Surveillance for antimicrobial resistance in enteric commensals and pathogens in the Australian commercial egg industry

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Project Title: Surveillance for antimicrobial resistance in enteric commensals and pathogens in the Australian commercial egg industry

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Foreword

The Australian Government has been actively progressing the development of a coordinated plan for the management of antimicrobial resistance and antimicrobial use (AMU) in humans and animals. Broad support for the development of the 'National Antimicrobial Resistance Strategy' was obtained from key stakeholders across the medical, health, veterinary, agricultural and pharmaceutical communities at the 'Australian One Health Antimicrobial Resistance Colloquium' in 2013.

A surveillance model for use in the Australian egg industry was developed and implemented, which is closely in-line with the OIE Chapter 6.8 recommendations.

Acknowledgements

Australian Eggs would like to thank the Australian Government's *Stronger Farmers, Stronger Economy – Improvements to access premium markets – improve Biosecurity measure of the Agricultural Competitiveness White Paper* funding to complete this survey, and all the people involved in sample collection, bacterial isolation, resistance profiling and analysis - Birling Avian Laboratories staff, Dr David Jordan and Dr Sam Abraham and his research team including Rebecca Abraham, Terence Lee, Kelly Hunt, John Blinco, Soraya Haynes and Ali Harb. Australian Eggs would like to thank Tecan for their contribution to the project.

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Abbreviations

AEL	Australian Eggs Limited
ami	Aminoglycosides
AMR	Antimicrobial resistance
AMS	Antimicrobial stewardship
AMU	Antimicrobial usage
APVMA	Australian Pesticides and Veterinary Medicines Authority
AVA	Australian Veterinary Association
AVPA	Australasian Veterinary Poultry Association
BEA	Bile esculin azide
bla	Beta lactams
BPW	buffered peptone water
c1g	First generation cephalosporins
CLSI	Clinical and Laboratory Standards Institute
CR	Clinically resistant
DAFF	Commonwealth Department of Agriculture, Fisheries and Forestry
DAWE	Commonwealth Department of Agriculture, Water and the Environment
ECOFF	Epidemiological Cut-off Values
ESA	Egg Standards of Australia
EUCAST	European Committee on Antimicrobial Susceptibility Testing
fpi	Folate pathway inhibitors
mac	Macrolides
MALDI-TOF MS	Matrix assisted laser desorption ionisation-time of flight mass spectrometry
MDR	Multi-drug resistance (clinical resistance to three or more classes)
MIC	minimum inhibitory concentration
MLST	Multilocus sequence type
MR	Microbiologically-Resistant
MS	Microbiologically-Susceptible
OIE	World Organisation for Animal Health
oxa	Oxazolidinones
phe	Phenicols
pol	Polymixins
qui	Quinolones
ST	Sequence type
str	Streptogramins
tet	Tetracyclines

Glossary

Aligned chicken egg producers	Defined as producers that have the industry voluntary quality assurance program – Egg Standards of Australia (ESA) – in place.
Clinically-Resistant	Bacterial isolates are not inhibited by the usually achievable concentrations of antimicrobials which indicates there is a low probability of a favourable treatment outcome.
Microbiologically-Resistant	Non-wild-type isolates which are expressing resistance to a drug at a higher level than isolates that have no mutations known to confer resistance. These isolates may or may not be clinically resistant.
Microbiologically-Susceptible	Wild type isolates which are the typical form of bacteria as it occurs in nature. These bacteria have not been exposed to antimicrobial selection or acquired any resistance.
Multi-drug resistance	Isolates that are resistant to three or more classes of antimicrobials based on microbiological breakpoint (where one is available) is classified as multi-drug resistant (MDR) phenotype.
Non-aligned poultry farm production	Defined as producers that have full-time labour in place and are geared toward producing on a sufficient scale for the sale of eggs (FAO, 2003), but do not have the industry voluntary quality assurance program – Egg Standards of Australia (ESA) – in place.

EXECUTIVE SUMMARY

Background

Surveillance for antimicrobial resistance (AMR) can provide valuable feedback on the efficacy of antimicrobial stewardship (AMS) programs and improvements that should be implemented. Since 2003, the Australian Government has been actively progressing the development of a coordinated plan for the management of AMR and antimicrobial use in animals. Collaborations between the livestock industries and the Australian Government have led to a number of projects on AMR and AMS that aim to gather information about the status of animals in Australia, but also guide the Australian Government and industry AMR and AMS efforts and support international and national discussions. In 2019, a proof-of-concept AMR surveillance study identified the most appropriate mechanisms for obtaining and performing antimicrobial susceptibility testing on *Salmonella* isolates obtained from Australian layer shed environments. Overall, the results confirmed the low antimicrobial resistance status of *Salmonella.* An extension of this work is presented here, which assessed the AMR prevalence in *E. coli* and *Enterococcus* spp. obtained from the environment of Australian layer flocks. This report defines a surveillance model for use in the Australian egg industry based on the recommendations in OIE Chapter 6.8 "Harmonisation of national AMR surveillance and monitoring programmes" (OIE, 2018), and is closely in line with the surveillance project undertaken in other industries such as pig and chicken meat.

Approach

A cross-sectional survey was conducted, between August 2019 and January 2020, of commercial chicken egg producers aligned and non-aligned to the Australian egg industry's voluntary quality assurance program (ESA) from New South Wales/Australian Capital Territory, Victoria, Western Australia, Queensland, Tasmania, and South Australia. Three hundred samples were collected from individual 'production units' which consisted of one or more sheds (or enclosures) of layer chickens similarly managed with respect to type of bird, stage of production, feed and housing attributes. *E. coli* and *Enterococcus* isolation and typing was conducted on a total of 511 bacterial isolates with susceptibility to antimicrobials determined using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method and followed with genetic analysis for all *E. faecium* isolates and any *E. coli* or *E. faecalis* isolates that exhibited resistance to highly important antimicrobials (ASTAG, 2018) or multi-drug resistance phenotypes.

E.coli

Of all *E. coli* isolates tested, 52.0% were microbiologically susceptible to all of the tested antimicrobials. All *E. coli* isolates were microbiologically susceptible to ceftiofur, chloramphenicol and colistin. Microbiological resistance was observed for ampicillin (16.2%), cefoxitin (1.4%), ciprofloxacin (2.7%), florfenicol (2.4%), gentamicin (1%), streptomycin (4.7%), tetracycline (37.8%) and trimethoprim/sulfamethoxazole (10.5%). Multi-drug resistance was observed in 21 isolates (7.0%), with one isolate exhibiting resistance to five antimicrobial classes. Not all phenotypically observed resistance was confirmed by the detection of corresponding antimicrobial resistance genes or mutations and confirms previous work in *E. coli*. The findings are consistent with other recent studies demonstrating low levels of antimicrobial resistance to high or medium importance antimicrobials among *E. coli* isolated from Australian livestock (Abraham, 2019; Barlow, 2015; Kidsley, 2018)

Enterococcus

E. faecium and *E. faecalis* were the dominant *Enterococcus* species isolated. No genotypic or phenotypic vancomycin resistance was observed for all enterococci isolates. 30% of *E. faecium* and 39.3% of *E. faecalis* isolates displayed phenotypic susceptibility to all antimicrobials tested. Six *E. faecium* isolates and three *E. faecalis* isolates displayed an MDR phenotype. All *E. faecium* isolates tested were microbiologically susceptible to benzylpenicillin, chloramphenicol, daptomycin, gentamicin, linezolid, teicoplanin, vancomycin and virginiamycin. *E. faecium* isolates displayed microbiological resistance for ampicillin (5.1%),

erythromycin (22.5%) and tetracycline (58.8%), however none belonged to the major sequence types responsible for sepsis in humans in Australia from 2015-2017. For *E. faecalis,* 57% were microbiologically resistant to tetracycline, but none were microbiologically resistant to benzylpenicillin, daptomycin, gentamicin, teicoplanin, vancomycin and virginiamycin. Microbiological resistance was observed in *E. faecalis* isolates for ampicillin (1.5%), chloramphenicol (1.5%), erythromycin (11.9%), linezolid (0.7%) and streptomycin (1.5%).

Conclusion

These results suggest the contribution of AMR from the layer industry in Australia to the prevalence of resistance to high and medium importance antimicrobials observed in humans, is likely to be low. However, more work is needed to understand the pathways for resistance. This includes understanding antimicrobial usage in the industry, and other transmission pathways of AMR bacteria into flocks, to identify the most relevant factors that need to be addressed to reduce the presence of AMR bacteria in Australian layer flocks. Industry is undertaking to clarify these issues and identify practical approaches to improving industry understanding of the risks associated with AMR bacteria. These include projects designed to further support adoption of biosecurity practices, and better clarify the potential sources and origins of critically important antimicrobial resistance among indicator bacteria in the absence of use. More recently, the findings from this project informed review of the industry's National Biosecurity Manual which was updated to include reference to the importance of biosecurity for minimising the risk of incursion of AMR bacteria into a flock (Australian Eggs, 2020b).

For the layer industry, the results reflect decades of stringent regulatory controls on antimicrobial use, biosecurity and infection prevention practices and the resultant favourable disease status of Australian layer farms.

INTRODUCTION

Antimicrobial resistance (AMR) is a serious threat to public health globally. National and international efforts to deal with AMR centre on antimicrobial stewardship (AMS) programs and activities which are designed to minimise the emergence of resistance in bacteria in humans and animals due to inappropriate use, and importantly, to minimise the risks of spread in, and between, animal and human populations. Antimicrobial stewardship programs are central to ensuring antimicrobials are used appropriately to manage animal and human health while minimising risks of AMR emergence. The main components of any AMS program involve monitoring and surveillance of antimicrobial usage (AMU) and AMR to guide what constitutes appropriate AMS measures and identify whether AMS programs are effective or require adjustment. The outcome of an AMS program is to minimise and reduce the presence of AMR bacteria. AMR surveillance, in particular, is important as it is a direct measure of this outcome, and this is why it should be conducted on a regular basis. Internationally, a few countries have advanced AMR surveillance systems, but all are based on different models because it is widely recognised that any AMR surveillance system needs to be tailored to a country, its industries and animal and human health systems (DANMAP, 2014; Government of Canada, 2015; CDC, 2013).

In Australia, the egg industry is an important intensive animal production system. The industry is comprised of a small number of large producers and many small and medium sized producers. There is also some backyard production. In 2019, Australian egg production was approximately 507 million dozen eggs valued at $832.9 million (Australian Eggs, 2020a) and the industry has experienced strong growth over the past decade with the latest data indicating the consumption of 246 eggs per person per annum (Australian Eggs, 2020a). Currently, Australia does not import shell eggs for human consumption, but imported egg products are either preserved, cooked, pulped or in powder form (Australian Eggs, 2020a). In Australia, there are three egg production systems: cage, barn (or cage-free) and free-range. Organic egg production is a niche segment within the free-range sector. Eggs are produced in all States and the Australian Capital Territory. New South Wales (~33%), Queensland (~25%) and Victoria (~26%) produce most of the overall egg production (Australian Eggs, 2020a).

Deregulation of the egg industry in the eastern states of Australia (1989-1996) changed the industry business model and allowed the industry to plan and build farms on new sites taking biosecurity, isolation, separation of rearing from laying facilities and housing single age flocks in each shed into consideration. Together with the improvements in husbandry, vaccination, biosecurity, nutrition and housing, there has been a growing emphasis by breeders on the care of the breeder birds so that they produce day old chicks with sound genetics and with immunity against a number of diseases, particularly those that cause immunosuppression (such as Marek's and infectious bursal disease). By 2004, the Australian Pesticides and Veterinary Medicines Authority (APVMA) had included antibiotic label restraints which further restricted the use of antibiotics in egg layers during rearing and lay (APVMA, 2017). Australia, compared to most other countries, has an extremely limited number of antibiotics approved for use in egg layers (ASTAG, 2018; Table 1). These restrictions mean there are few concerns about AMR bacteria of importance to human health emerging from the Australian egg industry for a wide spectrum of antibiotics.

In the 1970s, poultry veterinarians, via the Australasian Veterinary Poultry Association (AVPA), recommended tighter controls over antimicrobials by feed mills and farms that mix their own feed. The poultry industry and the AVPA jointly developed a "AVPA Code of Practice for the Use of Antibiotics in the Poultry Industry" to ensure a much tighter control of antibiotic use from the 1980s. In 2001, the Code was revised to include all antibiotics, not just Schedule 4 (prescription only) antibiotics and to include "Prudent Use Guidelines" (AVPA, 2001; Grimes, 2004). Under the various State Stock Medicine Acts, prescribing veterinarians must adhere to the "Guidelines for Prescribing, Authorising and Dispensing Veterinary Medicines (2005)" which is set by the Australian Veterinary Association (AVA) (Bond, 2008). In 2019, Australian Eggs published industry guidelines for AMS to be adopted by farmers and veterinarians overseeing flock health (Australian Eggs, 2019a).

Complementary to this, the industry has moved proactively whenever possible to limit the use of antimicrobials and use appropriate vaccines to minimise disease risks. Development and implementation of *Mycoplasma* and fowl cholera vaccines, in particular, were important steps to replacing antibiotic use.

