Development of an enhanced methodology for large-scale detection and quantification of antimicrobial resistant bacteria in livestock

-by-

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I, Zheng Zhou Lee, declare that:

This thesis is my account of my research and has been substantially accomplished during enrolment in this degree, except where other sources are fully acknowledged. All co-authors, where stated and certified by my Principal Supervisor or Executive Author, have agreed that the works presented in this thesis represent substantial contributions from myself. The thesis contains as its main content, work that has not previously been submitted for a degree at any other university. In the future, no part of this thesis will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of Murdoch University and where applicable, any partner institution responsible for the joint-award of this degree.

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1) RAMP Approval – RAMP0959_02_18
2) Working with Biological Materials Approval – WBM_18_257

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Date: 20th October 2021

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This thesis contains work that has been accepted for publication.

Details of the work: Zheng Zhou Lee designed and performed the experiment, data analysis and writing of the manuscript Rebecca Abraham reviewed and edited the manuscript Mark O'Dea reviewed and edited the manuscript Ali Harb, Kelly Hunt and Terence Lee assisted in the experiment Sam Abraham assisted in conceptualising the experiment, and reviewed and edited the manuscript David Jordan assisted in conceptualising the experiment, assisted in data analysis, and reviewed and edited the manuscript
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I, Sam Abraham certify that the student's statements regarding their contribution to each of the works listed above are correct. As all co-authors' signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis. Principal supervisor signature: Date: 21/10/21

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Thesis Abstract

Antimicrobial resistance (AMR) is a global health challenge for both humans and animals. A potential source of antimicrobial resistant bacteria is in livestock due to the widespread and unrestrained use of antimicrobials. This is further exacerbated by the presence of bacteria resistant to critically important antimicrobials (CIAs) that are classified as the last-line of treatment of infectious diseases in humans. AMR surveillance in livestock has become a key cornerstone of AMR control strategies by informing the presence and frequency of resistance including CIA-resistant bacteria. Established approach of AMR surveillance in livestock typically have a national-level focus that only acquire a maximum of 300 isolates nationwide for antimicrobial susceptibility testing (AST), with each isolate representing one sample from one farm. While this approach is sufficient for evaluating AMR at national-level, it is inadequate for AMR surveillance at herd-level as one isolate is not sufficient to represent AMR of each farm, leading to errors when implementing antimicrobial stewardship and AMR control measures at the herd-level. This project aimed to address this issue by developing an enhanced AMR surveillance method that combines a multiple samples per herd approach with automated laboratory robotics and selective agars incorporated with antimicrobials to provide accurate large-scale data on the presence, frequency and carriage levels of resistant bacteria within individual farms.

The first step in developing the enhanced method was validating suitable selective agars for enumeration of resistant *E. coli* colonies. Of the three *E. coli* selective agars compared, MacConkey agar was found to be consistently inferior in *E. coli* growth performance than the two modern commercially available *E. coli* selective agars, Brilliance™ *E. coli* and CHROMagar™ ECC. This inferiority in *E. coli* growth performance was consistently seen regardless of whether pure cultures or homogenised faecal samples were used for inoculation

onto *E. coli* selective agar with or without incorporation of antimicrobials. Brilliance™ ESBL and CHROMagar™ ESBL which are two modern commercially available selective agar targeting extended-spectrum cephalosporin (ESC)-resistant *E. coli* were also compared to determine which is better suited for quantifying ESC-resistant *E. coli*. The latter was found to be more suitable compared to the former due to being able to support a wider diversity of ESC-resistant *E. coli* strains.

The chosen selective agars were subsequently applied to the enhanced method to describe the CIA-resistance scenario of Australian pigs in order demonstrate its capability to provide a more accurate and detailed AMR data at the herd, state and national-level. A major finding was the detection of CIA-resistant *E. coli* in Australian pigs. Fluoroquinolone (FQ)-resistant *E. coli* was present among majority of Australian pig farms nationwide, while the presence of ESC-resistant *E. coli* was detected among eight Australian pig farms nationwide, with the former having a higher frequency compared to the latter. However, compared to the commensal *E. coli* population, carriage levels of both resistant *E. coli* were lower, indicating that CIA-resistant *E. coli* has not yet spread throughout the commensal *E. coli* population. When subjected to AST, CIA-resistant *E. coli* harbouring phenotypic resistance towards FQ and ESC was detected but due to the nature of FQ-resistance mechanisms, it has limited clinical relevance. Whole genome sequencing (WGS) was also performed on CIA-resistant *E. coli* which revealed that ST744 and ST4981 are the current dominant FQ-resistant *E. coli* and ESC-resistant *E. coli* sequence types (STs) respectively present among Australian pigs nationwide. Further analysis suggests that both STs were likely introduced into Australian pigs via external sources. Nonetheless, the multiple samples per herd approach and quantitative focus of the enhanced method demonstrated that it is capable of delivering a

more accurate and detailed AMR data at the herd-level compared to established AMR surveillance systems.

The adaptability of the enhanced method towards a different livestock species was demonstrated through the performance of AMR surveillance on ten Australian meat chicken farms. While ESC-resistant *E. coli* was not detected, ciprofloxacin-resistant *E. coli* was detected on all farms, with carriage levels that were lower than commensal *E. coli*. This indicates that FQ-resistant *E. coli* is present among all ten farms but has not yet spread throughout its commensal *E. coli* population. When subjected to AST, only 57.1% of FQ-resistant *E. coli* isolates were multi-class resistant, and that the most common phenotypic resistance profile was one with resistance towards two antimicrobial classes. Though WGS will be conducted to ascertain the genomic characteristics of FQ-resistant *E. coli* isolates in these ten farms, the findings demonstrated that the enhanced method is also capable of delivering the same accurate and detailed AMR data at the flock-level for meat chickens.

In conclusion, the findings demonstrated that the enhanced method is capable of delivering a more accurate and detailed AMR data than established AMR surveillance systems for livestock at all levels of governance, and with different livestock species. This ultimately leads to improved judgements when implementing AMR control strategies as part of biosecurity protocols to prevent further emergence and spread of CIA-resistant *E. coli*. Additionally, it provides further prospects for expanding the application of the enhanced method within the food and public health sectors, with further opportunities for enhancement via the inclusion of data pertaining to antimicrobial use and resistance transmission pathways.

Note on Thesis Layout

This thesis consists of a series of chapters prepared as individual manuscripts for publishing. Chapter 1 is prepared as a scientific literature review that provides an introductory overview of the thesis topic. Chapter 2 has been peer-reviewed and published in a scientific journal, while Chapters 3 to 5 are prepared as fully formed scientific manuscripts for publishing. To maintain formatting consistency throughout this thesis, Chapter 2 have been modified from their original published form.

Other Publications

The following publications arose during my candidature:

1. Robotic Antimicrobial Susceptibility Platform (RASP): A next-generation approach to One Health surveillance of antimicrobial resistance.

   Alec Truswell, Rebecca Abraham, Mark O'Dea, **Zheng Zhou Lee**, Terence Lee, Tanya Laird, John Blinco, Shai Kaplan, John Turnidge, Darren J Trott, David Jordan, Sam Abraham

   Journal of Antimicrobial Chemotherapy
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2. Diversity detected in commensals at host and farm level reveals implications for national antimicrobial resistance surveillance programs.

   Tanya Laird, David Jordan, **Zheng Zhou Lee**, Mark O'Dea, Marc Stegger, Alec Truswell, Shafi Sahibzada, Rebecca Abraham, Sam Abraham

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CHAPTER 1

General Introduction

1.1 The threat of antimicrobial resistance

The introduction of antimicrobials was heralded as one of the most important medical breakthroughs for public health, as the use of antimicrobials as therapeutic agents meant that common infectious diseases which used to have a high mortality rate were easily treated (1). Furthermore, the prophylactic use of antimicrobials also led to the introduction of more invasive and infection-prone surgical procedures which would otherwise lead to a high mortality rate due to infections (1). However, the effectiveness of antimicrobials has been threatened by the emergence of antimicrobial resistance (AMR) due to the widespread and unrestrained use of antimicrobials. In response to this, the World Health Organisation (WHO) has categorised antimicrobials based on their importance to human medicine as a framework for antimicrobial stewardship (the control on how antimicrobials are used) in order to prevent or slow down further emergence and spread of AMR (2). Antimicrobials which are used as the last line of treatment for bacterial infections in humans (fluoroquinolones [FQs], extended-spectrum cephalosporins[ESCs] and carbapenems) are categorised as critically important (2), and the development of resistance towards critically important antimicrobials (CIAs) have become a major source of concern for public health (3). This is further compounded by the fact that the rate of AMR development is faster than the discovery of new effective antimicrobials, prompting fears that we may return to the dark age of the pre-antibiotic era (4). With an estimated 700,000 deaths annually being attributed to resistant bacterial infections, the WHO has listed AMR as a major global health issue of the 21st century (3, 5).

Inappropriate use of antimicrobials is a big factor contributing to the emergence of AMR. In developed countries, excessive prescription leads to unnecessary widespread use of antimicrobials, while the failure to complete recommended doses of antimicrobials due to

insufficient awareness about AMR leads to the presence of antimicrobials at sub-therapeutic levels that are unable to kill pathogens yet exerts selective pressure on them to develop resistance (6). In developing countries, antimicrobials are readily available and easy to purchase without any prescription thus contributing to the widespread and unrestrained use of antimicrobials (6). The inappropriate use of antimicrobials also extends to livestock due to the widespread use of antimicrobials. In the United States (US) alone, it was estimated that approximately 70% of medically important antimicrobials for humans are sold for use to the livestock industry (5). This has led to resistant bacteria also emerging in livestock and making these animals become reservoirs for resistant bacteria and genes.

1.2 The use of antimicrobials in livestock

Antimicrobial uses in livestock are broadly divided into three categories - therapeutic use to treat existing disease conditions, preventative use to prevent future infections and non-therapeutic use as growth promotants (7, 8). Therapeutic treatment of disease follows the same approach as humans or companion animals where antimicrobials are provided after diagnosis. However, in the presence of an infected animal, metaphylactic antimicrobial treatment of the entire herd may be provided at a high dosage for a short period of time to contain and prevent dissemination of disease (9, 10). In contrast, prophylactic treatment provides antimicrobials at subtherapeutic levels for several weeks to the entire herd to prevent future infections (even if no clinical signs are present) particularly during stressful periods where the development of infections are likely to occur (9, 10).

The non-therapeutic use of antimicrobials as growth promotants became popular following the discovery that low doses of antimicrobials such as oxytetracycline and penicillin can lead to enhanced weight gain in animals (9-11). Though the exact mechanism of this effect has yet

to be elucidated, several hypotheses have been proposed which include the stimulation of vitamin and growth factor synthesis, the reduction of specific microflora in the gastrointestinal tract (GIT) and improvement of nutrient absorption (9). The benefits of growth promotants have made its usage a routine practise in livestock throughout many parts of the world especially following the intensification of modern food production (10).

1.3 Development of resistant bacteria in livestock

The method of antimicrobial delivery to livestock is a major factor contributing to the emergence of resistant bacteria in livestock. While antimicrobial treatments can be provided individually through oral or injection methods, the high density of animals within a modernised intensive farm makes such administration laborious and time-consuming, particularly for preventative (metaphylaxis or prophylaxis) and growth promotion treatments where antimicrobials are administered to the entire herd or flock (10). The use of medicated feeds became a solution but the biggest problem attributed to this approach was ensuring that each animal received the appropriate dosage of antimicrobials (10). As feed is often provided ad libitum to animals, animals become more selective during feeding thus leading to the ingestion of antimicrobials at inappropriate dosages (11). This is particularly evident with growth promotion treatments where subtherapeutic concentrations of antimicrobials are fed to livestock continuously throughout their lives. This is further exacerbated by growth promotant antimicrobials being easily accessible without veterinary intervention, leading to untrained farm workers having free access to purchase, mix and administer antimicrobials to feeds with inconsistent dosages (10-12). Nonetheless, the constant exposure of antimicrobials on bacteria within the gastrointestinal tract (GIT) of livestock, particularly at subtherapeutic levels, leads to the development of AMR as the bacteria adapts to its environment to survive.

Mutations. Mutations are the most direct way for bacteria to develop AMR. In the wild, such mutations do occur naturally when bacteria are presented with antimicrobial-secreting organisms (such as the fungus mould from which penicillin was derived) (13). However, as such mutations are often associated with fitness loss, the frequency of resistant bacteria in the wild are low as they are unable to compete against non-resistant bacteria for resources in an antimicrobial-free environment (13, 14). With the advent of unrestrained antimicrobial use in the modern era, what would be a rare occurrence in the wild has become commonplace as the widespread use of antimicrobials, both in the animal and human health sectors, creates multiple niche environments where only resistant bacteria can survive (14). Moreover, the constant presence of antimicrobials also exerts intense selective pressure thereby forcing bacteria to adapt in order to survive, leading to rapid emergence of AMR. An example of this is the emergence of FQ-resistance which is attributed to mutations within the quinolone-resistance determining regions (QRDRs) that arose due to selective pressure from FQ use (15). With FQ being a CIA, this has led to many countries like Australia removing FQ from use in livestock in order to prevent the emergence of FQ-resistance (16).

Horizontal gene transfer. Horizontal gene transfer (HGT) is another mechanism by which bacteria can acquire resistance genes and plays a crucial role in the dissemination of resistance genes particularly between commensal and pathogenic bacteria as it crosses genera and species boundaries (14). Thus, even though commensal bacteria in livestock have limited potential to cause diseases in humans, there is an inherent risk of transfer of resistance genes to pathogenic bacteria via HGT, making resistant commensal bacteria of all species equally concerning to animal and public health.

HGT functions through the transfer of mobile genetic elements (MGEs) such as plasmids, transposons and integrons between bacterial cells. Plasmids are self-replicating circular, double-stranded DNA molecules residing within bacteria, separate from the bacterial chromosomal DNA (17). Within the plasmid genome are backbone genes that are essential for the control of core plasmid functions as well as nonessential accessory genes that can confer potentially beneficial traits to bacteria such as AMR (14, 17). These accessory genes are integrated into the bacterial host chromosome through small mobile elements present on the plasmid (17). In addition, these mobile elements also allow the plasmid to transfer these accessory genes to other plasmids thereby further facilitating the dissemination of resistance genes (17). Specific plasmids known as conjugative plasmids are also able to mediate the transfer of genes (such as resistance genes) between bacteria through a process called conjugation. This process involves the plasmid establishing a link between bacterial cells that are in direct contact with each other via a pilus that allows the transfer of genes between bacteria (17). Many genes conferring resistance to CIAs are well-known to be disseminated via plasmids, with examples being the bla_{CTXM} and $bla_{\text{IMP-4}}$ genes conferring resistance to ESCs and carbapenems respectively (18-20).

Transposons are known as jumping gene systems due to their ability to jump from one site to another both within and between DNA molecules like plasmids or bacterial chromosomes (21). Most transposons jump randomly between sites rather than specific insertion sites (21). Consequently, transposons carrying resistance genes are able to transfer these genes freely either directly to bacteria or indirectly via plasmids (21). Integrons are described as natural gene capture systems as they are capable of acquiring or exchanging gene cassettes (22). Gene cassettes are small, circular DNA molecules containing a single gene that usually codes for products associated with AMR (21). When gene cassettes are integrated into integrons,

they facilitate the dissemination of AMR especially in Gram-negative bacteria or plasmids where integrons are widely found (23). Additionally, they also contribute to the emergence of multi-class resistant (MCR) bacteria as integrons are able to accumulate multiple gene cassettes. An example of this is the detection of MCR *Salmonella enterica* in Australian cats that harboured resistance towards nine antimicrobial classes and heavy metals due to the presence of plasmids with a cassette array composed of plasmid, transposon and integron AMR genes (19).

Co-selection. The mechanism of co-selection facilitates the development of multi-drug resistance whereby the selection of resistance towards one antimicrobial will also lead to the development of resistance to another (24). The two main mechanisms responsible for driving this process are co-resistance and cross-resistance (24). Co-resistance involves two or more unrelated resistance genes which are genetically linked within a mobile genetic element such as plasmids (24). Upon acquiring mobile genetic elements possessing such linkages via HGT, the bacteria gains resistance to multiple antimicrobials present in that genetic linkage (24, 25). Though it is possible that co-resistance can occur through mutations, the mechanism is typically associated with the acquirement of linked resistance genes from mobile genetic elements (25). Interestingly, co-resistance is often seen in resistance towards both heavy metals and antimicrobials where genes conferring resistance towards heavy metals are genetically linked to AMR genes (24). In contrast, cross-resistance involves resistance towards multiple antimicrobials that possess the same mode of action (24). The development of resistance towards one antimicrobial with a particular mode of action results in the bacteria gaining resistance towards any other antimicrobial that also possess the same mode of action (25). Therefore, cross-resistance is generally seen between antimicrobials of the same class as they share similar structures and mode of actions (25). One such example is the development

of vancomycin-resistant *Enterococci* (VRE) due to the use of avoparcin as a growth promotant in livestock as both antimicrobials belong to the same antimicrobial class (26).

1.4 Regulation of antimicrobial use in livestock

With the emergence of AMR in livestock being associated with unrestricted antimicrobial usage, many countries have implemented strict regulations on antimicrobial use in livestock. While regulations on antimicrobial use in livestock differ between countries, the banning of non-therapeutic use of antimicrobials as growth promotants is common in many countries. This began with the Swann Report of 1969 in the United Kingdom (UK), which recognised the possibility that antimicrobial growth promotants contribute to the emergence of AMR (12). In response, the UK banned the use of several antimicrobials including tetracycline and penicillin for use as growth promotants with several other countries including Australia following suit (10). Currently, the European Union has banned all growth promotant antimicrobial use in livestock while in the US all antimicrobials important for human medicine have ceased to be used as growth promotants (9, 27).

Regulation on the therapeutic and preventative uses of antimicrobials in livestock has not been as strict when compared to their use as growth promotants, although veterinary prescriptions are often required as part of regulations for the administration of antimicrobials in livestock for therapeutic and preventative uses. For example, growth promotants have been banned from use in Switzerland since 1999, but the preventative use of antimicrobials in medicated feeds are still allowed through veterinary prescriptions (28). However, prescription guidelines vary widely around the globe for the same antimicrobial (10). For instance, the UK still uses FQs widely as a therapeutic medicated feed in poultry while in the US, FQs were banned from animal use since 2005, and in Australia, FQs were never permitted for use in

livestock (16). The continued use of FQs in some countries is of particular concern due to this antimicrobial class being categorised as a CIA by the WHO (2). As mentioned previously, this categorisation was developed based on their importance to the treatment of bacterial infections in humans, and serves as a guideline for countries to formulate strategies regulating the use of antimicrobials in livestock (2). Part of this strategy includes the development of CIA lists within the national or regional context of a specific country or geographic region (a move encouraged by the WHO) (29). An example of this is the categorisation of streptogramins as a highly important antimicrobial class in the latest iteration of the WHO list as opposed to Australia which categorises it as a CIA due to the antimicrobial pristinamycin (which belongs to the streptogramin class) used as a reserve agent for treating methicillin-resistant *Staphylococcus aureus* (MRSA) infections in Australia (29). Nonetheless, the regulation on antimicrobial use in livestock has played important roles in controlling the frequency of AMR. Success stories of this regulation is seen in Australia where frequency levels of FQ-resistant *Enterobacteriaceae* stayed low due in part to the prevention of FQ use in Australian livestock, while in Denmark, the banning of avoparcin use as a growth promotant led to the reduction in the frequency of VRE among Danish livestock (12, 16, 26).

1.5 Antimicrobial resistance surveillance in livestock

With livestock being potential AMR reservoirs, the inclusion of AMR surveillance to monitor the emergence, frequency and spread of resistant bacteria in livestock forms a key part of AMR control strategies. In recent years, the need for AMR surveillance in livestock has become more pressing due to the detection of CIA-resistant bacteria even in the presence of strict regulation of CIA use (30-32). A prime example of this is in Australia where AMR surveillance in livestock is rarely done due to its unique AMR scenario. In addition to its geographical isolation, Australia has implemented strict biosecurity protocols to prevent the

emergence of CIA-resistance in Australian livestock, which includes strict regulations on the importation of live animals and unprocessed food products into Australia and the use of CIAs in Australian livestock (like the aforementioned prevention of FQ use in Australian livestock) (33-35). Despite these regulations, CIA-resistant *Enterobacteriaceae* have been detected among Australian livestock albeit at a low frequency (18, 36-39), which prompts the need for effective AMR surveillance for early detection of emerging CIA-resistance, regardless of whether CIA is used or not.

Data acquired from AMR surveillance are used to make judgements concerning the implementation of antimicrobial stewardship and AMR control measures in livestock at all levels of governance. However, the effectiveness of such policies are dependent on the quality of the AMR surveillance data which can be affected by a myriad of factors ranging from sampling techniques to the resolution of data (be it phenotypic resistance or genomic data), and even how well data from humans, animals, food and the environment are integrated as espoused by the One Health approach to AMR surveillance (40).

The indicator bacterium *Escherichia coli*. To facilitate easy detection and monitoring of resistance in livestock, *Escherichia coli* has been widely used as an indicator bacterium species by established AMR surveillance systems for livestock due to its ubiquity as a GIT commensal in animals, ease of isolation, and tendency to easily develop or acquire resistance (41). Having a standardised indicator for AMR surveillance in livestock is critical for comparing data between herds and countries. Data on the frequency of resistant *E. coli* can be used to monitor changes in AMR patterns, and predict the emergence of resistance in pathogenic bacteria through the potential transfer of resistance genes via HGT (42).

Additionally, it also assists in identifying potential risks contributing to the development or spread of AMR such as the intensiveness of selective pressure due to antimicrobial use (42).

The tendency to develop and acquire new resistances made *E. coli* one of the most common multi-drug resistant (MDR) bacterium with global reports identifying the presence of MDR *E. coli* with resistance towards CIAs in various livestock species (18, 43-46). While the emergence of CIA-resistant *E. coli* is attributed to the presence of selective pressure due to CIA use, the aforementioned detection of CIA-resistant *E. coli* in the absence of CIA use creates a new dimension to the threat of AMR, as it indicates that regulating the use of antimicrobials is not sufficient to prevent the emergence of CIA-resistance. Theories postulating how CIA-resistant *E. coli* would emerge in the absence of selective pressure point towards external sources introducing CIA-resistant *E. coli* into livestock herds although further investigation is still required to ascertain these sources (36, 47, 48). Nonetheless, this highlights the need for AMR surveillance even in the absence of direct antimicrobial usage, and the use of *E.coli* as an indicator to detect the emergence of CIA-resistance.

International antimicrobial surveillance systems. Throughout the world, many countries have developed their own national AMR surveillance system to counter the threat of AMR. At its core, the monitoring of AMR forms a key part of the surveillance system objective, though many also include the tracking of antimicrobial use as part of this objective. Of these systems, the National Antimicrobial Resistance Monitoring System (NARMS) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) are the two oldest national AMR surveillance systems developed in the US and Denmark respectively (49, 50).

NARMS was developed in 1996 to track resistant enteric bacteria from all levels of the food chain (humans, retail and livestock), and thus relies on the collaboration of three federal agencies – Centre for Disease Control and Prevention (CDC), US Food and Drug Administration (FDA) and the US Department of Agriculture (USDA), together with any participating state and local public health departments (49). Each federal agency is responsible for surveillance of a particular level of the food chain (such as USDA focusing on livestock) (49). As such, it is essential that AMR data can be accurately compared between all three agencies thus solidifying NARMS strong feature in having a robust methodology to standardise sampling and laboratory techniques (8). This ultimately leads into the ability to integrate AMR data for analysis based on the One Health approach.

In 1995, DANMAP was the first national AMR surveillance system to be developed worldwide (50). It is considered one of the most successful national AMR surveillance system due to its successful organisation blueprint where data is coordinated and integrated in a systematic manner at all levels of the food chain (8). This made DANMAP become a model for many countries to develop their own surveillance systems. Instead of having specific federal agencies handle AMR surveillance at specific levels of the food chain, DANMAP sent samples to specific institutions for isolation and antimicrobial susceptibility testing of bacteria (8, 50). Additionally, DANMAP also collects antimicrobial usage data through a national digital database monitoring program called VetStat, which continuously collects data on all prescribed antimicrobials used to treat animals (50). This is further integrated with antimicrobial use data in humans from the Danish Medicines Agency (50). The data of resistant bacteria and antimicrobial use is then consolidated and analysed as part of the One Health approach.

Sample size. Sample size is an important factor in determining the quality of AMR surveillance data as the number of samples should accurately represent the population being surveyed (51). For livestock, established AMR surveillance systems generally sample one isolate from one animal per herd with a maximum of 300 isolates representing 300 herds nationwide. This approach is based on the assumption that the total number of all samples is statistically sufficient to represent AMR at national-level and results are derived through the traditional broth microdilution technique used for evaluating antimicrobial susceptibility, a procedure that is expensive and laborious to set up on a large-scale (52-55). While this approach is sufficient to represent AMR at national-level, one isolate per sample does not accurately represent the AMR status of an entire herd (54, 55). It was concluded by Persoons et al. (2011) that variation in bacterial composition between individuals affects the overall representation of AMR within the herd thus necessitating the collection of multiple samples per herd to accurately evaluate AMR at herd-level (54). While acquiring national-level AMR surveillance data is crucial in guiding broad AMR control policies nationwide, a greater representation of AMR at herd-level is equally critical in developing adaptable AMR control measures tailored to each specific AMR scenario on the farm. However, an increase in sample numbers per herd presents another issue - namely the cost in time, labour and material to process all samples. Therefore, in order to implement a multiple samples per herd approach for AMR surveillance, new techniques for high throughput sample processing at an affordable cost is required. Such implementations would re-invent the methodology of AMR surveillance in livestock, to deliver data at national-level without sacrificing the detailed representation of AMR at herd-level.

Sample type. An ideal sample type for AMR surveillance is one that is easily collected, stored and processed in a standardised manner without affecting the microbiota of the sample

(56). With *E. coli* being used as an indicator bacterium, faeces became the core samples used in established AMR surveillance systems for livestock. Generally, faecal samples are collected after a fresh pass to minimise cross-contamination and to ensure the microbiota of the faeces is not affected by the environment or passage of time (56). However, this can be a laborious process especially when considering the increase in sample numbers for a multiple samples per herd approach for providing detailed representation of AMR at herd-level. Alternative sample types such as swabs can be considered as a way to improve the ease and efficiency for large-scale sample collection. Currently, there are no studies in the literature comparing the performance of swabs and faecal samples in the context of AMR surveillance in livestock. Most studies focused on comparing faecal samples with rectal swabs for the purpose of detecting enteric bacteria for clinical diagnosis (56-61). With rectal swabs requiring the animal to be restrained during sample collection, it would not be feasible for AMR surveillance in livestock as it is equally laborious and dangerous for both the animal and handler. An acceptable compromise for efficient collection would be faecal swabs, as swabs would only be required for insertion into fresh faeces instead of the rectum. Further investigation to assess the feasibility and performance of faecal swabs over faecal samples in the context of AMR surveillance for livestock would thus be required.

Pool samples which are tested as a group rather than individually present another potential method for improving the efficiency of sample collection for AMR surveillance in livestock. Though used for screening diseases with a low frequency, Schmidt et al. (2015) demonstrated that pooled samples are suitable for representing the frequency of AMR at herd-level when combined with real-time polymerase chain reaction (RT-PCR) (62). This was followed up by Clasen et al. (2016) who reported that five individual samples for pooling is sufficient to represent an entire herd when using the same RT-PCR method as Schmidt et al. (2015) (62,

63). Additionally, Clasen et al. (2016) also reported large variation in the presence of bacterial AMR genes between individuals which correlates with conclusions from Persoons et al. (2011) that variations in bacterial composition between individuals does affect the AMR representation of each herd (54, 63). However, one limitation arising from the Clasen et al. (2016) study was that only one pig farm was investigated thus their results may not apply to other pig herds or livestock species (63). Nevertheless, there is potential in combining sample pooling methods with faecal swabs for AMR surveillance in livestock. Though faecal swabs are easier to obtain, a major limitation of this sample type is the varying amounts of faeces swabbed which in turn may affect the number and variety of bacteria seen on cultures. By pooling faecal swab samples, such variation may be eliminated and provides an easy and efficient method for sample collection. Further investigation is thus warranted to assess the viability of using sample pooling methods with faecal swabs for AMR surveillance in livestock.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing (AST) comprises *in vitro* methods used to assess the phenotypic resistance of bacteria towards a specific antimicrobial and is thus a key component of the AMR surveillance process to detect and assess the emergence and frequency of resistance. Disc diffusion, broth microdilution and agar dilution are the three AST techniques used in laboratories, with broth microdilution considered the gold standard technique (Table 1.1) (64). An important aspect of AST is standardisation which ensures that all AST results (be it for clinical diagnosis or AMR surveillance) are comparable across all laboratories nationally and internationally. Such standardisation is facilitated through guidelines set by international reference bodies like the European Committee of Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) especially in regards to how resistance is determined

with each AST technique (65, 66). The existence of standardised guidelines is arguably the most important strength of AST techniques as the methodology of each technique has undergone stringent testing and validation to achieve the most consistent and accurate results.

