susceptibility Testing (AST) was performed by broth microdilution using Veterinary Reference Card panels for 12 antimicrobials (Sensititre®, Trek Diagnostics, East Grinstead, UK), and in-house broth microdilution panels were made according to CLSI standards (CLSI, 2015), and used to test susceptibility to colistin, florfenicol and kanamycin. Minimum inhibitory concentrations (MICs) were interpreted according to CLSI breakpoints and/or by using the recommended European Committee for Antimicrobial Susceptibility Testing epidemiological cut-off values (ECOFFs).

Three main serotypes comprised approximately one half of the collection – Typhimurium (61/307; 19.9%), Senftenberg (45/307; 14.7%) and Agona (37/307; 12.1%). Among the *S.* Typhimurium isolates, phage types 9, 170 and 135 were predominant (each representing 2.2% of total isolates). Non-susceptibility was observed to occur at a low level to streptomycin (7/307; 2.3%), sulfisoxazole (6/307; 2.0%), chloramphenicol (4/307; 1.3%) and tetracycline (3/307; 1%). Very low levels of non-susceptibility were observed to ampicillin (2/307; 0.7%) and cefoxitin (2/307; 0.7%). All isolates were susceptible to amoxicillin-clavulanate, azithromycin, ceftiofur, ceftriaxone, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin and trimethoprim-sulfamethoxazole. A very high proportion of isolates (293/307; 95.4%) among the 307 *Salmonella* spp. from Australian layer shed environments was susceptible to all 16 antimicrobial agents tested, as indicated below in Table 6. Fourteen isolates (4.6%) were resistant to one antimicrobial class, and two isolates (0.7%), were resistant to two antimicrobial classes (including aminoglycosides, beta lactams and tetracycline). Two isolates (one serotype Havana and one serotype Montevideo strain) exhibited a MDR phenotype (0.7%) to aminoglycosides (streptomycin), folate pathway inhibitors (sulfisoxazole) and tetracycline.

Whole genome sequencing identified the *S*. Typhimurium isolate resistant to two antimicrobial classes as a ST19 strain, the most common globally distributed *Salmonella* sequence type associated with poultry and foodborne gastroenteritis. The Havana and Montevideo isolates resistant to three antimicrobial classes were identified as rare sequence types (STs) (ST4 and ST578, respectively).

Overall, the results confirm the low antimicrobial resistance status of *Salmonella* isolated from Australian caged and free range layer farm environments, which likely reflects the combination of restrictions on antimicrobial use, and in particular, critically important antimicrobial agents including fluoroquinolones, 3rd generation cephalosporins and colistin, combined with effective non-antimicrobial disease control mechanisms.

Overall Conclusions and Recommendations

1) The large number of isolates expressing a wild-type phenotype with respect to antimicrobial susceptibility to the 16 tested antimicrobial agents and the low frequency of resistance to predominantly first line antimicrobial agents with a low ASTAG importance rating likely reflects the highly conservative antimicrobial use, disease control and biosecurity policies adopted by the Australian layer industry.
2) The three isolates exhibiting non-susceptibility to two or more antimicrobial classes were subjected to whole genome sequencing, which identified the genetic mechanism/s of resistance to ampicillin, tetracycline, sulfonamides and trimethoprim and streptomycin.
3) Future AMR surveillance studies could differentiate free range from caged shed environmental samples.
4) Future AMR surveillance studies could focus on screening commensal Escherichia coli (as undertaken in pilot surveys in the meat chicken and pig industries) isolated from shed drag swab samples to identify if the resistance profiles are similar.

1 Introduction

Globally *Salmonella enterica* subspecies *enterica* is a common cause of foodborne human gastroenteritis. In Australia, consumption of undercooked or cross-contaminated egg and/or egg products have been implicated in outbreaks of human salmonellosis (Chousalkar et al., 2018). *Salmonella* organisms are also useful as an indicator of the extent of exposure of enteric bacterial communities to antimicrobials because their primary reservoir is the gut of vertebrate hosts, where the organisms readily respond to selection pressures arising from administration of antimicrobials. Infection with non-typhoidal *Salmonella* causes mild gastroenteritis and is self-limiting, however, in cases of severe and systemic human salmonellosis, antimicrobials are required. In some countries, indiscriminate and/or poor regulated use of antimicrobial agents in both animal and human populations may have led to the emergence of multidrug-resistant (MDR) *Salmonella* strains that are also resistant to critically important antimicrobials used in human medicine (Campos et al., 2018). The emergence and dissemination of antibiotic resistance in *Salmonella* is therefore of significant global concern for both animal and public health.