These vaccines were developed in Australia, funded by the poultry industry, and are now sold internationally. This proactive strategy over the past twenty years was in response to concerns about antibiotic resistance in farm animals (Antunes, 2016; Woolhouse, 2015). Increasingly, the layer industry is establishing dedicated rearing farms and single age units, including separation of farms and different age flocks to reduce pathogen transmission. The industry is also using alternative preventative and therapeutic options such as probiotics, prebiotics, organic acids and other feed supplements in lieu of antimicrobial treatments. Testing for antibiotic residues in eggs is undertaken annually by the Department of Agriculture, Water and Environment's (DAWE) National Residue Survey (NRS). This helps to demonstrate that the egg industry adheres to withholding periods for antimicrobial use (DAWE, 2019).

In Australia, a pilot AMR surveillance program in food-producing animals was commissioned by the then DAFF (Department of Agriculture, Fisheries and Forestry) in 2003-2004 (Commonwealth of Australia, 2007), and has been followed by various activities since. This includes the formalisation of a national AMR strategy in 2015 (Commonwealth of Australia, 2015), which was updated in 2020 (Commonwealth of Australia, 2020). Broad support for the development of a national AMR strategy was obtained from key stakeholders across the medical, health, veterinary, agricultural and pharmaceutical communities in 2013 at the 'Australian One Health Antimicrobial Resistance Colloquium'. Collaborations between the Australian livestock industries and the Commonwealth Government have led to a number of projects on AMR and AMS that aim to gather information about the situation in Australia, but also guide Government and industry AMR and AMS efforts and support international and national discussions and initiatives. A review, 'Surveillance and reporting of antimicrobial resistance and antibiotic usage in animals and agriculture in Australia' (Shaban et al., 2014 [the AMRIA report]) sponsored by the then Department of Agriculture assessed Australian and international AMR and antimicrobial usage surveillance programs that were in place in animal industries at the time with a purpose to define a suitable Australian program. This process involved close consultation with key stakeholders in the agriculture and veterinary sectors. The review identified one of the major components of surveillance being the assessment of AMR in commensal bacteria and pathogens present in the gut of food animals at slaughter. In 2015, the recommendations from the AMRIA report were reviewed by government and industry stakeholders and used as the basis for the development of a plan to deliver a proof-of-concept project for AMR surveillance in pigs that may also be applied to other major food industries in the future. The Australian Government has been actively progressing a coordinated AMR surveillance and AMU plan for humans and animals and have identified sectors for which there is a paucity of AMR data, including in the layer industry.

In 2019, the Australian Government funded a proof-of-concept AMR surveillance study to identify the most appropriate mechanisms for obtaining and performing antimicrobial susceptibility testing on *Salmonella* isolates obtained from Australian layer shed environments (Trott et al., 2019). Overall, the results confirmed the low AMR 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, the non-use of high importance antimicrobial agents including fluoroquinolones, 3rd generation cephalosporins and colistin, combined with effective non-antimicrobial disease control mechanisms. DAWE and Australian Eggs subsequently funded an extension of this work to assess the AMR prevalence in *E. coli* and *Enterococcus* spp. in the Australian chicken egg (layer) industry. This report provides these additional results. Together, these results define a surveillance model for use in the Australian egg industry and is closely in line with the surveillance projects undertaken in other industries (pigs and chicken meat). The results of this project will assist the DAWE in discussions nationally and internationally concerning the AMR status of Australia's animal populations. The outcomes also provide vital information to support the Australian egg industry in defining cost-effective approaches to AMR and AMS.

Table 1 Antimicrobial agents approved for use in Australian layer hens

Antimicrobial Agent *[1]	Class	Importance [2]
Lincomycin	Lincosamide	Medium
Spectinomycin	Aminocyclitol	Medium
Trimethoprim + Sulfadimidine	Diaminopyrimidine + Sulfonamide	Medium (nhu)
Trimethoprim + Sulfadiazine	Diaminopyrimidine + Sulfonamide	Medium (nhu)
Amoxicillin	Penicillin	Low
Bacitracin	Polypeptide	Low
Chlortetracycline	Tetracycline	Low
Neomycin (feed)	Aminoglycoside	Low
Neomycin (water)	Aminoglycoside	Low
Flavophospholipol	Glycophospholipid	Low (nhu)[3]
Streptomycin	Aminoglycoside	Low
Lasalocid	Ionophore	Low (nhu)
Monensin	Ionophore	Low (nhu)
Salinomycin	Ionophore	Low (nhu)
Narasin	Ionophore	Low (nhu)
Sulfadimidine	Sulfonamide	Low (nhu)
Tylosin	Macrolide	Low (nhu)

*Information extracted from ASTAG, 2018 and cross-referenced with information available in PubCRIS (https://portal.apvma.gov.au/pubcris)

[1] not all registered antimicrobial agents are used or available for use.

[2] ASTAG importance rating (ASTAG, 2018)

[3] nhu = not used in humans

Objective

The aim of this study is to estimate the susceptibility profiles against specific antimicrobials in indicator bacteria (*E. coli* and *Enterococcus spp.*) in the Australian chicken egg industry.

Project team

Successful completion of this work required collaboration between several individuals and institutions. A number of people involved in the Technical Group and the Antimicrobial Resistance Surveillance Task Group have given freely of their time and expertise to assist this collaboration between the egg industry and the DAWE, and their contributions are gratefully acknowledged.

- Australian Eggs Limited (AEL), Dr Raymond Chia; Project coordinator
  Overall coordination of the project and first contact point for stakeholders. Establish and provide protocols to laboratories and for sample collection. Primary responsibility for the project and authorship of the report.
- Company coordinator for each company involved in the study
  Coordinated collection of cloacal swabs on each plant associated with that company. Trained livestock staff / manager or poultry veterinarians at the participating farms. Responsibility for ensuring samples are collected and shipped as per the protocol.
- Poultry veterinarians
  Collection of cloacal swabs from non-ESA accredited farms. Samples are collected and shipped as per the protocol.
- Birling Avian Laboratories, Drs Sue Sharpe and Tony Pavic; Primary laboratory
  They have NATA accreditation, general expertise in veterinary microbiology with capacity and infrastructure for collation of cloacal swab samples, isolation and identification of target organisms, storage of isolates, and collation of data sent to the AMR laboratories in coordination with the project coordinator. Responsibility for ensuring only five swabs from each farm collected was submitted, isolation protocol was followed, and isolates are characterised, stored and shipped appropriately. They maintain a copy of all isolates for reference. Sue_Sharpe@baiada.com.au; Tony_Pavic@baiada.com.au
- Antimicrobial Resistance and Infectious Diseases Laboratory, School of Veterinary Life Sciences, Murdoch University (Assoc. Prof. Sam Abraham); AMR testing laboratory – specialist ability at performing phenotypic AMR testing on bacterial isolates by broth microdilution. Responsible for providing scientific and technical advice to the project as requested and assist the project coordinator in analysis and interpretation of results and compilation of the report. Additional technical support was provided by Dr David Jordan (NSW Department of Primary Industries). S.Abraham@murdoch.edu.au.
- Drs Leigh Nind (DAWE), David Jordan (NSW DPI) and Laura Macfarlane-Berry (DAWE); Management group – General oversight and epidemiological input for the design and reporting of the project. Leigh.Nind@awe.gov.au; david.jordan@dpi.nsw.gov.au and Laura.Macfarlane-Berry@awe.gov.au.

METHODS AND MATERIALS

Study design

A cross-sectional survey was conducted of commercial chicken egg producers aligned and non-aligned to the AEL voluntary quality assurance program (ESA), from New South Wales/Australian Capital Territory, Victoria, Western Australia, Queensland, Tasmania, and South Australia. The Northern Territory was not sampled, as there are no commercial chicken egg producers within this jurisdiction. Unlike the poultry meat industry, where birds must be brought to a central processing point for slaughter, there is no equivalent processing chain to implement a simple and convenient sample collection process that is probabilistic (statistically correct) for the egg industry. For this reason, sampling was conducted on a farm-to-farm basis centred on 'production units' (Figure 1).

Sampling design

Selection of farms

For the purposes of sampling, the chicken egg industry was defined as being comprised of multiple commercial entities often in a number of localities and within each enterprise there are one or more farms at specific geographic locations. Within each farm, there may be more than one production system (e.g. cage, barn and free range).

A sampling frame for aligned egg-producing farms was obtained from the list of farms participating in the industry quality assurance scheme (Egg Standards of Australia; ESA; Australian Eggs, 2019b). Each farm on this list was assigned a random identifier and the farms sorted in ascending order of their allocated random identifier. From this randomly ordered list, the required number of farms from each state and territory (see Table 2) was included in the sample by extracting from the top of the list (thus obtaining a completely random sample). An article was published in the industry Eggstra Eggstra June 2019 edition informing egg producers about the study and requesting their voluntary participation in the AMR survey if selected. Farms that were approached to be involved in the survey but were unable to participate, were replaced with the next farm in the randomly ordered list and not already included in the sample.

For non-aligned farms, one veterinarian per jurisdiction was approached following the same protocol as per aligned farmers.

Selection of production units

Each production system can have a "production unit" that consists of one or more sheds (or enclosures) of layer chickens similarly managed with respect to: type of bird, stage of production, feed and housing attributes (Figure 1). Production units effectively are groupings of layer birds on the same farm receiving similar management (e.g. cage hens). Sampling was conducted at the production unit level. A commercial entity was eligible in this survey if they have at least one commercial egg production unit.

In the event that a farm had all three different production systems on site (e.g. barn, free range and cage), 5 samples were collected from the largest (most hens) production system on that farm. If a farm had cage or barn hens as well as free range production system, 5 samples were collected from the larger (more hens) production system. If a farm had only one production system, 5 samples were collected from that production system. If a commercial entity had more than one location, each location was considered a separate entity. Although the management of both sites should be similar, they should be considered as two separate sets of data.

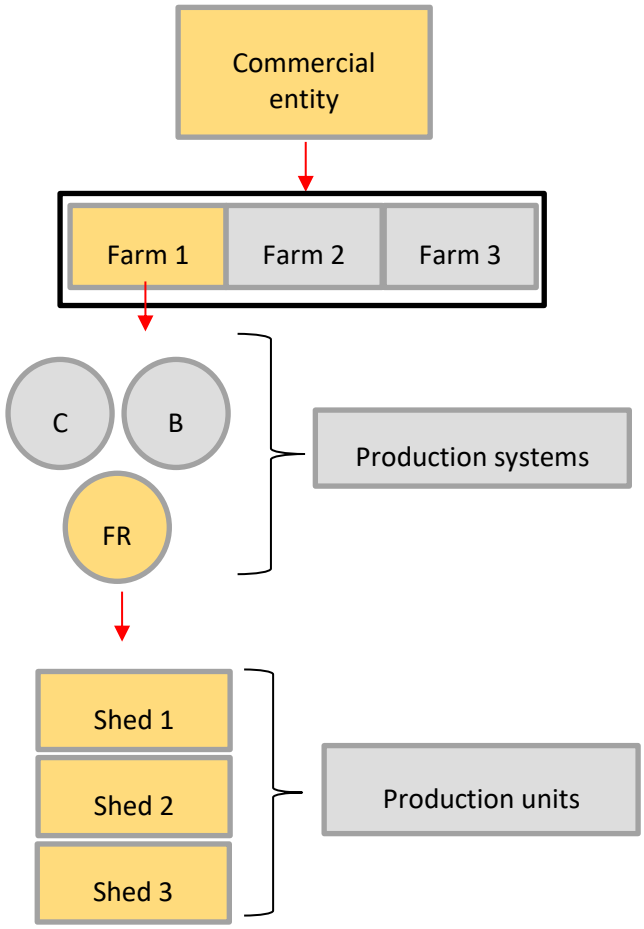

Figure 1: Structure of the egg industry. C=cage; B=barn; FR= free range

Selection of production units

Birds were selected from each production system as outlined in Appendices 1-3.

Collection of data and samples

Cloacal swabs were collected from healthy birds on participating farms. Different approaches were used for sample collection from aligned and non-aligned enterprises.

Aligned producers tend to be larger enterprises with a high resource base for managing quality control and biosecurity issues. Livestock managers or poultry veterinarians for industry aligned farms are experienced at sampling from poultry. These managers or veterinarians were provided with a sampling plan and requested to submit cloacal swab samples to the primary laboratory according to pre-supplied instructions (Appendices 1- 4).

Collection of swabs from the non-aligned flocks was performed by private veterinarians according to pre-supplied instructions (Appendix 4) during their routine visits to such flocks. Australian Eggs contacted one veterinarian from each jurisdiction and provided them with the same information for sample collection as used in the aligned flocks.

Farm managers and private veterinarians involved in sampling were sent laboratory submission forms to collate information about the flock including flock size.

Sample size calculation

Surveillance for AMR involves measurement of multiple outcomes determined by the various combinations of organisms of interest and antimicrobial agent present in the broth microdilution assay. The priority outcome was identification of the proportion of isolates expressing resistance to high importance antimicrobials such as ceftiofur, ciprofloxacin, colistin and vancomycin. Experience with earlier surveys of Australian livestock (especially AMR in *Salmonella* from layer hen environments [Trott, 2019], and the meat chicken proof of concept study [ACMF, 2018]), suggested that the proportion of isolates expressing resistance to high importance antimicrobials was expected to be very low or nil. Thus, the sample size had to be sufficient such that the upper 95% confidence limit in the case of nil positives provided an acceptable boundary of error from a policy and decision-making perspective.

