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Clarifying the origin and ecological reservoir of unusual forms of critically important antimicrobial resistant *E. coli* in Australian pigs

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Executive Summary

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. Resistance to critically important antimicrobials (CIAs) such as extended spectrum cephalosporins (ESCs) fluoroquinolones (FQs), and carbapenems among *Enterobacteriaceae* is of one of the greatest concerns.

CIA-resistant bacteria can be introduced into a food-animal system either from humans, water, soil, pest animals, wild animals etc and is expected to result in passive carriage of low numbers of organisms. This is in contrast to emergence and amplification within the production system as a result of the selective pressure of antimicrobial use. Antimicrobial resistance surveillance that is performed for AMR in food animals usually does not have the ability to discern these pathways.

This research conducted a proof-of-concept study of AMR surveillance in regard to the detection and quantification of CIA-resistant *E. coli* within pigs using the novel RASP Quantification protocol in combination with traditional antimicrobial susceptibility testing and genomic sequencing involving 10 pig farms.

The findings are divided into three parts: the first-year survey for CIA-resistant *E. coli* in WA pig farms as part of a proof-of-concept study, the second-year survey for CIA-resistant *E. coli* within the same WA pig farms for the proof-of-concept study, and lastly the detection and quantification of unusual forms of resistance among Australian pigs on a national scale using RASP Quantification.

In the first survey, a total of 160 faecal samples from 7 out of 10 farms were identified to have fluoroquinolone (FQ)-resistant *E. coli* isolates but no extended-spectrum cephalosporin (ESC)-resistant *E. coli* was identified. 50 representative FQ-resistant *E. coli* isolates from 160 FQ-resistant *E. coli* isolates across 7 farms were subjected to whole genome sequencing. Two primary STs [ST167 (*n* = 24) and ST744 (*n* = 22)] were identified from sequencing data and further genomic analysis of both STs were performed. Four other STs were identified (ST10, ST34, ST1161 and ST11612) with one isolate per ST.

In the second survey, the same 10 farms from the proof-of-concept study were surveyed again after one year. FQ-resistant *E. coli* was identified on all 10 farms from a total of 187 samples and ESC-resistant *E. coli* was identified on 2 farms from a total of 38 samples. 62 out of 187 representative FQ-resistant *E. coli* isolates across 10 farms and 15 out of 38 ESC-resistant *E. coli* across two farms were subjected to whole genome sequencing. FQ-resistant *E. coli*, ST744 (*n* = 36) was the primary ST identified followed by ST167 (*n* = 24) and ST11613 (*n* = 2) from the sequencing data. For ESC-resistant *E. coli*, ST1141(*n* = 9) and ST10 (*n* = 6) were the two STs identified from the sequencing data.

A total of 90 water samples and 70 environmental from 7 pig farms were collected during the second survey. Fluoroquinolone-resistant *E. coli* isolates were identified in 9 water samples, but no ESC-resistant *E. coli* isolates was identified. Fluoroquinolone-resistant *E. coli* isolate was identified from only one environmental sample and there were no ESC-resistant *E. coli* isolates.

Finally, a national survey of 30 farms across Australia (300 samples, 10 samples/farm) was conducted to evaluate the carriage of FQ and ESC-resistant *E. coli.* The results identified 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.

This comprehensive study does demonstrate the potential for the emergence of critically important antimicrobial resistant bacteria in the absence of direct antimicrobial use in the livestock and highlights the importance of on-going monitoring of antimicrobial resistant bacteria in livestock.

Also, we should thank DAWE for supporting first year of the project. Sample collection and analysis of initial 10 farms were supported by Australian Department of Agriculture, Water, and the Environment under the Animal Biosecurity and Response Reform program.

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1. Background to Research

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. Resistance to critically important antimicrobials (CIAs) such as extended spectrum cephalosporins (ESCs) fluoroquinolones (FQs), and carbapenems among *Enterobacteriaceae* is of one of the greatest concerns (Scott et al. 2019).

When such resistance occurs in food-producing animals, there is the potential risk of transmission to humans through the food chain and/or the environment. Food-producing animals colonised with CIA-resistant bacteria can act as reservoirs for both the resistant organisms and for the genetic material encoding for resistance that can be transferred to sensitive bacteria. CIA-resistant bacteria can be introduced into a food-animal system either from humans, water, soil, pest animals, wild animals etc and is expected to result in passive carriage of low numbers of organisms (Aarestrup et al., 2008; Abraham et al., 2015; Abraham et al., 2019; Nekouei et al., 2018). This is in contrast to emergence and amplification within the production system as a result of the selective pressure of antimicrobial use. Antimicrobial resistance surveillance that is performed for AMR in food animals usually does not have the ability to discern these pathways.

This report highlights the major findings of AMR surveillance in regard to the detection and quantification of CIA-resistant *E. coli* within pigs using the novel RASP Quantification protocol in combination with traditional antimicrobial susceptibility testing and genomic sequencing. The findings are divided into three parts: the first-year survey for CIA-resistant *E. coli* in WA pig farms as part of a proof-of-concept study, the second year survey for CIA-resistant *E. coli* within the same WA pig farms for the proof of concept study, and lastly the detection and quantification of unusual forms of resistance among Australian pigs on a national scale using RASP Quantification.