Compared with many other countries, Australia has a cautious approach to antimicrobial agent usage in food-producing animals. Antimicrobial agents such as fluoroquinolones and gentamicin, are prohibited for use in food-animals, colistin and 4th generation cephalosporins are not registered for animal use and ceftiofur, a 3rd generation cephalosporin, is not approved for mass administration in food-producing animals and is not used in poultry. There are further restrictions on the use of many antimicrobial agent classes in laying birds due to the risk of antimicrobial residues in eggs (AVA, 2015; Table 1). Additionally, effective vaccination programs and strict biosecurity limit the occurrence of endemic diseases and further reduce the requirement for antimicrobial treatments. Based on 2017 Australian Eggs statistics, 48% of Australian layers are in cages and over 52% are in alternative systems including barn, free range and speciality systems (Australian Eggs, 2017).

Table 1 Antimicrobial agents registered for use for the treatment of egg laying poultry in Australia

Antimicrobial agents			
First line	**Second line**	**Third line**	**Use prohibited**
Neomycin	Spectinomycin	Nil	Fluoroquinolones
Chlortetracycline	Lincomycin		Gentamicin
Zinc bacitracin			Chloramphenicol
Flavophospholipol			Nitrofurans
			Colistin
			Ceftiofur

Divided into first line, second line and third line, based on Australian Veterinary Association (AVA, 2015) – with the inclusion of Flavophospholipol.

In Australia, proof-of-concept surveys of antimicrobial resistance (AMR) in *Salmonella* isolates recovered from pigs and meat chickens have been completed, but are not yet published by the Department of Agriculture and Water Resources. Such data on AMR among *Salmonella* isolates from the chicken egg industry are limited. Pande et al. (2015) undertook an antimicrobial susceptibility study of *Salmonella* isolates derived from layer shed environments and egg shells from South Australia and New South Wales. A total of 91.7% of isolates were susceptible to all tested antimicrobials. Very low to low levels of resistance were observed to ampicillin (5.5%), tetracycline (4.1%), cephalothin (2.1%) and trimethoprim (0.8%) (Pande et al., 2015). Following on from this study, the current project aimed to undertake antimicrobial susceptibility testing (AST) among *Salmonella* isolates recovered from commercial egg farm environments throughout Australia using World Organisation for Animal

Health standards and guidelines. This project was funded by the Department of Agriculture and Water Resources as a part of the Australian Government's Agricultural Competitiveness White Paper, the government's plan for stronger farmers and a stronger economy, and was undertaken with the support and assistance of Australian Eggs and Scolexia.

2 Objectives

1) To determine antimicrobial susceptibility profiles among *Salmonella* isolates recovered from the Australian chicken egg industry layer shed environments, including determining the frequency of resistance to critically important classes of antimicrobials (hypothesised to be very low or negligible).
2) To screen selected *Salmonella* isolates found to be resistant to critically important antimicrobial agents, by whole genome sequence analysis to identify resistance mechanisms.

3 Materials and Methods

3.1 *Salmonella* isolate collection

Salmonella isolates (n = 307) obtained from layer flock environments were acquired from archived collections of co-operating *Salmonella* reference laboratories (University of Melbourne Microbiological Diagnostic Unit, Public Health Laboratory, and the *Salmonella* Reference Laboratory, SA Pathology, Adelaide), state-based food safety authorities and university research institutes, on transport medium swabs. A small number of isolates (n = 29) were obtained directly from drag swab samples from Tasmanian sheds submitted on behalf of industry veterinarians to the University of Adelaide, Veterinary Diagnostic Laboratory, for *Salmonella* culture as none of the collaborating laboratories had isolates from this state that matched the selection criteria. All isolates have either previously undergone serotyping (with some isolates also phage typed) or were serotyped by the Microbiological Diagnostic Unit, Public Health Laboratory, Department of Microbiology and Immunology, at the Doherty Institute for Infection and Immunity, the University of Melbourne.

The project was undertaken with the support of industry veterinarians (Scolexia Pty Ltd) providing services to a large proportion of the Australian industry, who undertake regular environmental swabbing of layer shed environments representing barn, cage and free range systems. It is assumed, therefore, that approximately 50% or more of the 307 isolates are derived from alternative systems (barn and free range), and the entire collection is representative of current *Salmonella* diversity within the Australian egg industry. Given the distribution of laying farms within Australia, an approximate total of 75 isolates each was requested from Victoria, New South Wales and Queensland, and 25 isolates each from South Australia, Western Australia, and Tasmania. The selection criteria for obtaining the isolates were as follows:

1) An isolation date no earlier than January 2015.
2) Isolate to be derived from the layer shed environment (i.e. via the drag swab method).
3) No more than a single isolate from each shed unless multiple serotypes were obtained.
4) Databases of isolates from reference laboratories were submitted on a confidential basis to Prof David Jordan, NSW DPI veterinary epidemiologist for random selection of isolates using random number generation. A list of isolate numbers for inclusion in the study was then sent back to the reference laboratory, and the isolates were retrieved

from -80°C stocks and transported to the Australian Centre for Antimicrobial Resistance Ecology (ACARE) AMR surveillance laboratory at the University of Adelaide for antimicrobial susceptibility testing (AST).