Figure 2 demonstrates that with 200 isolates, this upper confidence limit when nil positives detected is approximately 1.8%. When a target of 300 isolates was used, a preferred upper confidence limit of 1.2% was achieved (calculated using the Clopper-Pearson method for determining exact binomial confidence limits assuming no clustering). Within the financial limitations of the study, 300 *E. coli* and 300 *Enterococcus* spp. could be appraised for phenotypic resistance traits. The assumption was that each sample yielded at least one of each of the target commensals, although the yield may be less than 100%. Consequently, the distribution of samples between 62 production units resulted in five isolates, each covering *E. coli* and *Enterococcus* spp., per production unit. This required the collection of five swabs per production unit. This was also preferred as it enabled provision of more meaningful results to farm managers to inform their AMS programs. Five samples per production unit also permitted analysis of mixed effects linear models. Apart from these considerations, the number of isolates per production unit is somewhat arbitrary due to balancing the cost of collection and the number of flocks covered by the survey.

Approximately 85% of commercial egg production in Australia are aligned and the remainder are non-aligned. Hence, 255 of 300 required swabs were collected from aligned farms and 45 swabs collected from non-aligned farms.

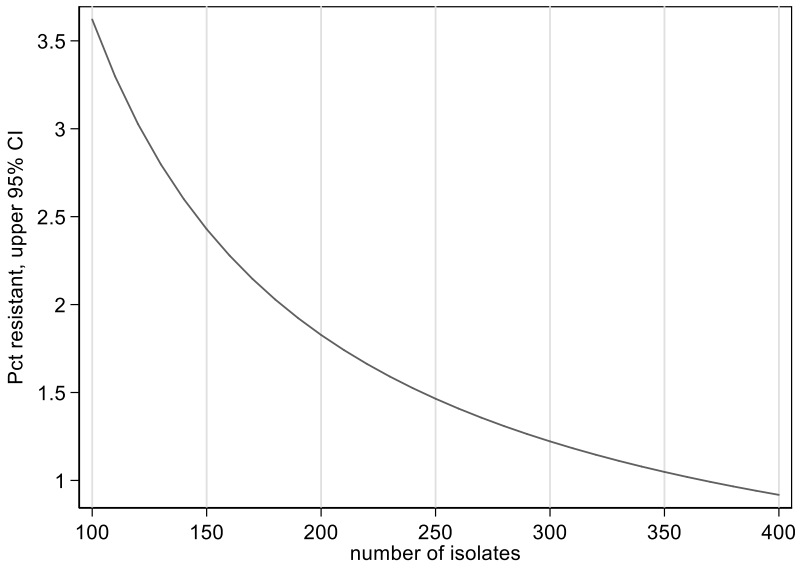

Figure 2. Determining the number of isolates. For a range of number of isolates to be included in the study (x-axis) the upper 95% exact binomial confidence limit for the percentage of isolates in the population expressing resistance to an antimicrobial of high importance when the observed number of positive isolates in the sample is zero (0).

Table 2: Number of aligned and non-aligned farms and the number of swabs needed from each state/territory

	NSW/ACT	QLD	Vic	Tas	SA	WA
% stock in the state/territory [1]	31.6	27.8	22.4	0.4	7.2	10.6
Total no. of swabs needed from each state/territory based on % of stock in the state for aligned farms (n=255)	81	71	57	1	18	27
Total no. of aligned farms needed if each farm is to conduct 5 swabs from each state/ territory	16	12	11	1	4	5
Total no. of swabs needed from each state/territory based on % of stock in the state for non-aligned farms (n=45)	14	13	10	1	3	4
Total no. of non-aligned farms needed if each farm is to conduct 5 swabs from each state/territory	3	6	2	1	1	1

[1] Australian Eggs, 2018

Laboratory activities

In the Australian context, there was a strong focus on estimating the level of resistance to antimicrobials of high importance, such as fluoroquinolones and extended spectrum cephalosporins.

Isolation and confirmation of target organisms (to species level) at the primary laboratory

Birling Avian Laboratories was the primary laboratory for this study. The processing of samples inevitably involved vortexing the samples with diluent so it was considered reasonable to assume the target organisms were completely randomly distributed throughout the test matrix. Duplicate copies of all isolates were retained in on-site storage at Birling Avian Laboratories with single copies dispatched to the AMR testing laboratory.

Upon receival of the samples at the primary laboratory, the time and temperature of the swabs was recorded. If the samples were outside the required temperature range, the collection person on the farm was notified and additional samples collected.

Enterococcus isolation and typing

The prepared samples were vortexed to resuspend the diluent and then streaked direct from BPW onto BEA agar. The agar plates were incubated at 42°C for 48 hrs and speciated using Vitek 2 (BioMerieux) mass spectrometry. From a pure subculture of the original colony, bacteria were harvested for storage at -20°C on cryo-beads (Cryobank, Mast Diagnostics) in two separate, identical containers labelled with the sample code and the laboratory reference number.

E. coli isolation and typing

The prepared samples were vortexed to resuspend the diluent, and then streaked direct from BPW onto *E. coli* chromogenic agar, which achieved both bacterial isolation and type confirmation. The agar plates were incubated at 37°C for 18 hrs and then one clone was selected and subcultured onto Coli ID for purity. *E. coli* isolation was confirmed using an indole test. From a pure subculture of the original colony, bacteria were harvested for storage at -20°C on cryo-beads (Cryobank, Mast Diagnostics) in two separate, identical containers labelled with the sample code and the laboratory reference number.

Dispatch to AMR laboratories

One vial of cryo-beads for each isolate was shipped to the AMR reference laboratory, which for this study was the Antimicrobial Resistance and Infectious Diseases Laboratory, School of Veterinary Life Sciences,

Murdoch University. The AMR reference laboratory conducted species identification/confirmation using MALDI-TOF MS (Microflex, Bruker, MA, USA) and antimicrobial susceptibility testing.

Recovery of isolates for AMR testing

For *E. coli* and *Enterococcus*, one cryo-bead from each vial is placed onto Columbia sheep blood agar (Micromedia, Australia) and rolled with a loop in a circle, to create the initial streak zone. Further streaking from the initial zone was done prior to aerobic incubation at 37°C for 24 hrs. A single colony was again sub-cultured on Columbia sheep blood agar at 37°C for 24 hrs before performing antimicrobial susceptibility testing. All isolates underwent species confirmation at this point using MALDI-TOFF. Bacteria from the sub-cultured plate were frozen at -80°C in Brain Heart Infusion Broth with 20% glycerol for further testing (such as sequencing or repeat MIC).

Susceptibility testing of isolates in specialist AMR laboratories

For *E. coli*, the antimicrobials tested were amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, colistin, streptomycin, tetracycline and trimethoprim/sulfamethoxazole.

For *Enterococcus*, the antimicrobials tested were ampicillin, chloramphenicol, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin, teicoplanin, tetracycline, vancomycin and virginiamycin.

Antimicrobial susceptibility for the isolates was determined by the broth microdilution method in-house panels prepared according to Clinical and Laboratory Standards Institute (CLSI) ISO 20776 standards.

Interpretation

Antimicrobial susceptibility testing is commonly undertaken for diagnostic or surveillance purposes and therefore it is important to appreciate the different ways in which the data can be interpreted. The overarching principle of interpreting susceptibility data is to classify data into distinct and meaningful categories by using breakpoint values. When laboratories measure the expression of resistance to a drug by a bacterial isolate, the results are given along a continuous scale. The breakpoint is an agreed position along that scale such that all isolates can be classified as being either above or below the breakpoint. The breakpoint classifies the isolate as 'sensitive' (susceptible) or 'resistant' to the tested antimicrobial. There are two types of breakpoints used for classifying antimicrobial susceptibility of a bacterial isolate – **Microbiological Cut-off Values** (also referred to as 'Epidemiological Cut-off Values' or ECOFFs) and **Clinical Breakpoints**. To allow for comparability between other studies that may only use one or the other of these, both have been used in this study, where available. Briefly, 'clinical resistance' to an antimicrobial refers to isolates that, in a clinical setting, would not be successfully removed by use of that antimicrobial. 'Microbiologically resistant' refers to isolates that have potentially been exposed to an antimicrobial and while potentially not clinically resistant, may show signs of emerging resistance.

Microbiological Cut-off Values (ECOFF)

'Microbiological Breakpoints' are referred to in this report for clarity (they are synonymous with ECOFF's). In recent years, 'Microbiological Breakpoint' values are encouraged to be used in AMR surveillance since they allow for the detection of emerging resistance in a bacterial population. It was developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2016). Both the clinical and microbiological breakpoints for *E.coli* and *Enterococcus* are listed in Tables 3 and 4, respectively. The microbiological breakpoint consists of a single breakpoint value which classifies isolates into two categories; microbiologically-susceptible (MS or wild type) and microbiologically-resistant (MR or non-wild type).

Microbiologically-Susceptible (MS): Wild type isolates which are the typical form of bacteria as it occurs in nature. These bacteria have not been exposed to antimicrobial selection or acquired any resistance.

Microbiologically-Resistant (MR): Non-wild-type isolates which are the mutated form of bacteria that are expressing some elevated levels of AMR. These isolates may or may not be also clinically resistant.

Clinical Breakpoints

'Clinical breakpoints' for each drug are a minimum inhibitory concentration (MIC) value above which the isolates are regarded as clinically resistant to that drug. These are values provided by CLSI in document VET01S (CLSI, 2015) that are used to guide clinicians with regards to antimicrobial treatment options for their patients. As such, they include considerations such as clinical outcome data and *in vitro* pharmacological properties of the antimicrobial drug in addition to susceptibility data. Therefore, clinical breakpoints have a limited role in surveillance studies looking for emerging resistances.

Clinically-Resistant (CR): Bacterial isolates are not inhibited by the usually achievable concentrations or when susceptibility results indicate the likelihood of specific AMR mechanisms and the success of treatment by the agent has not been reliably shown.

Multi-drug resistance (MDR): Isolates that are resistant to three or more classes of antimicrobials based on microbiological breakpoint (where one is available) is classified as multi-drug resistant (MDR) phenotype.

Table 3: Breakpoints used for susceptibility testing for *Escherichia coli*

Class	Agent	Range (mg/L)	Microbiological Breakpoint (mg/L) [c]	Clinical Breakpoint (mg/L) [a b]
Aminoglycosides	Gentamicin	0.5 - 16	2	>8
	Streptomycin	2 - 64	16	- [d]
β-lactam / β-lactam inhibitor combination	Amoxicillin-Clavulanate (2:1 ratio)	1 - 32	- [d]	>16
Cephems	Cefoxitin	0.5 - 32	8	>16
	Ceftiofur	0.13 - 8	1	>4[b]
	Ceftriaxone	0.25 - 64	0.13	>2
Fluoroquinolones	Ciprofloxacin	0.016 - 4	0.06	>0.5
Folate pathway inhibitors	Trimethoprim-Sulfamethoxazole (1:19)	0.13 - 4	0.25	>2
Penicillins	Ampicillin	1 - 32	8	>16
Phenicols	Chloramphenicol	2 - 32	16	>16
	Florfenicol	1 - 128	16	>8[b]
Polymyxins	Colistin	0.13 - 8	2	>2
Tetracyclines	Tetracycline	4 - 32	8	>8

[a] CLSI VET01S or [b]M100 30th Ed. breakpoints (mg/L);
[c] EUCAST epidemiological cut-off values (mg/L); [d] Not defined

Table 4: Breakpoints used for susceptibility testing for *Enterococcus* species

Class	Agent	Species	Range (mg/L)	Microbiological Breakpoint (mg/L)[c]	Clinical Breakpoint (mg/L) [a]
Aminoglycosides	Gentamicin	*E. faecium*	32 - 1024	32	-[d]
		E. faecalis	32 - 1024	64	-[d]
	Kanamycin	*E. faecium*, *E. faecalis*	128 - 1024	-[d]	-[d]
	Streptomycin	*E. faecium*	256 - 1024	128[e]	-[d]
		E. faecalis	256 - 1024	512	-[d]
Glycopeptides	Vancomycin	*E. faecium*, *E. faecalis*	0.25 - 32	4	>16
	Teicoplanin	*E. faecium*, *E. faecalis*	0.13 - 4	2	>16
Lincosamide	Lincomycin	*E. faecium*, *E. faecalis*	1 - 32	-[d]	-[d]
Lipopeptides	Daptomycin	*E. faecium*	0.13 - 4	8	>4
		E. faecalis	0.13 - 4	4	>4
Macrolides	Erythromycin	*E. faecium*, *E. faecalis*	0.25 - 8	4	>4
Oxazolidinones	Linezolid	*E. faecium*, *E. faecalis*	0.5 - 8	4	>4
Penicillins	Ampicillin	*E. faecium*, *E. faecalis*	0.5 - 16	4	>8
	Benzylpenicillin	*E. faecium*, *E. faecalis*	0.5 - 16	16	>8
Streptogramin	Quinupristin-Dalfopristin	*E. faecium*,	0.25 - 8	-[d]	>2
		E. faecalis	0.25 - 8	-[d]	-[d]
	Virginiamycin	*E. faecium*	1 - 32	4	-[d]
		E. faecalis	1 - 32	32	-[d]
Tetracyclines	Tetracycline	*E. faecium*, *E. faecalis*	2 – 16	4	>8
Phenicols	Chloramphenicol	*E. faecium*, *E. faecalis*	2 - 32	32	>16

[a] CLSI VETO1S or M100S breakpoints (mg/L); [c] EUCAST epidemiological cut-off values (mg/L); [d] Not defined; [e] Revised ECOFF breakpoint outside of tested range

Genetic analysis

Genetic analysis was undertaken for all *E. faecium* isolates in addition to *E. coli* and *E. faecalis* isolates exhibiting resistance to antimicrobials of high importance and MDR resistance phenotypes (≥ 3 classes of antimicrobials).