Table 1.1 General description of each antimicrobial susceptibility testing technique

Technique	Description
Disc Diffusion	1. Agar plates are lawn-inoculated with bacteria and a paper disc infused with a standardised concentration of an antimicrobial is placed onto the plate 2. The antimicrobial will diffuse into the agar to form a concentration gradient around the disc as the plate is being incubated 3. The diameter zone of bacterial growth is measured and resistance determined based on the length of the zone
Broth Microdilution	1. Broth is placed into each well of a microtiter plate 2. Antimicrobial is mixed into the first well with serial dilutions performed to acquire a range of antimicrobial concentrations 3. Bacteria is mixed into each well and the plate incubated 4. Each well is examined for bacterial growth which is indicated by opacity 5. Resistance is determined based on the lowest antimicrobial concentration required to inhibit bacterial growth
Agar Dilution	1. During agar preparation, antimicrobial is mixed with the agar to acquire agar incorporated with the target antimicrobial at a specific concentration 2. The same agar is prepared but with a range of concentrations of the target antimicrobial 3. Bacteria is spot-inoculated onto each agar and incubated 4. Plates are inspected for growth with resistance determined based on the lowest incorporated antimicrobial concentration inhibiting bacterial growth

Currently, CLSI and EUCAST are the two most popular reference bodies used globally. Previously, CLSI, being the first established institute to develop standardised guidelines for AST, was used more prominently but in recent years, many European countries have switched to EUCAST in order to harmonise AMR methodology across the region (67). While AST guidelines between both bodies are the same in concept, differences do exist in methodology that may pose issues when comparing AST results between countries that utilises different reference bodies - examples being discrepancies in clinical breakpoints between both bodies and how these breakpoints are determined (67-69). Clinical breakpoints are antimicrobial concentration thresholds where organisms growing at this threshold and above are considered clinically resistant thus making the antimicrobial clinically ineffective for treatment. This focus on clinical efficacy of antimicrobials has made CLSI appropriate for clinical settings, compared to EUCAST which also has an additional threshold termed the epidemiological cut-off (ECOFF) value (69). The ECOFF value is used to differentiate susceptible wild type populations of organisms from resistant non-wild type populations (69). Under ECOFF threshold, organisms growing above the threshold are considered resistant non-wild type regardless of how large or minute the effect is. This leads to situations where non-wild type organisms may lie below, at or even above the clinical breakpoints (Figure 1.1) (69, 70). Within clinical settings, the ECOFF value would not be suitable as it does not provide information as to whether said organism can resist antimicrobial treatments but in the context of AMR surveillance in livestock, the ECOFF value, especially when combined with clinical breakpoints, provides a spectrum determining how serious the progress of resistance within a herd or flock is (such as whether resistance has only started to emerge or progressed to the point of clinical relevance). However, as the ECOFF value is also utilised by EUCAST to determine clinical breakpoints, this has become a major factor in discrepancies in clinical

breakpoints between both bodies and needs to be taken into account whenever AST data for AMR surveillance is compared (71).

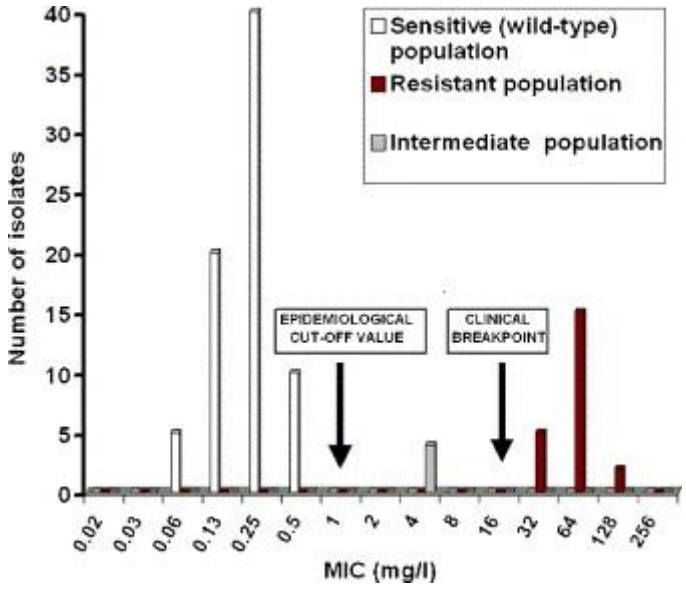

Figure 1.1 Distributions of susceptible and resistant organism populations. Epidemiological cut-off values are used to differentiate susceptible wild type populations from resistant non-wild type populations which may lie below, at or above clinical breakpoints that determine whether a specified antimicrobial is effective for treatment against the organism (70).

AST is expensive and labour intensive to perform which are major factors in the implementation of the one isolate per sample per herd approach used by established AMR surveillance systems for livestock. Consequently, these factors have made performing AMR surveillance in livestock on a large-scale difficult. Within clinical settings, laboratories (particularly those in hospitals) eliminated this issue through the use of automated equipment such as the Vitek 2 System and robotics which allows high throughput processing of bacterial cultures and reading of AST results in a standardised manner with minimum human input (72). Though such equipment was specifically designed for clinical settings, there lies the potential for adapting technology such as robotics for large-scale AMR surveillance in livestock although the cost to design, acquire and optimise one presents a major hurdle in unlocking that potential.

Whole genome sequencing. Whole genome sequencing (WGS) is a relatively new technology that enhances the resolution of AMR data through the acquirement of genomics data, resulting in a combine phenotypic and genotypic mapping of resistance. Phenotypic resistance data alone is only sufficient to provide information regarding the presence of resistance towards antimicrobials, and though crucial in managing further spread of AMR via antimicrobial stewardship, is not capable of elucidating resistance mechanisms or the origin and transmission pathways of resistance. By unfolding the genetic sequence of resistant bacteria, information pertaining to the strain or sequence type (ST) of the bacteria and the presence of AMR genes, virulence genes and plasmids can be acquired (73). The identification of AMR genes allows the identification of resistance mechanisms while virulence genes inform whether resistant commensal bacteria such as *E. coli* would potentially be pathogenic and become a threat to animal and public health (73). Moreover, the identification of bacterial strains or STs and key AMR genes provides the opportunity to use

phylogenetic analysis to map the source of resistance and its transmission pathways which is critical when implementing measures to prevent further emergence and spread of AMR (73). Examples include the discovery on the origin of MRSA (74) and its transmission pathways within UK hospitals (75).

1.6 Quantification of antimicrobial resistance

A key component currently missing in established AMR surveillance systems for livestock is the quantification of resistant bacteria. Without quantitative data, even when resistance is detected, it cannot be determined as to what extent it has spread throughout farms. The lack of quantitative data particularly impacts herd-level AMR surveillance (as there is no unit of inter-farm comparison), and impacts the implementation of herd-level AMR control measures, as the AMR scenario of each farm may vary thereby requiring different protocols for controlling AMR.

Despite the advantages quantitative data brings to AMR surveillance, there are two main reasons for its absence from established AMR surveillance systems for livestock. The first is the amount of labour involved to perform colony enumeration of target bacteria on agar for quantification. As the target bacteria such as *E. coli* is needed to be rapidly and accurately identified on agar, selective agars that target specific bacteria are required. Traditionally, MacConkey (MAC) agar has been used for AMR surveillance to isolate *E. coli* with studies also successfully isolating specific resistant *E. coli* (such as FQ-resistant *E. coli*) by incorporating MAC agar with FQ class antimicrobials (41, 76-78). Though this makes MAC agar incorporated with antimicrobials potentially useful for quantifying resistant *E. coli*, the process involve to acquire countable colonies for enumeration is extremely laborious when performed manually. Moreover, should AMR surveillance for livestock utilise the multiple

samples per herd approach recommended by Persoons et al. (2011) (54) to improve AMR representation at herd-level, it would not be feasible to perform such large-scale quantitative AMR surveillance manually. The second reason is the lack of validation of quantifying resistant bacteria on selective agars incorporated with antimicrobials, with little examination of potential interaction of agar components and antimicrobial efficacy. Currently, the validation of techniques used in AMR surveillance is focused on phenotypic AST to detect the presence of resistance rather than quantification. In particular, the validation of antimicrobial efficacy when incorporated into selective agars is essential as any changes to efficacy may impact colony growth and thus the number of colonies for enumeration, leading to inaccurate quantitative results.

1.7 Limitation of established antimicrobial surveillance systems

Currently, there are three main components absent in established AMR surveillance systems for livestock. Firstly, the single isolate per sample per herd approach is inadequate in representing AMR scenarios at the herd-level. Secondly, this approach also reduces the probability of detecting resistance with a low frequency, which is crucial when detecting early emergence of CIA-resistance particularly in the absence of CIA use. Lastly, the lack of quantitative data means that it is impossible to measure how extensive resistance has spread throughout the farms.

A multiple samples per herd approach would be ideal in addressing the first two missing components to provide greater representation of the AMR scenario in each farm while improving the probability of detecting emerging resistance. However, as mentioned

previously, the increase in sample numbers makes it expensive and laborious to perform AMR surveillance on a large-scale. The application of robotics with selective agars incorporated with antimicrobials is ideal in solving this issue. By designing a robot capable of high throughput sample processing, agar inoculation, and rapid identification of target resistant bacteria on selective agar incorporated with antimicrobials for quantification and isolation, it creates the potential to perform automated large-scale quantitative AMR surveillance with minimum human input (79). While the initial cost to design and set up the robot would be high, this can be offset by the long-term reduction in labour cost while providing a means to acquire a more accurate and detailed AMR data at the herd-level.

1.8 Project aims

The focus of this project is thus to develop an enhanced AMR surveillance method that centres on acquiring quantitative data through a combination of a multiple samples per herd approach with robotics and selective agar incorporated with antimicrobials. In developing this enhanced method, four aims were created. Chapter 2 addresses the first aim which is to validate suitable selective agars targeting the indicator bacterium *E. coli* with the best *E. coli* growth performance for quantifying *E. coli*. Next, Chapter 3 addresses the second aim to demonstrate the capability of the enhanced method to provide a more accurate and detailed AMR data compared to established AMR surveillance methods at the herd-level. This was followed-up in Chapter 4 and 5 that ensures the enhanced method works at all levels of governance with different livestock species. Given that Australian livestock were used as models for this project, the final aim is to investigate the current CIA-resistance scenario of Australian livestock (which comprises Chapters 3 to 5).

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CHAPTER 2

Validation of selective agars for detection and quantification of *Escherichia coli* resistant to critically important antimicrobials

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2.1 ABSTRACT

Success in the global fight against antimicrobial resistance (AMR) is likely to improve if surveillance can be performed on an epidemiological scale. An approach based on agars incorporated with antimicrobials has enormous potential to achieve this. However, there is a need to identify the combinations of selective agars and key antimicrobials yielding the most accurate counts of susceptible and resistant organisms. A series of experiments involving 1,202 plates identified the best candidate-combinations from six commercially available agars and five antimicrobials using 18 *Escherichia coli* strains as either pure cultures or inocula-spiked faeces. The effect of various design factors on colony counts were analysed in generalised linear models. Without antimicrobials, Brilliance™ *E. coli* (Brilliance) and CHROMagar™ ECC (CHROMagar) agars yielded 28.9% and 23.5% more colonies than MacConkey agar. The order of superiority of agars remained unchanged when faecal samples with and without spiking of resistant *E. coli* were inoculated onto agars with or without specific antimicrobials. When incorporating antimicrobials at varying concentrations, it was revealed that ampicillin, tetracycline and ciprofloxacin are suitable for incorporation into Brilliance and CHROMagar agars at all defined concentrations. Gentamicin was only suitable for incorporation at 8 and 16 µg/mL while ceftiofur was not suitable for incorporation. CHROMagar™ ESBL agar supported growth of a wider diversity of extended-spectrum cephalosporin-resistant *E. coli*. The findings demonstrate the potential for agars incorporated with antimicrobials to be combined with laboratory-based robotics to deliver AMR surveillance on a vast scale with greater sensitivity of detection and strategic relevance.

2.2 INTRODUCTION

Antimicrobial resistance (AMR) has been identified as one of the most serious threats to animal and human health in the current era (1). A key component for controlling AMR is the conduct of surveillance to inform the frequency of resistance and spread of resistant bacteria. The livestock sector has become a focus for surveillance because of the potential for AMR to transfer to humans along the food chain. Food products with a propensity to be contaminated with animal microflora such as ground meat are increasingly included in surveillance because of the risk of zoonotic pathogens undergoing selection for resistance in the animal gut or acquiring resistance via horizontal gene transfer (2-5). In both food and livestock, commensals such as *Escherichia coli* have been widely exploited for use in AMR surveillance since they readily develop resistance during *in-vivo* exposure to antimicrobials and are easily isolated as a ubiquitous component of the gut microflora (6). A barrier for improving surveillance in food and livestock is that the broth microdilution technique for evaluating antimicrobial susceptibility of bacterial colonies, as recommended by international reference organisations, are expensive and labour intensive though the process has adapted well to a clinical context (7, 8). In national surveillance programs (such as the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme), sampling must typically be constrained due to the aforementioned drawbacks of the broth microdilution technique (9). For example, fewer than 300 commensal *E. coli* isolates are obtained from the same number of herds or flocks of a given animal species in a year with food product surveys similarly affected (9). The inferences that can be drawn from surveillance results are thus often constrained in scope and frequently fail to support decision making at the coalface of animal and food production where changes to production management to control AMR arguably stands to have the greatest benefit. Therefore, an enhanced approach is needed that

can affordably assess a substantially larger number of isolates and samples within an authoritative design to produce evidence on an epidemiological rather than clinical scale.

One way to achieve the scale described above is through large-scale enumeration of resistant *E. coli* from food or faecal samples using a process akin to agar dilution technique for antimicrobial susceptibility testing (AST). Here, plating of diluted samples onto agars incorporated with antimicrobials is the foundation which can be further automated using laboratory-based robotics (10). However, conventional solid agar used for AST such as Mueller-Hinton agar (MHA) or traditional selective agar such as MacConkey (MAC) agar are unsuitable because they make it impossible to identify the target bacteria based solely on colony morphology, and especially in the case of MHA, the growth of non-target bacteria is not adequately suppressed. Fortunately, modern selective agars are now commercially available for isolating *E. coli*. These agars suppress most non-target organisms and achieve accurate colony identification using a chromogenic reaction (11). One key issue in the use of these agars is whether or not the activity of antimicrobials that are incorporated is compromised by other agar components leading to inaccurate counts of resistant *E. coli*. A second key issue is whether or not the plating of diluted samples containing all of the microbial genera that are naturally occurring in the original samples (faeces or food) interferes with the AST of the target organism (in this case commensal *E. coli*). Previous studies have shown that MAC agar incorporated with ciprofloxacin is able to selectively isolate ciprofloxacin-resistant *E. coli* (12-14), although tetracycline cannot be used with MAC in this way due to interference in antimicrobial activity by divalent cations (calcium and magnesium salts are an integral component of MAC agar) (15-19). Similarly, there is a need to evaluate the suitability of commercial selective agars targeting extended-spectrum

cephalosporin (ESC)-resistant *E. coli* such as Brilliance™ ESBL (Brilliance ESBL) and CHROMagar™ ESBL (CHROMagar ESBL) agars for detection and enumeration under the same conditions.

This study aims to address these issues through three objectives. The first is to compare colony counts of *E. coli* on selective agars (Brilliance™ *E. coli* [Brilliance] and CHROMagar™ ECC [CHROMagar] agars) to assess which has the best *E. coli* growth performance for accurate enumeration of *E. coli* colonies. Secondly, to identify which combination of specific concentrations of antimicrobials (ampicillin, tetracycline, gentamicin, ciprofloxacin and ceftiofur) and selective agars achieve the most accurate enumeration of resistant *E. coli* (this includes equivalent evaluation of commercial agars for isolation of ESC-resistant *E. coli*) via colony counts. Thirdly, to assess whether the ability to detect and quantify resistance is reduced when the target organisms are co-mingled with natural flora present in faecal samples. Together, the findings serve to identify the optimal selective agar for achieving large-scale detection and quantification of resistant *E. coli* in samples from the food chain using laboratory robotics.

2.3 MATERIALS AND METHODS

All agars used in this study were commercially available and were used as directed by the manufacturer with the exception of the incorporation of additional antimicrobials as demanded by study design.

Experiment A: Comparison of *E. coli* selective agars with and without incorporation of antimicrobials. The performance of growing *E. coli* on three *E. coli* selective agars and a fourth non-selective control agar with and without incorporation of antimicrobials were compared using pure cultures of diverse *E. coli* strains with an overview of the general procedure shown in Figure 2.1a. All agars without antimicrobials were purchased directly from suppliers. The three selective agars used were MAC (Edwards Group), Brilliance (Thermo Fisher Scientific) and CHROMagar (MicroMedia, Edwards Group) agars. MHA (Edwards Group) was used as the fourth agar and was chosen for comparison due to its status as the gold standard non-selective agar for routine AST (20). The same four agars incorporated with antimicrobials were prepared in-house using the agar dilution technique as per manufacturer instructions. Both Mueller-Hinton broth powder (Oxoid, Thermo Fisher Scientific) and agar No. 1 powder (Oxoid, Thermo Fisher Scientific) were used to prepare MHA. MacConkey No. 3 powder (Oxoid, Thermo Fisher Scientific) was used to prepare MAC agar. Brilliance agar was prepared using Brilliance™ *E. coli*/coliform selective medium powder (Oxoid, Thermo Fisher Scientific) while *E. coli*-coliforms chromogenic medium (Conda, Edwards Group) was used to prepare CHROMagar agar. The antimicrobials selected for incorporation into agars were ampicillin, tetracycline, gentamicin, ciprofloxacin and ceftiofur (from the penicillin, tetracycline, aminoglycoside, fluoroquinolone [FQ] and third-generation cephalosporin families respectively). These were included due to their importance in the livestock and public health sectors (particularly ciprofloxacin and ceftiofur which are CIAs for human medicine) and thus often included in AMR surveillance programs involving livestock and food products (3, 5, 21-24). All antimicrobial stocks were prepared using antimicrobial powders (Sigma-Aldrich) and stored following Clinical and Laboratory Standards Institute (CLSI) guidelines (20). All stocks were used within the shelf life detailed by the manufacturer. Prior to pouring into Petri dishes, antimicrobials were added to sterilised

agars after being cooled in a 60 ^{o}C water bath, to obtain specific concentrations for each respective antimicrobial. Three concentrations were chosen for ampicillin (8, 16 and 32 µg/mL), tetracycline (4, 8 and 16 µg/mL) and ciprofloxacin (1, 2 and 4 µg/mL) while four were chosen for gentamicin (2, 4, 8 and 16 µg/mL) and ceftiofur (1, 2, 4 and 8 µg/mL). All concentrations were chosen to cover the clinical breakpoints for *E. coli* as listed by CLSI with the epidemiological cut-off points (ECOFF) listed by the European Committee of Antimicrobial Susceptibility Testing (EUCAST) covered for ampicillin, tetracycline, gentamicin and ceftiofur (25, 26). After the addition of antimicrobials, 20 mL of the agar mixture was poured into 90 mm diameter circular Petri dishes and left to solidify under a laminar flow hood. All agars incorporated with antimicrobials were stored in the dark at 4 ^{o}C and used within two weeks of preparation.

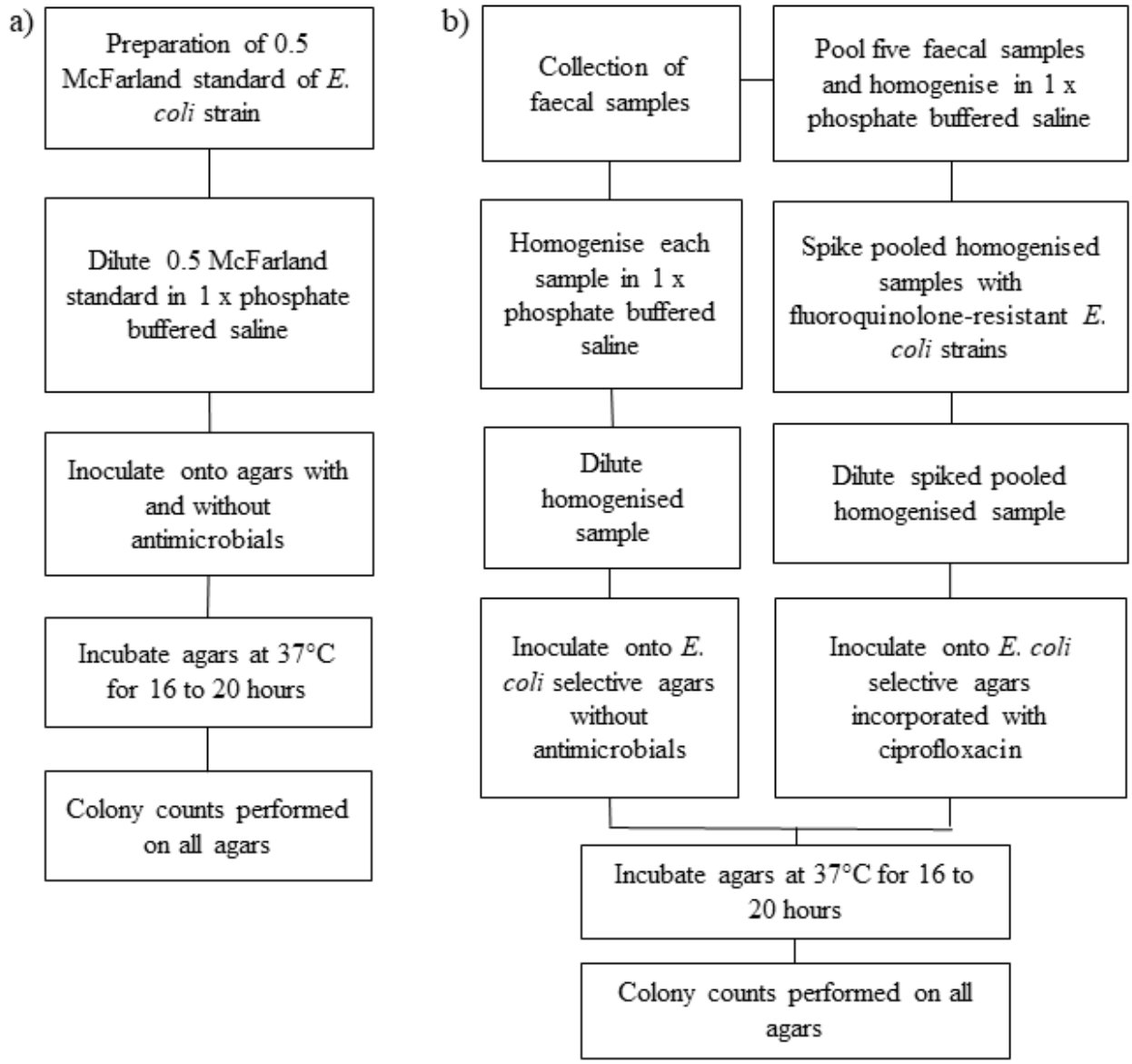

Figure 2.1 Overview of the general procedure for Experiment A. Figure 2.1a shows the procedure using pure cultures of *Escherichia coli* strains while Figure 2.1b shows the procedure using homogenised faecal samples with and without spiking of fluoroquinolone-resistant *E. coli* strains.

Eight *E. coli* strains were chosen with ATCC 25922 included as the quality control strain while the remaining seven strains were *E. coli* isolated from different animal species. SA44 was isolated from pigs (27), SA1000, SA1001 and SA1002 were isolated from Australian Silver Gulls (28), and SA1003, SA1004 and SA1005 were archival in-house strains (Table 2.1 - Supplementary). The rationale for selection of these strains was to achieve diversity in origin to capture variations potentially present in wild type populations of *E. coli*. Prior to the commencement of the experiment, minimal inhibitory concentration (MIC) testing using the broth microdilution method was performed on all *E. coli* strains according to CLSI guidelines to obtain the phenotypic resistance profile of each strain (20) (Table 2.1 - Supplementary). All growth observations on agars incorporated with antimicrobials were compared to the phenotypic resistance profile shown for each strain in order to determine any unexpected absence or presence of *E. coli* growth. After overnight growth on Columbia sheep blood agar (Edwards Group), a suspension of each *E. coli* strain meeting the 0.5 McFarland standard was prepared using a nephelometer (Sensititre). Each standardised inoculum underwent 10-fold serial dilution to 10^{-5} in sterile 1 x phosphate buffered saline. Inoculation was performed by dispensing 80 µL of the 10^{-5} inoculum onto agar without antimicrobials and spread evenly across the agar surface using a sterile loop. Inoculation on agars without antimicrobials was repeated for a total of five replicates per combination of agar and strain while inoculation on agars incorporated with antimicrobials was repeated for a total of two replicates per combination of agar, strain and antimicrobial concentration. All agars were incubated between 16 to 20 hours at 37 ^{o}C. Presumptive identification of *E. coli* on Brilliance and CHROMagar agars was performed based on colony colour as detailed by the manufacturer. For MAC agar, pink colonies were presumed to be *E. coli* due to most *E. coli* strains being known to be lactose fermenters. Being a non-selective agar, *E. coli* colonies on MHA appear colourless.

Homogenised bovine faecal samples were used as field samples to verify the performance for growing *E. coli* on the same three *E. coli* selective agars without antimicrobials with an overview of the general procedure shown in Figure 2.1b. All agars without antimicrobials were purchased from the same suppliers described above. Twenty bovine faecal samples from the Murdoch University farm were sampled. All faecal samples were collected from fresh faecal piles and processed on the same day of collection. Approximately 2 g of each faecal sample was homogenised for 30 seconds in 18 mL of sterile 1x phosphate buffered saline (PBS) using a BagMixer® 400 P laboratory blender (Interscience, Edwards Group). This was repeated two more times to obtain a total of three replicates per sample. The homogenised mixture of each replicate underwent a 10-fold serial dilution and 80 µL of each 10-fold dilution was inoculated onto each agar and spread evenly across the agar surface using a sterile loop. The procedure for agar incubation and presumptive identification of *E. coli* on agar were the same described above for agars without antimicrobials.

Homogenised bovine faecal samples spiked with FQ-resistant *E. coli* strains (Table 2.2 - Supplementary) was used as field samples to further evaluate the performance for growing FQ-resistant *E. coli* on the same three *E. coli* selective agars incorporated with 4 µg/mL of ciprofloxacin with an overview of the general procedure shown in Figure 2.1b. All agars were prepared in the same manner described above for agars incorporated with antimicrobials. A ST131 and ST744 *E. coli* strain isolated from Australian Silver Gulls was chosen for inoculation into faecal samples due to their ubiquity as FQ-resistant *E. coli* strains internationally in both humans and animals (28-32). The first ten bovine faecal samples used previously were chosen for pooling. Each pooled sample consists of five individual samples

to form a total of two pooled samples. For each pooled sample, approximately 2 g of each individual faecal sample (total of approximately 10 g) was homogenised for 30 seconds in 90 mL of sterile 1x phosphate buffered saline (PBS) using a BagMixer® 400 P laboratory blender (Interscience, Edwards Group). This was repeated two more times to obtain a total of three replicates per pool sample. After overnight growth on Columbia sheep blood agar (Edwards Group), a suspension of each *E. coli* strain meeting the 0.5 McFarland standard was prepared using a nephelometer (Sensititre) and inoculated into the homogenised mixture of each replicate to obtain bacterial concentrations of 10^3, 10^5 and 10^7 colony forming units per gram (CFU/g). Mixtures containing 10^5 and 10^7 CFU/g were serially diluted to 10^{-1} and 10^{-3} dilution factor respectively. 80 µL of 10^3, 10^5 and 10^7 at neat, 10^{-1} and 10^{-3} dilution factor respectively were inoculated onto each agar and spread evenly across the agar surface using a sterile loop. The procedure for agar incubation and presumptive identification of *E. coli* on agar were the same as described above for agars incorporated with antimicrobials. ATCC 25922 was also inoculated onto each agar as quality control.