Note: It could not be determined from the isolate submission information if the shed drag swab sample generating the isolate was obtained from caged birds or birds defined as free range according to the current code. Additionally, all state-based veterinary laboratories in Tasmania were contacted, but no Salmonella isolates from layer shed environments were available from archived collections that met the selection criteria. Therefore, drag swab samples were obtained from five layer enterprises in Tasmania and submitted to the University of Adelaide Veterinary Diagnostic Laboratory for Salmonella isolation.

Upon arrival to the ACARE laboratory all isolates were cultured using Horse Blood Agar (HBA – bioMérieux Australia Pty Ltd) and incubated at 37°C for 24 hrs. Colonies were selected for MALDI-TOF mass spectrometry confirmation of identification of genus and species, subcultured again on to HBA and incubated at 37°C for 24 hrs for AST. Cultures were then harvested using a sterile swab inoculated into 1 mL Tryptone Soya Broth plus 20% glycerol (prepared in-house) and stored at -80°C.

3.2 Phenotypic antimicrobial susceptibility testing

AST was performed by broth microdilution using Veterinary Reference Card panels (Sensititre®, Trek Diagnostics, East Grinstead, UK). The CMV3AGNF format was used to test *Salmonella* spp. In addition, in-house broth microdilution panels were made according to CLSI standards (CLSI, 2015), and used to test susceptibility to colistin, florfenicol and kanamycin. The antimicrobial concentration ranges for the agents are shown in Table 2. MIC endpoints were determined both visually and by assessing optic density at a 600 nm wavelength using a Sensititre™ Vizion™ MIC viewing system.

Quality control strains *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, *E. coli* ATCC 35218 *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212, were used throughout the study period.

3.3 Interpretation

Minimum inhibitory concentrations (MIC) were interpreted according to CLSI VET01S (CLSI, 2015b) and/or by using the recommended European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2018) epidemiological cut-off values (ECOFFs), indicated in Table 2. CLSI M100S (CLSI, 2016) breakpoints were used where animal species antimicrobial agent combinations were not available. Where no EUCAST or CLSI interpretative criteria were available, breakpoints were harmonised with those of the National Antimicrobial Resistance Monitoring System (NARMS), USA (CDC, 2015).

For each antimicrobial, the frequency of *Salmonella* isolates with MICs above the CLSI susceptible breakpoint was expressed as % non-susceptible (CLSI, 2011). Similarly, the frequency of *Salmonella* isolates with MICs above the EUCAST ECOFF was expressed as % non-wild-type (Simjee et al., 2018). It is noted, however, that the NARMS breakpoint for florfenicol (8 µg/mL; established for *S.* Choleraesuis isolates only) is below the EUCAST ECOFF (16 µg/mL), therefore isolates were defined as non-susceptible to this agent if they had a florfenicol MIC higher than 16 µg/mL. The frequency of non-susceptibility for each antimicrobial agent was described as rare: <0.1%; very low: 0.1% to 1.0%; low: >1.0% to 10.0%; moderate: >10.0% to 20.0%; high: >20.0% to 50.0%; very high: >50.0% to 70.0%; and extremely high: >70.0%; according to the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (EFSA and ECDC, 2015). Isolates showing non-susceptibility to one or more antimicrobial agents in three or more classes were classified as MDR

(Magiorakos et al., 2012), with the recommendation that all MDR *Salmonella* isolates should be subjected to further analysis by whole genome sequencing for resistance gene identification and comparison to internationally available sequence information (Table 2).

Table 2 Breakpoints used for Antimicrobial Susceptibility Testing of *Salmonella* spp. isolates

Antimicrobial Class	Antimicrobial Agent	Range (mg/L)	ECOFF > [a]	CLSI [b] or NARMS [c]		
				S	I	R
Aminoglycosides	Gentamicin	0.25 - 16	2	≤4	8	>8
	Kanamycin	2 - 256	-	≤16	32	>32
	Streptomycin	2 - 64	16	≤32	-	>32
β-lactam/β-lactam inhibitor combination	Amoxicillin-clavulanate (2:1 ratio)	1 - 32	-	≤8	16	>16
Cephems	Cefoxitin	0.5 - 32	8	≤8	16	>16
	Ceftiofur	0.12 - 8	2	≤2	4	>4
	Ceftriaxone	0.25 - 64	-	≤1	2	>2
Fluoroquinolones	Ciprofloxacin	0.015 - 4	0.06	≤0.06	0.12-0.5	>0.5
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (1:19)	0.12 - 4	1	≤2	-	>2
	Sulfisoxazole	16-256	256	-	-	>256
Macrolides	Azithromycin	0.12 - 16	-	≤16	-	>16
Penicillins	Ampicillin	1 - 32	8	≤8	16	>16
Phenicols	Chloramphenicol	2 - 32	16	≤8	16	>16
	Florfenicol	1 - 128	16	≤4 [d]	8	>8 [d]
Polymyxins	Colistin	0.12 - 8	-	-	-	-
Tetracyclines	Tetracycline	4 - 32	8	≤4	8	>8

[a] EUCAST epidemiological cut-off values (mg/L).