DNA extraction and library preparation

A total of 92 (*E. coli*, 11; *Enterococci*, 81) isolates were genetically sequenced.

DNA extraction was performed on all isolates using the MagMAX Multi-sample DNA extraction kit (ThermoFisher Scientific) as per the manufacturer's instructions. DNA library preparation was conducted using the Celero™ DNA-Seq Library Preparation Kit Preparation kit (NuGen, Tecan). Library preparations were sequenced on the Illumina Nextseq platform using a mid-output 2x150 kit. All read data will be deposited to the NCBI sequence read archive prior to submission of research manuscripts.

Bioinformatics analysis

Raw sequence reads were assembled using SPAdes denovo assemblier (Nurk et al.,2013) and AMR genes were identified are all based on known genes using Abricate (T. Seeman) with the ResFinder database (Zankari et al., 2012).

Data Management and Analysis

To manage farm confidentiality, a unique random identifier was allocated to each production unit recorded alongside results for every sample. This allowed reporting of results back to managers of production units via Australian Eggs Limited and private practitioners using the unique codes.

When the primary laboratory received swabs, the required sample identifying information was entered into the Laboratory Information Management System as well as the results from phenotypic and genetic assays. When all the results from all swabs were available, the complete data set was forwarded to Australian Eggs with farm identity anonymised (if not already) and the dataset passed to the epidemiologist for analysis.

Analysis consisted of deriving MIC tables for each target organism and demonstrating the percentage of isolates expressing resistance at the microbiological breakpoint. Multiple class resistance status of each isolate was derived and tabulated from microbiological breakpoints. Exact 95% confidence intervals for the percent of isolates resistant was obtained by the Clopper-Pearson method. All analysis was conducted using the Stata (version 15.1 or later) analysis package.

Deliverables (outputs)

1. Obtain antimicrobial susceptibility profiles among key indicator bacteria (*E. coli* and *Enterococcus*) recovered from the Australian chicken egg industry.
2. Determine the prevalence of resistance of the indicator bacteria to medically-important classes of antimicrobials.
3. Selected isolates are screened for AMR genes by whole genome sequencing.

RESULTS

Bacterial isolation

The flock size sampled ranged from 2400 – 760,000 birds. A total of 511 bacterial isolates were recovered from 62 sample kits between August 2019 and January 2020. *E. faecalis* was the predominant species of *Enterococcus* recovered, followed by *E. faecium* (Table 5).

Table 5: Isolates recovered

Genus	Species	Number isolated (% of genus)
Escherichia	*coli*	296 (100)
Enterococcus	*faecium*	80 (37.2)
	faecalis	135 (62.8)

Escherichia coli

E. coli resistance

The AMR patterns for *E. coli* based on microbiological break points are shown in Figure 3. Comprehensive distribution of MIC concentrations for *E. coli* including frequency of clinical resistance is shown in Table 6. All *E. coli* isolates tested were microbiologically susceptibile to ceftiofur, and chloramphenicol and colistin. Microbiological resistance was observed for ampicillin (16.2%), cefoxitin (1.4%), ciprofloxacin (2.7%), florfenicol (2.4%), gentamicin (1%), streptomycin (4.7%), tetracycline (37.8%), trimethoprim/sulfamethoxazole (10.5%). Currently, there are no microbiological breakpoints for amoxicillin-clavulanate, however; 2.7% of the isolates tested were clinically resistant. For ceftriaxone, the lowest dilution rate tested was above the microbiological breakpoint, hence, the proportion of colonies microbiologically resistant could not be determined.

Eight isolates demonstrated microbiological resistance to the fluoroquinolone class (ciprofloxacin MIC > 0.13 mg/L) (Table 6). However, none were classified as clinically resistant (ciprofloxacin MIC > 1 mg/L). Of the 296 *E. coli* isolates, 154 isolates (52.0%) were susceptible to all antimicrobials tested.

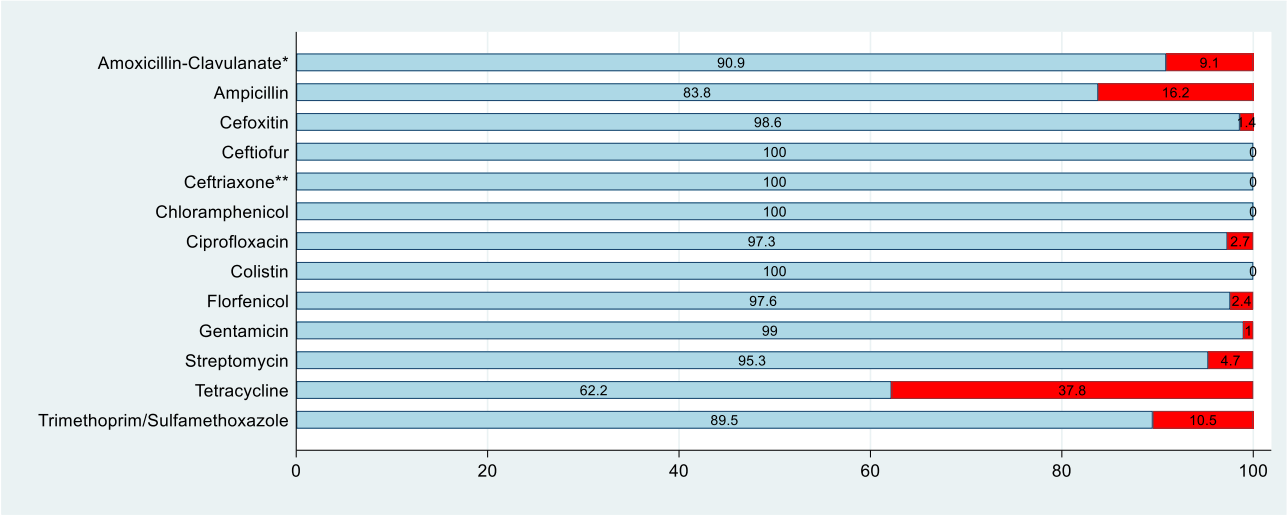

Figure 3. Antimicrobial resistance patterns for *Escherichia coli* (n=296) based on microbiological breakpoints. Percent of susceptible (blue) and percent resistant (red) unless otherwise indicated by footnotes. Percentage results were rounded to one decimal place. * when present, the data for this drug represents the percent non-susceptible due to a lack of breakpoint for wild type. ^ Clinical break points are used when microbiological breakpoints were unavailable for this drug. ** Data for this drug represents the percent non-susceptible due to the microbiological breakpoint being below the dilution range and could not be determined.

Table 6. Distribution of minimum inhibitory concentrations (mg/L) for *Escherichia coli* (n=296) isolates from layers on farm. Percentage of isolates classified as microbiologically resistant by EUCAST.

Antimicrobial	0.016	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	MR (%)	CR (%)
Amoxicillin-Clavulanate^							2.4	8.4	39.2	40.9	6.4	0.0	2.7			.	2.7
Ampicillin							0.7	10.5	54.1	18.6	3.7	3.0	9.5			16.2	12.5
Cefoxitin							3.4	43.9	40.2	11.1	1.4					1.4	0.0
Ceftiofur*				20.3	60.1	18.6	1.0									0.0	0.0
Ceftriaxone*					95.9	3.7	0.3									.	0.0
Chloramphenicol								1.0	24.3	67.9	6.8					0.0	0.0
Ciprofloxacin*	97.0	0.3		1.4	1.4											2.8	0.0
Colistin*				5.4	80.1	13.5	11									0.0	0.0
Florfenicol									4.4	49.7	43.6	2.4				2.4	0.0
Gentamicin					36.5	44.3	13.9	4.4	0.7			0.3				1.0	0.3
Streptomycin								33.8	50.3	9.8	1.4	1.4	2.0	1.4		4.7	.
Tetracycline									59.8	2.4	2.0	2.0	33.8			37.8	37.8
Trimethoprim / Sulfamethoxazole				87.8	1.7	1.0	0.3			9.1						10.5	9.1

*High importance antimicrobial according to ASTAG list. ^microbiological breakpoint is not defined. The shaded areas indicate the range of dilutions tested for each antimicrobial. Vertical lines indicate the microbiological breakpoint. Numbers outside the dilution range represent the percentage of isolates that were still growing at the highest dilution and were therefore assumed to have an MIC above this dilution. Where the dilution range does not cross the vertical line, or vertical line does not exist then the percentage of isolates not susceptible at the microbiological break point cannot be calculated. MR = microbiologically resistant - indicates the percent of colonies classed as resistant at the microbiological breakpoint; CR = clinically resistant - indicates the percent of colonies classed as clinically resistant. All numbers rounded to one decimal place which may result in slight rounding errors.

Multi-drug resistance profiles for E.coli

For *E. coli*, six MDR profiles (defined as being microbiologically resistant to three or more classes of antimicrobial) were identified accounting for 7.0% (n=21) of *E. coli* isolates. The most common MDR profile was bla/fpi/tet. One isolate demonstrated resistance to four classes of antimicrobials (ami_bla_fpi_tet). One isolate demonstrated resistance to five classes (ami_c1g_fpi_qui_tet) of antimicrobials. Of all *E. coli* isolates tested, 52.0% (n=154) were not resistant to any of the tested antimicrobials. The MDR profiles for commensal *E. coli* are shown in Table 7, and any isolates classified as MDR have been highlighted.

Table 7. MDR and non-MDR profiles of *Escherichia coli* isolates obtained from layer hen environments with resistance classification based on microbiological breakpoints (n = 296).

No. of resistances	phenotype	No. of isolates	% of total
0	nil	154	52.4
1	ami	2	0.7
1	bla	11	3.7
1	c1g	3	1.0
1	fpi	3	1.0
1	phe	4	1.4
1	qui	6	2.0
1	tet	52	17.7
2	ami_tet	6	2.0
2	bla_phe	1	0.3
2	bla_tet	20	6.8
2	fpi_tet	11	3.7
3	ami_bla_tet	3	1.0
3	ami_fpi_tet	3	1.0
3	bla_fpi_tet	12	4.1
3	bla_qui_tet	1	0.3
4	ami_bla_fpi_tet	1	0.3
5	ami_c1g_fpi_qui_tet	1	0.3

ami= aminoglycosides, bla= beta lactams, c1g=1st generation cephalosporins, fpi= folate pathway inhibitors, pol = polymixins, qui = quinolones, tet= tetracyclines; Isolates classified as MDR (≥ 3 resistances) have been shaded.

E.coli Genome Sequencing

Whole genome sequencing was undertaken on all *E. coli* isolates exhibiting phenotypic resistance to fluoroquinolones (high importance antimicrobial). These results are shown in Table 8. No mutations or AMR genes were detected for fluoroquinolones resistance with the exception of one isolate that carried *QnrS1*, a known fluoroquinolone resistance gene (Cerquetti, 2009). The two MDR *E. coli* isolates harbouring phenotypic quinolone resistances belonged to two different sequence types (ST746 and ST155) and carried AMR genes to first line antimicrobials such as tetracycline, and

aminoglycosides. Not all phenotypically detected resistance was confirmed by the detection of corresponding AMR genes or mutations as previously described (Ellington, 2017; Bortolaia, 2020).

Table 8: Phenotype and genotype data for quinolone resistant *E. coli* isolates

MLST	Phenotype	Genotype	No. of isolates
ST-746	c1g, qui, ami, tet, fpi	*aadA1, dfrA1, strA, strB, sul1, sul2, tet(A)*	1
ST-155	qui	*lnu(C)*	1
ST-3714	qui	*sul1*	1
ST-155	bla, qui, tet	*blaTEM-1B, QnrS1, tet(A)*	1
ST-155	qui	-	2
ST-355	qui	-	2

Enterococcus faecium

E. faecium resistance

All *E. faecium* isolates tested were microbiologically susceptible to benzylpenicillin, chloramphenicol, daptomycin, gentamicin, linezolid, teicoplanin, vancomycin and virginiamycin. Microbiological resistance was observed for ampicillin (5.1%), erythromycin (22.5%) and tetracycline (58.8%). Quinupristin-dalfopristin does not have an established microbiological breakpoint, but two isolates were clinically resistant (>2 mg/L). Kanamycin which also does not have an established microbiological breakpoint, had two isolates (>512mg/L) that were clinically resistant. For lincomycin, >50% of isolates and for streptomycin, 11 isolates were clinically resistant (Figure 4, Table 9).