Experiment B: Comparison of ESC-resistant *E. coli* selective agars. The performance of growing ESC-resistant *E. coli* on two ESC-resistant *E. coli* selective agars were compared using pure cultures of diverse ESC-resistant *E. coli* strains (Table 2.2 - Supplementary) with an overview of the general procedure shown in Figure 2.2a. All agars were purchased directly from suppliers. Brilliance ESBL (Thermo Fisher Scientific) and CHROMagar ESBL (MicroMedia, Edwards Group) agars were the two ESC-resistant *E. coli* selective agars while MHA (Thermo Fisher Scientific) was selected as the non-selective agar. The supplier for MHA in Experiment B differed from Experiment A, however the formulation of the agar was the same. Ten ESC-resistant *E. coli* strains were chosen with each strain harbouring a

different gene conferring resistance to ESCs in order to encompass the wide genotypic variations present in ESC-resistant *E. coli* strains (Table 2.2 - Supplementary). SA44 and SA1001 were the only two strains from Experiment A included in Experiment B (Table 2.2 - Supplementary). Of the remaining eight strains, SA27 was isolated from pigs (29) while SA1074, SA1075, SA1076, SA1077, SA1078, SA1079 and SA1080 were isolated from Australian Silver Gulls (28) (Table 2.2 - Supplementary). The procedure for culturing ESC-resistant *E. coli* strains, McFarland standard preparation, agar inoculation (including replicate numbers) and incubation, and presumptive identification of *E. coli* on MHA were the same as Experiment A using pure cultures of *E. coli* strains. Presumptive identification of ESC-resistant *E. coli* on Brilliance ESBL and CHROMagar ESBL agars were performed based on colony colour detailed by the manufacturer. ATCC 25922 was also inoculated onto each agar as quality control.

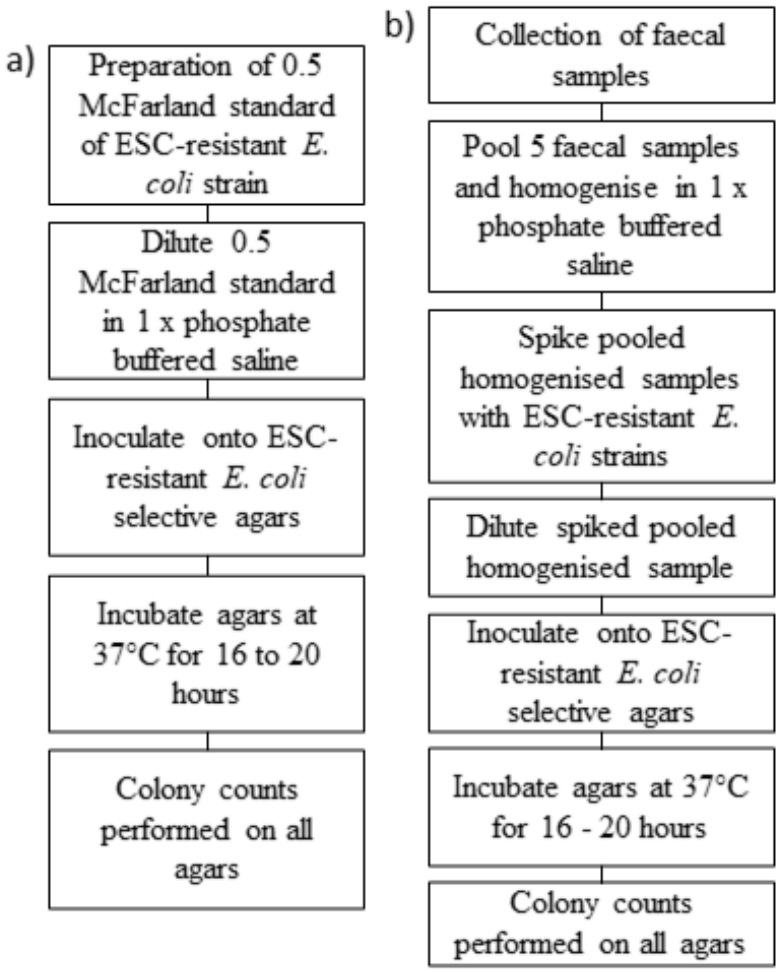

Figure 2.2 Overview of the general procedure for Experiment B. Figure 2.2a shows the procedure using pure cultures of extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* strains while Figure 2.2b shows the procedure using homogenised faecal samples spiked with ESC-resistant *E. coli* strains.

Homogenised bovine faecal samples spiked with ESC-resistant *E. coli* strains (Table 2.2 - Supplementary) was used as field samples to verify the performance for growing ESC-resistant *E. coli* on the same two ESC-resistant *E. coli* selective agars with an overview of the general procedure shown in Figure 2.2b. Ten ESC-resistant *E. coli* strains were also chosen with nine strains, SA27, SA44, SA1001, SA1074, SA1075, SA1076, SA1077, SA1079 and SA1080, being the same strains described above while the last strain, SA1083, was another strain previously isolated from Australian Silver Gulls (28) (Table 2.2 - Supplementary). The first five bovine faecal samples used previously in Experiment A were chosen for pooling. The procedure for pooling, strain inoculation into homogenised faecal mixture, agar inoculation (including replicate numbers) and incubation were the same as Experiment A when using homogenised bovine faecal samples spiked with FQ-resistant *E. coli* strains on agars incorporated with ciprofloxacin with the exception that only Brilliance ESBL and CHROMagar ESBL agars were used. Presumptive identification of ESC-resistant *E. coli* on agar was the same as described above. ATCC 25922 was also inoculated onto each agar as quality control.

Statistical analysis. Statistical analysis used the linear model framework in Stata version 16.0 (Stata Corporation, TX, USA). All analyses were fixed effect models with the count of *E. coli* colonies on each plate as the outcome. Factors in each model were determined by the design of each experiment and included type of agar, strain of *E. coli*, concentration of antimicrobial and their interactions. The results were derived as estimates of marginal effects and expressed (in text and figures) as the mean effect of each combination of agar and antimicrobial concentration adjusted for *E. coli* strains used in the particular experiment and interaction terms. For experiments based on pure cultures of *E. coli* strains, a model was

constructed for agars without antimicrobials, and one model for each antimicrobial when incorporated into agars. In the latter case, only *E. coli* strains resistant to the antimicrobial being evaluated was included in the linear model. For experiments based on faecal samples spiked with a mixture of *E. coli* strains, the analysis was similar although the factor representing *E. coli* strain was not required.

2.4 RESULTS

Experiment A: Comparison of *E. coli* growth on commercial *E. coli* selective agars. Three selective agars and one non-selective agar without incorporation of antimicrobials were compared for the ability to support growth of diverse *E. coli* strains (with and without resistance to various antimicrobials: Table 2.1 - Supplementary). All *E. coli* strains grew on agar without antimicrobials. Agar had a highly significant effect ($P<0.01$) on colony growth with the order of superiority being Brilliance agar (mean of 78.9 colonies per plate), CHROMagar agar (mean of 74.7 colonies per plate), MHA (mean of 60 colonies per plate) and MAC agar (mean of 59 colonies per plate) (Figure 2.3). However, though strain did have a significant effect on colony counts ($P<0.001$), it did not change the above order of superiority of agars for any strain (Figure 2.1 - Supplementary). In summary, *E. coli* counts on Brilliance, CHROMagar and MHA were on average 28.9%, 23.5% and 1.68% respectively higher than MAC agar (the worst performing).

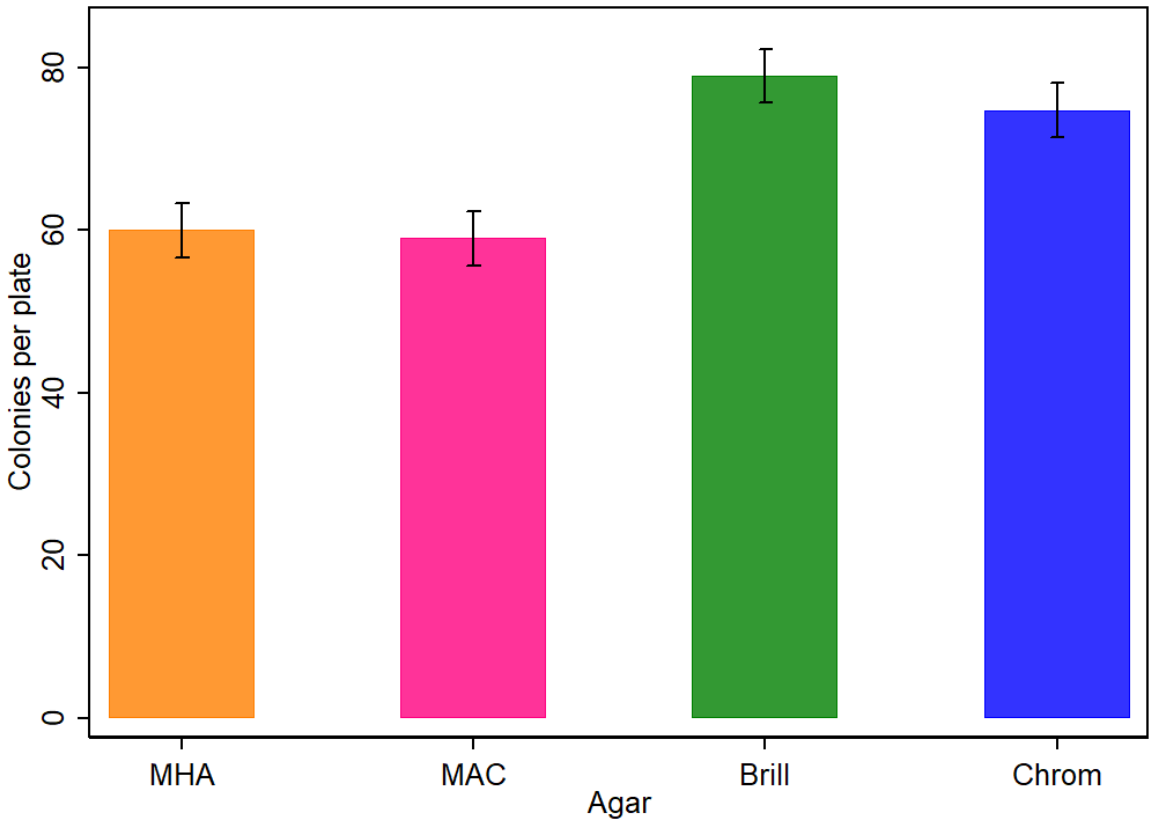

Figure 2.3 Comparisons of *Escherichia coli* growth performance (mean colony counts per plate ± se) on three *E. coli* selective agar and Mueller-Hinton agar (all without antimicrobials) (total number of plates = 160). Standardised inoculum across all agars consisted of diluted pure cultures of diverse *E. coli* strains. Means were calculated as marginal effects from linear model analysis. Key: MHA - Mueller-Hinton agar, MAC - MacConkey agar, Brill - Brilliance™ *E. coli* agar, Chrom - CHROMagar™ ECC agar.

All *E. coli* strains susceptible to ampicillin, tetracycline, ciprofloxacin and ceftiofur did not grow on agars with the corresponding incorporated antimicrobial (at any concentration). However, *E. coli* strains susceptible to gentamicin grew on MAC (2 and 4 µg/mL), Brilliance (2 µg/mL) and CHROMagar (2 µg/mL) agars incorporated with gentamicin. All *E. coli* strains resistant to ampicillin, tetracycline and ciprofloxacin grew on all agars with the corresponding incorporated antimicrobial (at any concentration). SA1001 was the only gentamicin-resistant *E. coli* strain that grew on all agars incorporated with gentamicin at all concentrations while growth of SA44 (also resistant to gentamicin) was not observed on MHA incorporated with 8 and 16 µg/mL of gentamicin. Ceftiofur-resistant *E. coli* strains grew on MAC agar when incorporated with ceftiofur (at any concentration). In contrast, growth was inconsistent but overall reduced on Brilliance and CHROMagar agars when incorporated ceftiofur concentrations climbed above 1 µg/mL (Figure 2.2 - Supplementary).

Separate linear models were constructed for each antimicrobial used. As with agar without antimicrobials, Brilliance and CHROMagar agars performed consistently better than MAC agar (Figure 2.4). This includes Brilliance and CHROMagar agars incorporated with ceftiofur which was superior to MAC agar incorporated with ceftiofur (Figure 2.4). Antimicrobial concentration was found to have a significant effect for all antimicrobials tested ($P<0.001$). Agar had a significant effect on all antimicrobials except tetracycline ($P<0.05$) and strain had significant effects on all except tetracycline and gentamicin ($P<0.01$). Significant interaction effects between strain and agar were found for tetracycline, gentamicin and ceftiofur ($P<0.05$), between agar and antimicrobial concentration for tetracycline ($P<0.01$) and ceftiofur ($P<0.001$), between strain and antimicrobial concentration for tetracycline and gentamicin ($P<0.05$) and between all three factors for gentamicin ($P<0.01$).

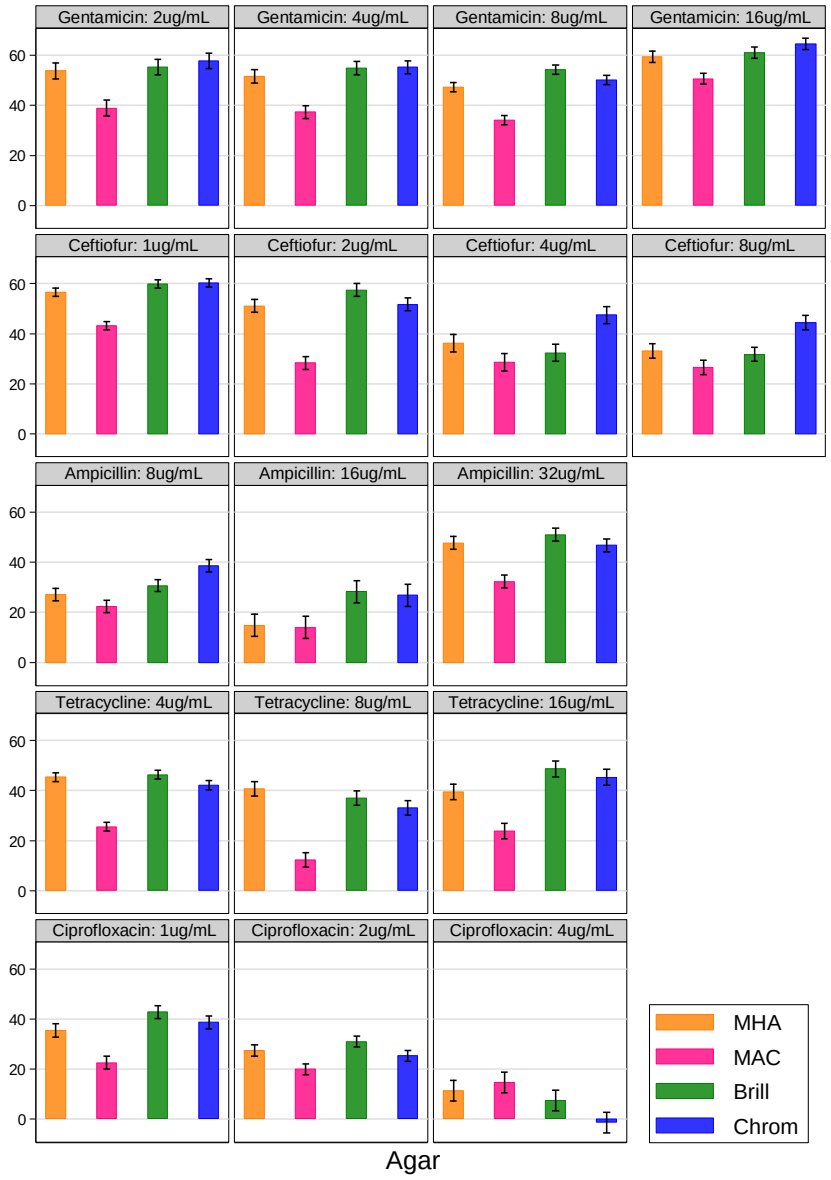

Figure 2.4 Comparisons of *Escherichia coli* growth performance (mean colony counts per plate ± se) on three *E. coli* selective agars and Mueller-Hinton agar each incorporated with ampicillin, tetracycline, gentamicin, ciprofloxacin or ceftiofur at three or four concentrations (total number of plates = 424). Standardised inoculum across all agars consisted of diluted pure cultures of diverse *E. coli* strains resistant to each antimicrobial. Means were calculated as marginal effects from linear model analysis. Key: MHA - Mueller-Hinton agar, MAC - MacConkey agar, Brill - Brilliance™ *E. coli* agar, Chrom - CHROMagar™ ECC agar.

Finally, the three *E. coli* selective agars with and without incorporation of antimicrobials was further tested using homogenised bovine faecal samples (with and without spiking of two FQ-resistant *E. coli* strains: Table 2.2 - Supplementary). For agars without antimicrobials, the order of superiority was CHROMagar (mean of 35.6 colonies per plate), Brilliance (mean of 34.2 colonies per plate) and MAC agars (mean of 29.1 colonies per plate) (Table 2.1). For agars incorporated with ciprofloxacin, growth of FQ-resistant *E. coli* strains was observed on all agars regardless of bacterial concentration and the order of superiority was Brilliance (mean of 32.8 colonies per plate), CHROMagar (mean of 28.3 colonies per plate) and MAC (mean of 22.8 colonies per plate) agars (Table 2.1). In this model, agar ($P<0.001$), strain ($P<0.05$), bacterial concentration ($P<0.001$) and interactions between agar and bacterial concentration had significant effects ($P<0.001$).

Table 2.1 Comparisons of wild type or endogenous *Escherichia coli* and fluoroquinolone-resistant *E. coli* growth performance (mean colony counts per plate) on *E. coli* selective agar with and without incorporation of ciprofloxacin (total number of plates = 288). Standardised inoculum consisted of homogenised bovine faecal samples with and without spiking of fluoroquinolone-resistant *E. coli* strains. Key: MAC - MacConkey agar, Brilliance - Brilliance™ *E. coli* agar, CHROMagar - CHROMagar™ ECC agar.

	MAC		**Brilliance**		**CHROMagar**	
Homogenised bovine faecal samples	**Without antimicrobials**	**With ciprofloxacin**	**Without antimicrobials**	**With ciprofloxacin**	**Without antimicrobials**	**With ciprofloxacin**
Without spiking[a]	29.1	-	34.2	-	35.6	-
Spiked with fluoroquinolone-resistant *E. coli* strains[b]	-	22.8	-	28.3	-	32.8

[a] Samples were not spiked with fluoroquinolone-resistant *E. coli* and thus were only inoculated onto agars without ciprofloxacin

[b] Samples were spiked with fluoroquinolone-resistant *E. coli* and were only inoculated onto agars incorporated with ciprofloxacin

Experiment B: Comparison of ESC-resistant *E. coli* growth on commercial ESC-resistant *E. coli* selective agars. Two ESC-resistant *E. coli* selective agars (Brilliance ESBL and CHROMagar ESBL agars) were compared for the ability to support growth of diverse ESC-resistant *E. coli* strains (Table 2.2 - Supplementary). The non-selective MHA without antimicrobials was used as a control agar. All ESC-resistant *E. coli* strains grew on all agars with the exception of SA27 which did not grow on Brilliance ESBL agar. CHROMagar ESBL agar (mean of 48.24 colonies per plate) best supported growth followed by MHA (mean of 42.72 colonies per plate) and Brilliance ESBL agar (mean of 28.74 colonies per plate) (Figure 2.5) and this order of superiority was also observed on each ESC-resistant *E. coli* strain (Figure 2.3 - Supplementary). In this model, all factors and their associated interactions ($P<0.001$) had significant effects on colony counts.

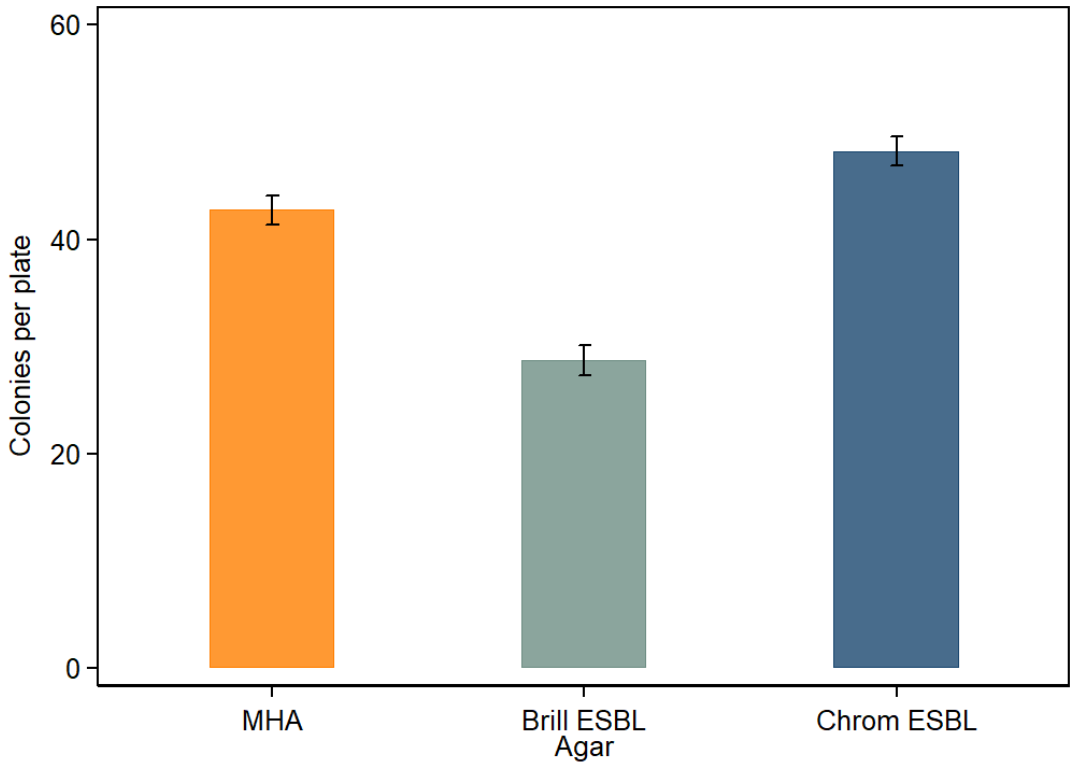

Figure 2.5 Comparisons of extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* growth performance (mean colony counts per plate ± se) on two ESC-resistant *E. coli* selective agars with Mueller-Hinton agar (without antimicrobials) present as a control agar (total number of plates = 150). Standardised inoculum across all agars consisted of diluted pure cultures of diverse ESC-resistant *E. coli* strains. Means were calculated as marginal effects from linear model analysis. Key: MHA - Mueller-Hinton agar, Brill ESBL - Brilliance™ ESBL agar, Chrom ESBL - CHROMagar™ ESBL agar.

Finally, Brilliance ESBL and CHROMagar ESBL agars were further tested using homogenised bovine faecal samples spiked with ten ESC-resistant *E. coli* strains (Table 2.2 - Supplementary). Brilliance ESBL agar (mean of 24.3 colonies per plate) was found to be superior to CHROMagar ESBL agar (mean of 14.9 colonies per plate) (Figure 2.6) with the same superiority order observed on each ESC-resistant *E. coli* strain (Figure 2.4 - Supplementary). The only exception was SA27 which did not grow on Brilliance ESBL agar regardless of bacterial concentration. All factors including associated interactions had significant effects ($P<0.001$) on colony counts.

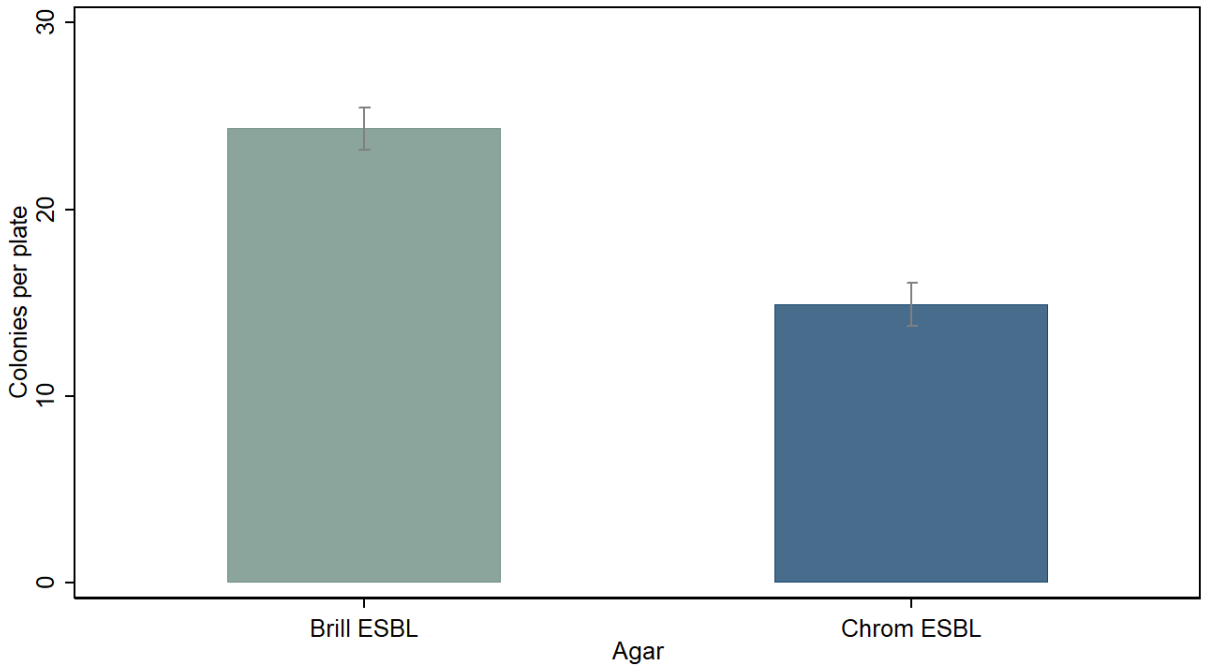

Figure 2.6 Comparisons of extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* growth performance (mean colony counts per plate ± se) on two ESC-resistant *E. coli* selective agars (total number of plates = 180). Standardised inoculum across all agars consisted of homogenised bovine faecal samples spiked with diverse ESC-resistant *E. coli* strains. Means were calculated as marginal effects from linear model analysis. Key: Brill ESBL - Brilliance™ ESBL agar, Chrom ESBL - CHROMagar™ ESBL agar.

2.5 DISCUSSION

AMR surveillance in livestock and food products is a critical tool for progressive antimicrobial stewardship, prevention of AMR spread and the preservation of effective antimicrobials. Through the combination of high-throughput robotics with selective agar incorporated with the antimicrobial of interest, it is possible to quantify carriage levels and frequency of resistance. With *E. coli* being used as a common indicator bacterium in AMR surveillance systems (6), this study aimed to identify the optimal selective agar and antimicrobial concentrations for quantifying populations of resistant *E. coli* for AMR surveillance in livestock.

In this study, three selective agars were tested (MAC, Brilliance and CHROMagar agars). Despite the presence of other experimental factors and interactions significantly affecting colony counts, both Brilliance and CHROMagar agars were comparable in performance and consistently superior to MAC agar in all situations (pure cultures, faecal samples and faecal samples spiked with FQ-resistant *E. coli* strains) as demonstrated through a higher number of *E. coli* colonies. The superior growth performance on Brilliance and CHROMagar agars can be attributed to the basic function and design of the agar. Both selective agars were specifically formulated for growing coliform bacteria and while the exact ingredients within the selective mix of both agars are undisclosed by the manufacturer, there may be components that provide specific growth support towards coliform bacteria including *E. coli*. In contrast, the consistently inferior performance of MAC agar could be attributed to its components that indiscriminately select for Gram-negative bacteria. Unlike Brilliance and CHROMagar agars, MAC agar possesses bile salts as its selective component to suppress

Gram-positive bacteria growth by induction of DNA damage (33). However, it is also likely that this bile salt mechanism also indirectly exerts a suppressive effect on *E. coli* growth due to *E. coli* constantly having to express genes that reduces growth rate in order to repair any DNA damage (34). Therefore, the indiscriminate selection combined with the suppressive effect of bile salts in MAC agar presents a more stressful environment for *E. coli* resulting in an inferior performance. Additionally, this consistent performance of Brilliance and CHROMagar agars also demonstrated that the capability of both agars in supporting susceptible and FQ-resistant *E. coli* growth for detection and quantification was not impeded by the co-presence of faecal microflora.

Ampicillin, tetracycline, gentamicin, ciprofloxacin and ceftiofur were incorporated into each agar to identify the best concentration for growing the corresponding resistant *E. coli* for quantification. MAC agar consistently supported less growth regardless of antimicrobial and concentration and thus is not considered appropriate for quantitative AMR surveillance. Only ampicillin, tetracycline and ciprofloxacin were found to be suitable for incorporation into Brilliance and CHROMagar agars at all defined concentrations with growth of all resistant strains observed. In contrast, gentamicin was only suitable for incorporation into Brilliance and CHROMagar agars at 8 and 16 µg/mL as growth of susceptible strains were observed at lower concentrations. A higher number of susceptible strains grew on CHROMagar agar than Brilliance agar which suggests a higher level of suppression of gentamicin activity with the former. Currently, it is difficult to ascertain the mechanism by which this suppression occurs, although one possibility could be due to the significant three-way interaction between all factors.