2. Objectives of the Research Project

This project sets out to:

1. Clarify the origins and source of the unusual forms of resistance
2. Determine how fluoroquinolones (FQ) resistant clones are entering the pig production units
3. Identify pathway to reduce the occurrence and spread of FQ or critically important antimicrobials (CIA) *E. coli* carriage in pigs
4. Identify the mechanisms resulting in high frequency carriage, persistence and maintenance in pig herds in the absence of specific drug use
5. Perform full genomic characterisation of FQ-resistant clones from pigs to map origin, transmission and co-selection potential

3. Research Methodology

3.1 Sample collection to detect and quantify unusual forms of resistance in pigs

From 17 Aug 2020 to 17 Nov 2020, 30 pig farms across Australia each submitted 10 faecal swabs from finisher pigs at slaughter from export abattoirs. The number of Farms from each state were determined by total number of pigs/ state. Farm enrolment are as follows: QLD (n=8); VIC (n=7); NSW (n=6); SA (n=5); WA (n=4). Specimens were processed at the Antimicrobial Resistance and Infectious Disease Laboratory at Murdoch University using the Robotic Antimicrobial Susceptibility Platform (RASP).

Due to COVID-19 outbreak our ability to recruit additional farms from different geographical location was limited. As a result, in consultation with Dr. van Breda and Prof. Jordan a modified design using samples collected for RRDP grant from abattoir was used for quantification of CIA-resistance from Australian pig farms. The modified version will investigate CIA-resistance quantification from 300 faecal samples representing 30 farms. Please note that quantification of CIA-resistance is part of this project and not part of RRDP robotics grant. See below for study design.

3.2 Bacterial isolation and quantification

Each faecal sample was subjected to quantification (10 samples/ Farm) using *E. coli* selective agar to quantify the total *E. coli* within a sample (*E. coli* isolation (ECC) agar without antimicrobial) and *E. coli* selective agar infused with antimicrobials to select for critically important resistance to ciprofloxacin (ECC with CIP) or extended spectrum cephalosporins (Chrome ESBL).

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.

Briefly, 75 µL of diluted homogenised samples were inoculated onto CHROMagar™ ECC (CHROMagar) (MicroMedia, Edwards Group) agar with and without incorporation of antimicrobials (4 µg/mL ciprofloxacin) and CHROMagar™ ESBL (CHROMagar ESBL) (MicroMedia, Edwards Group) agar. 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.

Antimicrobial concentrations were selected based on clinical breakpoints listed by the Clinical and Laboratory Standards Institute (CLSI) and previously validated to be suitable for incorporation into CHROMagar agar for quantifying resistant *E. coli (*Clinical and Laboratory Standards Institute, 2018).

3.3 Characterisation of FQ-resistant E. coli

The MIC for 710 ciprofloxacin-resistant *E. coli* originating from CHROMagar incorporated with ciprofloxacin was collected using the RASP_MIC protocol described above.

3.4 Characterisation of ESC-resistant E. coli

The MIC of 149 ESC-resistant *E. coli* originating from ESBL CHROMagar were determined using RASP_MIC protocols described above.

3.5 Interpretation

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).

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.

3.6 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) (Abraham et al., 2018).

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) (Bankevich et al., 2012).

Multi-locus sequence typing (MLST) was performed, with sequence types (STs) identified using the PubMLST database (Jolley et al., 2018). 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 (Carattoli et al., 2014), ResFinder (Aankari et al., 2012) and VFDB (Chen et al., 2016)) 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 (Zhou et al., 2020) and NCBI Sequence Read Archive (Leinonen et al., 2011) 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) (Didelot and Wilson, 2015) before a maximum-likelihood phylogeny was constructed via RAxML (v8.0.0) (Stamatakis, 2014). Annotation of phylogenetic trees was performed using the ggtree package (v3.0.4) in R (v4.1.1) (Yu et al., 2017).

4. Results

4.1 Genotypic characterisation of ciprofloxacin-resistant E. coli.

A representative subset of 50 ciprofloxacin-resistant *E. coli* isolates were selected for WGS based on phenotypic MDR and RAPD PCR profiles. Following WGS the 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 ST11512 (*n* =1). The latter four STs were all from isolates on Farm A.

All STs contained MDR isolates, with resistance towards three (*n* = 19, 79.2%: aminoglycosides, beta-lactams and quinolones) and six (*n* = 14, 63.6%; aminoglycosides, beta-lactams, folate pathway inhibitors, phenicols, quinolones and tetracyclines) antimicrobial classes being the most frequent profiles for ST167 and ST744 respectively. The number of MDR profiles and the AMR and virulence genes identified amongst isolates belonging to each ST are shown in Table 1.

Table 1: Number of isolates, multi-drug resistant profiles and detection of know antimicrobial resistant genes for each ST containing ciprofloxacin resistant E. coli

ST	No. of isolates	No. MDR Profiles	Antimicrobial resistant 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 the *gyrA* subunit while two isolates harboured both aforementioned substitutions in *gyrA* and either the G239T or the T240C substitution in the *parC* subunit. No known plasmid-mediated FQ-resistance genes were identified.