[b] CLSI VETO1S (CLSI, 2015b) or M100S (CLSI, 2016) breakpoints (mg/L), S = sensitive; I = intermediate; R = resistant.

[c] NARMS (CDC, 2016) breakpoints (mg/L) (orange text).

[d] *Salmonella* Choleraesuis only. Therefore non-susceptibility was defined according to the ECOFF.

3.4 Resistance profiles

Resistance profiles to each of the antimicrobial classes were generated to examine co-resistance among *Salmonella* spp. isolates. As defined in Section 5.3, MDR isolates possessed a resistance profile comprising non-susceptibility to at least one agent in three or more antimicrobial classes as listed below in Table 3.

Table 3 Definitions of antimicrobial classes for determining multidrug-resistance of *Salmonella* spp. isolates

Antimicrobial class	Antimicrobial agents
Aminoglycosides	Gentamicin, kanamycin, streptomycin
β-lactam / β-lactam inhibitor combination	Amoxicillin-clavulanate
Cephems (extended spectrum cephalosporins)	Ceftriaxone, ceftiofur
Cephems (cephamycins)	Cefoxitin
Fluoroquinolones	Ciprofloxacin
Folate/pathway inhibitors	Trimethoprim-sulfamethoxazole, sulfisoxazole
Penicillins	Ampicillin
Phenicols	Chloramphenicol, florfenicol
Polymyxins	Colistin
Tetracyclines	Tetracycline

3.5 Whole genome sequencing

Whole genome sequencing was performed on selected isolates (n = 3) using Illumina MiSeq. Briefly, samples underwent library preparation using the Nextera XT DNA library preparation kit according to the manufacturer's instructions, and sequencing was performed on a MiSeq V3 2x300 flow cell. The Nullarbor pipeline v1.01 (https://github.com/tseemann/nullarbor) was used to assemble the sequenced strains. The resulting FASTA files were analysed using the ResFinder, VirulenceFinder and additional functions of the Centre for Genomic Epidemiology database – http://www.genomicepidemiology.org/

3.6 Statistical analysis

Confidence intervals of proportions were calculated where appropriate using GraphPad Prism version 7.01 for Windows, GraphPad Software, La Jolla California, USA – www.graphpad.com.

4 Results

4.1 Isolate origin

The origin of the 307 *Salmonella* isolates received, re-identified and subjected to AST is shown in Table 4.

Table 4 Number of *Salmonella* spp. isolates received from layer shed environments by state

State	Number
New South Wales	77
Queensland	76
Victoria	78
South Australia	25
Tasmania	29
Western Australia	22
Total	307

4.2 Serotype distributions

Salmonella isolate serotypes and *S.* Typhimurium phage types are summarised in Figure 1 and Figure 2, respectively. Three main serotypes comprised approximately one half of the collection – Typhimurium (61/307; 19.9%), Senftenberg (45/307; 14.7%) and Agona (37/307; 12.1%). Among the *S.* Typhimurium isolates, phage types 9, 170 and 135 were predominant (each representing 2.2% of total isolates).

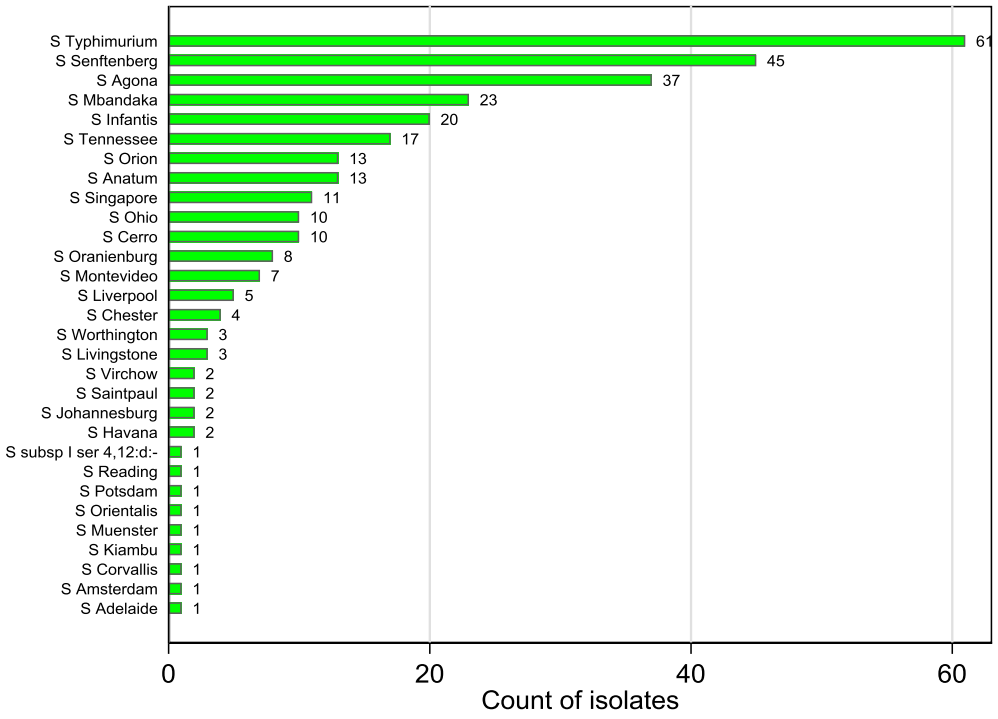

Figure 1 Distribution of serotypes among the 307 *Salmonella* spp. isolates from commercial layer shed environments in Australia