A significant interaction between agars and ceftiofur was identified which, given the unexpected growth inhibition of some ceftiofur-resistant *E. coli* strains at higher ceftiofur concentrations, indicate a likely synergistic effect of ceftiofur with agar resulting in greater ceftiofur activity. It is also possible that this synergy also extends to 1 µg/mL despite all ceftiofur-resistant *E. coli* strains growing at this concentration. With the lack of information in the current literature pertaining to interactions between agar and ceftiofur, further investigation is needed to explain this phenomenon. Nonetheless, this indicates that ceftiofur is not suitable for incorporation into Brilliance and CHROMagar agars and we suggest that either ESC-resistant *E. coli* selective agar such as CHROMagar ESBL agar be used for quantitative AMR surveillance of ESC-resistant *E. coli* or further investigation into the viability of using other third-generation cephalosporin antimicrobials such as cefotaxime or ceftriaxone for incorporation into selective agar.

Finally, both Brilliance ESBL and CHROMagar ESBL agars had unique advantages. While Brilliance ESBL agar was superior in supporting growth of ESC-resistant *E. coli* strains from spiked homogenised faecal samples, CHROMagar ESBL agar was able to support a wider diversity of ESC-resistant *E. coli* strains. This was evident from the absence of SA27 growth on Brilliance agar as opposed to its presence of growth on CHROMagar agar regardless if it was from a pure culture or spiked homogenised faecal sample which also serves to demonstrate that the interference of SA27 (and thus ESC-resistant *E. coli*) growth on both agars may likely be due to interactions between strain and agar rather than the co-presence of faecal microflora. Nevertheless, the capability of CHROMagar ESBL agar to capture a wider diversity of ESC-resistant *E. coli* makes it better suited for AMR surveillance than Brilliance ESBL agar as it would increase the probability of detecting ESC-resistant *E. coli*.

The reason for growth variation between strains was not clear as it was not the principal feature being evaluated. Most data in the current literature focuses on growth rate of *E. coli* strains under specific environmental conditions but none have evaluated possible factors influencing growth rates between *E. coli* strains (35-37). Significant interactions between strain with agar or antimicrobial are one such factor affecting growth rate but given the uniformity in performance across all agars with and without incorporation of antimicrobials, it suggests that this influence towards growth was minimal and not enough to affect the performance outcome of each agar.

This study represents the first step towards establishing an enhanced AMR surveillance method for assessing AMR in livestock and food products. As opposed to the established method of AMR surveillance, this enhanced method is both qualitative and quantitative in nature and is built on the capacity to rapidly identify *E. coli* colonies on agars for colony enumeration. When combined with robotics, it provides exciting opportunities for up-scaling based on programming and machine learning pathways to allow the identification of *E. coli* colonies based on colony colour for enumeration with reduced human input and potentially greater accuracy (10). The practical ramifications for this are that more accurate information can be obtained from a greater number of samples that increases the sensitivity of detecting a given phenotype across a population of animals and herds. It is an especially relevant technique for early detection of resistance to critically important antimicrobials (CIAs) since it cannot be assumed that either the level of colonisation is uniform across animals or herds (38), or that the phenotypes of interest are present at a high enough concentration to be found by traditional AST means. Moreover, any positive colonies detected can be preserved for

genomic interrogation to understand their ecological origins as demonstrated in studies of human-wildlife-livestock transmission (28).

Based on this study, we recommend the use of Brilliance and CHROMagar agars with and without incorporation of antimicrobials as well as CHROMagar ESBL agar in combination with robotics to evaluate the feasibility of this enhanced AMR surveillance method. Additionally, this enhanced AMR surveillance method also has promising applications within food, clinical and public health settings through large-scale qualitative and quantitative AMR surveillance of CIA-resistant bacteria to support infection control and evaluation of the effectiveness of antimicrobial stewardship (39).

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2.7 SUPPLEMENTARY MATERIALS

Table 2.1 - Supplementary Phenotypic resistance profiles for eight *Escherichia coli* strains used in Experiment A, measured as minimum inhibitory concentration (MIC) and corresponding known critically important antimicrobial(CIA)-resistance genes. The profile was used to compare the presence or absence of growth of each *E. coli* strain on agar incorporated with antimicrobials at defined concentrations.

	Ampicillin		**Tetracycline**		**Gentamicin**		**Ciprofloxacin**		**Ceftiofur**		
Strain	**MIC (µg/mL)**	**Resistant**	**MIC (µg/mL)**	**Resistant**	**MIC (µg/mL)**	**Resistant**	**MIC (µg/mL)**	**Resistant**	**MIC (µg/mL)**	**Resistant**	**Known CIA Resistant Genes**
ATCC 25922	4	No	2	No	0.5	No	0.064	No	0.5	No	-
SA44	>128	Yes	>128	Yes	32	Yes	0.064	No	>16	Yes	$bla_{\text{CTXM-1}}$
SA1000	4	No	2	No	1	No	>4	Yes	0.25	No	QRDR mutation
SA1001	>128	Yes	128	Yes	>32	Yes	>4	Yes	>16	Yes	$bla_{\text{CTXM-14}}$, QRDR mutation
SA1002	>128	Yes	>128	Yes	1	No	>4	Yes	>16	Yes	$bla_{\text{CTXM-14}}$, QRDR mutation
SA1003	8	No	2	No	1	No	0.064	No	0.5	No	-
SA1004	>128	Yes	>128	Yes	1	No	0.125	No	0.5	No	QRDR mutation
SA1005	4	No	2	No	0.5	No	0.064	No	0.25	No	-

CIA critically important antimicrobials
QRDR quinolone resistance determining region
- no resistant genes present

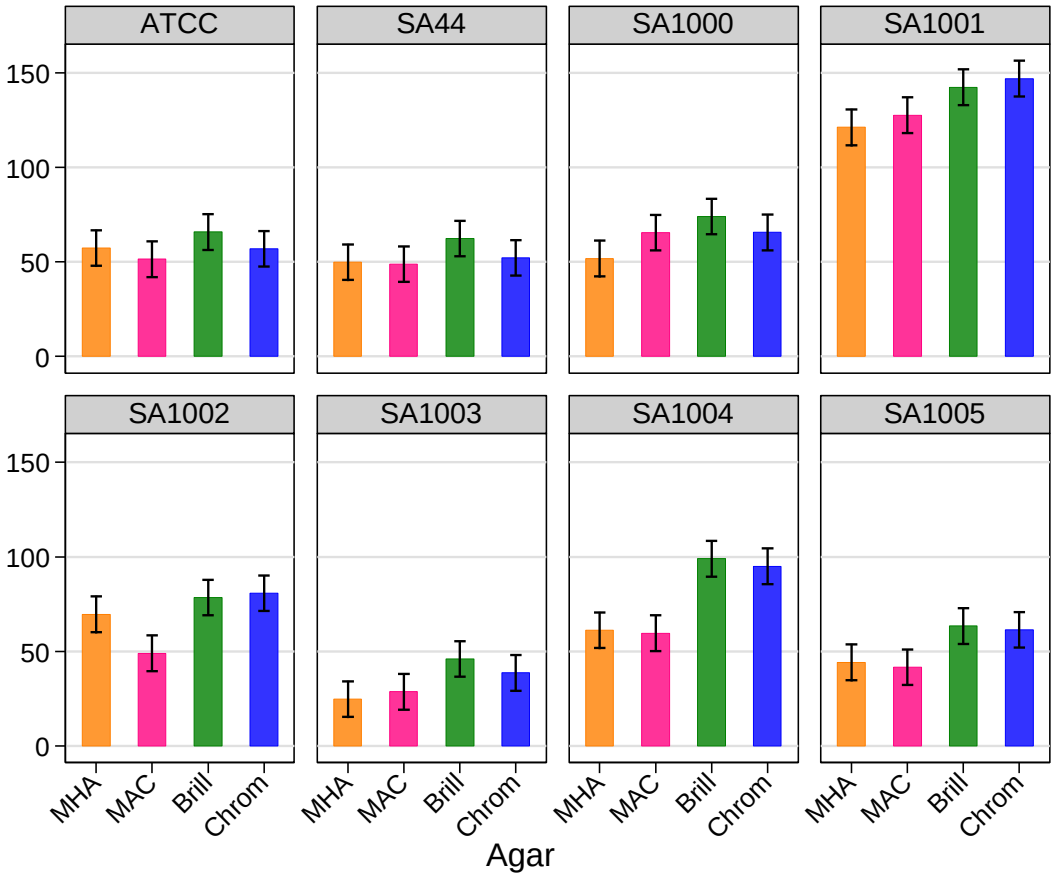

Figure 2.1 - Supplementary Comparisons of growth performance (mean colony counts per plate ± se) of each *Escherichia coli* strain on three *E. coli* selective agars and Mueller-Hinton agar (all without antimicrobials) (total number of plates = 160). Standardised inoculum across all agars consisted of diluted pure cultures of each *E. coli* strain. Key: MHA - Mueller-Hinton agar, MAC - MacConkey agar, Brill - Brilliance™ *E. coli* agar, Chrom - CHROMagar™ ECC agar.

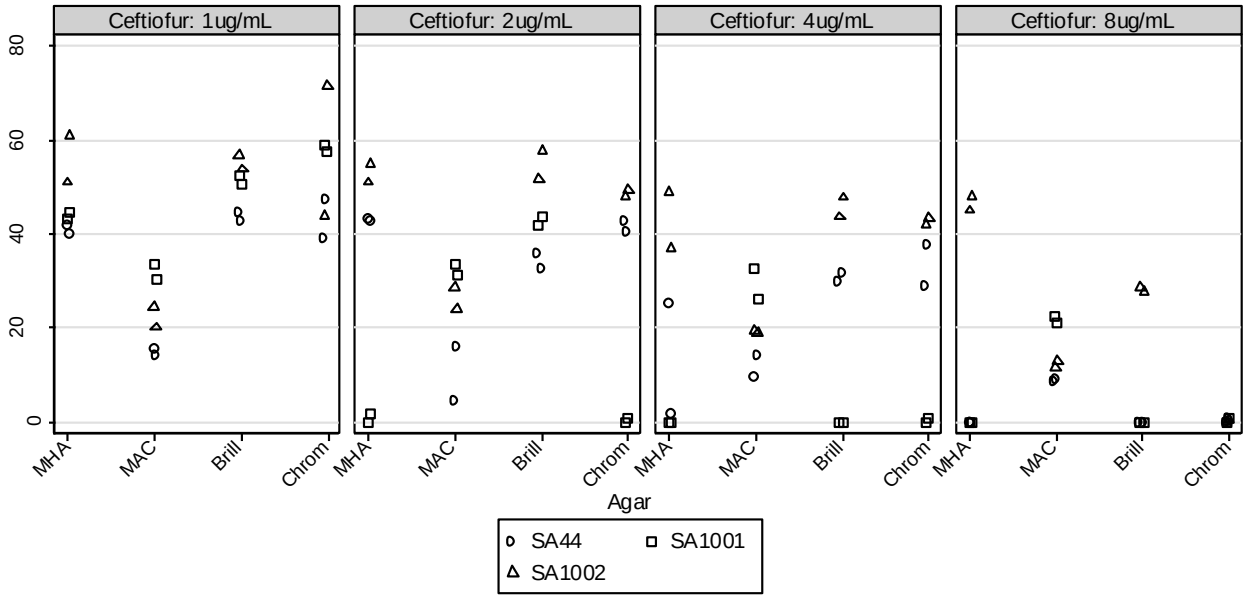

Figure 2.2 - Supplementary Individual colony counts per plate of ceftiofur-resistant *Escherichia coli* strains obtained when inoculated from dilutions of pure culture onto three *E. coli* selective agars and Mueller-Hinton agar incorporated with ceftiofur at each of four concentrations. Key: MHA - Mueller-Hinton agar, MAC - MacConkey agar, Brill - Brilliance™ *E. coli* agar, Chrom - CHROMagar™ ECC agar.

Table 2.2 - Supplementary List of critically important antimicrobial (CIA)-resistant *Escherichia coli* strains used in this study. For different experiments, pure cultures of fluoroquinolone-resistant *E. coli* were used. Prior to inoculation, they were diluted and spiked into homogenised faecal samples. Assessment of growth occurred on *E. coli* selective agars incorporated with ciprofloxacin. Similarly, pure cultures of extended-spectrum cephalosporin (ESC)-resistant *E. coli* were diluted and spiked into homogenised faecal samples for plating onto ESC-resistant *E. coli* selective agars.

		Experiment A		**Experiment B**		
Strain	**Resistance type**	**Pure cultures**	**Spiked into faecal samples**	**Pure cultures**	**Spiked into faecal samples**	**Known CIA-Resistant Genes**
ST131	Fluoroquinolone-resistant	No	Yes	No	No	QRDR mutation
ST744	Fluoroquinolone-resistant	No	Yes	No	No	QRDR mutation
SA27	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CMY-2}}$
SA44	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CTXM-1}}$
SA1001	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CTXM-14}}$
SA1074	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CMY-13}}$, $bla_{\text{TEM-1B}}$
SA1075	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CTXM-27}}$
SA1076	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CTXM-55}}$
SA1077	ESC-resistant	No	No	Yes	Yes	$bla_{\text{SHV-12}}$, $bla_{\text{CTXM-15}}$
SA1078	ESC-resistant	No	No	Yes	No	$bla_{\text{CTXM-15}}$

SA1079	ESC-resistant	No	No	Yes	Yes	$bla_{\text{TEM-1B}}$
SA1080	ESC-resistant	No	No	Yes	Yes	$bla_{\text{CMY-42}}$, $bla_{\text{CTXM-15}}$
SA1083	ESC-resistant	No	No	No	Yes	$bla_{\text{CMY-2}}$

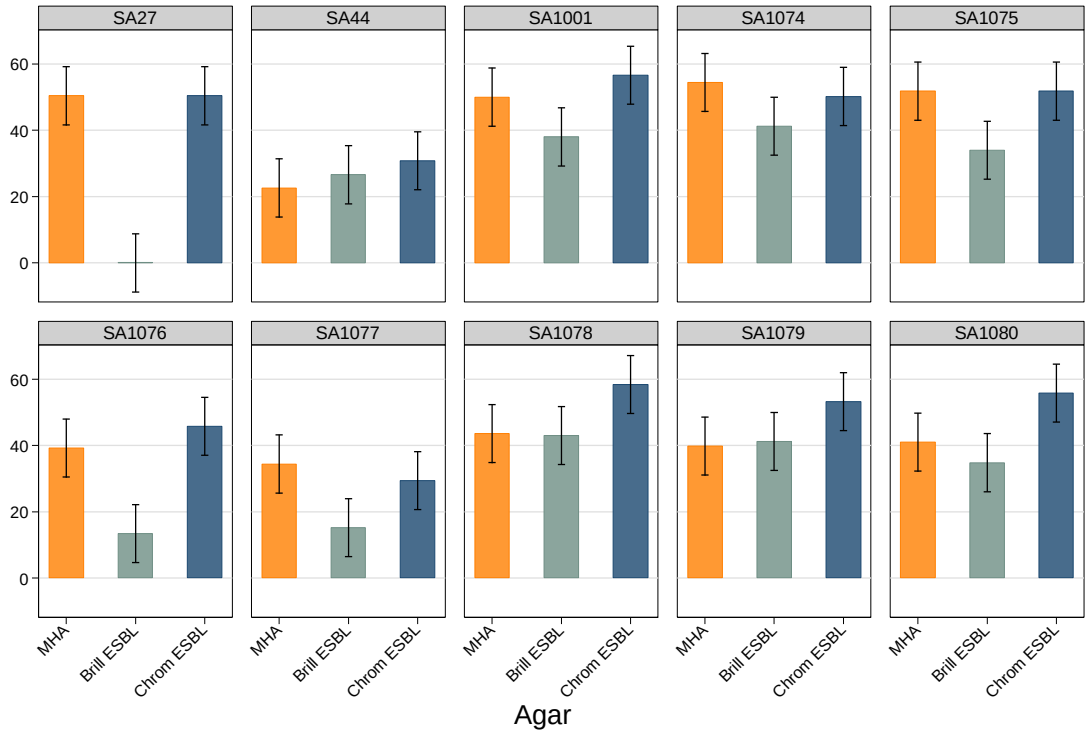

Figure 2.3 - Supplementary Comparisons of growth performance (mean colony counts per plate ± se) of each extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* strain on two ESC-resistant *E. coli* selective agars with Mueller-Hinton agar (without antimicrobials) present as control (total number of plates = 150). Standardised inoculum across all agars consisted of diluted pure cultures of diverse ESC-resistant *E. coli* strains. Key: MHA - Mueller-Hinton agar, Brill ESBL - Brilliance™ ESBL agar, Chrom ESBL - CHROMagar™ ESBL agar.

CHAPTER 3

Application of an enhanced antimicrobial resistance surveillance method to detect and quantify fluoroquinolone-resistant *Escherichia coli* in pigs in the absence of direct fluoroquinolone use

3.1 ABSTRACT

The emergence of critically important antimicrobial (CIA)-resistant *Enterobacteriaceae* even in the presence of strict regulations on CIA use adds a new dimension to the threat from resistant bacteria. In this study, an enhanced antimicrobial resistance (AMR) surveillance method was developed that combines a multiple samples per herd approach with laboratory robotics and selective agars incorporated with antimicrobials. This approach was applied to faecal samples from ten Australian pig farms to detect and quantify the extent of resistance in commensal *E. coli* to key antimicrobials including CIAs. All CIA-resistant *E. coli* were subjected to antimicrobial susceptibility testing by broth microdilution and sequencing to identify entire resistomes and genomic characteristics. Over 299 faecal samples were inoculated onto CHROMagar™ ESBL and CHROMagar™ ECC agar with and without antimicrobials. Extended-spectrum cephalosporin-resistant *E. coli* were not detected, but on seven farms, ciprofloxacin-resistant *E. coli* were detected with differing frequency of positive samples and low carriage levels compared to general commensal *E. coli*. Genomic analysis revealed ST167 and ST744 to be the dominant fluoroquinolone-resistant *E. coli* sequence types among the seven farms, and were possibly introduced via human or wild bird carriers. Resistance towards ampicillin, tetracycline and gentamicin was frequent among all ten farms, with carriage levels of the former two being comparable to commensal *E. coli* unlike gentamicin which was at least several orders of magnitude lower. These findings highlight the importance of highly sensitive, high throughput AMR surveillance as a biosecurity tool to prevent the exacerbation of CIA-resistance even in the presence of strict regulation on CIA use.

3.2 INTRODUCTION

Antimicrobial resistance (AMR) is one of the most pressing and globally significant animal and public health issues, with an estimated 700,000 human deaths annually being attributed to infections with resistant bacteria (1, 2). Gram-negative bacteria, particularly pathogenic *Escherichia coli*, are a major concern especially when resistance involves critically important antimicrobials (CIAs) (extended-spectrum cephalosporins [ESCs], fluoroquinolones [FQs] and carbapenems) since these are heavily relied upon as the last line of defence for bacterial infections in humans (3). Livestock are a potential reservoir for the development and spread of CIA-resistant bacteria that can be transmitted to humans via the food chain or other ecological pathways (4-7). While commensal *E. coli* from livestock have limited potential to cause diseases in humans due to the absence of key virulence genes (8), they are capable of transferring CIA-resistance to human pathogens via horizontal transfer of mobile genetic elements harbouring CIA-resistance genes (4).

The emergence of CIA resistance in livestock has largely been attributed to the direct use of CIAs in these animals (9-14), most notably FQs and ESCs. Additionally, once emerged, ESC-resistance can persist in the microbiota of the gut of livestock for a protracted period of time after removal of direct selective pressure (15). Consequently, this has led to a significant global effort to limit the use of CIAs in livestock (2).

The emergence of CIA-resistant *Enterobacteriaceae* in the absence of direct CIA use has become a problematic phenomenon. Carbapenem-resistant *E. coli* and *Salmonella enterica* carrying VIM-1 carbapenemase-encoding IncHI2 plasmids were reported in German pig farms even though carbapenems are not legally licensed for use in European livestock (16-18). In Australia, despite FQ not being registered for use in livestock, two studies in pigs

have reported the detection of FQ-resistant *E. coli* potentially having a broad host range and belonging to clonal lineages that are suggestive of being introduced into Australian pig herds via human or wild bird carriers (5, 19). These findings added a further dimension to the threat of AMR that necessitates the development of improved surveillance methods that enable early detection so that establishment of CIA-resistance in livestock can be prevented and its origins understood.

The application of the Robotic Antimicrobial Susceptibility Platform (RASP) in microbiology has potential to drive powerful reforms in AMR surveillance (20). Robotics delivers cost-effective and high-throughput processing of samples including bacterial isolation and identification, antimicrobial susceptibility testing (AST), and automated reporting of results (20). Broth microdilution sensitivity testing is conveniently integrated into RASP and performed without sacrificing accuracy (20). Because the approach permits tremendous expansion in the number of herds, animals and isolates being evaluated, the surveillance system can detect key forms of resistance earlier and better define its epidemiological significance (20). Moreover, meaningful results can be provided to specific herds as soon as one week following sample collection whereas under established AMR surveillance systems, herds may receive little (if any useful data) within a reasonable timeframe (20). Augmentations to RASP are described in this study, consisting of validated protocols based on agar dilution assays that were described and objectively assessed in Chapter 2. The result is a capacity for direct enumeration of resistant *E. coli* colonies and quantification of their carriage levels in faeces, with this performed on a pace and scale previously unimagined. As with earlier RASP developments, phenotypic or genomic investigation including whole genome sequencing (WGS) is optionally appended where this is needed to further elucidate the resistome or ecological heritage of contentious isolates. This

study demonstrates how this new component of RASP can further enhance AMR surveillance through a high-resolution assessment of the AMR status of ten commercial pig herds by quantifying the extent of resistance in commensal *E. coli* to key antimicrobials including CIAs, using an enhanced AMR surveillance method that combines a multiple samples per herd approach with RASP and selective agars incorporated with antimicrobials.

3.3 MATERIALS AND METHODS

Sample collection. Invitation to participate in this study was sent to commercial pig farms from one Australian state spanning a geographical region of approximately 113,000 km^2. Farm selection was based on the first ten farms that responded. Thirty fresh faecal samples from finisher pigs on each farm were collected, except for a single farm from which 29 samples were obtained (total n = 299). All samples were collected from pen floors by veterinarians or under veterinary supervision, with collection distributed across all housing systems containing finisher pigs on the farm. Samples were transported on ice and processed within 16 hours of collection.

Sample processing and inoculation. Approximately 2 g of each faecal sample was homogenised for 30 seconds in 18 mL of sterile 1x phosphate buffered saline (PBS) using a BagMixer® 400 P laboratory blender (Interscience, Edwards Group). Post homogenisation samples were processed using RASP (20). Briefly, 75 µL of diluted homogenised samples were inoculated onto CHROMagar™ ECC (CHROMagar) (MicroMedia, Edwards Group) agar with and without incorporation of antimicrobials (32 µg/mL ampicillin, 16 µg/mL tetracycline, 16 µg/mL gentamicin and 4 µg/mL ciprofloxacin) and CHROMagar™ ESBL (CHROMagar ESBL) (MicroMedia, Edwards Group) agar. The importance of these antimicrobials for human medicine follows the classification by the Australian Strategic and

Technical Advisory Group on Antimicrobial Resistance (ASTAG) (21). All agar inoculation was performed using RASP's two-zone spiral plating protocol (two dilutions of each homogenised sample per agar) that imitates the standard lawn spread technique to obtain countable colonies on agar (20). Antimicrobial concentrations were selected based on clinical breakpoints listed by the Clinical and Laboratory Standards Institute (CLSI) and previously validated in Chapter 2 to be suitable for incorporation into CHROMagar agar for quantifying resistant *E. coli* (22). All agars were incubated at 37 ºC for 16 to 20 hours.

Quantification and isolation of *E. coli* colonies. Presumptive identification of *E. coli* colonies for the purpose of quantification, isolation and storage was performed based on chromogenic reaction of each agar (as detailed by the manufacturer) and captured on digital images and analysed using the Pickolo^{TM} software with RASP (20). All *E. coli* colonies on CHROMagar agar without antimicrobials and CHROMagar ESBL agar were presumed to be general commensal *E. coli* and ESC-resistant *E. coli* respectively, while *E. coli* colonies on CHROMagar agar incorporated with antimicrobials were presumed to be *E. coli* resistant to the incorporated antimicrobial. Carriage level of *E. coli* was expressed in colony forming units per gram (CFU/g) of faeces. A single *E. coli* colony (if present) from CHROMagar agar incorporated with ciprofloxacin and CHROMagar ESBL agar was isolated by RASP and inoculated into a well of a 96-well plate containing 150 µL of Luria-Bertani (LB) broth. All 96-well plates were incubated at 37 ºC for 16 to 20 hours, before 150 µL of LB broth with 40% glycerol was added into each well and the 96-well plate was stored at -80 ºC.

Antimicrobial sensitivity testing of CIA-resistant *E. coli*. Prior to AST, MALDI-TOF (Bruker Microflex) from broth cultures was used to confirm identity of *E. coli* isolates (20). AST using the broth microdilution method was performed on RASP according to CLSI

guidelines (20, 23). All AST plates were imaged using the Sensititre™ Vizion™ Digital MIC Viewing System (Thermo Fisher Scientific) for minimal inhibitory concentration (MIC) interpretation. Susceptibility to thirteen antimicrobials representing ten classes were assessed and includes ampicillin (beta-lactam), apramycin (aminocyclitol), cefoxitin (first-generation cephalosporin), ceftiofur (third-generation cephalosporin), ceftriaxone (third-generation cephalosporin), chloramphenicol (phenicol), ciprofloxacin (quinolone), colistin (polymixin), florfenicol (phenicol), gentamicin (aminoglycoside), streptomycin (aminoglycoside), tetracycline (tetracycline) and trimethoprim-sulfamethoxazole (folate pathway inhibitor). The concentration range for each antimicrobial was selected based on Sensititre™ NARMS CMV3AGNF Gram Negative Plate (24) and follow the importance classification set by ASTAG (21). MIC results were interpreted using epidemiological cut-off (ECOFF) values according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), with the exception of ceftriaxone which was interpreted using CLSI clinical breakpoints as the concentration range for ceftriaxone did not include the ECOFF value (25, 26), and apramycin which was interpreted based on the ECOFF value for *E. coli* validated by Yang et al. (2020) (27). In this study, when interpreting based on ECOFF values, isolates classified as wild type are referred as susceptible while those classified as non-wild type are referred as resistant. Isolates resistant to at least three antimicrobial classes are categorised as multi-class resistant (MCR).

Whole genome sequencing of CIA-resistant *E. coli*. Random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) was performed on all confirmed *E. coli* isolates to identify distinct *E. coli* clones. DNA extraction for RAPD PCR was performed using the 6% Chelex (Bio-Rad) method with PCR performed using 1254 primer (5′-CCGCAGCCAA-3′; Sigma-Aldrich) and GoTaq® Green Master Mix (Promega) (15). Distinct *E. coli* clones based

on RAPD profiles and phenotypic AMR profiles from each farm were selected for WGS. DNA extraction for WGS was performed using the MagMax™-96 DNA Multi-Sample kit (Applied Bio Systems, Thermo Fisher Scientific) according to manufacturer instructions on a MagMax™ 96-well automated extraction platform (Life Technologies). DNA library preparations were conducted using the Celero™ DNA-Seq kit (NuGEN) according to manufacturer instructions, with sequencing performed using the NextSeq™ 500/550 Mid Output 2x150 Reagent Cartridge v2 (Illumina). Sequencing data was *de novo* assembled using SPAdes (v3.14.1) (28). Multi-locus sequence typing (MLST) was performed, with sequence types (STs) identified using the PubMLST database (29). Plasmids, AMR and virulence genes were identified based on the *de novo* assembled genomes using ABRicate (v1.0.1) (https://github.com/tseemann/abricate) using the publicly available PlasmidFinder (30), ResFinder (31) and VFDB (32) databases respectively. Identified plasmids, AMR and virulence genes were considered present if they were at greater than 95% coverage and identity. Single nucleotide polymorphisms (SNPs) within quinolone resistance-determining regions (QRDRs) were identified using Snippy (v4.1.0) (https://github.com/tseemann/snippy). Dominant STs of *E. coli* that were identified were compared to an international collection of the same STs within the EnteroBase (33) and NCBI Sequence Read Archive (34) databases, both accessed June 5th 2021. STs with an ERR or SRR accession on the international database were downloaded, with MLST performed to ascertain their STs. Any isolates that were not the same dominant STs identified in this study or did not contain information pertaining to country or continent were excluded. Phylogenetic trees of each dominant ST were constructed by producing a core genome SNP alignment using Snippy (v4.1.0) (https://github.com/tseemann/snippy) followed by removal of putative recombinant DNA segments using ClonalFrameML (v1.11) (35) before a

maximum-likelihood phylogeny was constructed via RAxML (v8.0.0) (36). Annotation of phylogenetic trees was performed using the ggtree package (v3.0.4) in R (v4.1.1) (37).