The only beta-lactam-resistant gene identified was *bla*$_{\text{TEM-1B}}$ which was present in 49 of the sequenced isolates, with one ST744 isolate being negative. Three sulphonamide-resistance genes (*sul1*, *sul2* and *sul3)* were identified, with 49 isolates 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-resistant 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 1). Moreover, the study isolates shared the same branch with international ST167 isolates (*n* = 135) originating from North America and Europe (Figure 1).

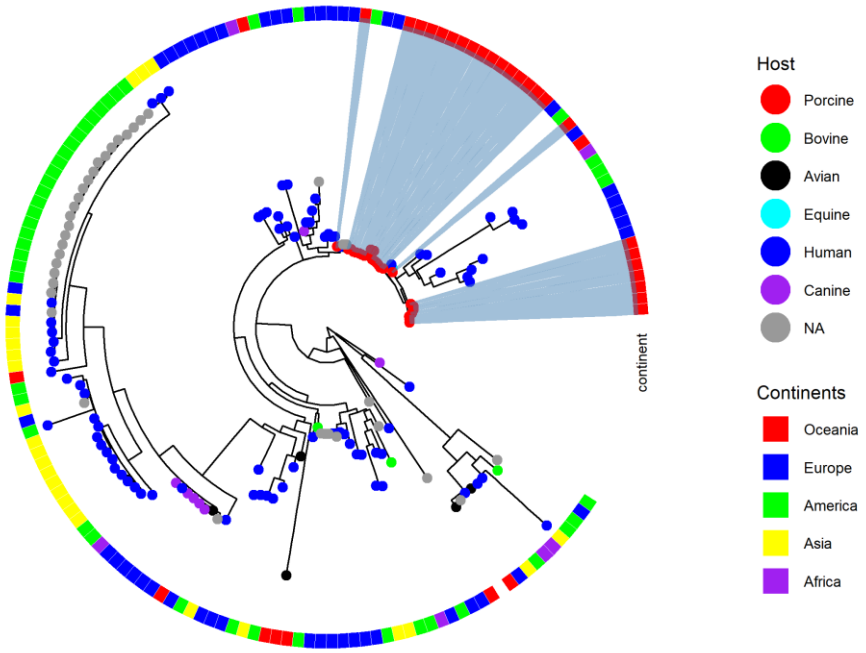

Figure 1: Mid-point rooted maximum likelihood phylogenetic tree of ST167 using 159 genomes (international isolates n=135, study isolates n=24) with 9423 SNP sites. Coloured circles of each node represent the host from which the isolate originated while the coloured squares represent the continent from where the host originated. The 24 isolates from this study are highlighted with a blue background and are clustered along the same branch of the phylogenetic tree.

In contrast, the ST744 isolates identified in this study (*n* = 22) were dispersed in small clusters throughout different branches of the tree located with international ST744 isolates (*n* = 214) from a wider range of countries and regions including North America, Europe, Asia and Africa (Figure 2).

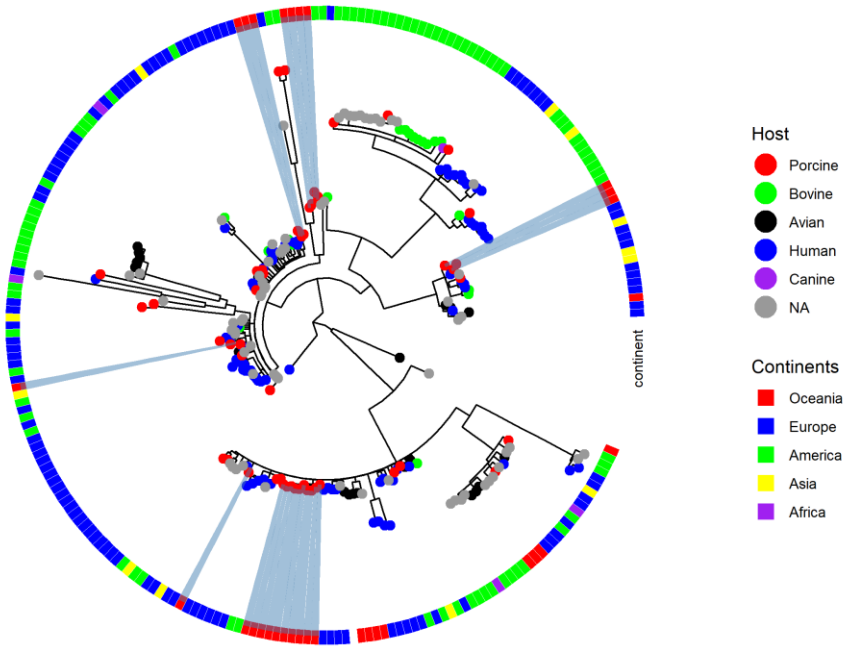

Figure 2: Mid-point rooted maximum likelihood phylogenetic tree of ST744 using 236 genomes (international isolates n = 214, study isolates n = 22) with 6441 SNP sites. Coloured circles on each node represent the host from which the isolate originated while the coloured squares represent the continent from where the host originated. The 22 isolates from this study are highlighted with a blue background and are scattered in small clusters throughout the phylogenetic tree.

The following scatterplot (Figure 3) shows the range of CFU/g of *E. coli* for each sample from every farm during both survey period.