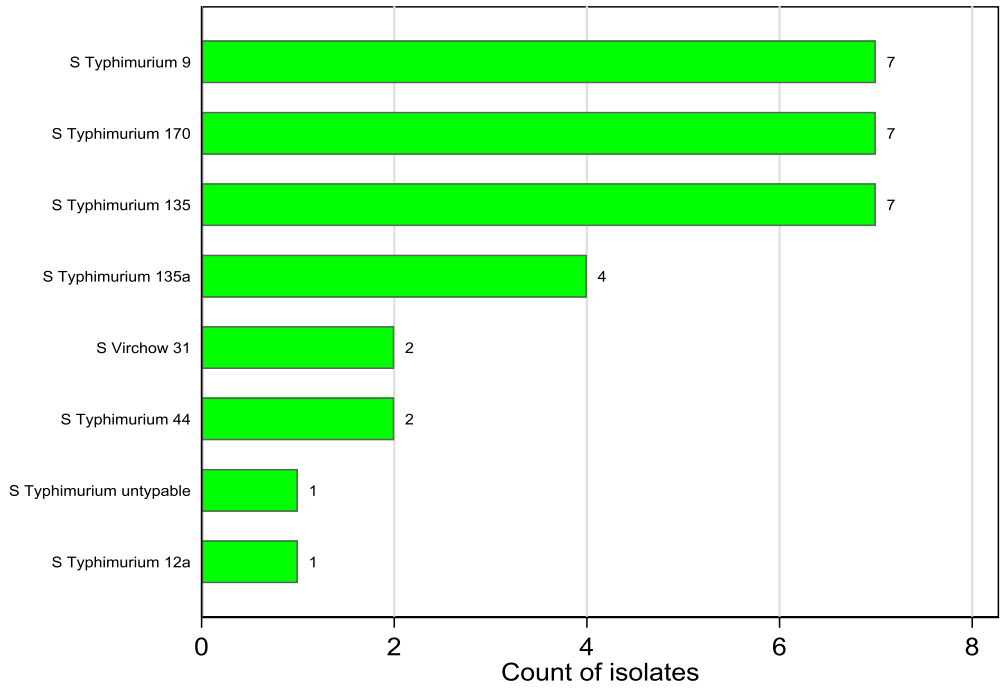

Figure 2 Frequency of occurrence of significant *Salmonella* spp. phage types among the 307 *Salmonella* isolates from commercial layer shed environments in Australia

Note: Some *S.* Typhimurium isolates were not subjected to phage typing.

4.3 MIC distributions

MIC distributions, percentage non-wild-type according to ECOFFs and percentage non-susceptible according to CLSI/NARMs clinical breakpoints for the 307 *Salmonella* isolates are shown in Table 5.

Table 5 Minimum inhibitory concentration (MIC) distributions established for 16 antimicrobials against 307 *Salmonella* spp. isolates from commercial layer shed environments in Australia

Antimicrobial agent	Number and percentage of isolates with MICs (mg/L) at: [a]																	%NWT [b] [95% CI]	%NS [c]
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256		
Amoxicillin-clavulanate								290	15	0	2	0	0						
								94.5	4.9	–	0.7	–	–[d]					—[e]	0.0
Ampicillin								276	26	3	0	0	0	2					
								89.9	8.5	1	–	–	–	0.7				0.7 [0.1 – 2.3]	0.7
Azithromycin					0	0	0	1	39	238	28	1							
					–	–	–	0.3	12.7	77.5	9.1	0.3						—	0.0
Cefoxitin							0	3	107	183	12	2	0						
							–	1	34.9	59.6	3.9	0.7	–					0.7 [0.1 – 2.2]	0.7
Ceftiofur					0	1	26	270	10	0	0								
					–	0.3	8.5	87.9	3.3	–	–							0.0 [0.0 – 1.5]	—
Ceftriaxone						307	0	0	0	0	0	0	0	0					
						100	–	–	–	–	–	–	–	–				—	0.0
Chloramphenicol									4	123	176	4	0						
									1.3	40.1	57.3	1.3	–					0.0 [0.0 – 1.5]	1.3
Ciprofloxacin		237	70	0	0	0	0	0	0	0									
		77.2	22.8	–	–	–	–	–	–	–								0.0 [0.0 – 1.5]	0.0
Colistin					0	42	164	96	5	0									
					–	13.7	53.4	31.3	1.6	–								—	0.0
Florfenicol								0	5	147	152	3	0	0	0				
								–	1.6	47.9	49.5	1	–	–	–			0.0 [0.0 – 1.5]	0.0
Gentamicin						122	169	15	1	0	0	0							
						37.7	55	4.9	0.3	–	–	–						0.0 [0.0 – 1.5]	0.0
Kanamycin									294	9	4	0	0	0	0	0			
									95.8	2.9	1.3	–	–	–	–	–		—	0.0
Streptomycin									5	54	197	44	5	0	2				
									1.6	17.6	64.1	14.3	1.6	–	0.7			2.3 [1.1 – 5.1]	2.3
Sulfisoxazole												16	32	174	67	12	6		
												5.2	10.4	56.7	21.8	3.9	2.0	—	2.0
Tetracycline										304	0	0	0	3					
										99	–	–	–	1				1 [0.2 – 2.8]	1
Trimethoprim/sulfamethoxazole					305	2	0	0	0	0	0								
					99.3	0.7	–	–	–	–	–							0.0 [0.0 – 1.5]	0.0