Statistical analysis. Under the RASP protocol, data from digital imaging of colonies on selective agars were all electronically captured for processing and descriptive analysis in the Stata analysis package (version 16.0, Stata Corporation, Texas, USA). Quantitative counts of *E. coli* in CFU/g of faeces were $\log_{10}$ transformed where required for interpretation and analysis. Similarly, MIC data from CIA-resistant *E. coli* isolates were captured by RASP and processed within Stata to obtain MIC tables with exact confidence intervals for proportion of resistant colonies derived by the Clopper-Pearson method.

3.4 RESULTS

Quantification of resistant *E. coli*. The presence and extent of AMR within Australian pig herds was described through the detection and quantification of resistant *E. coli* towards CIAs, first and second-line antimicrobials on ten Australian pig farms. The frequencies and carriage levels of commensal *E. coli* and each resistant *E. coli* detected in this study are shown in Figure 3.1. Across all farms, average carriage level of commensal *E. coli* was at 6 $\log_{10}$ CFU/g, with four farms (Farms B, G, I and J) having an average of 5 $\log_{10}$ CFU/g and one (Farm F) having 7 $\log_{10}$ CFU/g. No ESC-resistant *E. coli* were detected. Ciprofloxacin-resistant *E. coli* were detected on seven farms with an average of 1 $\log_{10}$ CFU/g. Resistance towards first (ampicillin and tetracycline) and second-line (gentamicin) antimicrobials was detected on all farms. Carriage levels of gentamicin-resistant *E. coli* was comparatively low, with an average 2 $\log_{10}$ CFU/g, compared to ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* with an average of 6 $\log_{10}$ CFU/g and 5 $\log_{10}$ CFU/g respectively.

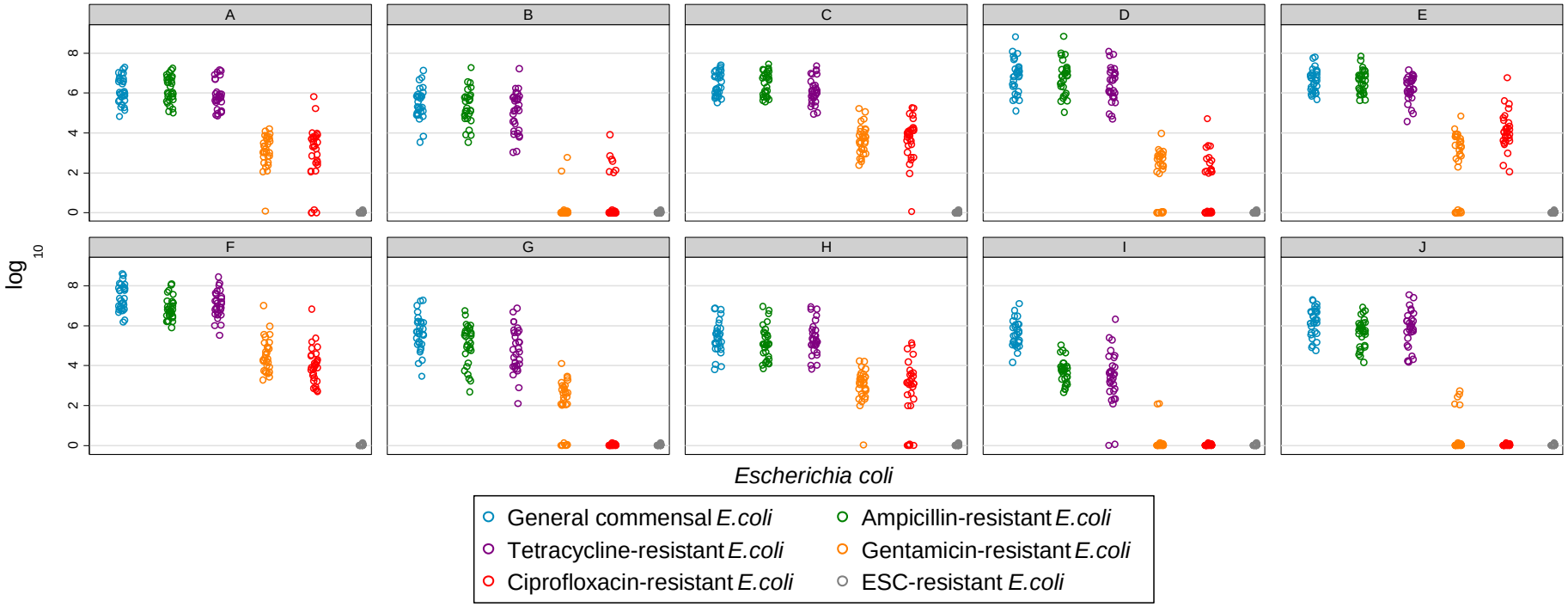

Figure 3.1 Farm-level and animal-level variation in concentration (log_{10} CFU/g) of commensal *Escherichia coli* phenotypes in pig faeces. Data represents 1794 observations from 299 individual faecal samples across 10 farms (labelled A to J). Each faecal sample was enumerated six times: once on CHROMagar™ ECC agar without antimicrobials, once on each of five CHROMagar™ ECC agar incorporated either with ampicillin, tetracycline, gentamicin or ciprofloxacin, and once on CHROMagar™ ESBL agar. Enumeration was by image capture and digital analysis of chromogenic traits of individual colonies.

Notable differences in frequency of positive samples with ciprofloxacin-resistant *E. coli* were observed between farms, with Farms B and D having a low to moderate frequency of seven and 15 samples respectively compared to the other five farms with a high frequency of 24 to 30 samples. However, for all farms with ciprofloxacin resistance, the average carriage level of ciprofloxacin-resistant *E. coli* in positive samples was about 2 logs lower than the average carriage level of general commensal *E. coli* on each farm.

The frequency of positive samples with gentamicin-resistant *E. coli* also varied widely between farms, with Farms B, I and J having a frequency of two to five samples with an average carriage level of less than 1 $\log_{10}$ CFU/g in each farm as opposed to the remaining farms which had 20 to 30 samples with an average carriage level of 1 to 4 $\log_{10}$ CFU/g. However, much like ciprofloxacin-resistant *E. coli*, the average carriage level of gentamicin-resistant *E. coli* of each farm was at least 2 $\log_{10}$ CFU/g lower than the average carriage level of general commensal *E. coli*. In contrast, the frequency of positive samples with ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* between farms were consistent at 30 samples with Farm I the only exception with a frequency of 28 samples for tetracycline-resistant *E. coli*. Farm I was also the only farm that had an average carriage level of both resistant *E. coli* that was lower than general commensal *E. coli* by 2 $\log_{10}$ CFU/g, compared to other farms where the average carriage level of both resistant *E. coli* and general commensal *E. coli* were similar.

Phenotypic characterisation of ciprofloxacin-resistant *E. coli*. A total of 160 ciprofloxacin-resistant *E. coli* isolates from CHROMagar agar incorporated with ciprofloxacin were subjected to AST using the broth microdilution technique. The

percentages of resistance for all isolates are shown in Figure 3.2. All isolates were confirmed to be resistant towards ciprofloxacin (100%) with MIC value above the CLSI clinical breakpoint of 1 µg/mL (25). Additionally, high levels of resistance towards ampicillin (99.4%), streptomycin (73.1%), tetracycline (68.1%), trimethoprim-sulfamethoxazole (57.5%) and chloramphenicol (55.6%), and low levels of resistance towards florfenicol (5.6%) were observed. All isolates were susceptible to apramycin, cefoxitin, ceftiofur, ceftriaxone, colistin and gentamicin.

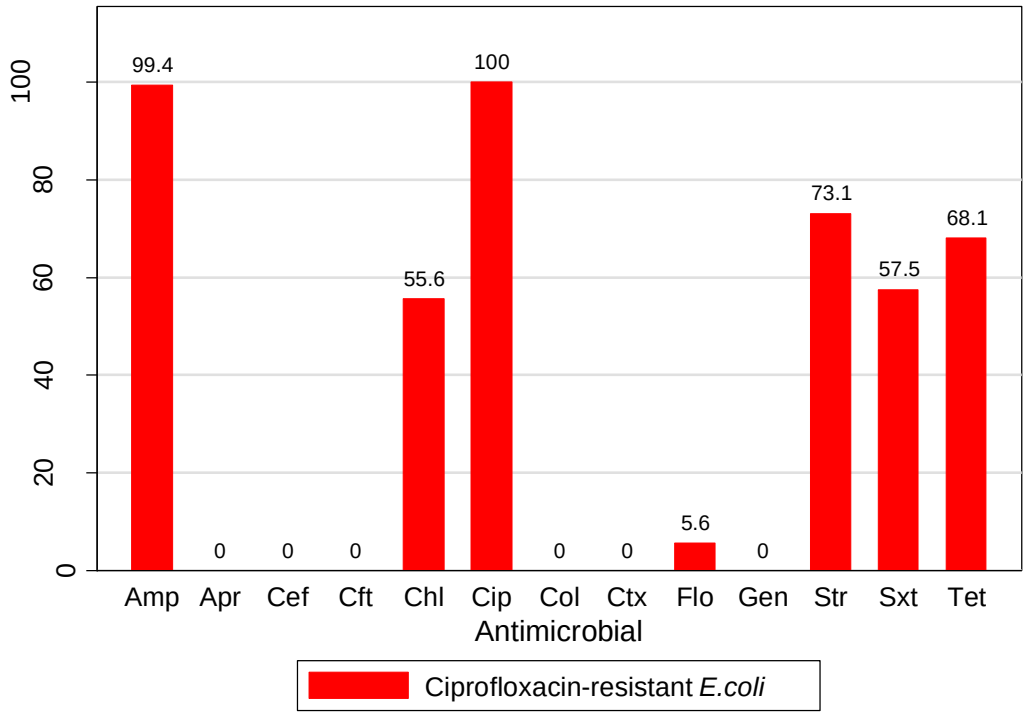

Figure 3.2 Percentage of ciprofloxacin-resistant *Escherichia coli* isolates (*n* = 160) resistant to 13 antimicrobials based on the epidemiological cut-off (ECOFF from EUCAST) value, with the exception of ceftriaxone and apramycin that was based on the clinical breakpoint (from CLSI) and ECOFF value validated by Yang et al. (2020) (27) respectively. Key: Amp - Ampicillin, Apr - Apramycin, Cef - Cefoxitin, Cft - Ceftiofur, Chl - Chloramphenicol, Cip - Ciprofloxacin, Col - Colistin, Ctx - Ceftriaxone, Flo - Florfenicol, Gen - Gentamicin, Str - Streptomycin, Sxt - Trimethoprim-sulfamethoxazole, Tet - Tetracycline.

All 160 ciprofloxacin-resistant isolates were classified as MCR, with ten different profiles identified (Table 3.1). The most common profile was resistance towards six antimicrobial classes (*n* = 51, 31.9%: aminoglycoside, beta-lactam, folate pathway inhibitor, phenicol, quinolone and tetracycline) followed by profiles with three (*n* = 48, 30.0%: aminoglycoside, beta-lactam and quinolone) and five (*n* = 23, 14.4%: beta-lactam, folate pathway inhibitor, phenicol, quinolone and tetracycline) antimicrobial classes (Table 3.1). The number of MCR profiles on each farm did not increase with the frequency of ciprofloxacin-resistant *E. coli* present (Table 3.1 - Supplementary). For example, Farm D with 15 ciprofloxacin-resistant *E. coli* isolates had five MCR profiles, Farm E with 30 ciprofloxacin-resistant *E. coli* isolates had two MCR profiles, and Farm F with 29 ciprofloxacin-resistant *E. coli* isolates had six MCR profiles (Table 3.1 - Supplementary).

Table 3.1 Multi-class resistance profiles of ciprofloxacin-resistant *Escherichia coli* isolates based on interpretive breakpoints applied in Figure 3.2. Isolates with resistance towards three or more antimicrobial classes are categorised as multi-class resistant.

Multi-class resistance profile	Number of antimicrobial classes	Number of isolates	% of total
ami-bla-qui	3	48	30.0
bla-qui-tet	3	7	4.4
phe-qui-tet	3	1	0.6
ami-bla-qui-tet	4	1	0.6
bla-fpi-phe-qui	4	1	0.6
bla-phe-qui-tet	4	11	6.9
ami-bla-fpi-phe-qui	5	2	1.3
ami-bla-fpi-qui-tet	5	15	9.4
bla-fpi-phe-qui-tet	5	23	14.4
ami-bla-fpi-phe-qui-tet	6	51	31.9

ami: aminoglycoside, bla: beta-lactam, fpi: folate pathway inhibitor, phe: phenicol, qui: quinolone, tet: tetracycline

Genotypic characterisation of ciprofloxacin-resistant *E. coli*. A subset of 50 ciprofloxacin-resistant *E. coli* isolates was selected for WGS to achieve inclusion in the majority of distinct phenotypic MCR and RAPD PCR profiles. Following WGS, majority of the isolates were found to belong to ST167 (*n* = 24) and ST744 (*n* = 22), with the others belonging to ST10 (*n* = 1), ST34 (*n* = 1), ST11611 (*n* =1) and ST11612 (*n* =1) (Table 3.2 - Supplementary). The latter four STs were all from isolates on Farm A. All STs were MCR isolates, with resistance towards three (*n* = 19, 79.2%: aminoglycoside, beta-lactam and quinolone) and six (*n* = 14, 63.6%; aminoglycoside, beta-lactam, folate pathway inhibitor, phenicol, quinolone and tetracycline) antimicrobial classes being the most frequent profiles for ST167 and ST744 respectively. The number of MCR profiles and the AMR genes identified amongst isolates belonging to each ST are shown in Table 3.2.

Table 3.2 Number of isolates, multi-class resistant profiles and known antimicrobial resistance genes detected for each ciprofloxacin-resistant *Escherichia coli* sequence type.

ST	Number of isolates	Number of MCR Profiles	Antimicrobial resistance genes
167	24	2	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*
744	22	5	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, *aadA2*, *aadA5*, *aph(3'')-Ib*, *aph(3')-Ia*, *aph(6)-Id*, *floR*
10	1	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul3*, *tet(B)*, *aadA2*, *aadA5*
34	1	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul3*, *tet(B)*, *aadA2*, *aadA5*
11611	1	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul3*, *tet(B)*, *aadA2*, *aadA5*
11612	1	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *tet(A6*, *aph(3'')-Ib*, *aph(6)-Id*

All isolates displayed substitutions within the quinolone-resistance determining regions (QRDR) with 44 isolates (88.0%) possessing C248T, G259A, A2034C and T2482G substitutions in *gyrA*, and a G239T substitution in *parC*. Of the remaining isolates, a subset (*n* = 4) harboured only the A2034C and T2482G substitutions in *gyrA* while two isolates harboured both aforementioned substitutions in *gyrA* and either the G239T or the T240C substitution in *parC*. No known plasmid-mediated FQ-resistance genes were identified. The only beta-lactam-resistance gene identified was $bla_{\text{TEM-1B}}$ which was present in 49 of the sequenced isolates (98.0%), with one ST744 isolate being negative. Three sulphonamide-resistance genes (*sul1*, *sul2* and *sul3)* were identified with each isolate harbouring at least one of these genes, and only one ST11612 isolate being negative. Two tetracycline-resistance genes (*tet*[A] and *tet*[B]) were identified, with 29 isolates (58.0%) harbouring one or both genes. All isolates also harboured at least one aminoglycoside-resistance gene (from a total of five identified genes). However, despite five STs (ST167, ST744, ST10, ST34 and ST11611) having isolates displaying phenotypic resistance towards phenicol class antimicrobials, only four ST744 isolates (8.0%) harboured the phenicol-resistance gene *floR*. No known virulence genes consistent with pathogenic *E. coli* were identified. A total of 20 plasmids were identified with IncFIB(AP001918) being the most frequently found plasmid across all STs (*n* = 43, 86.0%) followed by IncX (*n* = 27, 54.0%), IncFIC(FII) (*n* = 26, 52.0%) and IncFII(pRSB107) (*n* = 21, 42.0%) .

Phylogenetic analysis of the two dominant STs revealed that all ST167 isolates identified in this study (*n* = 24) were closely related in one cluster, indicating a close phylogenetic relationship (Figure 3.3). Moreover, this study isolates shared the same branch with international ST167 isolates (*n* = 135) originating from the Americas and Europe (Figure 3.3). In contrast, the ST744 isolates identified in this study (*n* = 22) were dispersed in small

clusters throughout different branches of the tree, with international ST744 isolates (*n* = 214) originating from a wider range of continents - the Americas, Europe, Asia and Africa (Figure 3.4).

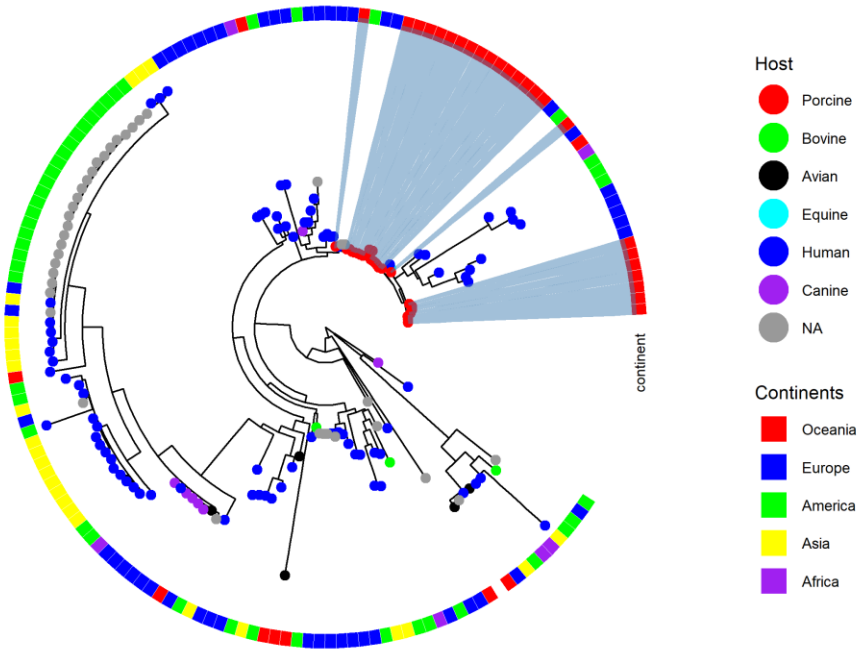

Figure 3.3 Mid-point rooted maximum likelihood phylogenetic tree of ST167 using 159 genome (international isolates *n* = 135, study isolates *n* = 24) with 9423 SNP sites. Coloured circles on each node represent the host from which the isolate originated while the coloured squares represent the continent from where the host originated from. The 24 isolates from this study are highlighted with a blue background and are clustered along the same branch of the phylogenetic tree.

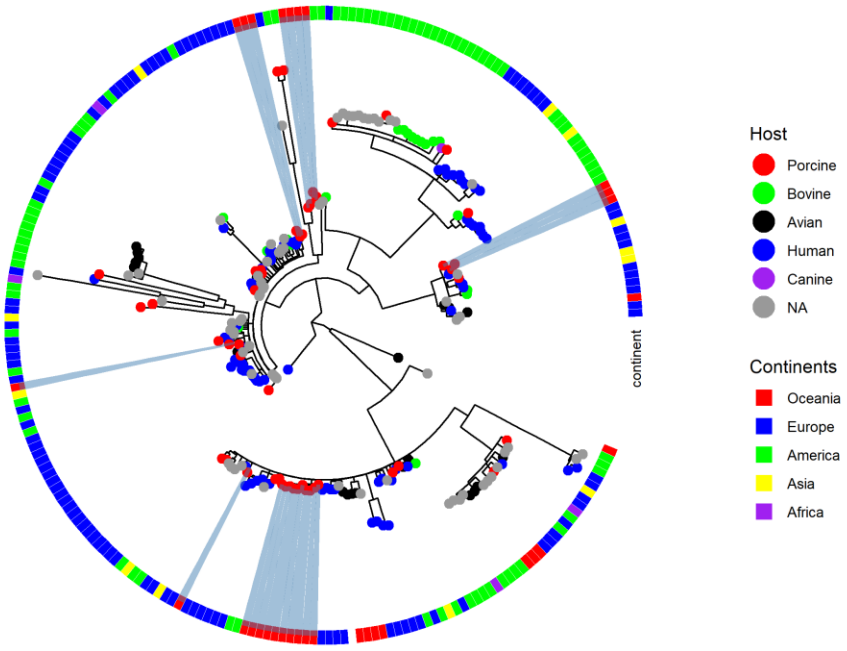

Figure 3.4 Mid-point rooted maximum likelihood phylogenetic tree of ST744 using 236 genome (international isolates *n* = 214, study isolates *n* = 22) with 6441 SNP sites. Coloured circles on each node represent the host from which the isolate originated while the coloured squares represent the continent from where the host originated from. The 22 isolates from this study are highlighted with a blue background and are scattered in small clusters throughout the phylogenetic tree.

3.5 DISCUSSION

AMR surveillance of livestock serves as a critical tool for monitoring the emergence, presence and frequencies of resistance (particularly CIA-resistance) in order to prevent further spreading of resistance. Through the application of an enhanced AMR surveillance method utilising RASP in combination with a multiple samples per herd approach and selective agars incorporated with antimicrobials, the presence and extent of CIA-resistant *E. coli* within ten Australian pig farms was described. While no ESC-resistant *E. coli* were detected, ciprofloxacin-resistant *E. coli* were detected in seven farms with low carriage levels. Multiple MCR profiles were also identified among FQ-resistant *E. coli* with genomic analysis revealing two dominant STs (ST167 and ST744) currently present among the seven farms. With first and second-line antimicrobials, resistance towards ampicillin, tetracycline and gentamicin was highly frequent among all ten farms although only *E. coli* resistant to ampicillin and tetracycline had carriage levels comparable to the general commensal *E. coli* population. Though the findings of this study were limited geographically, it conclusively demonstrated how the inclusion of validated enumeration assays based on agar dilution can enhance AMR surveillance by delivering a more detailed description of AMR (especially FQ-resistance with a low frequency) at the herd-level that would not be possible with established approaches to AMR surveillance based on a single isolate per herd (38).

While this study was not the first to detect FQ-resistant *E. coli* within Australian pigs (5, 19), it represents the first to quantify the frequency and carriage levels of FQ-resistance within Australian pig herds. This also extends to the identification of dominant FQ-resistant *E. coli* STs currently present among the seven Australian pig farms in this study with FQ-resistant *E. coli*. Given that FQ is not registered for use in Australian livestock, the presence of the two dominant FQ-resistant *E. coli* STs (ST167 and ST744) in these seven farms were likely due

to introduction through external sources. ST744 has previously been reported to occur at low frequency in Australian seagulls, cats and dogs (39, 40), and both STs have also previously been widely reported internationally in humans, livestock and wild birds (41-48). Considering the close phylogenetic association between this study isolates with other international isolates, it is possible that FQ-resistant *E. coli* may have been introduced into these seven farms through farm workers returning from overseas or incursions of wild birds (5). An introduction through livestock is unlikely given the strict national biosecurity regulations surrounding the importation of livestock and unprocessed animal products into Australia (49). With regard to ST167, the phylogenetic clustering suggests that only one ST167 clone closely related to ST167 isolates from the Americas or Europe is present within these seven farms, and may have been introduced at a single time-point. In contrast, the dispersal of ST744 isolates from this study into small clusters throughout the phylogenetic tree suggests the presence of multiple ST744 clones closely related to ST744 isolates from the Americas, Europe, Asia and Africa that may have been introduced at differing times.

Once introduced, factors that contribute to the spread of FQ-resistant *E. coli* clones between pig herds (such as proximity to other livestock farms, bird habitat and migration routes, and the movement of breeding stock and workers between farms) require further investigation to better understand CIA-resistance transmission pathways between farms and the surrounding ecological systems in the presence of strict regulation on CIA use, and its risk towards public health. Such an investigation would also rely on a thorough understanding of the CIA-resistance status of each farm, which was made possible in this study through the sampling of healthy pigs directly from farms instead of using diagnostic samples from diseased pigs or at abattoirs without regards to the origin of the animal (as performed with established AMR surveillance systems for livestock) (38). A clearer understanding of the links between CIA-

resistance transmission pathways and the emergence of CIA-resistant *E. coli* on farms would also impact the implementation of biosecurity measures at the herd-level. Traditionally, the focus of biosecurity on farms has been to prevent the introduction of pathogens that cause clinical disease to livestock and the public via the food chain (50). This study introduces a new aspect of biosecurity for farms, namely the need to prevent the introduction or spread of CIA-resistant bacteria. While the concept remains the same as the traditional focus of biosecurity, the fact that CIA-resistant bacteria can be transmitted among commensal bacteria in healthy pigs provides a new challenge, as no external signs indicating the introduction or presence of CIA-resistance are present. In the long term, control might best be achieved through a combination of quarantine and hygiene protocols, early detection of CIA-resistance incursion using techniques described in this study, plus additional scrutiny of the local ecosystem.

Though FQ-resistant *E. coli* were detected within seven Australian pig farms, its present impact on public health is likely low. Carriage levels of FQ-resistant *E. coli* in this study were always lower than general commensal *E. coli*, indicating that FQ-resistance within each herd has not yet spread throughout the entire commensal *E. coli* population. Furthermore, the potential for FQ-resistance genes to be transferred to other human pathogens via horizontal transfer is negated, as all genes harbouring FQ-resistance were identified within the *gyrA* and *parC* QRDR regions which arise through specific mutations and are thus chromosomally-mediated (unlike plasmid-mediated FQ-resistance genes) (51).

The presence of several MCR profiles amongst FQ-resistant *E. coli* has implications towards animal production and management due to potential co-selection of CIA-resistant *E. coli* strains through the use of first and second-line antimicrobials since all other antimicrobial

classes within the MCR profiles are registered for use in Australian pigs (52). While MCR *E. coli* profiles with resistance towards five or more antimicrobial classes (including FQs) have been reported internationally, none have evaluated the proportion of profiles present in livestock in the context of AMR surveillance (53-56). As demonstrated with findings in this study, the proportion of profiles present in each farm varies widely, with no clear pattern, and could have been missed when using the established approach of AMR surveillance of a single isolate per herd. Nonetheless, this highlights the advantage of the multiple samples per herd approach and the importance of on-going AMR surveillance to monitor the emergence or spread of FQ-resistant *E. coli* with MCR profiles.

Recent international studies have identified MCR *E. coli* with resistance towards multiple CIA classes including FQs, ESCs and polymyxins (57-59). The absence of resistance towards ceftriaxone, ceftiofur and colistin among FQ-resistant *E. coli* from this study was thus encouraging. This reflects the strict regulation of antimicrobial use in Australia as colistin is also not registered for use in Australian livestock, while the only ESC (ceftiofur) available for use in Australian pigs is as an off-label treatment (60). However, since FQ-resistant *E. coli* occurred in the absence of direct FQ use, the emergence of ESC-resistant *E. coli* via introduction through external sources is also a threat. Recent studies have identified wild birds carrying ESC-resistant *E. coli* which could potentially transmit ESC-resistance into Australian pig herds via scavenging of feed and fouling of water (40, 61). Cases of reverse zoonosis (human to animal transmission) have been documented with the emergence and spread of methicillin-resistant *Staphylococcus aureus* ST398 in Australian pigs which reinforces the likelihood of ESC-resistant *E. coli* emerging in Australian pig herds through the same route (62). To prevent further spread among Australian livestock, on-going AMR surveillance, antimicrobial stewardship and improving biosecurity measures on farms are

paramount for prevention, management and early detection of emerging ESC and colistin-resistance (even in the presence of strict regulations of CIA use).

The high frequency and high carriage levels of *E. coli* with resistance towards ampicillin and tetracycline among all ten Australian pig farms reflects the regular use of both antimicrobial classes in Australian pigs (63). However, the high frequency of gentamicin-resistant *E. coli* between farms was unexpected since the aminoglycoside is not a registered antimicrobial for use in Australian livestock (60). Cross-resistance may be an important attributing factor as other aminoglycosides such as neomycin, apramycin and spectinomycin are registered for use in Australian pigs (60, 63). Importantly, given that FQ-resistant *E. coli* isolates identified in this study displayed phenotypic resistance towards streptomycin but not to apramycin and gentamicin, while harbouring at least one type of aminoglycoside-resistance gene, the continued use of registered aminoglycosides in Australian pigs may lead to FQ-resistant *E. coli* also developing apramycin and gentamicin resistance through the cross-resistance mechanism.