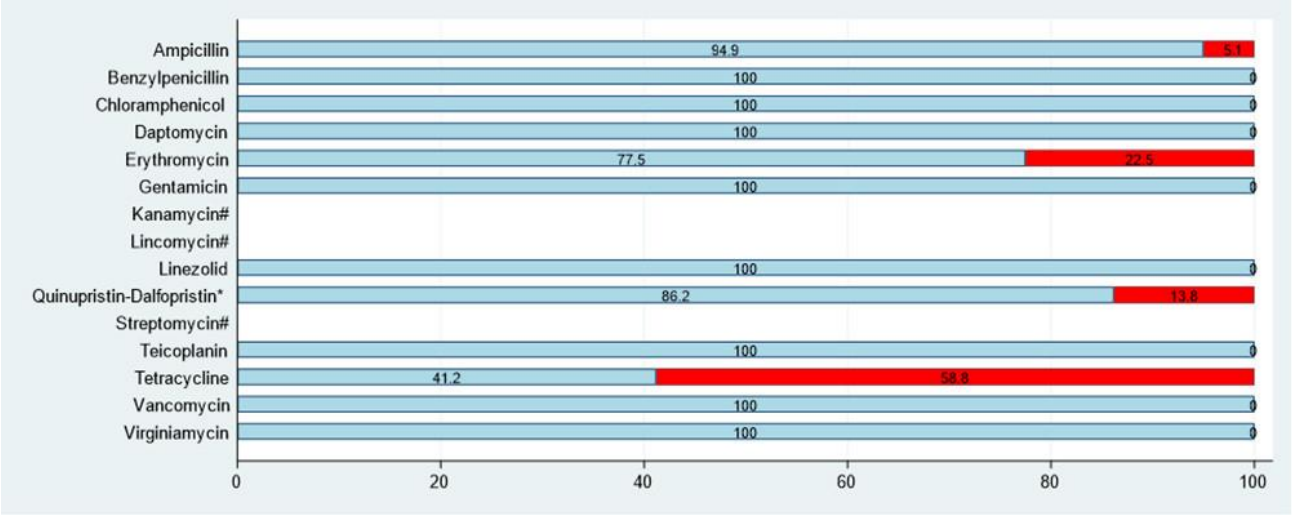

Figure 4. Antimicrobial resistance patterns for *Enterococcus faecium* (n=80) based on microbiological breakpoints. Clinical breakpoints are used when microbiological breakpoints were unavailable. Percent of susceptible (blue) and percent resistant (red) unless otherwise indicated by footnotes. Percentage results were rounded to one decimal place. * Denotes the data for this drug represents the percent non-susceptible at the CLSI non-susceptible breakpoint due to a lack of a microbiological breakpoint. # Denotes no data are presented for this drug due to lack of microbiological, CLSI susceptible and clinical breakpoints.

Table 9. Distribution of minimum inhibitory concentrations (mg/L) for *Enterococcus faecium* (n=80) isolates from layers on farm. Percentage of isolates classified as microbiologically resistant by EUCAST.

Antimicrobials	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	MR (%)	CR (%)
Ampicillin					40.0	25.0	17.5	12.5	3.8		1.3							5.1	1.3
Benzylpenicillin					43.8	23.8	18.8	7.5	6.3									0.0	0.0
Chloramphenicol							55.0	11.3	23.8	8.8	1.3							0.0	1.3
Daptomycin*			2.5	3.8	2.5	20.0	48.8	22.5										0.0	0.0
Erythromycin				57.5	5.0	5.0	5.0	5.0		22.5								22.5	22.5
Gentamicin											100							0.0	.
Kanamycin^													61.3	31.3	5.0	1.3	1.3	.	.
Lincomycin^						26.3	2.5	5.0	10.0	3.8	6.3	46.3						.	.
Linezolid*					35.0	41.3	17.5	6.3										0.0	0.0
Quinupristin-Dalfopristin*^				7.5	40.0	38.8	11.3	1.3	1.3									.	2.5
Streptomycin**														81.3	5.0	7.5	6.3	.	.
Teicoplanin*			45.0	42.5	12.5													0.0	0.0
Tetracycline							40.0	1.3		1.3	57.5							58.8	58.8
Vancomycin*				47.5	45.0	7.5												0.0	0.0
Virginiamycin*						98.8		1.3										0.0	.

*High importance antimicrobial according to ASTAG list. ^microbiological breakpoint is not defined. The shaded areas indicate the range of dilutions tested for each antimicrobial. Vertical lines indicate the microbiological breakpoint. Numbers outside the dilution range represent the percentage of isolates that were still growing at the highest dilution and were therefore assumed to have an MIC above this dilution. Where the dilution range does not cross the vertical line, or vertical line does not exist then the percentage of isolates not susceptible at the microbiological break point cannot be calculated. MR = microbiologically resistant - indicates the percent of colonies classed as resistant at the microbiological breakpoint; CR = clinically resistant - indicates the percent of colonies classed as clinically resistant. **The *E. faecium* breakpoint for streptomycin is lower than the lowest concentration tested and therefore prevalence of resistance could not be determined. All numbers rounded to one decimal place which may result in slight rounding errors.

Multi-drug resistance profiles for E.faecium

Three MDR profiles were identified for *E. faecium* accounting for 7.5% (n=6) of isolates. Three of these MDR isolates harboured resistance to three classes of antimicrobials (mac_str_tet). 30% (n=24) of the isolates were not resistant to any of the tested antimicrobials (Table 10).

Table 10. MDR and non-MDR profiles of *Enterococcus faecium* isolates obtained from layer hen environments with resistance classification based on microbiological breakpoints (n = 80).

No. of resistances	phenotype	No. of isolates	% of total *
0	nil	24	30.0
1	bla	1	1.3
1	mac	5	6.3
1	str	1	1.3
1	tet	31	38.8
2	bla_tet	1	1.3
2	mac_str	2	2.5
2	mac_tet	6	7.5
2	str_tet	3	3.8
3	bla_mac_tet	1	1.3
3	bla_str_tet	1	1.3
3	mac_str_tet	4	5.0

bla= beta lactams, mac= macrolides, tet= tetracyclines; Isolates classified as MDR (≥ 3 resistances) have been shaded. *Numbers rounded to one decimal place which may result in rounding errors.

Enterococcus faecium genome sequencing

Whole genome sequencing was performed on all *E. faecium* isolates. No genotypic or phenotypic vancomycin resistance was observed for all *E. faecium* sequenced (Table 11). Genomic characterisation of *E. faecium* isolates showed that all *E. faecium* phenotypically resistant to tetracycline harboured the *tet(M)* gene (Akhtar, 2009), however, most *E. faecium* harboured the *erm(B)* and/or the *msr(C)* gene known to convey macrolide resistance (Yu, 1997), without an accompanying phenotype. As the *E. faecium* genome is very plastic and subjected to mutations (Zhi Zhong, 2019), not all genes identified are functional or expressed. Therefore, the presence of resistance genes may not necessarily result in the expression of phenotypic resistance.

Table 11: *E. faecium* combined phenotype and genotype data – all isolates

MLST	Phenotype	Genotype	No. of isolates
ST-157	nil	-	1
ST-915	nil	-	1
Unknown ST	nil	*aadE,lnu(B)*	1
ST-32	nil	*lnu(A),msr(C)*	1
ST-22	nil	*msr(C)*	1
ST-26	nil	*msr(C)*	1

MLST	Phenotype	Genotype	No. of isolates
Unknown ST	nil	*msr(C)*	1
ST-245	nil	*msr(C)*	1
ST-195	nil	*msr(C)*	1
ST-640	nil	*msr(C)*	1
ST-10	nil	*msr(C)*	1
ST-666	nil	*msr(C)*	1
ST-640	nil	*msr(C), tet(M)*	1
ST-9	nil	*lnu(A),msr(C)*	2
ST-8	nil	*msr(C)*	2
ST-12	nil	*msr(C)*	2
ST-944	nil	*msr(C)*	2
ST-241	nil	*msr(C)*	3
ST-32	bla	*msr(C)*	1
ST-32	mac	*erm(B),msr(C)*	1
Unknown ST	mac	*msr(C),tet(L),tet(M)*	1
ST-445	mac	*aadE,erm(B),lnu(B),msr(C)*	3
ST-236	str	*msr(C),spc,tet(M)*	1
ST-195	tet	*aadE,ant(6)-Ia,erm(B),lnu(B),tet(L),tet(M)*	1
ST-195	tet	*aadE,erm(B),lnu(B),tet(L),tet(M)*	1
ST-195	tet	*aadE,erm(B),lnu(B),tet(M)*	1
Unknown ST	tet	*aadE,lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	tet	*aadE,lnu(B),msr(C),tet(M)*	1
ST-245	tet	*aadE,lnu(B),tet(M)*	1
ST-32	tet	*aadE,msr(C),tet(L),tet(M)*	1
ST-26	tet	*aadE,spc,tet(L),tet(M)*	1
ST-241	tet	*cat,lnu(A),msr(C),tet(L),tet(M)*	1
ST-8	tet	*erm(A),msr(C),spc,tet(M)*	1
Unknown ST	tet	*erm(B),msr(C),tet(M)*	1
ST-8	tet	*msr(C)*	1
ST-9	tet	*msr(C)*	1
ST-236	tet	*msr(C),spc,tet(M)*	1
ST-873	tet	*msr(C),tet(L),tet(M)*	1
ST-190	tet	*msr(C),tet(L),tet(M)*	1
ST-190	tet	*msr(C),tet(L),tet(M)*	1
ST-10	tet	*tet(L),tet(M)*	1
ST-26	tet	*tet(M)*	1
ST-241	tet	*msr(C),tet(L),tet(M)*	2
Unknown ST	tet	*msr(C),tet(M)*	2
Unknown ST	tet	*cat,lnu(A),msr(C),tet(L),tet(M)*	3
Unknown ST	tet	*msr(C),tet(L),tet(M)*	5
ST-11	bla_tet	*erm(A),spc,tet(L),tet(M)*	1
Unknown ST	mac_str	-	1
ST-445	mac_str	*aadE,erm(B),lnu(B),msr(C)*	1
ST-1099	mac_tet	*aadE,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	mac_tet	*aadE,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	mac_tet	*erm(B),lnu(A),msr(C),tet(L),tet(M)*	1
Unknown ST	mac_tet	*erm(B),msr(C),tet(L),tet(M)*	1

MLST	Phenotype	Genotype	No. of isolates
ST-1122	mac_tet	*erm(B),msr(C),tet(M)*	1
Unknown ST	mac_tet	*msr(C),tet(M)*	1
ST-640	str_tet	*aadE,cat,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
ST-445	str_tet	*aadE,erm(B),lnu(A),lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	str_tet	*erm(B),msr(C),tet(L),tet(M)*	1
Unknown ST	bla_mac_tet	*aph(3')-III,dfrG,erm(B),msr(C),tet(L),tet(M)*	1
ST-640	bla_str_tet	*erm(A),msr(C),spc,tet(L),tet(M)*	1
ST-10	mac_str_tet	*aadE,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	mac_str_tet	*aadE,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
Unknown ST	mac_str_tet	*aadE,ant(6)-Ia,erm(B),lnu(B),msr(C),tet(L),tet(M)*	1
ST-241	mac_str_tet	*erm(B),msr(C)*	1

Enterococcus faecalis

E. faecalis resistance

None of the *E. faecalis* isolates tested were microbiologically resistant to benzylpenicillin, daptomycin, gentamicin, teicoplanin, vancomycin and virginiamycin. Microbiological resistance was observed for ampicillin (1.5%), chloramphenicol (1.5%), erythromycin (11.9%), linezolid (0.7%), streptomycin (1.5%) and tetracycline (57.0%). Although two isolates were microbiologically resistant to ampicillin, they were not clinically resistant. Currently, there is no microbiological breakpoint for kanamycin, but five isolates were clinically resistant (Figure 5, Table 12). There are also no microbiological breakpoints for lincomycin and quinupristin-dalfopristin. No phenotypic vancomycin resistance was observed for all *E. faecalis*.