[a] Unshaded areas indicate MIC range for each agent available on the Sensititre CMV3AGNF card. MICs > than highest concentration available are indicated in the shaded region. Vertical green lines indicate EUCAST epidemiological cut-off (ECOFF) values; CLSI VET01S susceptible (blue) and resistant (red) breakpoints; NARMS breakpoint (red dashes).

[b] Percentage non-wild-type (EUCAST).

[c] Percentage non-susceptible, CLSI or NARMS (orange).

[d] Not applicable.

[e] Not defined.

4.4 MIC results

The *Salmonella* isolate collection from Australian commercial layer shed environments was 100% susceptible to over half of the 16 antimicrobial agents tested (amoxicillin-clavulanate, ceftiofur, ceftriaxone, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin and trimethoprim-sulfamethoxazole). For the remaining antimicrobials, non-susceptibility (i.e. isolates classified as either intermediate or resistant according to CLSI clinical breakpoints) was observed to occur at a low level to streptomycin (7/307; 2.3%), sulfisoxazole (6/307; 2.0%), chloramphenicol (4/307; 1.3%) and tetracycline (3/307; 1%). Very low levels of non-susceptibility were observed to ampicillin (2/307; 0.7%) and cefoxitin (2/307; 0.7%). For all antimicrobials with an established ECOFF, % non-wild-type was always the same value as % non-susceptible, except for chloramphenicol, where the ECOFF is one dilution higher than the CLSI susceptible breakpoint and florfenicol, where the ECOFF is one dilution higher than the NARMS resistant breakpoint for *S.* Choleraesuis. No isolate had an extended-spectrum β-lactamase (ESBL) phenotype (ceftriaxone MIC > 1 mg/L).

For each antimicrobial agent tested, the overwhelming majority of isolates possessed a wild-type phenotype according to EUCAST ECOFFs (range 97.4%-100%). These values are graphically represented in Figure 3.

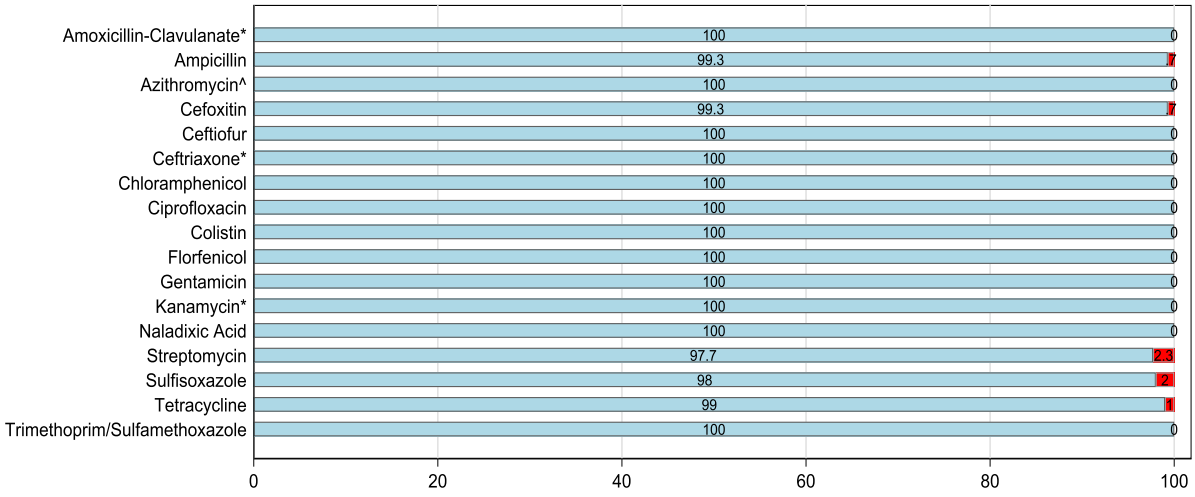

Figure 3 Antimicrobial resistance frequencies among the 307 *Salmonella* spp. isolates from commercial layer shed environments in Australia

Indicating % wild type (blue) and % non-wild type (red) based on EUCAST epidemiological cut-off values (ECOFFs).