In conclusion, this study achieved the aim of demonstrating the applicability of the enhanced AMR surveillance method to deliver a more accurate and detailed description of the presence and extent of AMR (including FQ-resistance with a low frequency) at the herd-level. It revealed a high frequency of *E. coli* with resistance towards ampicillin, tetracycline and gentamicin among all ten Australian pig farms. Carriage levels of ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* were comparable to the commensal *E. coli* population whilst gentamicin-resistant *E. coli* was lower. While no ESC-resistant *E. coli* were detected, FQ-resistant *E. coli* was detected on seven Australian pig farms with differing frequencies even in the absence of direct FQ use. Carriage levels of FQ-resistant *E. coli* were lower than the

commensal *E. coli* population. Genomic analysis revealed ST167 and ST744 as the dominant FQ-resistant *E. coli* STs among the seven Australian pig farms, with indication that they perhaps originated in humans or wild birds. These findings represent a baseline for on-going qualitative and quantitative CIA-resistance surveillance even in the presence of strict regulation of CIA use as an effective biosecurity assessment tool to detect the introduction and spread of CIA-resistance in Australian pigs.

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3.7 SUPPLEMENTARY MATERIALS

Table 3.1 - Supplementary Number of ciprofloxacin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to antimicrobial susceptibility testing using the broth microdilution technique, and the number of distinct multi-class resistant profiles identified on each farm. Each isolate from each farm corresponds to one sample containing ciprofloxacin-resistant *E. coli*.

Farm	Number of samples	Number of samples with ciprofloxacin-resistant *E. coli*	Number of MCR profiles
A	30	26	4
B	30	7	1
C	30	29	4
D	30	15	5
E	30	30	2
F	29	29	6
G	30	0	0
H	30	24	2
I	30	0	0
J	30	0	0

Table 3.2 - Supplementary Number of ciprofloxacin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to whole genome sequencing and the number of sequence types identified on each farm following sequencing. Each isolate from each farm corresponds to one sample containing ciprofloxacin-resistant *E. coli*.

Farm	Number of samples with ciprofloxacin-resistant *E. coli*	Number of sequenced isolates	ST167	ST744	ST10	ST34	ST11611	ST11612
A	26	6	**2**	0	**1**	**1**	**1**	**1**
B	7	2	**2**	0	0	0	0	0
C	29	13	**7**	**6**	0	0	0	0
D	15	7	**5**	**2**	0	0	0	0
E	30	10	**7**	**3**	0	0	0	0
F	29	6	**1**	**5**	0	0	0	0
H	24	6	0	**6**	0	0	0	0
Total	160	50	24	22	1	1	1	1

CHAPTER 4

Application of an enhanced antimicrobial resistance surveillance method to detect and quantify critically important antimicrobial-resistant *Escherichia coli* in Australian pigs on a national scale

4.1 ABSTRACT

Despite strict regulation, critically important antimicrobial (CIA)-resistant *Escherichia coli* have been detected in Australian pigs but their current prevalence is not known. Using an enhanced antimicrobial resistance (AMR) surveillance method combining a multiple samples per herd approach with laboratory robotics and selective agars incorporated with antimicrobials, this study investigated the presence and extent of CIA-resistant *E. coli* in Australian pigs on a national scale. All CIA-resistant *E. coli* were subjected to antimicrobial susceptibility testing by broth microdilution and sequencing to identify resistomes and genomic characteristics. Over 300 faecal samples from 30 pig farms across five Australian states were inoculated onto CHROMagar™ ESBL and CHROMagar™ ECC agars with and without ciprofloxacin. Ciprofloxacin-resistant *E. coli* was detected on 23 farms at a higher frequency than extended-spectrum cephalosporin (ESC)-resistant *E. coli* which was detected on eight farms. Carriage levels for CIA-resistant *E. coli* were lower than general commensal *E. coli*. ST744 was the most dominant FQ-resistant *E. coli* ST while ST4981 was found to be the most dominant ESC-resistant *E. coli* ST, with both STs likely introduced into Australian pigs from external sources. These findings highlight the capability of the enhanced AMR surveillance method to deliver substantially more accurate and detailed data at the herd-level. The approach can be affordably implemented on a national-scale, and leads to a more robust intelligence on the emergence and transmission of AMR at national and herd-level compared to the established approach for AMR surveillance in livestock.

4.2 INTRODUCTION

Critically important antimicrobials (CIAs) are the last line of defence against serious human bacterial infections (1). Thus, the development of resistance towards CIAs such as extended-spectrum cephalosporins (ESCs) and fluoroquinolones (FQs) is a significant public health concern (1). The emergence of CIA-resistant *Enterobacteriaceae* such as *Escherichia coli*, in livestock are of special interest due to these animals becoming potential reservoirs for CIA-resistance genes and facilitating the transmission of CIA-resistance to human pathogens via the food chain or other ecological pathways (2-5). While the emergence of CIA-resistance has generally been associated with selective pressure from direct CIA use, international reports of CIA-resistance emerging without direct CIA use in livestock and other animal species added a new dimension to the threat of CIA-resistant bacteria (3, 6-9) .

Unlike other parts of the world, Australia enjoys a unique antimicrobial resistance (AMR) status due to its geographical isolation, strict regulations on livestock importation and CIA usage in livestock (10, 11). The latter are of particular importance as FQ has never been registered for use in Australian livestock while ESC usage has been restricted by constraints applied during registration and product labelling (3, 10). However, despite such factors, CIA-resistant *Enterobacteriaceae* have been identified in Australian livestock albeit at a low frequency (3, 4, 10, 12, 13).

In Chapter 3, FQ-resistant *E. coli* was detected in seven of ten Australian pig farms in the absence of direct FQ use. FQ-resistant *E. coli* had an average carriage level of 5.7×10^4 colony forming units per gram (CFU/g) of faeces, although this average is well below the population level of general commensal *E. coli* of 1.35×10^7 CFU/g. Further genomic characterisation revealed ST167 and ST744 were the two dominant FQ-resistant *E. coli* sequence types (STs)

currently present among these seven farms, with both STs belonging to globally disseminated multi-class resistant (MCR, resistant to three or more antimicrobial classes) pathogenic clonal lineages (14-17). With recent Australian studies also identifying CIA-resistant *E. coli* in Australian silver gulls (18), and discovering evidence of methicillin-resistant *Staphylococcus aureus* transmission pathways between humans to pigs (19), Chapter 3 theorised that the presence of FQ-resistant *E. coli* in these seven farms was likely introduced by human carriers or wild birds transmitting CIA-resistant *E. coli* to transient bird species. However, due to the constrained geographical context of Chapter 3, it is unclear if the observed level of FQ-resistant *E. coli* is a national phenomenon affecting majority of Australian pig farms, and if the two dominant STs are responsible for the presence of FQ-resistant *E. coli* among Australian pigs nationwide. To address this, this study uses the same enhanced AMR surveillance method in Chapter 3 to investigate the presence and extent of CIA-resistant *E. coli* in Australian pig herds nationwide, followed by antimicrobial susceptibility testing (AST) and sequencing to identify MCR profiles and genomic characteristics of CIA-resistant *E. coli*.

4.3 MATERIALS AND METHODS

Sample collection. A total of 300 faecal samples from finisher pigs, comprised of ten samples each from 30 farms located across five Australian states, were collected at abattoirs between August to November 2020. The number of farms sampled from each state was in proportion to the size of the pig population indicated by data provided by the industry body (Australian Pork Limited). Samples were collected by veterinarians or under veterinary supervision by making an incision in the rectal wall post-evisceration using sterilised equipment to gather faeces into sterile containers. Individual samples were obtained at ten-minute intervals on the slaughter line until all ten samples were collected for a farm. If a

selected pig did not have any rectal contents, a replacement sample was collected from the next available pig in the slaughter sequence. Samples were transported to the laboratory on ice and processed within 24 hours of collection.

Bacterial isolation and quantification. Sample processing, agar inoculation and incubation, and presumptive identification of *E. coli* colonies on agar for quantification were performed as per the Robotic Antimicrobial Susceptibility Platform (RASP) protocols described in Chapter 3 with the exception that only CHROMagar™ ECC (CHROMagar) (MicroMedia, Edwards Group) with and without incorporation of ciprofloxacin (4 µg/mL) and CHROMagar™ ESBL (CHROMagar ESBL) (MicroMedia, Edwards Group) agars were used in this study. A subset of up to eight *E. coli* colonies from CHROMagar agar incorporated with ciprofloxacin (presumed ciprofloxacin-resistant *E. coli*) and a subset of up to eight colonies from CHROMagar ESBL agar (presumed ESC-resistant *E. coli*) were isolated from every positive agar by RASP and stored using the same protocols described in Chapter 3.

Antimicrobial susceptibility testing and genomic sequencing. Prior to AST, MALDI-TOF (Bruker Microflex) was used to confirm the identity of *E. coli* colonies as per protocols described in Chapter 3. Susceptibility to eight antimicrobials representing eight classes were assessed and performed on RASP as described in Chapter 3, and includes ampicillin (beta-lactam), apramycin (aminocyclitol), cefotaxime (third-generation cephalosporin), ciprofloxacin (quinolone), florfenicol (phenicol), gentamicin (aminoglycoside), tetracycline (tetracycline) and trimethoprim-sulfamethoxazole (folate pathway inhibitor). Minimal inhibitory concentration (MIC) interpretation of each antimicrobial and MCR categorisation followed the same protocol described in Chapter 3. An AMR index scheme, which rates antimicrobials based on their public health significance, was used as a summary measure of

resistance to compare isolated colonies within and between samples and farms. The scoring of antimicrobials was based on the Australian Strategic and Technical Advisory Group (ASTAG) importance rating (20). Antimicrobials of low, medium and high importance received a weighting of one, two and three respectively. The weighting of each resistance harboured by each isolate were tallied to acquire an AMR index score. Isolates that were susceptible to all antimicrobials received an index score of zero while those with resistance towards all eight antimicrobials received an index score of 15.

Isolates from each farm of each state were purposefully selected to ensure representation of each unique MCR profile, and whole genome sequencing and genomic characterisation of isolates performed as described in Chapter 3.

Statistical analysis. Under the RASP protocol, data from digital imaging of colonies on selective agars were all electronically captured for processing and descriptive analysis in the Stata analysis package (version 16.0, Stata Corporation, Texas, USA). Quantitative counts of *E. coli* in CFU/g of faeces were $\log_{10}$ transformed where required for interpretation and analysis. Similarly, MIC data from CIA-resistant *E. coli* isolates were captured by RASP and processed within Stata to obtain MIC tables with exact confidence intervals for proportion of resistant colonies derived by the Clopper-Pearson method.

4.4 RESULTS

Ciprofloxacin-resistant *E. coli*. The frequencies and carriage level of ciprofloxacin-resistant *E. coli* in each of 30 pig farms across five Australian states is shown in Figure 4.1. Overall, a total of 120 (40.0%) samples were detected with ciprofloxacin-resistant *E. coli* across 23 (76.7%) pig farms. Among the farms with FQ-resistant *E. coli*, variation in frequency of

positive samples was observed, with nine farms (Farms 7, 9, 12, 14, 16, 27, 28, 29 and 30) having more than five samples while two farms (Farms 3 and 21) only had one sample (Table 4.1 - Supplementary). Average carriage level of ciprofloxacin-resistant *E. coli* across positive farms was 1 $\log_{10}$ CFU/g. However, the overall average carriage level of general commensal *E. coli* was 6 $\log_{10}$ CFU/g across all farms thus making the average carriage level of ciprofloxacin-resistant *E. coli*, regardless of farm, consistently lower than general commensal *E. coli* by at least 3 $\log_{10}$ CFU/g.

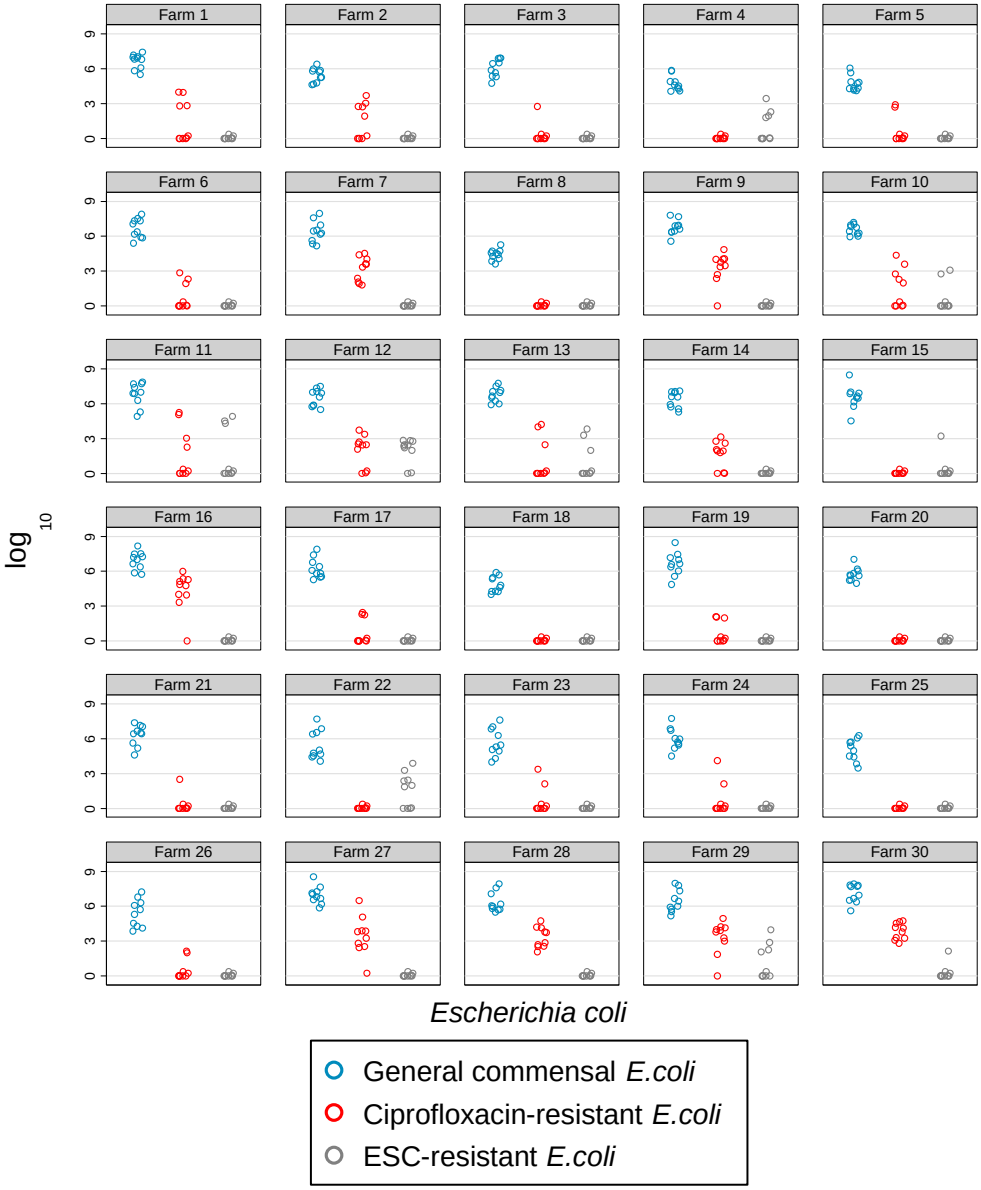

Figure 4.1 Farm-level and animal-level variation in concentration (log_{10} CFU/g) of commensal *Escherichia coli* phenotypes in pig faeces. Data represents 900 observations from 300 individual faecal samples across 30 farms. Each faecal sample was enumerated three times: once on CHROMagar™ ECC agar without antimicrobials, once on CHROMagar™ ECC agar incorporated with ciprofloxacin, and once on CHROMagar™ ESBL agar. Enumeration was by image capture and digital analysis of chromogenic traits of individual colonies.

A total of 710 ciprofloxacin-resistant *E. coli* isolates from 120 CHROMagar agars incorporated with ciprofloxacin (up to eight colonies for each positive sample) were subjected to AST in RASP using the broth microdilution technique. The percentages of resistance for all ciprofloxacin-resistant *E. coli* are shown in Figure 4.2. All presumptive ciprofloxacin-resistant *E. coli* were confirmed to be resistant to ciprofloxacin (100%), with MIC values at or above the Clinical and Laboratory Standards Institute (CLSI) clinical breakpoint of 1 µg/mL (21), except for one isolate which had a lower MIC value that exceeded the European Committee of Antimicrobial Susceptibility Testing (EUCAST) ECOFF value of 0.064 µg/mL (22). Additionally, these isolates were also resistant towards ampicillin (98.6%), tetracycline (86.9%), trimethoprim-sulfamethoxazole (79.4%), florfenicol (30.8%), gentamicin (10.0%), cefotaxime (1.8%) and apramycin (0.1%). All isolates exhibiting resistance towards cefotaxime had MIC values at the CLSI clinical breakpoint of 4 µg/mL (21). Overall, there was little variation in the AMR index score of ciprofloxacin-resistant *E. coli* isolates originating from the same pig, although inter-pig and intra-farm variations existed as demonstrated by five farms (Farms 7, 9, 10 and 29) having a uniform AMR index score compared to other farms (Farms 11, 16, 27 and 28) (Figure 4.3). Between farms, each had their own AMR index score pattern that differs from other farms (Figure 4.3).

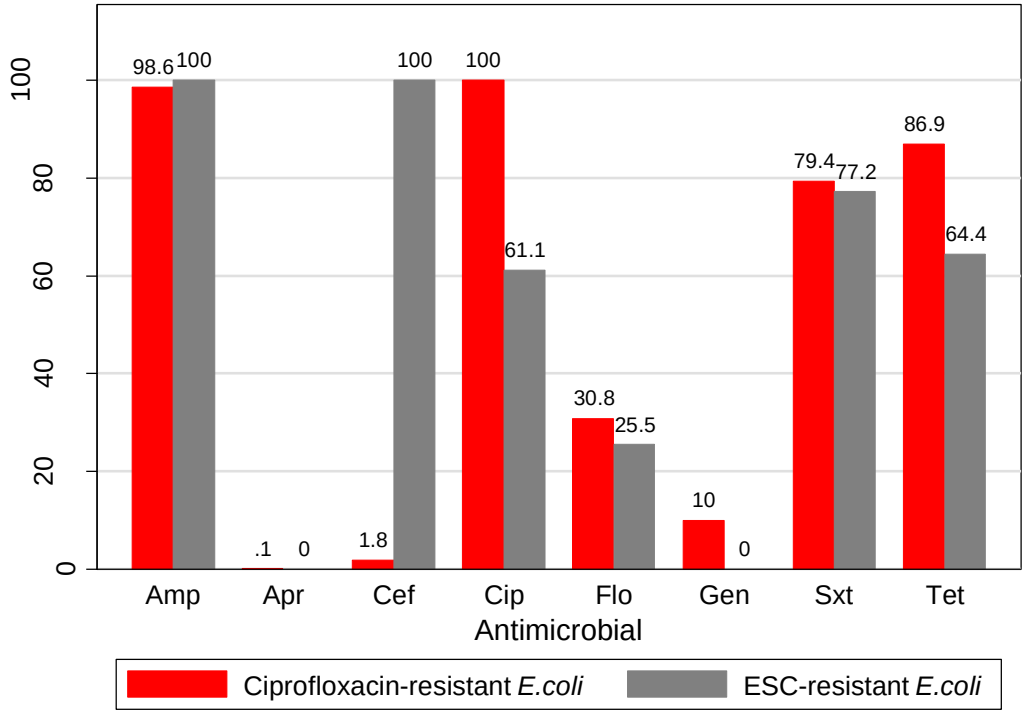

Figure 4.2 Percentage of ciprofloxacin-resistant *Escherichia coli* (*n* = 710) and extended-spectrum cephalosporin-resistant *E. coli* (*n* = 149) isolates resistant to eight antimicrobials based on the epidemiological cut-off (ECOFF from EUCAST) value, with the exception of apramycin that was based on the ECOFF value validated by Yang et al. (2020) (23). Key: Amp - Ampicillin, Apr - Apramycin, Cef - Cefotaxime, Cip - Ciprofloxacin, Flo - Florfenicol, Gen - Gentamicin, Sxt - Trimethoprim-sulfamethoxazole, Tet - Tetracycline.

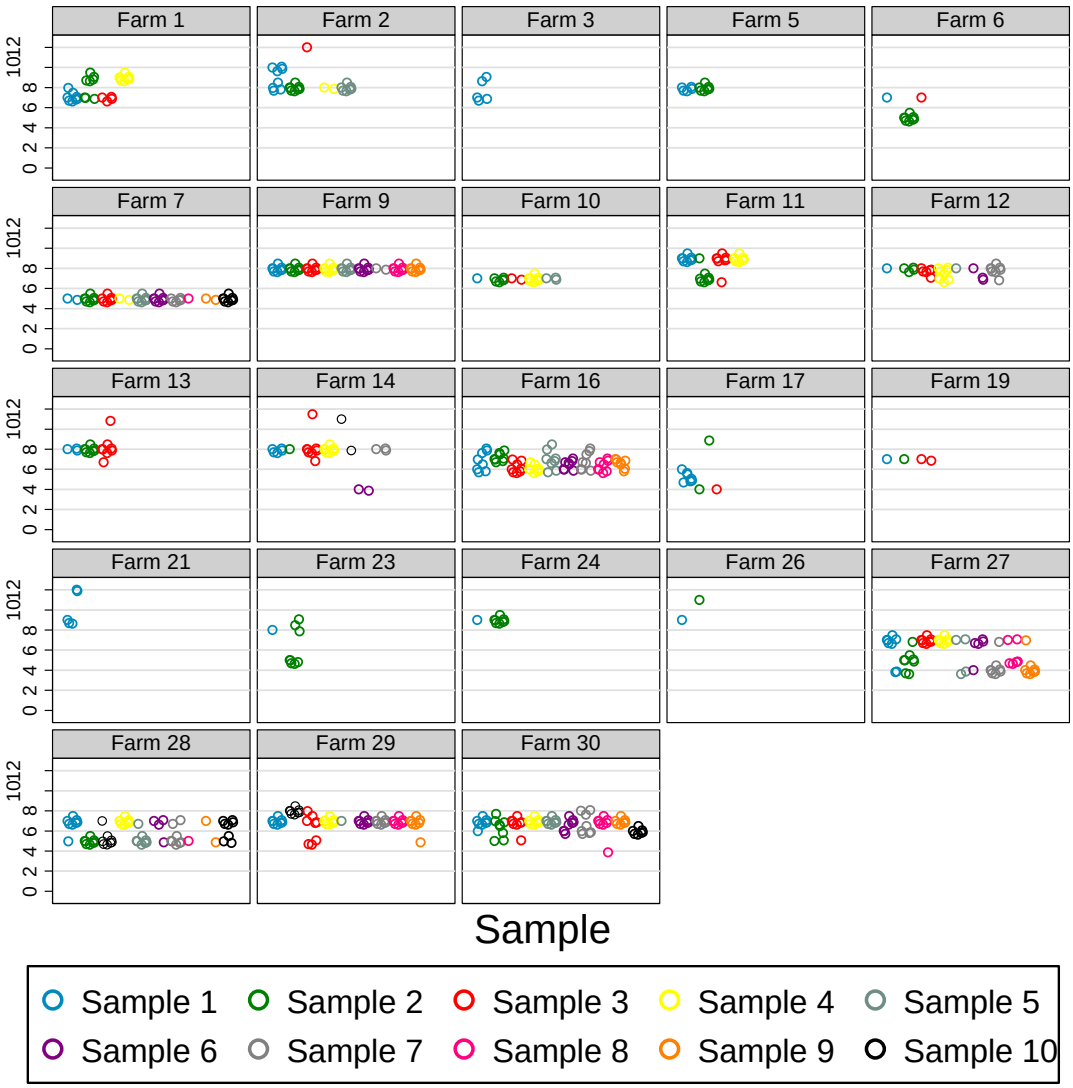

Figure 4.3 Antimicrobial resistance index score for each of 710 ciprofloxacin-resistant *Escherichia coli* isolates from 23 pig farms positive for ciprofloxacin-resistance across five Australian states. Samples within farm are colour coded to show extent of variation within and between isolates and samples on each farm. Farms without ciprofloxacin-resistance are excluded. Index scores are the sum of weights for each form of single resistance present in each isolate (see text).

The majority of ciprofloxacin-resistant *E. coli* isolates (*n* = 676, 95.2%) were classified as MCR (resistant to three or more antimicrobial classes) with 11 MCR profiles identified (Table 4.1). The most common profile was resistance towards four antimicrobial classes (*n* = 247, 34.8%: beta-lactam, folate pathway inhibitor, quinolone and tetracycline), followed by resistance towards five (*n* = 189, 26.6%: beta-lactam, folate pathway inhibitor, phenicol, quinolone and tetracycline), and three (*n* = 113, 15.9%: beta-lactam, quinolone and tetracycline) antimicrobial classes (Table 4.1).

Table 4.1 Multi-class resistance profiles of ciprofloxacin-resistant *Escherichia coli* and extended-spectrum cephalosporin-resistant *E. coli* isolates based on interpretive breakpoints applied in Figure 4.2. Isolates with resistance towards three or more antimicrobial classes are classified as multi-class resistant.

		Ciprofloxacin-resistant *E. coli*		**ESC-resistant *E. coli***	
Multi-class resistance profile	**Number of antimicrobial classes**	**Number of isolates**	**% of total**	**Number of isolates**	**% of total**
bla-c3g-fpi	3	0	0	1	0.7
bla-c3g-phe	3	0	0	3	2.0
bla-c3g-qui	3	7	1.0	22	14.8
bla-fpi-qui	3	54	7.6	0	0
bla-qui-tet	3	113	15.9	0	0
bla-c3g-fpi-phe	4	0	0	26	17.4
bla-c3g-fpi-qui	4	0	0	1	0.7
bla-c3g-fpi-tet	4	0	0	19	12.8
bla-c3g-phe-tet	4	0	0	9	6.0
bla-fpi-phe-qui	4	1	0.1	0	0
bla-fpi-qui-tet	4	247	34.8	0	0
ami-bla-fpi-qui-tet	5	54	7.6	0	0
bla-c3g-fpi-qui-tet	5	0	0	68	45.6
bla-fpi-phe-qui-tet	5	189	26.6	0	0
ami-bla-c3g-fpi-qui-tet	6	2	0.3	0	0
ami-bla-fpi-phe-qui-tet	6	4	0.6	0	0
bla-c3g-fpi-phe-qui-tet	6	4	0.6	0	0
acy-ami-bla-fpi-phe-qui-tet	7	1	0.1	0	0
Total		676	95.2	149	100

acy: aminocyclitol, ami: aminoglycoside, bla: beta-lactam, c3g: third-generation cephalosporin, fpi: folate pathway inhibitor, phe: phenicol, qui: quinolone, tet: tetracycline

A subset of 76 ciprofloxacin-resistant *E. coli* isolates were sequenced, with majority of isolates found to belong to ST744 (*n* = 29), with 12 other STs identified (Table 4.2 - Supplementary). The number of MCR profiles and the AMR genes identified amongst isolates belonging to each ST are shown in Table 4.2. All isolates displayed substitutions within the quinolone-resistance determining regions (QRDR) with 60 isolates (78.9%) possessing C248T, G259A, A2034C and T2482G substitutions in *gyrA*, and G239T substitution in *parC*. In addition to these substitutions, one isolate harboured the T240C substitution in *parC*, and three isolates (3.9%) also possessed the T240C substitution in *parC* and T1372G substitutions in *parE*.

No known ESC-resistance genes were detected in any sequenced ciprofloxacin-resistant *E. coli* isolates. The plasmid-mediated FQ-resistance (PMQR) gene *qnrS* was found in 15 (19.7%) isolates while 19 (25.0%) isolates harboured the phenicol-resistance gene *floR*. Two types of beta-lactam-resistance genes ($bla_{\text{TEM-1B}}$ and $bla_{\text{TEM-176}}$) were identified with $bla_{\text{TEM-1B}}$ present in 68 (89.5%) isolates and $bla_{\text{TEM-176}}$ present only in three (3.9%) isolates. Three types of sulphonamide-resistance genes were identified (*sul1*, *sul2* and *sul3*) with 67 (88.2%) isolates harbouring at least one sulphonamide-resistance gene. Four types of tetracycline-resistance genes were identified (*tet[A]*, *tet[B]*, *tet[H]* and *tet[M]*) with 62 (81.6%) isolates harbouring at least one tetracycline-resistance gene. Seven aminoglycoside-resistance genes comprising five different families (*aac[3]*, *aadA2*, *aadA5*, *aph[3"]*, *aph[3']* and *aph[6]*) were identified with 71 (93.4%) isolates harbouring at least one aminoglycoside-resistance family gene. No known virulence genes consistent with pathogenic *E. coli* were identified. A total of 29 plasmids were identified with IncFIB(AP001918) being the most frequently found plasmid across all STs (*n* = 44, 57.9%) followed by IncX1 (*n* = 33, 43.4%), IncFIC(FII) (*n* =

21, 27.6%), Incl1_Alpha (*n* = 17, 22.4%), IncFIA(HI1) (*n* = 16, 21.1%) and IncFIB(K)_Kpn3 (*n* = 15, 19.7%).