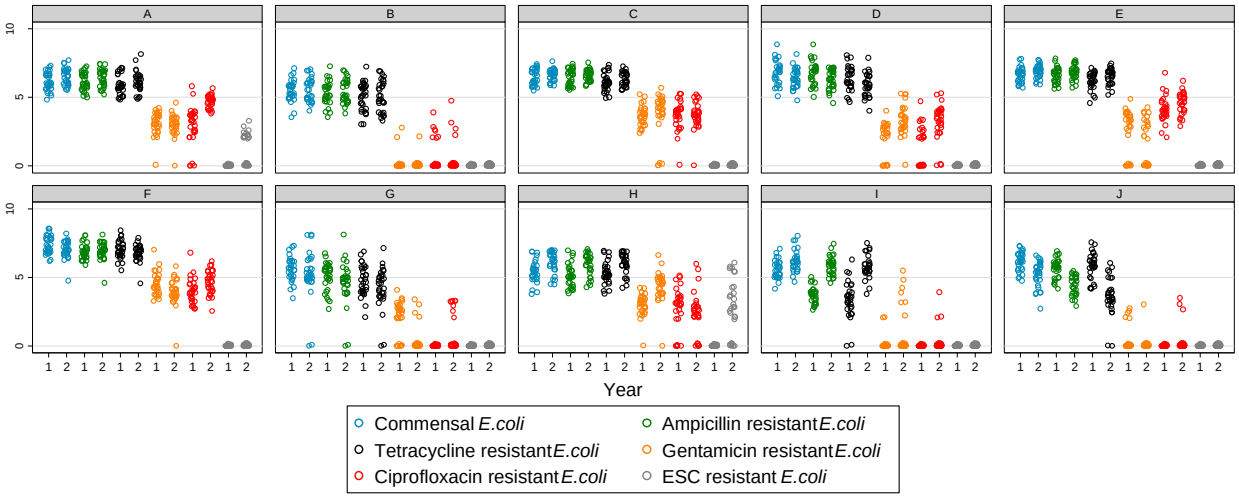

Figure 3: Colony forming units per gram (CFU/g) of faeces for general commensal and each type of resistant E. coli for all 10 farms (A to J) from two-year period Colonies were identified on CHROMagar™ ECC agars with and without incorporation of antimicrobials (ampicillin, tetracycline, gentamicin, ciprofloxacin) and CHROMagar™ ESBL agar based on colour according to the manufacturer's instructions.

4.2 Genomic analysis of second year isolates

Among the FQ-resistant *E. coli* isolates subjected to whole genome sequencing, ST744 (n = 36) was the primary ST identified followed by ST167 (n = 24) and ST11613 (n = 2). Among the ESC-resistant *E. coli*, ST1141(n = 9) and ST10 (n = 6) were the two STs identified from sequencing data.

All FQ-resistant *E. coli* isolates displayed substitutions within the QRDR, with all isolates possessing C248T, G259A, A2034C and T2482G substitutions in *gyrA*, and 61 isolates (98.3%) possessing G239T substitution in *parC*. Five isolates (8.1%) possessed the plasmid-mediated FQ-resistance gene *qnrS*. No known ESC-resistance genes were identified although the beta-lactam-resistance gene $bla_{\text{TEM-1B}}$ was present in all FQ-resistant *E. coli* isolates. Eleven isolates (17.7%) also harboured the phenicol-resistance gene *floR*. Three sulphonamide-resistance genes (*sul1*, *sul2* and *sul3)* were identified, with 59 isolates (95.2%) harbouring at least one of the genes, and three ST744 isolates being negative.

All ESC-resistant *E. coli* isolates also displayed substitutions within the QRDR. All isolates possessed A2034C and T2482G substitutions in *gyrA*, with one isolate possessing substitutions in C248T, G259A, A2034C and T2482G in *gyrA* and G239T in *parC*. Additionally, 14 isolates (22.6%) also harboured T240C substitutions in *parC* as well. The ESC-resistance gene $bla_{\text{CTXM-1}}$ was found on 14 isolates (93.3%) with one ST10 isolate not harbouring any known ESC-resistance gene. The beta-lactam-resistance gene $bla_{\text{TEM-1}}$ was also found on 14 isolates (93.3%).

The only ST1141 isolate without $bla_{\text{TEM-1}}$ did not possess any known beta-lactam-resistance gene. Three sulphonamide-resistance genes (*sul1*, *sul2* and *sul3)* were identified, with all isolates harbouring at least one of the genes. Further genomic analysis to identify known virulence genes and plasmids will be performed in addition to phylogenetic analysis.

4.3 Genomic characterisation of water and environmental samples

The number of water samples obtained from each farm and the corresponding number of samples identified with CIA-resistant *E. coli* (Table 2).

Table 2: CIA-resistant E. coli from water samples

Farm	Water samples	Samples with ciprofloxacin-resistant *E. coli*	Samples with ESC-resistant *E. coli*
A	11	3	0
B	13	0	0
C	11	3	0
D	11	0	0
E	21	3	0
H	12	0	0
J	11	0	0

The number of environmental samples obtained from each farm and the corresponding number of samples identified with CIA-resistant *E. coli* (Table 3).