* Data represents the percent non-susceptible due to unavailability of a wild type ECOFF.

^ Data represents the percent resistant due to unavailability of both a wild type ECOFF and susceptible breakpoint.

4.5 Resistance profiles

A very high proportion of isolates (293/307; 95.4%) among the 307 *Salmonella* spp. from Australian layer shed environments were susceptible to all 16 antimicrobial agents tested, as indicated below in Table 6. Fourteen isolates (4.6%) were resistant to one antimicrobial class, and two isolates (0.7%), were resistant to two antimicrobial classes (including aminogylcosides, beta lactams and tetracycline). Two isolates (serotype Havana, serotype Montevideo) exhibited an MDR phenotype (0.7%) being non-susceptible to only three classes that include streptomycin, sulfisoxazole and tetracycline.

Table 6 Resistance profiles established for the 307 *Salmonella* spp. isolates from Australian layer shed environments

No. of Classes	Phenotype*	Serotype	No. of isolates	%
0	No resistance		293	95.4
1	bla	*S.* Typhimurium, *S.* Orion	2	0.7
1	ami	*S.* Oranienburg, *S.* Agona, *S.* Montevideo, *S.* Amsterdam	4	1.3
1	fpi	*S.* Tennessee, *S.* Infantis, *S.* Anatum, *S.* Singapore	4	1.3
2	ami bla	*S.* Agona	1	0.3
2	bla tet	*S.* Typhimurium	1	0.3
3	ami fpi tet	*S.* Havana, *S.* Montevideo	2	0.7

*bla = beta lactam, tet = tetracycline, ami = aminoglycoside, fpi = folate pathway inhibitors, mac = macrolide.

4.6 Whole genome sequence analysis

In the case of three (of four) *Salmonella* isolates subjected to whole genome sequence analysis, the resistant phenotype matched the resistance genes identified in each isolate. The *S*. Typhimurium isolate resistant to two antimicrobial classes was identified as a ST19 strain, the most common globally distributed sequence type associated with poultry and foodborne gastroenteritis. The two MDR isolates belonged to rarely isolated STs. Their resistance gene profile (association of *sul1* with streptomycin resistance gene/s) suggests they may possess a class 1 integron.

Table 7 Serotype, sequence type (ST), resistance phenotype and resistance genotype of three layer shed environment *Salmonella* isolates subjected to whole genome sequencing

Serotype	Sequence type	Resistance phenotype	Resistance genotype	Notes
S. Typhimurium	19	Tetracycline, Ampicillin	*tetA* , bla_{TEM-1B}	Common Poultry ST
S. Havana	4	Streptomycin, Sulfisoxazole Tetracycline	*aadA4, aac(6')-Iaa, sul1,** tetB*	Rare
S. Montevideo	578	Streptomycin Sulfisoxazole Tetracycline	*aadA4, sul1,** tetB*	Rare

** Suggestive of a *sul1*-containing class 1 integron containing a streptomycin resistance gene.

5 Discussion

This proof-of-concept AMR survey among *Salmonella* isolates from Australian layer shed environments was undertaken with the support of Australian Eggs, Scolexia and national *Salmonella* reference laboratories to determine the best way to develop a viable and ongoing AMR surveillance system for the Australian layer industry. Sourcing isolates from the reference and research laboratories proved to be a practical, expedient and cost effective methodology, especially as serotyping of the isolates had already been undertaken. A robust and blinded random selection process was then undertaken to ensure the isolate collection was unbiased and representative. Unfortunately, none of the participating reference/research laboratories had any isolates from Tasmania or Western Australia that satisfied the selection criteria and contingencies were required. Shed environment isolates were eventually obtained from the WA Health Department, and collaborating industry veterinarians collected and submitted Tasmanian layer shed drag swab samples directly to the University of Adelaide Veterinary Diagnostic Laboratory for *Salmonella* isolation. These additional processes required coordination and an extension to the project reporting period. Future surveys can now be more streamlined.

Whilst it could not be determined, based on the information provided with the swab, whether it was from a caged or alternative housing shed, it is important to note that most cage sheds in Australia hold between 50,000 and 100,000 birds, and each shed is usually covered by a single drag swab sample. However, free range facilities usually hold between 6,000 and 40,000 birds, and require multiple drag swab samples, which could potentially bias towards more *Salmonella* isolates being obtained from alternative housing systems than the estimated 50%. Although it is recommended that future prospective AMR surveys could make this distinction, the uniformity of the current results suggests that there is little difference in the AMR status of *Salmonella* isolated from free range vs caged layer environments in Australia. In line with current research findings, *S.* Typhimurium was the most common serotype identified.