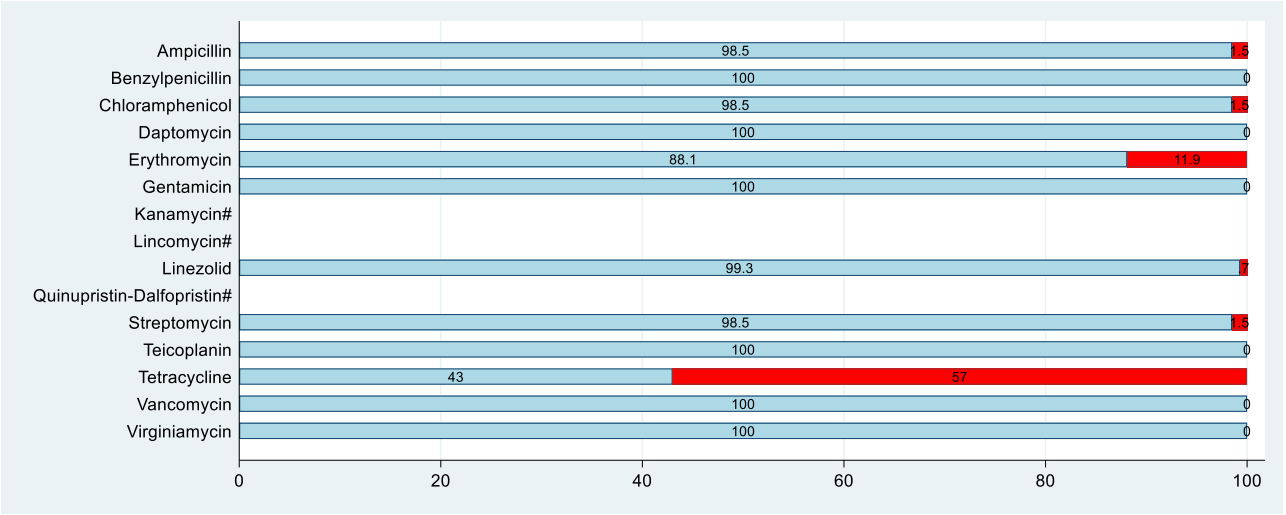

Figure 5. Antimicrobial resistance patterns for *Enterococcus faecalis* (n=135) based on microbiological breakpoints. Clinical break points are used when microbiological breakpoints were unavailable. Percent of susceptible (blue) and percent resistant (red). Percentage results were rounded to one decimal place. # Denotes no data are presented for this drug due to lack of microbiological, CLSI susceptible and clinical breakpoints.

Table 12. Distribution of minimum inhibitory concentrations (mg/L) for *Enterococcus faecalis* (n=135) isolates from layers on farm. Percentage of isolates classified as microbiologically resistant by EUCAST.

Antimicrobial	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	MR (%)	CR (%)
Ampicillin					8.1	45.9	41.5	3.0	1.5									1.5	0.0
Benzylpenicillin					0.7	11.1	80.7	7.4										0.0	0.0
Chloramphenicol							3.7	30.4	62.2	2.2		1.5						1.5	1.5
Daptomycin*			0.7	0.7	3.7	12.6	54.1	28.1										0.0	0.0
Erythromycin				26.7	19.3	37.8	4.4		0.7	11.1								11.9	11.8
Gentamicin											100							0.0	.
Kanamycin^													93.3	3.0			3.7	.	.
Lincomycin^						0.7			2.2	23.0	57.8	16.3						.	.
Linezolid*					3.0	20.0	74.8	1.5	0.7									0.7	0.7
Quinupristin-Dalfopristin*^							3.0	46.7	48.1	2.2								.	.
Streptomycin														97.8	0.7		1.5	1.5	.
Teicoplanin*			1.5	56.3	41.5	0.7												0.0	0.0
Tetracycline							43.0			0.7	56.3							57.0	57.0
Vancomycin*					13.3	53.3	33.3											0.0	0.0
Virginiamycin*						2.2	18.5	71.1	8.1									0.0	.

*High importance antimicrobial according to ASTAG list. ^microbiological breakpoint is not defined. The shaded areas indicate the range of dilutions tested for each antimicrobial. Vertical lines indicate the microbiological breakpoint. Numbers outside the dilution range represent the percentage of isolates that were still growing at the highest dilution and were therefore assumed to have an MIC above this dilution. Where the dilution range does not cross the vertical line, or vertical line does not exist then the percentage of isolates not susceptible at the microbiological break point cannot be calculated. MR = microbiologically resistant - indicates the percent of colonies classed as resistant at the microbiological breakpoint; CR = clinically resistant - indicates the percent of colonies classed as clinically resistant. All numbers rounded to one decimal place which may result in slight rounding errors.

Multi-drug resistance profiles for E.faecalis

Three MDR profiles were identified for *E. faecalis* accounting for 2.1% (n=3) isolates. No particular MDR profile was dominant. Two isolates demonstrated resistance to three classes of antimicrobials (ami_mac_tet and mac_phe_tet) and one isolate demonstrated resistance to four classes of antimicrobials (mac_oxa_phe_tet). Of the *E. faecalis* isolates tested, 39.3% (n=53) were not resistant to any of the tested antimicrobials (Table 13).

Table 13. MDR and non-MDR profiles of *Enterococcus faecalis* isolates obtained from layer hen environments with resistance classification based on microbiological breakpoints (n = 135).

No. of resistances	phenotype	No. of isolates	% of total
0	nil	53	39.3
1	ami	1	0.7
1	bla	1	0.7
1	mac	3	2.2
1	tet	63	46.7
2	bla_tet	1	0.7
2	mac_tet	10	7.4
3	ami_mac_tet	1	0.7
3	mac_phe_tet	1	0.7
4	mac_oxa_phe_tet	1	0.7

ami= aminoglycosides, bla= beta lactams, mac= macrolides, oxa= oxazolidinones, phe= phenicols, tet= tetracyclines; Isolates classified as MDR (≥ 3 resistances) have been shaded.

Enterococcus faecalis genome sequencing

Whole genome sequencing was performed on an *E. faecalis* with phenotypic resistance to oxacillin, macrolides, tetracycline and phenicols (Table 14). Whole genome sequencing identified ten antimicrobial resistance genes from this isolate including aph(3')-III, dfrG, erm(A), erm(B)-like, fexA-like, lsa(A)-like, optrA, spc, tet(L) and tet(M)-like genes (Treu-Cuot, 1983; Sekiguchi, 2005; Yu, 1997; Malhotra-Kumar, 2009; Poole, 2005; Wang, 2015; Murphy, 1985; McMurry, 1987; Akhta, 2009). No genotypic or phenotypic vancomycin resistance was observed in the sequenced E. faecalis (Table 14).

Table 14: *E. faecalis* combined phenotype and genotype data

MLST	Phenotype	Genotype	No. of isolates
ST-708	mac,oxa,tet,phe	*aph(3')-III, dfrG, erm(A), erm(B), fexA, lsa(A), optrA, spc, tet(L), tet(M)*	1

DISCUSSION

This study investigated AMR among *E. coli*, *E. faecium* and *E. faecalis* in Australian layer hens. Key observations from the study are as follows.

E. coli

More than half of the isolates were phenotypically and genotypically susceptible to all antimicrobials tested. The current study demonstrated low levels of resistance to antimicrobials with less critical ratings such as cefoxitin, trimethoprim/sulfamethoxazole, and moderate levels of resistance to tetracycline (37.8%) among *E. coli*, potentially reflective of industry usage, although usage data is not published. MDR phenotypes were observed only among a small number of *E. coli* isolates (7.0%), with one isolate exhibiting resistance to five antimicrobial classes (aminoglycosides, first generation cephalosporins, folate pathway inhibitors, quinolones and tetracyclines). The results indicate that significant resistance to antimicrobials of high importance were absent in commensal *E. coli* isolated from Australian layer hens. Where phenotypic results suggested resistance, this was not supported by the presence of known resistance genes, except in a single isolate that harboured a known quinolone resistance gene. The findings are consistent with other recent studies demonstrating low levels of AMR to high or medium importance antimicrobials among *E. coli* isolated from Australian livestock (Barlow, 2015; Pande, 2015; ACMF, 2018; Kidsley, 2018).

Enterococci

The results indicate that none of the *E. faecium* and *E. faecalis* isolated from Australian layer hens were resistant to high importance antimicrobials such as vancomycin and teicoplanin. However, some antimicrobial resistance to erythromycin (*E. faecium*, 22.5%; *E. faecalis,* 11.9%) and ampicillin (*E. faecium*, 5.1%; *E. faecalis,* 1.5%) was observed. Half the *E. faecalis* isolates (57%) were microbiologically resistant to tetracycline. 30% of *E. faecium* and 39.3% of *E. faecalis* isolates were phenotypically susceptible to all antimicrobials tested. Three *E. faecalis* isolates displayed a MDR phenotype which were confirmed by the presence of known resistance genes. The single *E. faecalis* isolate that exhibited an MDR phenotype to four antimicrobials also harboured the *optrA* gene known for conferring transferable linezolid resistance, which is an antimicrobial of high importance. The extremely low prevalence of *optrA* identified in this study suggests that it is a rare occurrence in laying hens at present, however, further analysis should be conducted to clarify the pathways for incursion of *optrA* into Australian layer flocks. Six *E. faecium* displayed an MDR phenotype. Of the *E. faecium* isolates with known resistance genes present, the majority displayed genes to only one antimicrobial, in most cases, tetracycline. *E. faecium* from this study did not belong to the major sequence types responsible for sepsis in humans in Australia from 2015-2017 (Lee, 2020).

Conclusion

The presence of resistance genes to antimicrobials not approved for use in the Australian poultry industry was surprising given the strict regulatory controls and integral veterinary involvement in flock health management. There is increasing evidence that resistant bacteria may transfer from humans or other animals (e.g. wild birds) to poultry, and other livestock, and it is highly possible that these transmission pathways have resulted in layer hens becoming infected with resistant bacteria (Sahibzada, 2017; Mukerji 2019, 2020; Abraham, 2020). Transmission of resistant bacteria from humans and other animals to flocks presents a biosecurity risk for the industry, as entry into a flock potentially amplifies populations of resistant bacteria, which ultimately presents a greater risk to human and animal health. Future investigations should consider prevalence of resistance to antimicrobials of animal health significance and continued work on minimising flock disease and adoption of antibiotic alternative therapies.

More work is needed to understand the pathways for tetracycline resistance. This includes understanding the level, and the reasons for, tetracycline usage in the industry to identify the most relevant factors that need to be addressed to reduce the observed levels of tetracycline resistance. Further, the lack of appropriate breakpoints for amoxicillin and lincomycin antimicrobials also presents a barrier to improved

understanding of bacterial resistance profiles. Industry is undertaking to clarify these issues and identify practical approaches to improving industry understanding of the risks associated with AMR bacteria. These include projects designed to further support adoption of biosecurity practices, and better clarify the potential sources and origins of critically important antimicrobial resistance among indicator bacteria in the absence of use. More recently, the findings from this project informed review of the industry's National Biosecurity Manual which was updated to include reference to the importance of biosecurity for minimising the risk of incursion of AMR bacteria into a flock (Australian Eggs, 2020b).

Overall, these results support similar findings from recent studies of Australian pigs and meat chickens (Kidsley, 2018; Abraham, 2019), which suggest the current contribution of AMR from food animals in Australia to the prevalence of resistance to antimicrobials of high and medium importance observed in humans, is likely to be low. For the layer industry, the results reflect decades of stringent regulatory controls on antimicrobial use, biosecurity and infection prevention practices, and the resultant favourable disease status of Australian layer farms.

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Appendix 1 - SOP for barn / aviary system sampling

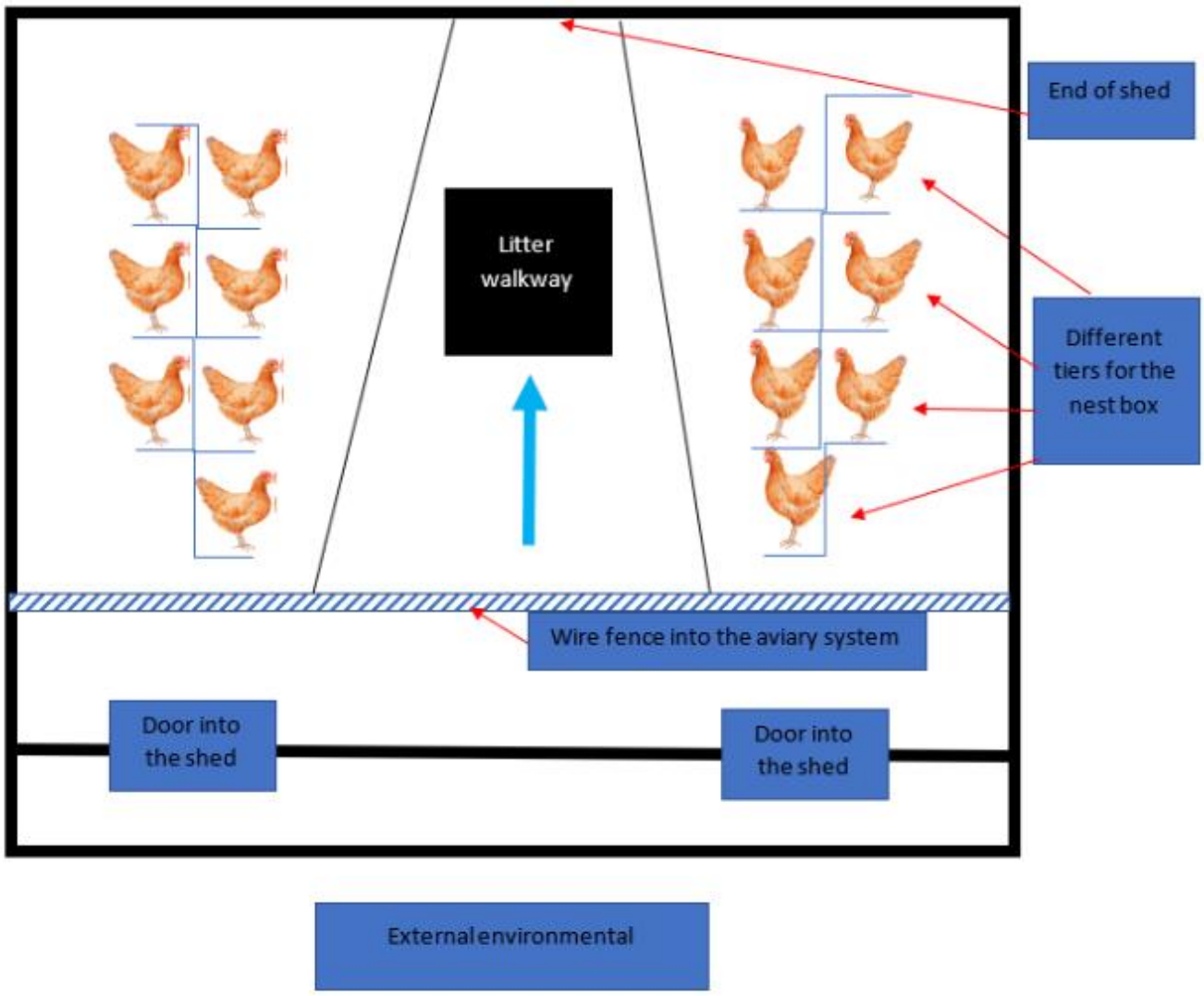


Figure 1 – Aviary system

1. From the wire fence inside the shed, walk 20 steps towards the end of the shed as indicated by the blue arrow in Figure 1.
2. Pick up a chicken and collect cloaca swab sample as per SOP “Appendix 1 - SOP for collecting cloaca swab samples”
3. Put the chicken down.
4. Next walk another 20 steps and repeat step 2.
5. Repeat step 2 until all 5 swabs have been collected