Table 3: CIA-resistant E. coli from environmental samples

Farm	Environmental Samples	Samples with ciprofloxacin-resistant *E. coli*	Samples with ESC-resistant *E. coli*
A	10	0	0
B	10	0	0
C	10	0	0
D	10	0	0
E	10	1	0
G	10	0	0
J	10	0	0

4.4 FQ and ESC-resistant E. coli in Australian pigs

The CFU/g of total *E. .coli*, ciprofloxacin resistant *E. coli* and extended spectrum cephalosporin (ESC) resistant *E. coli* was calculated (Figure 4 and 5) from 30 farms across Australia. Total *E. coli* per sample was very consistent within and between farms ranging from $1x10^4$ – $1x10^8$ CFU/g faeces. Ciprofloxacin (FQ) resistance was detected in 73% of farms at up to 10^4 CFU/g faeces. ESC resistance was detected on 40% of farms with at least one animal positive for ESC resistance. ESC resistance ranged from 0 – $1x10^4$ CFU/g faeces.

Figure 4: Distribution of total commensal E. coli count expressed as log10 cfu per gram of faeces (30 farms, 10 samples per farm), ciprofloxacin resistant count (Cip) and extended spectrum cephalosporin resistant count (ESC).

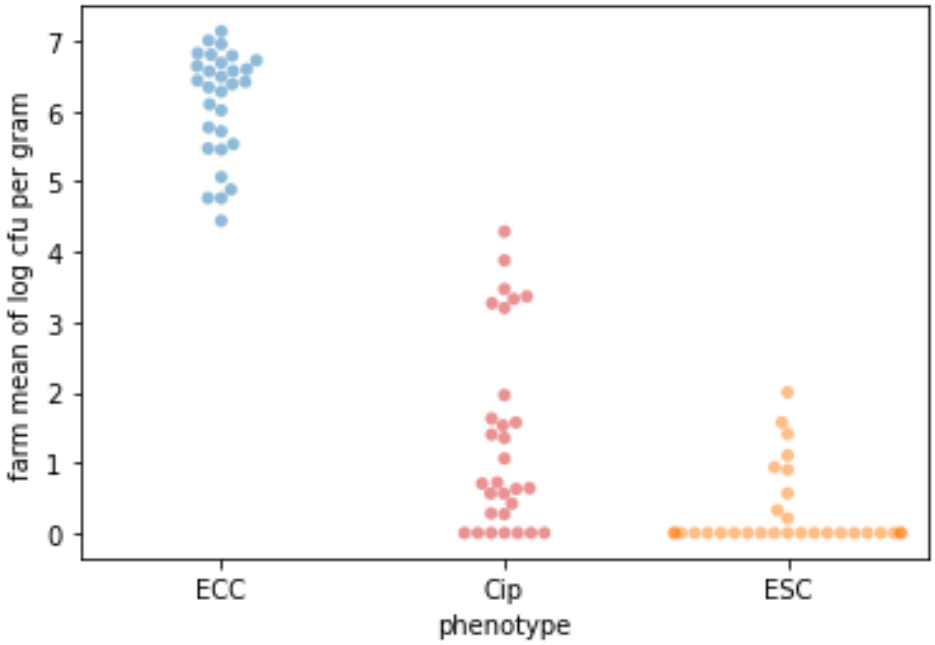

Figure 5: Distribution of the farm mean of log10 counts, total commensal E. coli (ECC), ciprofloxacin resistant (Cip) and extended spectrum cephalosporin resistant (ESC). Each dot represents a farm.

4.5 Characterisation of FQ-resistant E. coli

The percentages of resistance towards seven antimicrobials for all ciprofloxacin-resistant *E. coli* are shown in Figure 6. All presumptive ciprofloxacin-resistant *E. coli* were confirmed to be resistant to ciprofloxacin (100%) with MIC values of 1 µg/mL or higher except for one isolate which had a MIC value of 0.12 µg/mL. Additionally, high levels of resistance towards ampicillin (98.6%), tetracycline (86.9%) and trimethoprim-sulfamethoxazole (79.4%), moderate levels of resistance towards florfenicol (30.8%), and low levels of resistance towards gentamicin (10.0%) and cefotaxime (1.8%) were exhibited.

Most of the ciprofloxacin-resistant *E. coli* (95.2%; n= 676) were classified as multi-class resistant (MCR) with over ten different MCR profiles identified (Table 4). 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.

A total of 76 ciprofloxacin-resistant *E. coli* were selected for whole genome sequencing (WGS). Thirteen STs were identified with ST744 (*n* = 30) being the most dominant with the majority of the isolates originating from a single state (*n* = 11). The numbers of MDR profiles for each ST alongside any known AMR and virulence genes detected among the isolates are shown in Table 5.

All isolates displayed substitutions within the quinolone-resistance determining regions (QRDR) with 60 isolates (78.9%) displaying C248T, G259A, A2034C and T2482G substitutions in *gyrA*, and G239T substitution in *parC* subunits within the quinolone-resistance determining regions (QRDR) with one isolate displaying all aforementioned substitutions in addition to T240C substitution in the *parC* subunits.