The absence of non-wild type isolates among the large majority (96.4%) of the *Salmonella* isolate collection from Australian layer shed environments indicates that these isolates likely contain no genetic elements (i.e. plasmids or other mobile genetic elements) encoding resistance to the 16 antimicrobial agents tested. Only 2.9% of isolates were non-susceptible to one (eight isolates in total) or two antimicrobial agents (a single isolate), and only two isolates were classified as MDR. None of the isolates was non-susceptible to the tested antimicrobials considered highly important to human health by the Australian Strategic and Technical Advisory Group (ASTAG) on AMR (fluoroquinolones, third generation cephalosporins and colistin) (ASTAG, 2015). Overall, the results confirm the low antimicrobial resistance status of *Salmonella* isolated from Australian caged and free range layer farm environments, which likely reflects the combination of restrictions on antimicrobial use, and in particular, critically important antimicrobial agents including fluoroquinolones, 3rd generation cephalosporins and colistin, combined with effective non-antimicrobial disease control mechanisms.

Based on the phenotype of the low to very low proportion of isolates resistant to one, two or three antimicrobial agents, ampicillin-resistant isolates were hypothesised to possess SHV or TEM beta-lactamases and tetracycline-resistant isolates may possess *tet* resistance genes either on a plasmid or within the bacterial chromosome. Resistance to sulphonamides and streptomycin could indicate the presence of a *sul* gene, possibly within a class 1 integron (plasmid-mediated or within the bacterial chromosome), which may contain *an aad* (streptomycin) resistance gene within its variable region structure. This was confirmed by whole genome sequencing, which identified *tetA* and $bla_{\text{TEM-1B}}$ genes in the *S*. Typhimurium ST19 isolate, and streptomycin (sulphonamide and tetracycline resistance genes

in the *S.* Havana (*aadA4, aac(6')-Iaa, sul1*, *tetB*) and *S.* Montevideo isolates (*aadA4, aac(6')-Iaa, sul1, tetB*).

In comparison to the previous study of Pande et al. (2015), which examined AMR in 145 *Salmonella* isolates from two Australian states (SA and NSW), results were very similar on a national level, though a slightly lower percentage of total isolates (91.7%) were susceptible to all antimicrobial agents tested in the previous study. It is important to note in this previous study, however, that the rates of resistance were determined on the basis of isolate MICs attaining or exceeding the CLSI, NARMS or SVARM resistance breakpoints used. Resistance rates are, however, comparable with the present study on the basis of overlapping confidence intervals.

Relatively few international AMR surveys have focused solely on *Salmonella* isolated from poultry layer shed environments. In most studies, isolates sourced from layers and/or their environment are included with broilers. Iwabuchi et al. (2010) undertook a national survey of *Salmonella* prevalence in layer shed airborne dust samples in Japan and conducted AST on the isolates. At the time, there were an estimated 4,090 layer farms in Japan and 203 were surveyed, with 48 (23.6%) positive for *Salmonella.* A total of 380 *Salmonella* isolates were obtained, representing 34 serotypes with *S.* Infantis (11.0%), *S.* Agona (10.3%) and *S.* Mbandaka (9.7%) predominating. No *S.* Typhimurium and very low numbers of *S.* Enteritidis were detected. The frequency of resistance among the isolates was very high for streptomycin (64.2%), moderate for oxytetracycline (14.7%), low for fosfomycin (9.2%), colistin (5.5%), nalidixic acid (2.4%), cefuroxime (1.3%), kanamycin (1.3%) and cephalothin (1.1%), and very low for chloramphenicol (0.8%), ampicillin (0.5%), gentamicin (0.5%), and norfloxacin (0.5%).

Li et al. (2007) conducted a survey of *Salmonella* carriage in a commercial layer facility containing 12 high-rise houses. Approximately 300 g of fresh faeces was collected under the cages across the entire length of each six rows. Forty-five *Salmonella* isolates (approximately 50% of total isolates) were serotyped and subjected to AST for 15 antimicrobials. Thirty-five percent (16 of 45) of the *Salmonella* isolates were resistant to at least one antibiotic and a high proportion of isolates were resistant to tetracycline, ampicillin, streptomycin and ceftiofur. A small study comparing commercial and organic layer farms in Germany also identified quite high rates of antimicrobial resistance in *Salmonella* isolates (Schwaiger et al., 2008). All isolates were resistant to spectinomycin and a high proportion were also resistant to ampicillin, cefuroxime and doxycycline.

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) undertook a survey of antimicrobial resistance in *Salmonella* Heidelberg (the most common serotype associated with foodborne disease) in poultry from 1996-2010, with 41.2% of the 951 isolates sourced from layer sheds (St Amand et al., 2013). It is difficult to separate layers from meat birds in this study, but isolates from turkey shed environments were significantly more likely to be resistant to ceftiofur compared to layer shed environments. In total, 63% of isolates exhibited resistance to at least one antimicrobial agent and 9.3% of isolates were multidrug-resistant, with ceftiofur resistance ranging from 0-10.5% annually. Comparison is difficult with this study, as AST results were not clearly distinguishable between meat chicken and layer isolates.

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