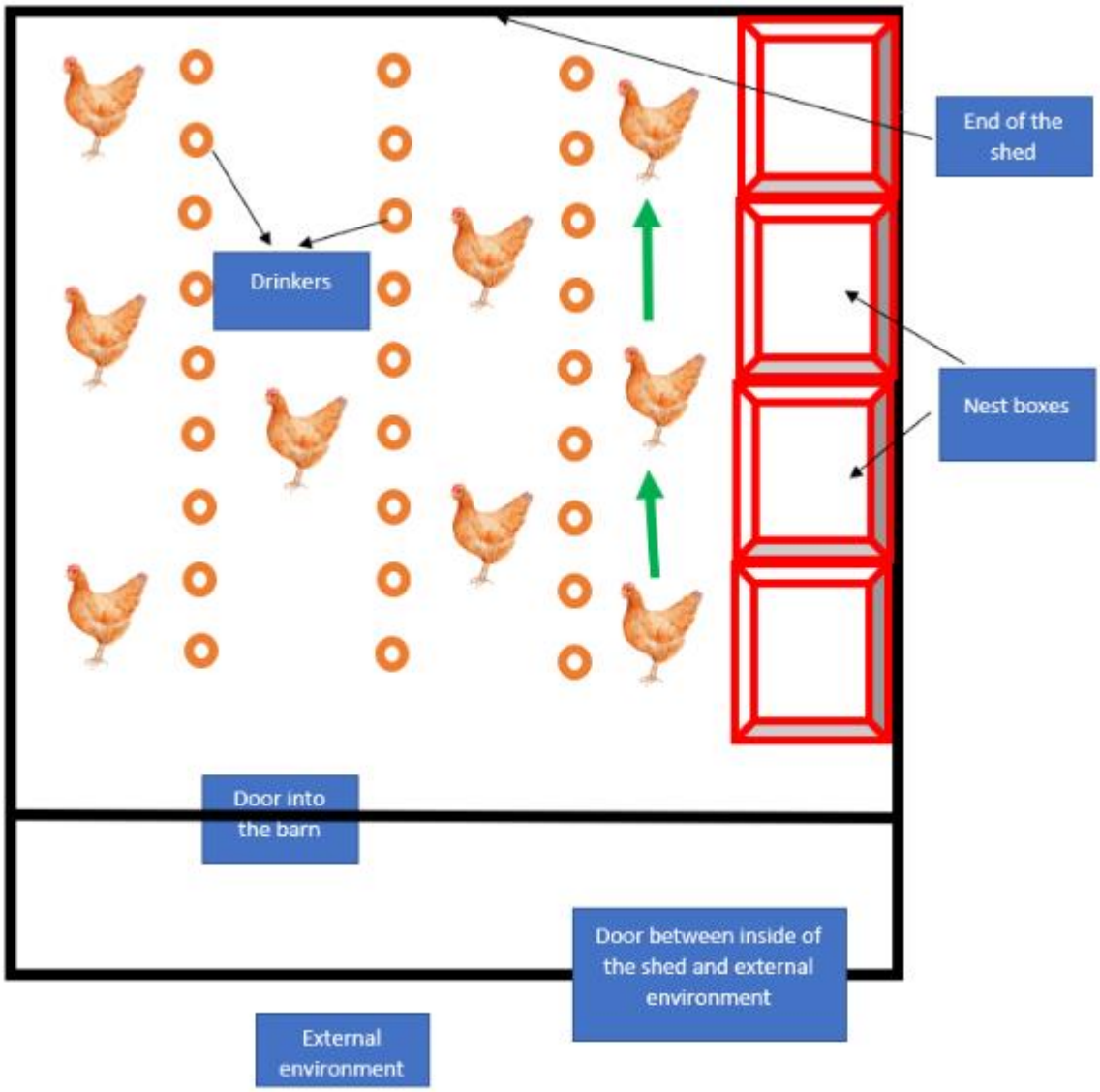


Figure 2 – Barn system

1. From the wall of the shed where the door is, in front of the next box, walk 20 steps towards the end of the shed as indicated by the green arrow in Figure 1.
2. Pick up a chicken and collect cloaca swab sample as per SOP “Appendix 1 - SOP for collecting cloaca swab samples”
3. Put the chicken down.
4. Next walk another 20 steps and repeat step 2.
5. Repeat step 2 until all 5 swabs have been collected

Appendix 2 - SOP for cage system sampling

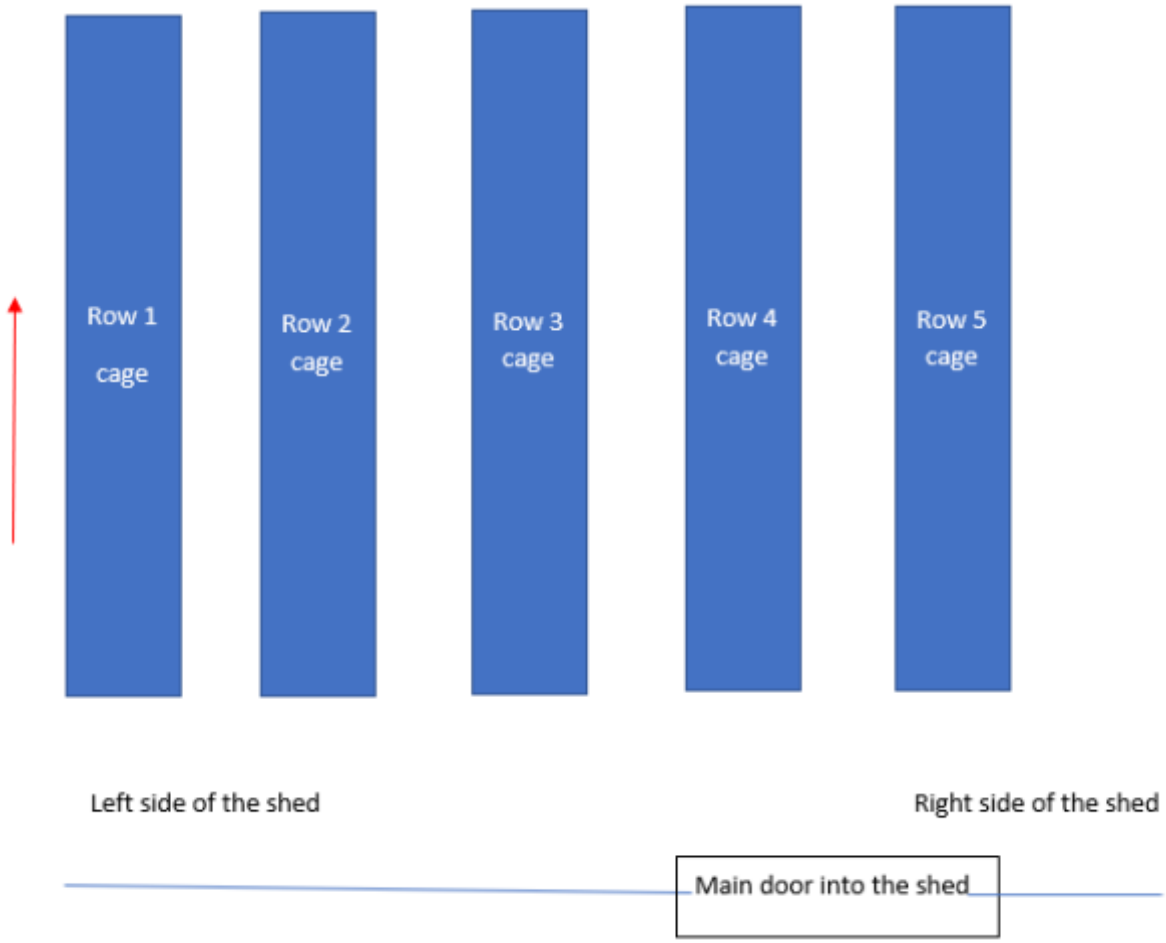


Figure 1 – shed design

Top most tier									
Bottom most tier	Number 1 cage		Number 25 cage		Number 50 cage		Number 75 cage		Number 100 cage

Direction as the arrow in Figure 1

Figure 2 – cage to choose to select chickens to be swabbed

1. All 5 swab samples are to be collected from row 1 as indicated by the red arrow (Figure1).
2. Select chickens from the bottom most tier of row 1 (Figure 2).
3. Walk to cage 1, pick a bird from cage 1 and collect cloaca swab sample as per SOP "Appendix 1 - SOP for collecting cloaca swab samples"
4. Put the bird back into the cage after swabbing and walk to cage 25 to collect another cloaca swab sample.
5. Next walk to cage 50, 75 and 100 and repeat step 3 and 4 until all 5 swabs are collected.

Appendix 3 - SOP for free range sampling

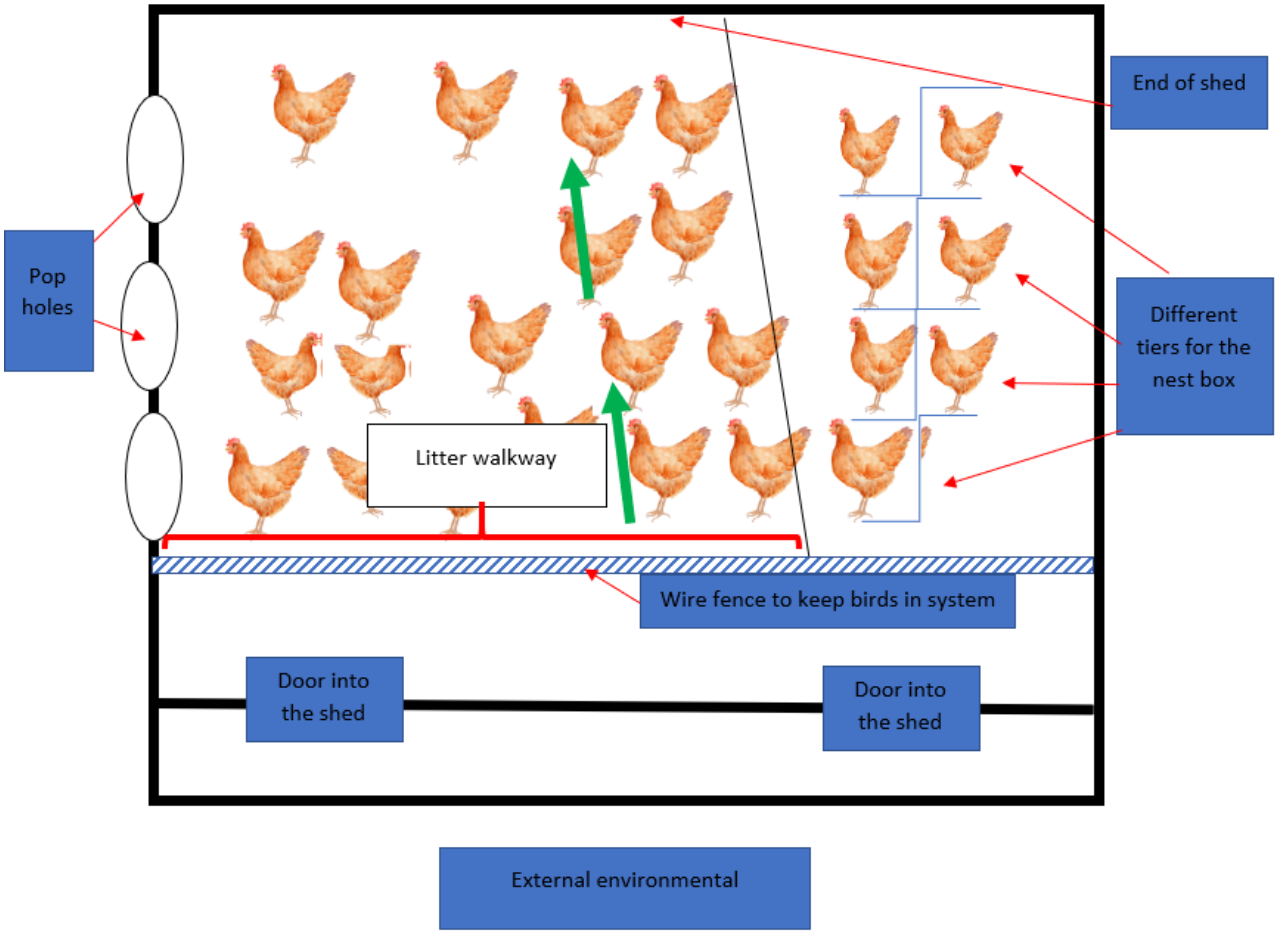


Figure 1 – Aviary system

1. From the wire fence inside the shed, walk 20 steps towards the end of the shed as indicated by the green arrow in Figure 1.
2. Pick up a chicken and collect cloaca swab sample as per SOP “Appendix 1 - SOP for collecting cloaca swab samples”.
3. Put the chicken down.
4. Next walk another 20 steps and repeat step 2.
5. Repeat step 2 until all 5 swabs have been collected.