A small subset of isolates (*n* = 3, 3.9%) also displayed T1372G substitutions in the *parE* subunits in addition to the other aforementioned substitutions in *gyrA* and *parC* subunits. No ESC-resistant genes were detected but the plasmid-mediated FQ-resistant (PMQR) gene *qnrS* was found in 15 (19.7%)

isolates while 19 (25.0%) isolates harboured the phenicol-resistant gene *floR*. Two types of beta-lactam-resistant genes (*bla*$_{TEM-1B}$ and *bla*$_{TEM-176}$) were identified with *bla*$_{TEM-1B}$ present in 68 (89.5%) isolates and *bla*$_{TEM-176}$ present only in three (3.9%) ST542 isolates.

Three types of sulphonamide-resistant genes were identified (*sul1*, *sul2* and *sul3*) with 67 (88.2%) isolates harbouring at least one sulphonamide-resistant gene while four types of tetracycline-resistant genes were identified (*tet[A]*, *tet[B]*, *tet[H]* and *tet[M]*) with 62 (81.6%) isolates harbouring at least one tetracycline-resistant gene. Seven aminoglycoside-resistant 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-resistant gene family.

4.6 Characterisation of ESC-resistant E. coli

The percentages of resistance towards seven antimicrobials for all ESC-resistant *E. coli* are also shown in Figure 6. All presumptive ESC-resistant *E. coli* were confirmed to be resistant to cefotaxime (100%) with a MIC value of 4 µg/mL. High levels of resistance towards ampicillin (100%), trimethoprim-sulfamethoxazole (77.2%), tetracycline (64.4%), ciprofloxacin (61.1%) and moderate levels of resistance towards florfenicol (25.5%) were also exhibited. Isolates exhibiting resistance towards ciprofloxacin MIC values ranging between 0.12 to more than 1 µg/mL. All ESC-resistant *E. coli* were susceptible to gentamicin.

All ESC-resistant *E. coli* were classified as MCR with eight MCR profiles identified (Table 6). The most common profile having 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.

A total of 21 ESC-resistant *E. coli* were selected for WGS. Six STs were identified with ST4981 (*n* = 7) being the most the dominant ST although it was only found within one state. The numbers of MCR profiles for each ST alongside any known AMR and virulence genes detected among the isolates are shown in Table 7. All isolates displayed at least one known substitutions 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* subunits while all ST10 (*n* = 4, 19.0%) isolates displayed A2034C and T2482G substitutions in *gyrA*, and T240C substitution in *parC* subunits. The PMQR gene *qnrS* was also found in four isolates (19.0%).

Three types of ESC-resistant genes were identified with the *bla*$_{CTXM-1}$ (*n* = 6, 28.6%) gene found in all ST10 and ST196 isolates, the *bla*$_{CTXM-14}$ gene (*n* = 5, 23.8%) in all ST88 and ST117 isolates and lastly, the *bla*$_{CTXM-15}$ (*n* = 10, 47.6%) gene in all ST2325 and ST4981 isolates. The phenicol-resistant gene *floR* was identified in five (23.8%) isolates while the beta-lactam-resistant genes *bla*$_{TEM-1B}$ and *bla*$_{TEM-106}$ was found in seven (33.3%) and one (4.8%) isolates respectively.

Two types of sulphonamide-resistant genes (*sul2* and *sul3*) were identified with each isolate harbouring either one of the genes while two types of tetracycline-resistant genes (*tet[A]* and *tet[M]*) were identified with 14 (66.7%) isolates harbouring at least one tetracycline-resistant gene. Five aminoglycoside-resistant 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-resistant gene family.