Table 4.2 Number of isolates, multi-class resistant profiles and known antimicrobial resistance genes detected for each ciprofloxacin-resistant *Escherichia coli* sequence type.

ST	Number of isolates	Number of MCR Profiles	Antimicrobial resistance genes
10	2	0	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul2*, *tet(B)*
44	3	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul3*, *aadA2*
155	2	1	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *aac(3)*, *aadA2*
167	9	3	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *aac(3)*
361	4	2	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *tet(M)*, *aadA2*
542	4	2	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-176}}$, *sul3*, *tet(A)*, *tet(B)*, *aadA2*
617	1	1	QRDR mutation, *qnrS*, *bla*$_{\text{TEM-1B}}$, *sul3*, *tet(B)*, *aadA2*
744	29	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, *tet(H)* , *tet(M)*, *aadA2*
1642	10	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *sul1*, *sul3*, *tet(A)*, *aac(3)*, *aadA2*
5909	4	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *sul3*, *tet(A)*, *tet(B)*, *tet(M)*, *aadA2*
11613	2	1	QRDR mutation, *qnrS*, *floR*, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *tet(M)*, *aadA2*
11916	1	1	QRDR mutation, *floR*, *bla*$_{\text{TEM-1B}}$, *sul3*, *tet(A)*, *tet(M)*, *aadA2*
11917	5	2	QRDR mutation, *bla*$_{\text{TEM-1B}}$, *sul3*, *tet(A)*, *aadA2*

ESC-resistant *E. coli*. The frequencies and carriage level of ESC-resistant *E. coli* in each of 30 pig farms across five Australian states is shown in Figure 4.1. Overall, a total of 32 (10.7%) samples were detected with ESC-resistant *E. coli* across eight (26.7%) pig farms. Farms 12 and 22 had the highest frequencies of positive samples with eight and six samples respectively, while Farm 10 and 15 had the lowest with two and one samples respectively (Table 4.3 - Supplementary). Overall, average carriage level of ESC-resistant *E. coli* across positive farms was 1 $\log_{10}$ CFU/g. Therefore, this also makes average carriage level of ESC-resistant *E. coli*, regardless of farm, consistently lower than general commensal *E. coli* by at least 4 $\log_{10}$ CFU/g.

A total of 149 ESC-resistant *E. coli* isolates from 32 CHROMagar ESBL agars (up to eight colonies per positive sample) were subjected to AST in RASP using the broth microdilution technique. The percentages of resistance for all ESC-resistant *E. coli* are also shown in Figure 4.2. All presumptive ESC-resistant *E. coli* were confirmed to be resistant to cefotaxime (100%), with MIC values at the CLSI clinical breakpoint of 4 µg/mL (21). Additionally, these isolates were also resistant towards ampicillin (100%), trimethoprim-sulfamethoxazole (77.2%), tetracycline (64.4%), ciprofloxacin (61.1%) and florfenicol (25.5%). Of the isolates exhibiting ciprofloxacin-resistance, 20 (13.4%) isolates had MIC values exceeding the EUCAST ECOFF value of 0.064 µg/mL (22), while 71 (47.7%) isolates had MIC values exceeding the CLSI clinical breakpoint of 1 µg/mL (21). Overall, there was little variation in the AMR index score of isolates originating from the same pig, with this same pattern observed between pigs of the same farm particularly Farms 12, 13 and 22 (Figure 4.4). However, between farms, the AMR index score pattern differs especially with Farms 12 and 13 having a score of ten compared to Farm 22 with a score of seven (Figure 4.4).

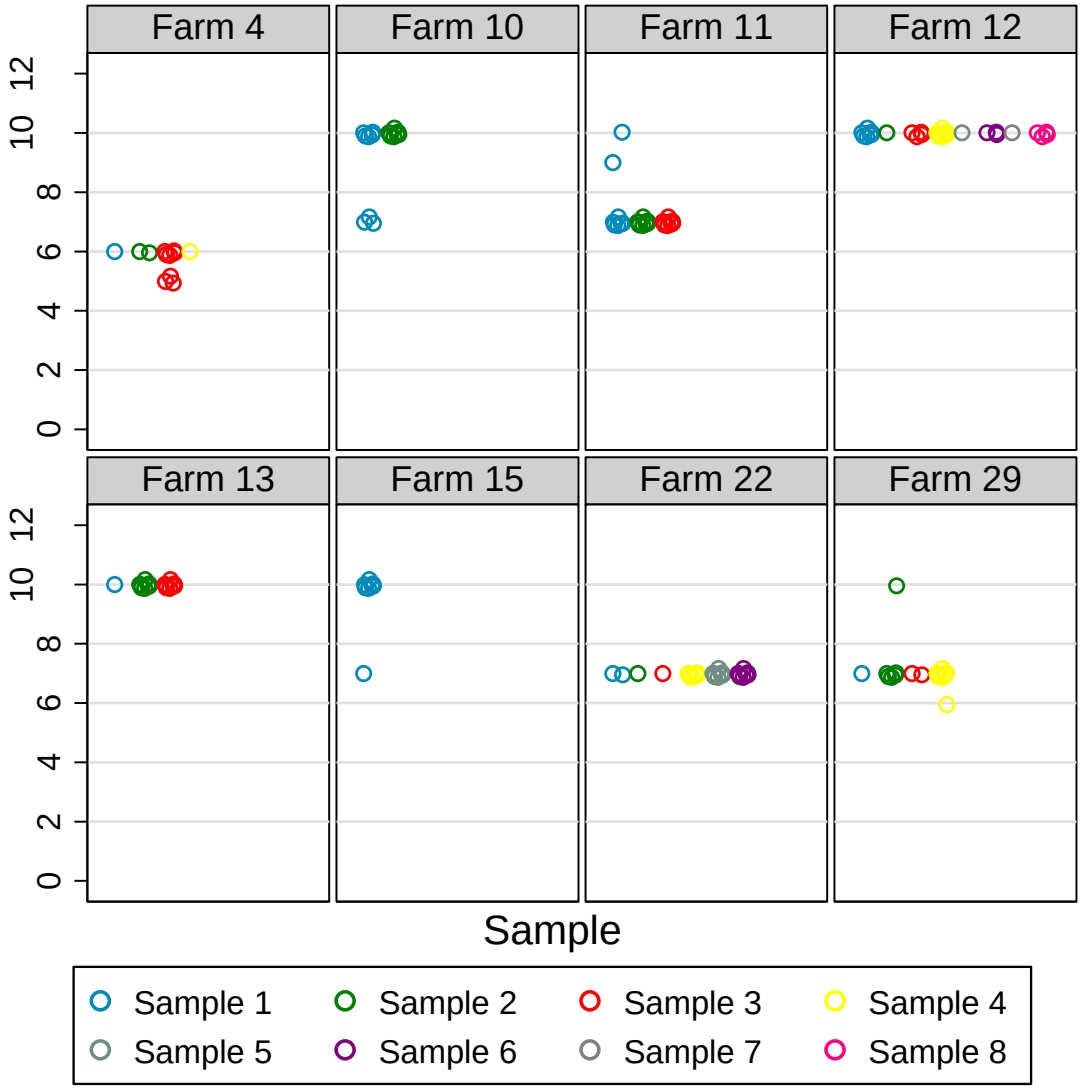

Figure 4.4 Antimicrobial resistance index score for each of 149 extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* isolates from 30 pig farms positive for ESC-resistance across five Australian states. Samples within farm are colour coded to show extent of variation within and between isolates and samples on each farm. Farms without ESC-resistance are excluded. Index scores are the sum of weights for each form of single resistance present in each isolate (see text).

All 149 ESC-resistant *E. coli* were classified as MCR with eight MCR profiles identified (Table 4.1). The most common profile was resistance towards five antimicrobial classes (*n* = 68, 45.6%: beta-lactam, third-generation cephalosporin, folate pathway inhibitor, quinolone and tetracycline), followed by resistance towards four (*n* = 26, 17.4%: beta-lactam, third-generation cephalosporin, folate pathway inhibitor and phenicol), and three (*n* = 22, 14.8%: beta-lactam, third-generation cephalosporin and quinolone) antimicrobial classes (Table 4.1).

A subset of 21 ESC-resistant *E. coli* isolates were sequenced, with majority of isolates found to belong to ST4981 (*n* = 7), with five other STs identified (Table 4.4 - Supplementary). The number of MCR profiles and the AMR genes identified amongst isolates belonging to each ST are shown in Table 4.3. All isolates displayed at least one known substitution within the QRDRs. Notably, all ST4981 (*n* = 7, 33.3%) isolates displayed C248T, G259A and T2482G substitutions in *gyrA*, G239T substitutions in *parC* and T1372G substitutions in *parE*.

The PMQR gene *qnrS* was found in four (19.0%) isolates. Three types of ESC-resistance genes were identified, with $bla_{\text{CTXM-1}}$ gene identified in six (28.6%) isolates, the $bla_{\text{CTXM-14}}$ gene in five (23.8%) isolates, and the $bla_{\text{CTXM-15}}$ gene in ten (47.6%) isolates. The phenicol-resistance gene *floR* was identified in five (23.8%) isolates while the beta-lactam-resistance genes $bla_{\text{TEM-1B}}$ and $bla_{\text{TEM-106}}$ was found in seven (33.3%) and one (4.8%) isolates respectively. Two types of sulphonamide-resistance genes (*sul2* and *sul3*) were identified with each isolate harbouring at least one sulphonamide-resistance gene. Two types of tetracycline-resistance genes (*tet[A]* and *tet[M]*) were identified with 14 (66.7%) isolates harbouring at least one of tetracycline-resistance gene. Five aminoglycoside-resistance genes comprising five different families (*aac[3]*, *aadA2*, *aadA5*, *aph[3")*, *aph[3']* and *aph[6]*) were identified with 16 (76.2%) isolates harbouring at least one aminoglycoside-resistance

family gene. No known virulence genes consistent with pathogenic *E. coli* were identified. A total of 14 plasmids were identified with IncFIB(AP001918) being the most frequently found plasmid across all STs (*n* = 13, 61.9%) followed by IncR (*n* = 7, 33.3%), IncFII (*n* = 6, 28.6%) and Incl1_Alpha (*n* = 6, 28.6%).

Table 4.3 Number of isolates, multi-class resistant profiles and known antimicrobial resistance genes detected for each extended-spectrum cephalosporin-resistant *Escherichia coli* sequence type.

ST	Number of isolates	Number of MCR Profiles	Antimicrobial resistance genes
10	4	3	QRDR mutation, *bla*$_{\text{CTXM-1}}$, *bla*$_{\text{TEM-1B}}$, *sul2*, *sul3*, *tet(A)*, *aadA2*, *aadA5*, *aph(3')*
88	1	1	QRDR mutation, *qnrS*, *bla*$_{\text{CTXM-14}}$, *bla*$_{\text{TEM-106}}$, *sul3*, *tet(A)*
117	4	3	QRDR mutation, *bla*$_{\text{CTXM-14}}$, *floR*, *bla*$_{\text{TEM-1B}}$, *sul2*, *tet(A)*, *aph(3'')*, *aph(3')*, *aph(6)*
196	2	1	QRDR mutation, *bla*$_{\text{CTXM-1}}$, *floR*, *sul2*, *aadA5*
2325	3	2	QRDR mutation, *qnrS*, *bla*$_{\text{CTXM-15}}$, *bla*$_{\text{TEM-1B}}$, *sul2*, *tet(A)*, *aph(3'')*, *aph(6)*
4981	7	3	QRDR mutation, *bla*$_{\text{CTXM-15}}$, *sul3*, *tet(A)*, *tet(M)*, *aadA2*

4.5 DISCUSSION

This study investigated the presence and extent of CIA-resistant *E. coli* among Australian pigs nationwide using the enhanced AMR surveillance method described in Chapter 3. Widespread presence of FQ-resistant *E. coli* among the majority of pig farms (*n* = 23, 76.7%) in this study was detected, and attributed to the presence of globally disseminated dominant FQ-resistant *E. coli* ST744 clones in Australian pigs. The presence of ESC-resistant *E. coli* among pig farms in this study was also detected albeit at a lower frequency (*n* = 8, 26.7%), and attributed to the presence of dominant ESC-resistant *E. coli* ST4981. However, carriage levels of CIA-resistant *E. coli* were consistently lower than the commensal *E. coli* population by at least 3 $\log_{10}$ CFU/g. Overall, the findings demonstrate the capability of the enhanced AMR surveillance method to provide quality state and national-level AMR data through a combination of sensitivity in the laboratory combined with a much higher intensity of isolate, animal and farm sampling. The result is a more accurate and detailed description on the presence and extent of AMR at the herd-level with profound improvement in capacity for early detection of CIA-resistance.

The detection of FQ-resistant *E. coli* in majority of pig farms in this study indicate that FQ-resistance is well established in the Australian pig population even though FQ is not registered for use. Despite this, carriage level of FQ-resistant *E. coli* is many orders of magnitude lower than general commensal *E. coli*, indicating that regardless of farm, FQ-resistance has not yet spread throughout the commensal *E. coli* population. Moreover, the chromosomal-mediated nature of FQ-resistance, where it only arises from specific mutations within the QRDRs, means that FQ-resistant *E. coli* is incapable of transferring FQ-resistance to pathogenic bacteria via horizontal transfer (24). This study was also the first to identify the PMQR *qnrS* gene among FQ-resistant *E. coli* isolates in Australian pigs. While PMQR genes

are transferrable via plasmids, they are of limited clinical relevance to humans and animals for several reasons (25). By itself, PMQR genes only confer low-levels of FQ-resistance that are below the clinical breakpoints (26, 27). Moreover, though PMQR genes also facilitate the selection of chromosomal-mediated FQ-resistance, this can only occur in the presence of selective pressure from FQ use (26, 27). This facilitation of chromosomal-mediated FQ-resistance is not a threat to animal and public health due to regulations preventing FQ use in Australian livestock. Nevertheless, plasmids harbouring PMQR genes may also harbour genes conferring resistance to other antimicrobials such as ESCs, which a subset of FQ-resistant *E. coli* in this study displayed phenotypically. For this reason, on-going antimicrobial stewardship in the livestock sector is essential for preventing the spread of FQ-resistant *E. coli* with plasmids harbouring CIA-resistance genes.

While ST744 was detected previously in an Australian pig (3), this study represents the first Australian study to identify ST744 as the current dominant FQ-resistant *E. coli* ST among majority of Australian pig herds nationwide. ST744 is well-known to be a FQ-resistant clone found globally (28-32). In Chapter 3, it was concluded that ST744 did not emerge locally, and most likely was introduced to Australian pigs via human carriers or migratory wild birds due to the close phylogenetic association of ST744 isolates from the seven Australian pig farms sampled in Chapter 3 with international ST744 isolates. The fact that all ST744 isolates (both in Chapter 3 and this study) harboured QRDR mutations, which is only possible via selective pressure from FQ use (24), reinforces the theory of introduction from external sources since FQ is not registered for use in Australian livestock. However, without any selective pressure from FQ use, it is interesting to note that FQ-resistant *E. coli* has persisted in Australian pigs since its first detection in 2015 (3). Considering that FQ-resistant *E. coli* ST744 displayed phenotypic resistance towards antimicrobial classes that are registered for

use in Australian pigs (beta-lactam, phenicol and tetracycline) (33), it is possible that the use of these antimicrobials is creating a niche environment for FQ-resistant *E. coli* ST744 to survive and proliferate. While further temporal studies are required to investigate this, it again highlights the importance of antimicrobial stewardship to control further spread of globally disseminated FQ-resistant *E. coli* clones.

In contrast to Chapter 3 findings, ESC-resistant *E. coli* were detected on eight farms, indicating that ESC-resistance is also present in Australian pig herds nationwide, albeit at low levels, despite the constraints on ESC use in Australian livestock (33). However, although the frequency and carriage level of ESC-resistant *E. coli* is even lower than FQ-resistant *E. coli*, the threat to public health is not necessarily low. It is well accepted that ESC-resistance spreads widely via plasmids (34-37) suggesting future potential for ESC-resistant *E. coli* to spread more extensively through the commensal *E. coli* population. There is thus a role for using highly sensitive techniques demonstrated in this study to continue monitoring the ESC-resistance burden in the gut of livestock.

The presence of QRDR mutations within ESC-resistant *E. coli* indicates a likely origin from external sources as local emergence of these mutations is not possible with the absence of FQ use. Given that this study is not the first to detect ESC-resistant *E. coli* (3, 38), this suggests that ESC-resistant *E. coli* has persisted among Australian pigs for a period of time. In fact, the ESC-resistance genes identified in this study were the same genes previously reported among Southeast Australian pig herds (38). This persistence may be attributed to ceftiofur use as it is the only ESC available for use in Australian pigs as an off-label treatment (10). In the last national survey on antimicrobial use in Australian pig farms, it was revealed that ceftiofur was used in 25% of farms (39), which provides a niche environment for ESC-

resistant *E. coli* to survive and proliferate. Though the restricted use of ESCs has likely contributed to the low frequency and carriage level of ESC-resistant *E. coli*, the use of ceftiofur still presents opportunities for ESC-resistance to persist for up to four years even when ceftiofur use is removed (34). Further temporal investigations into the relationship of ceftiofur use with the frequency and carriage level of ESC-resistant *E. coli* among Australian pigs is required to ascertain the effects of restricted ESC use on the persistence of ESC-resistant *E. coli*. Additionally, further phylogenetic studies investigating the relationship of dominant ESC-resistant *E. coli* ST4981 in this study with international isolates and identified ESC-resistant *E. coli* from other Australian pig studies would also help provide more evidence on how ESC-resistant *E. coli* was introduced into Australian pigs.

While the enhanced AMR surveillance method was initially developed to deliver a more accurate and detailed description of AMR at the herd-level, this also leads to a higher quality AMR data at the state and national-level. However, with CIA-resistant *E. coli* within Australian pigs likely persisting due to antimicrobial use, this indicates that the inclusion of antimicrobial usage data is also essential for further enhancement of AMR surveillance data. Moreover, with the introduction of CIA-resistant *E. coli* within farms also being affected by herd-level biosecurity protocols, it is also critical to consider animal and production management systems, movement of farm workers and animals, and the presence of wild birds around farms as part of the AMR surveillance data. Doing so would not only improve the accuracy and detail of AMR scenarios in each farm but also increase the understanding of CIA-resistance transmission pathways and selective pressure. Ultimately, this serves to further improve judgements for implementing effective AMR control strategies as part of biosecurity protocols to prevent further introduction and spread of CIA-resistance among Australian livestock.

In conclusion, this study reports widespread presence of FQ-resistant *E. coli* and the presence of ESC-resistant *E. coli* in Australian pig herds on a national scale. Although FQ-resistant *E. coli* had a higher frequency and carriage level than ESC-resistant *E. coli*, carriage levels of CIA-resistant *E. coli* were consistently several orders of magnitude lower than the commensal *E. coli* population. CIA-resistant *E. coli* with resistance towards both FQ and ESC were also detected at low levels. Although the public health significance of these findings is uncertain, they suggest that continued monitoring of the extent of FQ and ESC-resistance is needed and could be based on the demonstrably sensitive techniques used in this study.

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4.7 SUPPLEMENTARY MATERIALS

Table 4.1 - Supplementary Number of ciprofloxacin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to antimicrobial susceptibility testing using the broth microdilution technique, and the number of distinct multi-class resistant profiles identified on each farm.

Farm	Number of samples	Number of samples with ciprofloxacin-resistant *E. coli*	Number of isolates subjected to AST	Number of multi-class resistant profiles
1	30	4	28	3
2	30	5	26	3
3	30	1	5	2
5	30	2	12	1
6	30	3	10	2
7	30	10	52	1
9	30	9	66	1
10	30	5	20	1
11	30	4	32	3
12	30	7	31	2
13	30	3	19	3
14	30	7	29	3
16	30	9	72	3
17	30	3	10	3
19	30	3	4	1
21	30	1	5	2
23	30	2	9	3
24	30	2	9	1
26	30	2	2	2
27	30	9	63	2
28	30	10	63	2
29	30	9	65	2
30	30	10	78	4

Table 4.2 - Supplementary Number of ciprofloxacin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to whole genome sequencing and the number of sequence types identified on each farm following sequencing.

Farm	Number of samples with ciprofloxacin-resistant *E. coli*	Number of sequenced isolates	ST10	ST44	ST155	ST167	ST361	ST542	ST617	ST744	ST1642	ST5909	ST11613	ST11916	ST11917
1	4	4	0	**1**	0	0	**1**	0	0	0	**2**	0	0	0	0
2	5	3	0	0	0	0	0	**1**	0	0	**2**	0	0	0	0
3	1	2	0	0	0	0	0	0	1	0	0	0	0	**1**	0
5	2	2	0	0	0	0	0	**2**	0	0	0	0	0	0	0
6	3	3	0	0	0	0	0	0	0	**3**	0	0	0	0	0
7	10	3	0	0	0	0	0	0	0	**3**	0	0	0	0	0
9	9	2	0	0	0	0	0	0	0	**2**	0	0	0	0	0
10	5	2	0	0	0	0	0	0	0	**2**	0	0	0	0	0
11	4	2	0	0	**2**	0	0	0	0	0	0	0	0	0	0
12	7	3	0	0	0	0	0	0	0	**1**	0	**2**	0	0	0
13	3	2	0	0	0	0	0	0	0	0	0	**2**	0	0	0
14	7	6	0	0	0	**1**	**3**	0	0	**2**	0	0	0	0	0
16	9	4	0	0	0	0	0	0	0	0	0	0	0	0	**4**
17	3	5	0	0	0	0	0	0	0	**3**	**1**	0	0	0	**1**
19	3	2	0	0	0	0	0	0	0	**2**	0	0	0	0	0
21	1	2	0	0	0	0	0	0	0	0	**2**	0	0	0	0
23	2	2	0	**1**	0	0	0	0	0	0	**1**	0	0	0	0
24	2	1	0	0	0	0	0	0	0	0	**1**	0	0	0	0
26	2	2	0	0	0	0	0	**1**	0	0	**1**	0	0	0	0
27	9	6	0	0	0	**4**	0	0	0	**2**	0	0	0	0	0
28	10	4	0	0	0	0	0	0	0	**4**	0	0	0	0	0
29	9	6	**2**	0	0	0	0	0	0	**2**	0	0	**2**	0	0
30	10	8	0	**1**	0	**4**	0	0	0	**3**	0	0	0	0	0
Total	120	76	**2**	**3**	**2**	**9**	**4**	**4**	**1**	**29**	**10**	**4**	**2**	**1**	**5**

Table 4.3 - Supplementary Number of extended-spectrum cephalosporin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to antimicrobial susceptibility testing using the broth microdilution technique, and the number of distinct multi-class resistant profiles identified on each farm.

Farm	Number of samples	Number of samples with ESC-resistant *E. coli*	Number of isolates subjected to AST	Number of multi-class resistant profiles
4	30	4	12	2
10	30	2	16	2
11	30	3	24	3
12	30	8	29	1
13	30	3	17	1
15	30	1	8	2
22	30	6	26	1
29	30	4	17	3

Table 4.4 - Supplementary Number of extended-spectrum cephalosporin-resistant *Escherichia coli* isolates from each Australian pig farm subjected to whole genome sequencing and the number of sequence types identified on each farm following sequencing.

Farm	Number of samples with ESC-resistant *E. coli*	Number of sequenced isolates	ST10	ST88	ST117	ST196	ST2325	ST4981
4	4	3	0	0	**3**	0	0	0
10	2	3	0	0	0	0	**3**	0
11	3	3	0	0	0	0	0	**3**
12	8	2	0	0	0	0	0	**2**
13	3	2	0	0	0	0	0	**2**
15	1	2	0	**1**	**1**	0	0	0
22	6	2	0	0	0	**2**	0	0
29	4	4	**4**	0	0	0	0	0
Total	31	21	**4**	**1**	**4**	**2**	**3**	**7**

CHAPTER 5

Application of an enhanced antimicrobial resistance surveillance method to detect and quantify critically important antimicrobial-resistant *Escherichia coli* in Australian meat chickens

5.1 ABSTRACT

Discovery of critically important antimicrobial (CIA)-resistant *Escherichia coli* in Australian pigs at levels below the detection capability of conventional surveillance raises questions about the CIA-resistance status of other Australian livestock. Owing to recent detection of fluoroquinolone (FQ)-resistant *Campylobacter* spp. in Australian meat chickens, and because chicken is currently the most consumed meat in Australia, there is a need to understand the levels and distribution of FQ-resistant *E. coli* in meat chickens. To do so, we applied an enhanced antimicrobial resistance (AMR) surveillance method based on multiple samples per flock, combined with laboratory robotics and selective agars incorporated with antimicrobials. From ten commercial chicken flocks, approximately 30 caecum samples were obtained from each flock (total n = 295), and for each sample, commensal *E. coli* were enumerated on CHROMagar™ ESBL and CHROMagar™ ECC agar with and without incorporation of antimicrobials. Any isolate presumptively resistant to CIAs were subjected to antimicrobial susceptibility testing by broth microdilution. Extended-spectrum cephalosporin-resistant *E. coli* were not detected but ciprofloxacin-resistant *E. coli* were detected on all farms, ranging from one to 28 positive samples per farm, and typically at concentrations at least several logs lower than commensal *E. coli*. Not all FQ-resistant *E. coli* were found to be multi-class resistant. Resistance towards ampicillin and tetracycline was frequent among the ten farms, with carriage levels that were comparable to commensal *E. coli*, while resistance towards gentamicin was found to have a lower frequency and carriage level. These findings highlight the adaptability of the enhanced AMR surveillance method to another livestock species by delivering accurate and detailed AMR data at the flock-level for meat chickens.

5.2 INTRODUCTION

Antimicrobial resistance (AMR) has become a major global health issue of the 21st century due to the emergence of resistant bacteria threatening the effectiveness of therapy for treatment of human and animal infections (1). This is further exacerbated by the emergence of resistance towards critically important antimicrobials (CIAs), such as fluoroquinolones (FQs) and extended-spectrum cephalosporins (ESCs) that are the last line of treatments used in human medicine (2). Recent studies detecting CIA-resistant *Enterobacteriaceae* in livestock even in the presence of strict regulation on CIA use have prompted the need for more effective AMR surveillance to detect and quantify these organisms if they are present with low sample frequencies or at low carriage levels, or both (3-5).

In Australia, the implementation of AMR control measures also extends to the promotion of livestock biosecurity (especially at the national and flock-level). CIA-resistant *Enterobacteriaceae* have been detected in Australian livestock albeit at low frequencies (6-9) despite Australia's geographic isolation, a virtual prohibition on importation of livestock, and highly regulated antimicrobial use in livestock. In particular, the presence of resistance to FQs and ESCs are a source of concern since FQs are not registered for use in Australian livestock, while ESC use is substantially constrained by regulation (10). Previously, in Chapter 3, the study sought to investigate the presence and extent of CIA-resistance in ten Australian pig herds in the presence of strict regulation on CIA use, which was further extended in Chapter 4 to include 30 Australian pig herds across five Australian states. Findings in Chapter 4 showed that FQ-resistant *Escherichia coli* have a widespread presence throughout Australian pig farms (n = 23, 76.7%) while ESC-resistant *E. coli* was present at a lower frequency than FQ-resistant *E. coli* in Australian pig farms (n = 8, 26.7%). Quantification of CIA-resistant commensal *E. coli* also revealed that carriage levels of FQ-

resistant *E. coli* and ESC-resistant *E. coli* are, at present, lower than commensal *E. coli* by at least 3 and 4 $\log_{10}$ CFU/g respectively. The detection of CIA-resistant commensal *E. coli* in Australian pigs is therefore likely to go undetected in established AMR surveillance systems due to reliance on low intensity of sampling combined with low concentration of the target organism in faeces. This highlights the need to derive more sensitive laboratory methods, such as selective agars for detecting and quantifying resistance, more extensive sampling of colonies using laboratory robotics, and a more comprehensive collection of samples. Further, genomic characterisation of the pig isolates revealed ST744 and ST4981 to be the dominant sequence types (STs) of CIA-resistant *E. coli*. For reasons outlined above, it is likely these forms of resistance were introduced into Australian pigs via human carriers or wild birds (6).

It is currently unclear whether CIA-resistant *E. coli* might possibly be entering the food chain in Australia from other animal production systems. Chicken meat is currently the most consumed meat in Australia (11), with FQ and ESC not registered for use in Australian meat chickens (12). Nevertheless, FQ-resistant *Campylobacter* spp. are known to occur in Australian meat chickens (7), suggesting that the presence and extent of CIA-resistant *E. coli* in these livestock needs investigating. In this study, the same enhanced AMR surveillance method described in Chapter 3 was applied in the meat chicken sector to investigate the presence and extent of *E. coli* with resistance towards CIA, first and second-line antimicrobials. Further antimicrobial susceptibility testing (AST) was performed on CIA-resistant *E. coli* to determine their phenotypic resistance profiles.