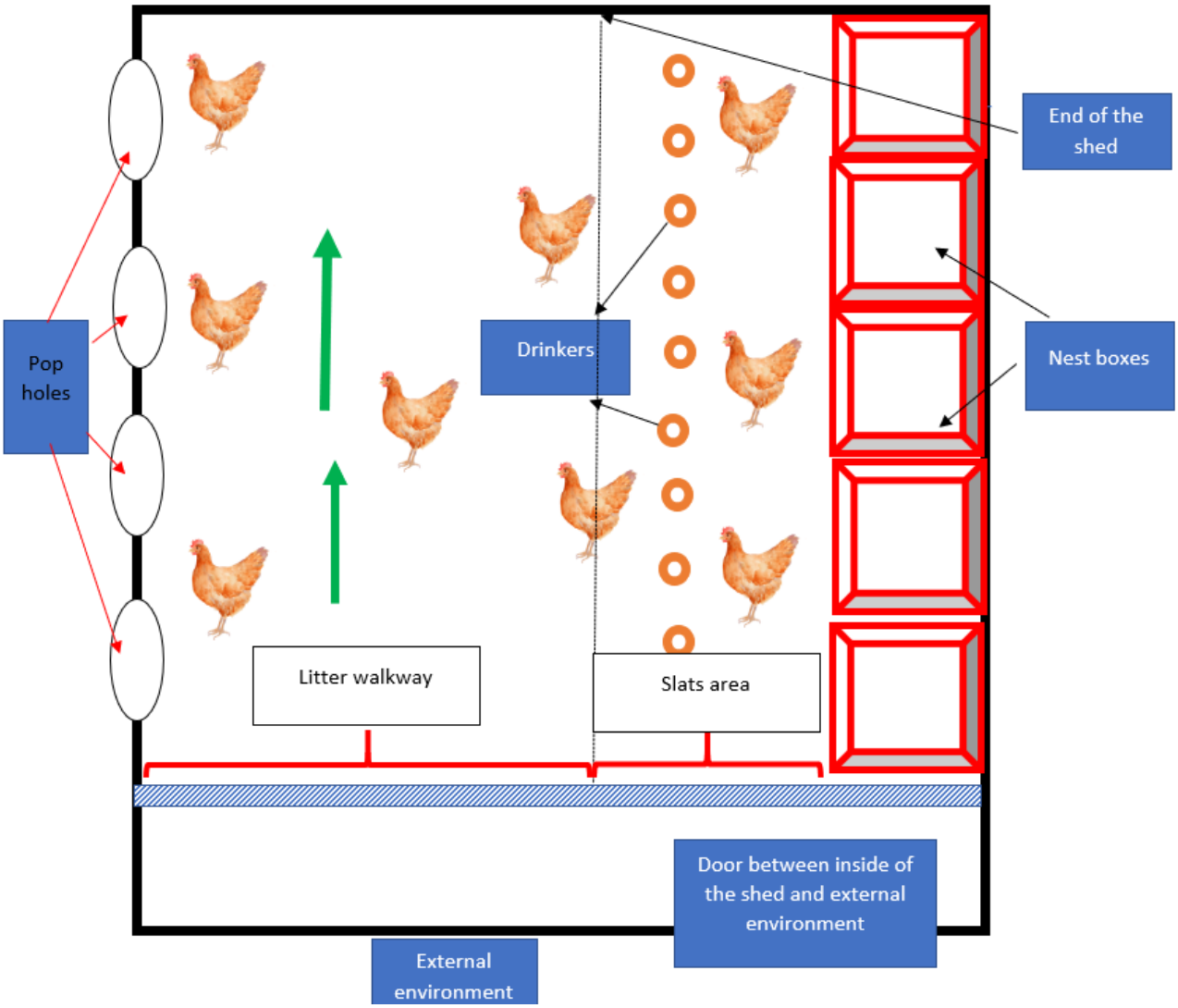


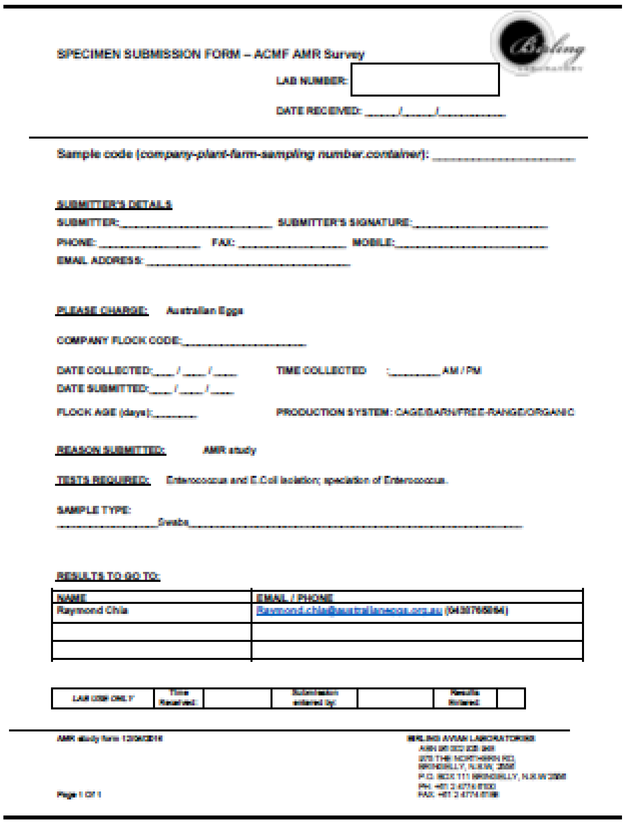
Figure 2 – free range system

1. From the wire fence inside the shed, walk 20 steps towards the end of the shed as indicated by the green arrow in Figure 1.
2. Pick up a chicken and collect cloaca swab sample as per SOP “Appendix 1 - SOP for collecting cloaca swab samples”.
3. Put the chicken down.
4. Next walk another 20 steps and repeat step 2.
5. Repeat step 2 until all 5 swabs have been collected.

Appendix 4 - SOP for collecting cloaca swab samples

Each collection kit contains: 5 amine swabs; 2 pairs of examination gloves; 1 plastic sleeve (for the sample collection form); 2 sample collection forms and 1 stamped envelope addressed to Birling Avian Laboratories.

1. Fill in the sample submission form.



SPECIMEN SUBMISSION FORM – ACMF AMR Survey

LAB NUMBER:

DATE RECEIVED: _____/_____/__________

Sample code (*company-plant-farm-sampling number.container*): ____________________

SUBMITTER'S DETAILS

SUBMITTER:______________________ SUBMITTER'S SIGNATURE:____________________

PHONE: _______________ FAX: ________________ MOBILE:______________________

EMAIL ADDRESS: ______________________________

PLEASE CHARGE: Australian Eggs

COMPANY FLOCK CODE:____________________

DATE COLLECTED:____ / ____ / ____ TIME COLLECTED :________AM / PM

DATE SUBMITTED:____ / ____ / ____

FLOCK AGE (days):_______ PRODUCTION SYSTEM : CAGE/BARN/FREE-RANGE/ORGANIC

REASON SUBMITTED: AMR study

TESTS REQUIRED: Enterococcus and E.Coli isolation; speciation of Enterococcus.

SAMPLE TYPE:
________________Swabs____

RESULTS TO GO TO:

NAME	EMAIL / PHONE
Raymond Chia	Raymond.chia@australianeggs.org.au (0430765064)

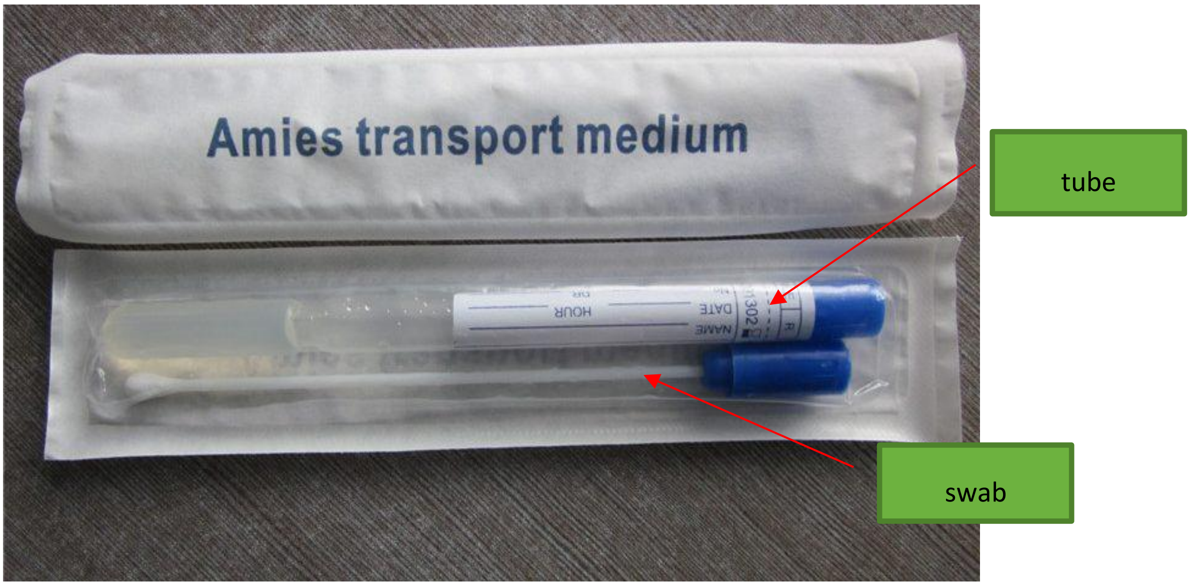
LAB USE ONLY	Time Received:		Submission entered by:		Results Entered:	

AMR study form [illegible]

Page 1 Of 1

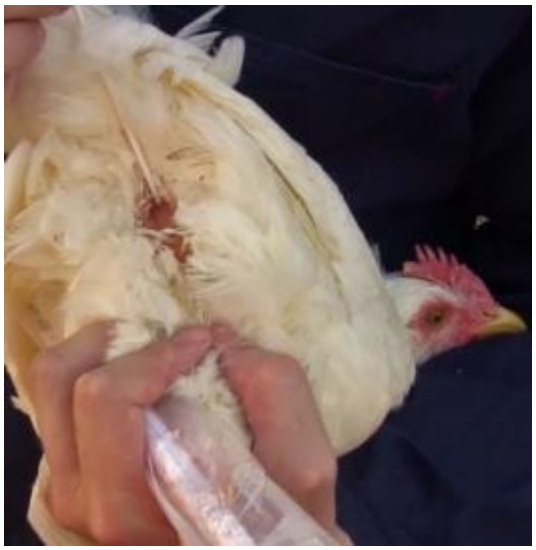
BIRLING AVIAN LABORATORIES
ABN [illegible]
975 THE NORTHERN RD
BRINGELLY, N.S.W. 2556
P.O. BOX 111 BRINGELLY, N.S.W 2556
PH: +61 2 [illegible]
FAX: +61 2 [illegible]

2. Put on gloves.
3. Remove the swab from the plastic / paper envelope and then remove the top of the tube (popoff).



4. Select a chicken using the approach outlined in Appendix 2,3 or 4 depending on your production system.

5. Pick up a bird gently. Do not catch a bird by holding tail or feathers.
6. Ensure you are holding the birds wings close to their body.
7. To lift, tuck the birds legs underneath and close to their body, keeping the wings compressed.
8. The bird should be positioned by your side with its head facing towards the back.
9. Restrain the bird by keeping one wing flush against your body and the other wing compressed with your arm (not too tight).
10. Insert a sterile cotton-tipped applicator or swab is into the cloaca/vent of the bird. Rotate the swabs gently in the cloacal 2 or 3 times to ensure the collection of faecal material.



11. Inset the swab into the tube.
12. Keep the birds legs restrained at all times.
13. Release a bird after completion of a procedure.
14. Repeat steps 3-13 for an addition four birds (five birds in total).
15. Put the swabs and the sample submission form into the self-addressed envelope and send the package to the laboratory by express post.