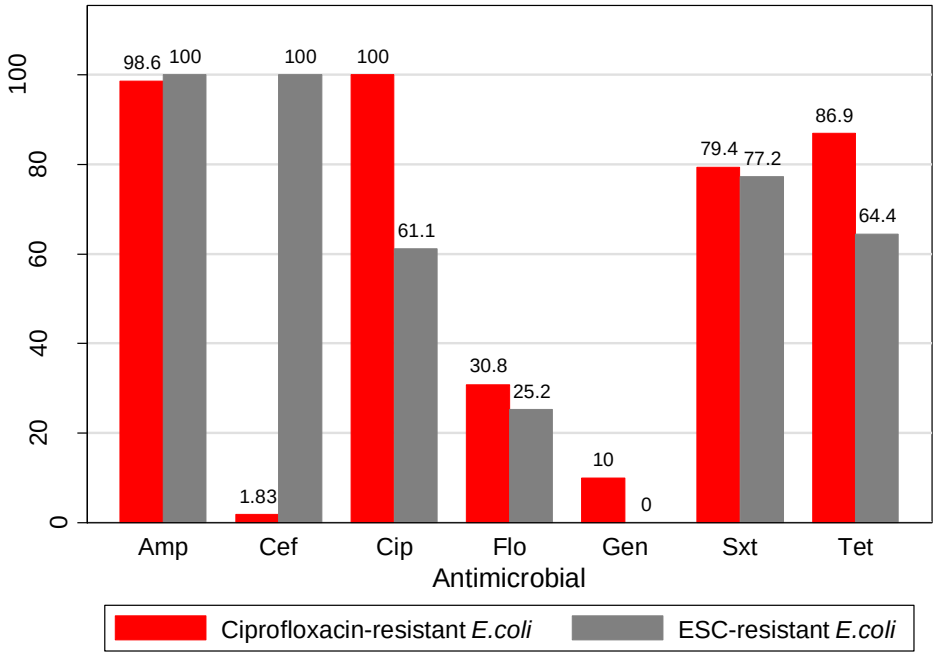

Figure 6: Resistance profiles of E. coli selected from pig faeces using antimicrobial infused agar. A total of 710 ciprofloxacin-resistant E. coli and 149 ESC-resistant E. coli across five states were subjected to MIC testing. Key: Amp - Ampicillin, Cef - Cefotaxime, Cip - Ciprofloxacin, Flo - Florfenicol, Gen - Gentamicin, Sxt - Trimethoprim-sulfamethoxazole, Tet - Tetracycline.

Table 4: Multi-drug resistance profiles of ciprofloxacin-resistant E. coli isolated from pig faeces using selective agar. Resistance to antimicrobials of each class was determined based on the epidemiological cut-off (ECOFF) value set by EUCAST with non-wild type isolates referred to as resistant. Isolates with resistance towards three or more antimicrobial classes are classified as multi-class resistant.

MDR profile	No. of isolates	% of total
bla_c3g_qui	7	1.0
bla_fpi_qui	54	7.6
bla_qui_tet	113	15.9
bla_fpi_phe_qui	1	0.1
bla_fpi_qui_tet	247	34.8
ami_bla_fpi_qui_tet	54	7.6
bla_fpi_phe_qui_tet	189	26.6
ami_bla_c3g_fpi_qui_tet	2	0.3
ami_bla_fpi_phe_qui_tet	5	0.7
bla_c3g_fpi_phe_qui_tet	4	0.6

bla – beta-lactam, c3g – third generation cephalosporins, qui – quinolone, fpi – folate pathway, ami – aminoglycoside, phe – phenicol, tet-tetracycline

Table 5: Number of isolates and multi-drug resistant profiles identified for each ciprofloxacin-resistant E. coli sequence types and the detection of any known antimicrobial resistant genes and virulence genes among these isolates.

ST	No. isolates	No. MCR Profiles	Antimicrobial resistant genes	Virulence genes
10	2	0	QRDR mutation, *bla*$_{TEM-1B}$, *sul1*, *sul2*, *tet(B)*	*fim*, *iroN*, *astA*
44	2	1	QRDR mutation, *bla*$_{TEM-1B}$, *sul3*, *aadA2*	*fim*, *astA*
155	2	1	QRDR mutation, *bla*$_{TEM-1B}$, *sul2*, *sul3*, *tet(A)*, *aac(3)*, *aadA2*	*fim*, *astA*
167	9	3	QRDR mutation, *bla*$_{TEM-1B}$, *sul2*, *sul3*, *tet(A)*, *aac(3)*	*pap*, *iroN*
361	4	2	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *sul2*, *sul3*, *tet(A)*, *tet(M)*, *aadA2*	*fim*, *astA*
542	4	2	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *bla*$_{TEM-176}$, *sul3*, *tet(A)*, *tet(B)*, *aadA2*	*fim*, *astA*
617	1	1	QRDR mutation, *qnrS*, *bla*$_{TEM-1B}$, *sul3*, *tet(B)*, *aadA2*	-
744	30	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, *tet(H)* , *tet(M)*, *aadA2*	*fim*, *pap*, *iroN*, *astA*
1642	10	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *sul1*, *sul3*, *tet(A)*, *aac(3)*, *aadA2*	*fim*, *astA*
5909	4	3	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *sul3*, *tet(A)*, *tet(B)*, *tet(M)*, *aadA2*	*fim*
11613	2	1	QRDR mutation, *qnrS*, *floR*, *bla*$_{TEM-1B}$, *sul2*, *sul3*, *tet(A)*, *tet(M)*, *aadA2*	*fim*
11916	1	1	QRDR mutation, *floR*, *bla*$_{TEM-1B}$, *sul3*, *tet(A)*, *tet(M)*, *aadA2*	*fim*, *astA*
11917	5	2	QRDR mutation, *bla*$_{TEM-1B}$, *sul3*, *tet(A)*, *aadA2*	*fim*, *astA*

Table 6: Multi-drug resistance profiles of ESC-resistant E. coli isolated from pig faeces using selective agar. Resistance to antimicrobials of each class was determined based on the epidemiological cut-off (ECOFF) value set by EUCAST with non-wild type isolates referred to as resistant. Isolates with resistance towards three or more antimicrobial classes are classified as multi-drug resistant.

MDR profile	No. of isolates	% of total
bla_c3g_qui	1	0.7
bla_c3g_phe	3	2.0
bla_c3g_qui	22	14.8
bla_c3g_fpi_phe	26	17.4
bla_c3g_fpi_qui	1	0.7
bla_c3g_fpi_tet	19	12.7
bla_c3g_phe_tet	9	6.0
bla_c3g_fpi_qui_tet	68	45.6

bla – beta-lactam, c3g – third generation cephalosporins, qui – quinolone, fpi – folate pathway, ami – aminoglycoside, phe – phenicol, tet-tetracycline

Table 7: Number of isolates and multi-drug resistant profiles identified for each ESC-resistant E. coli sequence types and the detection of any known antimicrobial resistant genes and virulence genes among these isolates.