5.3 MATERIALS AND METHODS

Sample collection. Caecum samples of meat chickens from ten Australian meat chicken farms were collected from two abattoirs between February 2020 to June 2021. All ten

sampled farms were distributed across one Australian state spanning a geographical region of approximately 113,000 km^2. Chickens are sent for processing at abattoirs on pre-planned schedules and at each abattoir, birds from five farms were selected based on availability on sample collection days. Thirty fresh caecum samples representing 30 meat chickens from each farm were collected after slaughter, except for two farms from which 27 and 28 samples respectively were obtained (total n = 295). Caecum samples were collected by abattoir workers by gathering the caecum of individual chickens using sterilised equipment. Samples were transported on ice and processed within 16 hours of collection.

Sample processing, quantification and isolation of *E. coli* colonies. Faecal contents of each caecum were aseptically obtained by incising the distal extremity with sterile instruments and expressing approximately 1 g of content into 9 mL of sterile 1x phosphate buffered saline (PBS). After vortexing, serial ten-fold dilutions, agar inoculation and incubation, presumptive identification of *E. coli* colonies on CHROMagar™ ECC (CHROMagar) with and without incorporation of antimicrobials (32 µg/mL ampicillin, 16 µg/mL tetracycline, 16 µg/mL gentamicin and 4 µg/mL ciprofloxacin) and CHROMagar™ ESBL (CHROMagar ESBL) (MicroMedia, Edwards Group) agars for quantification, and isolation of CIA-resistant *E. coli* on agars were all performed as per the Robotic Antimicrobial Susceptibility Platform (RASP) protocols described in Chapter 3.

Antimicrobial susceptibility testing of CIA-resistant *E. coli*. MALDI-TOF mass spectrometry was used to confirm the identity of *E. coli* colonies as per protocols described in Chapter 3, with *E. coli* colonies subjected to AST using the broth microdilution method. Susceptibility to eight antimicrobials representing eight classes were assessed and performed by RASP as described in Chapter 3, and includes ampicillin (beta-lactam), apramycin

(aminocyclitol), cefotaxime (third-generation cephalosporin), ciprofloxacin (quinolone), florfenicol (phenicol), gentamicin (aminoglycoside), tetracycline (tetracycline) and trimethoprim-sulfamethoxazole (folate pathway inhibitor). Minimal inhibitory concentration (MIC) interpretation and multi-class resistant (MCR) categorisation follows the same protocol described in Chapter 3.

Statistical analysis. Under the RASP protocol, data from digital imaging of colonies on selective agars were all electronically captured for processing and descriptive analysis in the Stata analysis package (version 16.0, Stata Corporation, Texas, USA). Quantitative counts of *E. coli* in CFU/g of faeces were $\log_{10}$ transformed where required for interpretation and analysis. Similarly, MIC data from CIA-resistant *E. coli* isolates were captured by RASP and processed within Stata to obtain MIC tables with exact confidence intervals for proportion of resistant colonies derived by the Clopper-Pearson method.

5.4 RESULTS

Quantification of resistant *E. coli*. The presence and extent of AMR within Australian meat chicken flocks was investigated through the detection and quantification of resistant *E. coli* towards CIAs, first and second-line antimicrobials on ten Australian meat chicken farms. The frequencies and carriage levels of commensal *E. coli* and each resistant *E. coli* detected in this study are shown in Figure 5.1. Across all farms, average carriage level of commensal *E. coli* was at 6 $\log_{10}$ CFU/g with one farm (Farm E) having an average of 5 $\log_{10}$ CFU/g and two farms (Farms F and G) at 7 $\log_{10}$ CFU/g. No ESC-resistant *E. coli* were detected. Ciprofloxacin-resistant *E. coli* were detected on all farms with an average of 1 $\log_{10}$ CFU/g. Resistance towards first-line antimicrobials (ampicillin and tetracycline) was detected on all farms, with ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* both having an

average carriage level of 5 $\log_{10}$ CFU/g. Resistance to second-line (gentamicin) antimicrobials was detected on seven farms with an average of 1 $\log_{10}$ CFU/g.

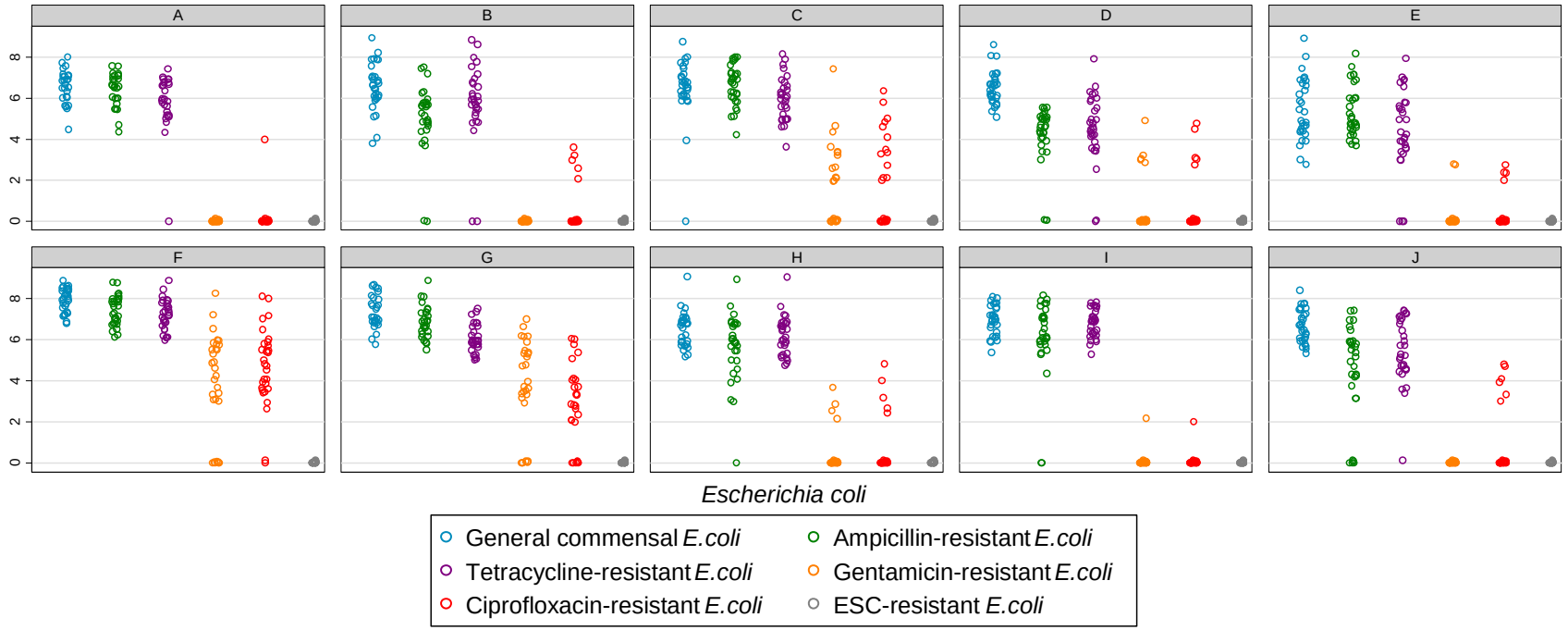

Figure 5.1 Farm-level and animal-level variation in concentration ($\log_{10}$ CFU/g) of commensal *Escherichia coli* phenotypes in chicken faeces. Data represents 1770 observations from 295 individual faecal samples across ten farms (labelled A to J). Each faecal sample was enumerated six times: once on CHROMagar™ ECC agar without antimicrobials, once on each of five CHROMagar™ ECC agar incorporated either with ampicillin, tetracycline, gentamicin or ciprofloxacin, and once on CHROMagar™ ESBL agar. Enumeration was by image capture and digital analysis of chromogenic traits of individual colonies.

Notable differences in frequency of positive samples with ciprofloxacin-resistant *E. coli* were observed between farms, with Farms F and G having a high frequency of 28 and 21 samples respectively, compared to Farm C with a moderate frequency of 13 samples, and remaining farms having a low frequency of one to six samples. However, regardless of farms, average carriage level of ciprofloxacin-resistant *E. coli* was always at least 3 $\log_{10}$ CFU/g lower than average carriage level of general commensal *E. coli* within each farm.

The frequency pattern of positive samples for gentamicin-resistant *E. coli* in each farm was similar to ciprofloxacin-resistant *E. coli,* with Farms F and G having the highest frequency at 23 and 22 samples respectively, Farm C with a moderate frequency of 13 samples and the remaining four farms with a frequency of one to five samples. However, regardless of farms, the average carriage level of gentamicin-resistant *E. coli* was lower than general commensal *E. coli* by at least 4 $\log_{10}$ CFU/g. In contrast, frequency of positive samples with ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* were consistently high among farms, with the lowest frequency at 25 (Farm J) and 27 (Farms B and E) samples respectively. Farm D was the only farm where the average carriage levels of ampicillin-resistant *E. coli* and tetracycline-resistant *E. coli* were lower than general commensal *E. coli* by 2 $\log_{10}$ CFU/g compared to other farms that had average carriage levels of both resistant *E. coli* at comparable levels with general commensal *E. coli*.

Phenotypic characterisation of ciprofloxacin-resistant *E. coli*. A total of 91 ciprofloxacin-resistant *E. coli* isolates from CHROMagar agar incorporated with ciprofloxacin were subjected to AST using the broth microdilution technique. The percentages of resistance for all isolates are shown in Figure 5.2. All isolates were confirmed to be resistant towards ciprofloxacin (100%), with MIC values above the Clinical and Laboratory Standards Institute

(CLSI) clinical breakpoint of 1 µg/mL (13). Additionally, these isolates were also resistant towards ampicillin (71.4%), tetracycline (52.7%), gentamicin (39.6%), trimethoprim-sulfamethoxazole (42.9%) and apramycin (12.1%). All isolates were susceptible to cefotaxime and florfenicol.

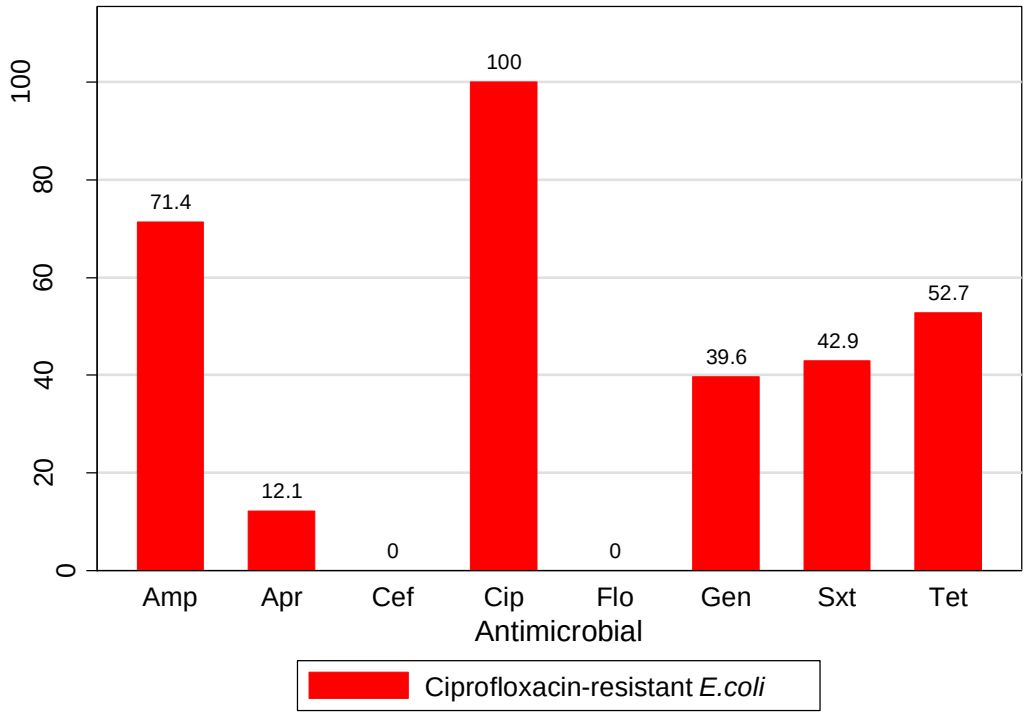

Figure 5.2 Percentage of ciprofloxacin-resistant *Escherichia coli* isolates (*n* = 91) resistant to eight antimicrobials based on the epidemiological cut-off (ECOFF from EUCAST) value, with the exception of apramycin that was based on the ECOFF value validated by Yang et al. (2020) (14). Key: Amp - Ampicillin, Apr - Apramycin, Cef - Cefotaxime, Cip - Ciprofloxacin, Flo - Florfenicol, Gen - Gentamicin, Sxt - Trimethoprim-sulfamethoxazole, Tet - Tetracycline.

Ten phenotypic resistance profiles were identified (Table 5.1) with seven profiles being MCR profiles. Only 57.1% of ciprofloxacin-resistant *E. coli* isolates (*n* = 52) were classified as MCR, with the proportion of MCR ciprofloxacin-resistant *E. coli* isolates spread across the farms (Table 5.2). Notably, Farm E was the only farm where all isolates were MCR with the remaining farms each having a disproportionate number of MCR isolates (Table 5.2). The two most common profiles were resistance towards five antimicrobial classes (*n* = 26, 28.6%: aminoglycoside, beta-lactam, folate pathway inhibitor, quinolone and tetracycline) and resistance towards three antimicrobial classes (*n* = 10, 11.0%: folate pathway inhibitor, quinolone and tetracycline) (Table 5.1). It was also noted that the most common phenotypic resistance profile was not a MCR profile with resistance towards two antimicrobial classes (*n* = 33, 36.3%: beta-lactam and quinolone), while three isolates (3.3%) only harboured quinolone as their phenotypic resistance profile (Table 5.1).

Table 5.1 Phenotypic resistance profiles of ciprofloxacin-resistant *Escherichia coli* isolates based on interpretive breakpoints applied in Figure 5.2. Isolates with resistance towards three or more antimicrobial classes are classified as multi-class resistant.

Phenotypic resistance profile	Number of antimicrobial classes	Number of isolates	% of total
qui	1	3	3.3
bla-qui	2	33	36.3
qui-tet	2	3	3.3
acy-ami-qui	3	7	7.7
bla-qui-tet	3	3	3.3
fpi-qui-tet	3	10	11.0
acy-ami-qui-tet	4	3	3.3
bla-fpi-qui-tet	4	3	3.3
ami-bla-fpi-qui-tet	5	25	27.5
acy-ami-bla-fpi-qui-tet	6	1	1.1

acy: aminocyclitol ami: aminoglycoside, bla: beta-lactam, fpi: folate pathway inhibitor, qui: quinolone, tet: tetracycline

Table 5.2 Number of ciprofloxacin-resistant *Escherichia coli* isolates from each Australian meat chicken farm subjected to antimicrobial susceptibility testing using the broth microdilution technique, and the number of distinct multi-class resistant (MCR) isolates and MCR profiles identified on each farm. Each isolate from each farm corresponds to one sample containing ciprofloxacin-resistant *E. coli*.

Farm	Number of samples with ciprofloxacin-resistant *E. coli*	Number of MCR isolates	Number of MCR profiles
A	1	0	0
B	5	2	1
C	14	11	3
D	5	3	2
E	4	4	2
F	28	11	3
G	22	16	4
H	5	1	1
I	1	0	0
J	6	4	2

5.5 DISCUSSION

Chapter 4 set a precedence in the study of epidemiology of resistant *E. coli* in Australian pigs by demonstrating in a structured, nationwide survey that resistance to FQs (and to a lesser extent ESCs) is widespread at levels below that are likely to be detected and quantified by established AMR surveillance methods. This chapter now extends this approach with the aim of describing the distribution of the same forms of resistance in commensal *E. coli* in Australian meat chickens. Additional motivations for this chapter are that CIA-resistant *Campylobacter* spp. have been recently found in Australian meat chickens (7), and secondly, the fact that chicken is currently the most consumed meat in Australia (11). While no ESC-resistant *E. coli* were detected, ciprofloxacin-resistant *E. coli* was detected on all ten farms. However, low carriage levels of FQ-resistant *E. coli* suggest that they may not have sufficient fitness to dominate the commensal *E. coli* population of each flock. Seven MCR profiles were identified though not all FQ-resistant *E. coli* isolates were found to be MCR. Finally, among the first and second-line antimicrobials, resistance towards ampicillin and tetracycline was present on all ten farms with high carriage levels approaching the commensal *E. coli* population, while resistance towards gentamicin was only present on seven farms with low carriage levels.

The main mechanism conferring FQ-resistance is the presence of mutations within the quinolone-resistance determining regions (QRDRs) that can only arise through selective pressure from FQ use (15). Given that FQ is not registered for use in Australian livestock, the presence of some FQ-resistant *E. coli* isolates with only a single phenotypic resistance profile towards FQs suggest that FQ-resistance did not emerge locally (10). The possibility of FQ-resistance emerging due to plasmid-mediated FQ-resistance (PMQR) genes is unlikely as these genes only conferring low-levels of FQ-resistance with limited clinical significance

(16-18). Furthermore, the conferring of low-level FQ-resistance does not reflect the MIC values of all FQ-resistant *E. coli* isolates in this study which exceeded the CLSI clinical breakpoint (13). The possibility of FQ-resistance arising from co-selection is also unlikely, as the *aac(6')-Ib-cr* gene which is a variant of the aminoglycoside-resistance gene conferring FQ-resistance, is also a PMQR gene that confers low-levels of FQ-resistance (19). Additionally, the *aac(6')-Ib-cr* gene was theorised to have emerged due to aminoglycoside-resistant *E.coli* being exposed to selective pressure from FQ use which would not be possible in the absence of FQ use within this study (20). Therefore, much like in Australian pigs, the presence of FQ-resistant *E. coli* in these ten Australian meat chicken farms were likely due to introduction by another host species such as humans or wild birds (6). However, further genomic analysis using whole genomic sequencing is required to identify the dominant sequence types (STs) of FQ-resistant *E. coli* among these farms, as a step towards elucidating their origins.

The low frequency and carriage level of FQ-resistant *E. coli* among the ten Australian meat chicken farms reflects findings from other Australian studies detecting low frequencies of FQ-resistant *E. coli* in Australian chickens (21, 22). Additionally, these findings were also similar to the frequency of FQ-resistant *Campylobacter* spp. detected in Australian meat chickens (7). It is tempting to regard this low frequency and carriage level of FQ-resistant *E. coli* as not a major concern to public health, however, there appears to have been little research to support such a position especially in light of studies identifying chicken meat as potential reservoirs for extraintestinal pathogenic *E. coli* (23-25). Maintaining vigilance through on-going surveillance appears prudent so that factors promoting the spread of FQ-resistance amongst farms and other species might be understood to the extent that prevention measures can be devised. The experience of this study, and also of the related studies on pigs

(Chapter 3 and 4), indicates that very high sensitivity must be designed into the surveillance effort and can be achieved by strategically combining intensive sampling of the animal population in question with processing of samples in the laboratory using high throughput robotics such as RASP.

In earlier work, the detection of ESC-resistant *E. coli* in Australian pigs reflects an historical reliance on ceftiofur in some herds through constrained access to this antimicrobial via off-label prescribing by veterinarians (26). In contrast, the Australian meat chicken industry effectively does not have access to any ESC antimicrobials because of a label constraint prohibiting the use of ceftiofur for mass medication (12). The absence of ESC-resistance among the ten farms in this study can thus be explained by exclusion of ceftiofur from this industry. While one other Australian study involving a much smaller number of isolates has detected ESC-resistant *E. coli* in retail chicken meat, there is the possibility that this arose from cross contamination from other products or humans in the post-processing and retail sectors (27). Nonetheless, on-going AMR surveillance of Australian meat chickens with the highly sensitive methods demonstrated here appears attractive for early detection of emerging ESC-resistance while also serving to support the integrity of the production process.

The frequency and carriage level of resistant *E. coli* towards first (ampicillin and tetracycline) and second-line (gentamicin) antimicrobials among the ten Australian meat chicken farms were similar to Australian pigs. The presence of these resistances is likely due to historical antimicrobial use over the long-term since antimicrobials from the corresponding classes have all been registered for use in Australian livestock (gentamicin is prohibited from use in Australian livestock although other aminoglycosides have been used) (28). Additionally,

resistance to all three classes were also previously detected in Australian meat chickens thus suggesting that the presence of these resistances is not new (21).

In conclusion, this study produced unique, high-resolution depictions of key forms of AMR in commensal *E. coli* in Australian meat chickens. Specifically, ampicillin and tetracycline-resistance is widespread within and between farms, and occurs with high carriage levels, gentamicin-resistance is commonly present at the flock-level but carriage in individuals is typically infrequent and at low levels. For CIAs, ESC-resistance is completely absent, and despite FQ having been excluded from use in Australian livestock, FQ-resistance was found in all flocks but typically present at low carriage levels in a varying proportion of individuals. These results demonstrated the enormous capacity that laboratory robotics have for producing epidemiologically-relevant intelligence on AMR, most critically addressing the need for sensitive warning of early emergence of resistance to CIAs. Further genomic investigation is easily applied to identify dominant STs currently present among these ten farms, and to elucidate the origins of FQ-resistance, a microbiological blemish that has arisen without the provocation of FQ use. Strong prospects exist to expand the enhanced method applied here to other livestock species, companion animals and perhaps humans.

5.6 REFERENCES

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CHAPTER 6

General conclusion

Surveillance for antimicrobial resistance (AMR) plays a critical role in defining the presence, geographic and temporal distribution of AMR, which then informs control strategies. The quality of surveillance outputs affects the ability to make informed decisions about antimicrobial stewardship and other control measures that prevent further emergence and spread of resistant bacteria. However, the established approach for national AMR surveillance programs has shortfalls in quality. That is because national programs rely on a single isolate per epidemiological unit (usually a herd, flock or production lot) for making inferences about the AMR status of livestock. Sampling for AMR in this way presents several limitations. Foremost is the decreased probability of detecting emerging resistance that might be present at only a low frequency or carriage level. Secondly, the number of isolates per animal, and per herd is far too low to provide meaningful quantitative data that could be judged essential for performing risk assessment. Thirdly, the inadequate representation of AMR scenarios amongst farms – the data neither show how much variations exists between farms nor do they provide any individual farm with the information needed to support decision making within that enterprise. The laboratory elements of an enhanced AMR surveillance method were therefore developed and form the core of this thesis. They address the fundamental problem of low sampling density of isolates (in the laboratory), animals and herds (in the field). The various chapters describe, validate and justify these technological developments in the laboratory and demonstrate their application to generating data from populations of animals and herds. In Chapter 2, the study identified suitable selective agars for quantifying the indicator bacterium *Escherichia coli* for acquiring quantitative AMR data (density of organisms per unit weight of faeces) using the enhanced method. A pilot study was then designed in Chapter 3 to demonstrate the capability of the enhanced method by investigating the presence and extent of AMR in ten Australian pig farms. This was followed with an upscale study in Chapter 4 that demonstrated the capability of the enhanced method

to provide the same accurate and detailed description of AMR scenarios on a national scale. Lastly, Chapter 5 demonstrated that the enhanced method could be applied to other livestock species by providing a detailed examination of the presence and extent of AMR in ten Australian meat chicken farms.

Of the three *E. coli* selective agars compared in Chapter 2, MacConkey (MAC) agar was found to be demonstrably inferior to Brilliance™ *E. coli* (Brilliance) and CHROMagar™ ECC (CHROMagar) agars. This inferior performance of MAC agar was consistent regardless of whether agars were incorporated with antimicrobials (ampicillin, tetracycline, gentamicin, ciprofloxacin and ceftiofur) or not, or whether faecal samples were spiked with fluoroquinolone (FQ)-resistant *E. coli* or not. Therefore, even though MAC agar is still capable of isolating *E. coli* for clinical diagnosis, its inferior *E. coli* growth performance (yielding fewer colonies) indicates that it is not suited for quantifying *E. coli* in the enhanced method. Two extended-spectrum cephalosporin (ESC)-resistant *E. coli* selective agars - Brilliance™ ESBL (Brilliance ESBL) and CHROMagar™ ESBL (CHROMagar ESBL) agars were also compared for their suitability in quantifying ESC-resistant *E. coli* in the enhanced method. It was found that not all ESC-resistant *E. coli* strains grew on Brilliance ESBL agar regardless of whether it was inoculated with pure cultures or homogenised faecal samples. The data from this study indicates that CHROMagar ESBL was more suitable for the enhanced method due to its capacity to support growth of a wider variety of ESC-resistant *E. coli* strains.

A major finding of Chapters 3 and 4 was the detection of FQ-resistant *E. coli* and ESC-resistant *E. coli* among sampled Australian pig farms albeit not in all farms and in all samples, and at low carriage levels. The data show that despite strict regulations governing

antimicrobial use in Australian livestock, FQ and ESC-resistance are present among pigs but at concentrations in individual samples well below that of the commensal *E. coli* population. Further genomic characterisation revealed that ST744 and ST4981 were the dominant FQ-resistant *E. coli* and ESC-resistant *E. coli* sequence types (STs) respectively present among positive farms, and were likely introduced via external sources rather than having emerged from the local population of microbes. Besides demonstrating the capability of the enhanced method to provide a more accurate and detailed description of AMR scenarios at national and herd-level, it also highlights two key elements for AMR surveillance. The first is the need to utilise highly sensitive methods such as selective agars, with intensive sampling and high throughput sample processing using laboratory robotics to detect and quantify resistance with low frequencies or carriage levels, which would have been missed or underestimated using the established single isolate per herd approach. The second is the inclusion of whole genome sequencing on subset of critically important antimicrobial (CIA)-resistant *E. coli* and data pertaining to transmission pathways of CIA-resistance to clarify its origins and spread in the presence of strict regulated use of antimicrobials.

While no ESC-resistant *E. coli* were detected among Australian meat chicken flocks in Chapter 5, ciprofloxacin-resistant *E. coli* were detected at low carriage levels, indicating that despite the absence of FQ use due to FQ not being registered for use in Australian livestock, FQ-resistant *E. coli* are present among sampled flocks but have yet to spread throughout the commensal *E. coli* population. It is reasonable to expect that the absence of selection pressure will to some extent, big or small, constrain the future level of colonisation of herds and individual pigs with FQ-resistant *E. coli*. The presence of some FQ-resistant *E. coli* isolates harbouring only a single phenotypic resistance towards quinolone class suggests that FQ-resistance was also likely introduced into positive farms via external sources and did not

emerge locally. Though additional genome sequencing is required to further elucidate the origin of FQ-resistance, the findings also provided the same detailed description of AMR scenarios as in Chapter 3 and 4 and highlighted the same key elements necessary for enhanced AMR surveillance. Moreover, it also demonstrated the flexibility of the enhanced method for application in other species of livestock, with encouraging potential to expand to more livestock species, companion animals, and even humans. With this expansion lies further potential to elucidate resistance transmission pathways between different industries or sectors via the integration of detailed and accurate AMR surveillance data using the One Health approach.

Overall, the findings in this thesis provides a comprehensive demonstration of the capabilities of the enhanced method and how it can be used to promote the effectiveness of AMR surveillance at national and herd-level with greater accuracy and detail than the established approach. In particular, it highlights the importance of using laboratory robotics as a central focus for large-scale quantitative AMR surveillance, since the enhanced method (multiple samples per herd approach combined with selective agar for enumerating resistant *E. coli*) would not be possible on a practical scale without a high level of automation. The rewards are data that allow for more well-informed judgements and decisions about the control of AMR, especially in relation to early detection of emerging resistance. In light of the findings arising from this thesis, recommendations for further studies to improve or expand the potential of the enhanced method are required:

1) Further validation of selective agars targeting different bacterial species using the same methodology used in Chapter 2 to expand the use of the enhanced method to quantify other indicator bacterium species (*Enterococci*) and zoonotic pathogens such as *Campylobacter* and *Salmonella* species.

2) Validating the incorporation of other CIAs, such as carbapenems into selective agars for use in the enhanced method.

3) Incorporation of quantification into national AMR surveillance systems with a multiple samples per herd approach to acquire more accurate and detailed description of AMR epidemiology.

4) Longitudinal studies using the enhanced method are needed to track changes in carriage levels of resistant bacteria through time and to monitor the effectiveness of AMR control strategies.

5) Evaluation of all potential niches for AMR within the farm precinct including farm workers, wild birds, pest species and the environment using the enhanced method, and reinforced with antimicrobial usage data and whole genome sequencing to understand the source, persistence and transmission of resistance.

6) Further investigation into the viability of using the enhanced method in other animal species including companion animals and wildlife, and to other sectors such as food and public health.

7) A cost comparison study of the enhanced method with national AMR surveillance programs in other countries to determine the financial viability of using the enhanced method on a national-scale