ST	No. isolates	No. MCR Profiles	Antimicrobial resistant genes	Virulence genes
10	4	3	QRDR mutation, *bla*$_{CTXM-1}$, *bla*$_{TEM-1B}$, *sul2*, *sul3*, *tet(A)*, *aadA2*, *aadA5*, *aph(3')*	*fim*, *astA*
88	1	1	QRDR mutation, *qnrS*, *bla*$_{CTXM-14}$, *bla*$_{TEM-106}$, *sul3*, *tet(A)*	*fim*, *pap*, *sfa*, *foc*, *iroN*
117	4	3	QRDR mutation, *bla*$_{CTXM-14}$, *floR*, *bla*$_{TEM-1B}$, *sul2*, *tet(A)*, *aph(3")*, *aph(3')*, *aph(6)*	*fim*, *pap*, *sfa*, *foc*, *iroN*, *vat*
196	2	1	QRDR mutation, *bla*$_{CTXM-1}$, *floR*, *sul2*, *aadA5*	*fim*, *iroN*
2325	3	2	QRDR mutation, *qnrS*, *bla*$_{CTXM-15}$, *bla*$_{TEM-1B}$, *sul2*, *tet(A)*, *aph(3")*, *aph(6)*	*fim*
4981	7	3	QRDR mutation, *bla*$_{CTXM-15}$, *sul3*, *tet(A)*, *tet(M)*, *aadA2*	*astA*

5. Discussion

5.1 Major findings of two year proof of concept study

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. In the first year, no ESC-resistant *E. coli* were detected but ciprofloxacin-resistant *E. coli* were detected in seven farms with low carriage levels. In the second year, ciprofloxacin-resistant *E. coli* was detected in all farms while two farms were detected with ESC-resistant *E. coli*, although carriage levels of both resistant *E. coli* were low.

Across both years, ST744 was found to be the primary FQ-resistant *E. coli* ST across the ten farms followed by ST167, while ST10 and ST1141 were the two primary ESC-resistant *E. coli* ST present among the two positive farms. With first and second-line antimicrobials, resistance towards ampicillin, tetracycline and gentamicin were 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 (DANMAP, 2018).

While this study was not the first to detect FQ-resistant *E. coli* within Australian pigs (Abraham et al., 2015; Kidsley et al., 2018), 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 primary FQ-resistant *E. coli* STs (ST167 and ST744) in these ten 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 (Kidsely et al., 2020, Mukerji et al., 2019), and both STs have also previously been widely reported internationally in humans, livestock and wild birds (Garcia-Fernandez et al., 2020, Gronthal et al., 2018, Guenther et al., 2012, Haenni et al., 2018, Hasan et al., 2012, Kindle et al., 2019, Shen et al., 2017, Su et al., 2017).

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 (Abraham et al., 2015). An introduction through livestock is unlikely given the strict national biosecurity regulations surrounding the importation of livestock and unprocessed animal products into Australia (Turner, 2011).

Though phylogenetic analysis was only performed on the first year isolates, the phylogenetic clustering suggests that only one ST167 clone closely related to ST167 isolates from the Americas or Europe is present within the seven positive 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.

Further phylogenetic analysis examining the phylogenetic relationships of FQ-resistant *E. coli* isolates between both years and the international database would provide further elucidation regarding the introduction and persistence of FQ-resistant *E. coli* in Australian pigs without direct FQ use.

5.2 RASP Quantification of unusual forms of resistance in E. coli within Australian pigs on a national scale

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 (Ruiz, 2003).

This study (together with findings from the second year of the proof-of-concept study) were 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 (Martinez-Martinez et al., 1998).

By itself, PMQR genes only confer low-levels of FQ-resistance that are below the clinical breakpoints. 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 (Poirel et al., 2012, Ruiz et al., 2012). 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 (Abraham et al., 2015), 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. The fact that all ST744 isolates (both in the proof-of-concept study and in RASP Quantification study) harboured QRDR mutations, which is only possible via selective pressure from FQ use (Ruiz, 2003), 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 (Abraham et al., 2015).

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) (Cutler et al., 2020), 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.

The detection of ESC-resistant *E. coli* on eight farms, indicates that ESC-resistance is also present in Australian pig herds nationwide, albeit at low levels, despite the constraints on ESC use in Australian livestock (Cutler et al., 2020). 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 (Abraham et al., 2018, Darphorn et al., 2021, de Lagarde et al., 2020, Moffat et al., 2020) 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 by RASP Quantification 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* (Abraham et al., 2015, van Breda et al., 2018), 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 RASP Quantification were the same genes previously reported among Southeast Australian pig herds (van Breda et al., 2018). 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 (Smith et al., 2016).

In the last national survey on antimicrobial use in Australian pig farms, it was revealed that ceftiofur was used in 25% of farms (Jordan et al., 2009), 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 (Abraham et al., 2018).

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.

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7. Publications Arising

Two publications are now being prepared form this work. In consultation with Prof. David Jordan, it is best to have this report released on a need-to-know basis until a research publication are reviewed and published due to the sensitive nature of outcomes from this study