**Appendix A**

**Surveillance for antimicrobial resistance in enteric commensals and zoonotic pathogens in Australian pigs 2022**

**Final Report**

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# Executive Summary

A total of 300 faecal samples from finisher pigs, comprised of ten samples each from 30 farms located across five Australian states (four from Western Australia, five from South Australia, six from New South Wales, seven from Victoria and eight from Queensland), were collected at abattoirs between July to November 2022. The faecal samples found 2730 *E. coli isolates, 81 Salmonella* spp. isolates, 124 *Enterococcus* spp. isolates, and *Campylobacter* spp. Isolates.

Although there is a 13-fold increase in *E. coli* isolates tested, and the overall resistance profiles remained similar to the 2015 AMR study. Resistance to chloramphenicol remained the similar (2015 42.0%, 2022 44.13%), as did tetracycline resistance (2015 75.0%, 2022 78.6%). Resistance to gentamicin and ampicillin increased slightly from 0.5% to 1.93% and 61.5% to 76.02% respectively. Very little resistance to cephams was observed in either study 0.5 % (n=1) resistant to cefoxitin in 2015 and 0.4% (n=1) resistant to cefotaxime in 2022, however no known resistance gene was identified in this isolate. A small number of isolates were microbiologically resistant to the carbapenem, meropenem. This is likely due to the phenomenon known as MIC drift, as the MIC is just above the ECOFF breakpoint for meropenem and no known resistance genes or mutations were identified in the subset sequenced.

The recovery of *Salmonella* spp. was similar to the 2015 study. The prevalence of ampicillin non-wildtype has increased slightly since the last study from 61.9% to 76.5%. An increase in chloramphenicol non-wildtype (17.9% increased to 45.68%), as well as florfenicol non-wildtype (8.3% increased to 40.74%) was also observed in this study compared to the last (17.9% increased to 45.68%). Tetracycline resistance decreased slightly compared to the last study (77.4% down to 66.67%). A small number of isolates were microbiologically resistant to the carbapenem, meropenem. This is likely due to the phenomenon known as MIC drift, as the MIC is just above the ECOFF breakpoint for meropenem and no known carbapenem resistance genes were identified in sequencing. The *Salmonella* isolates were serotyped using genome sequencing and the dominant serovar detected was S. Rissen. Interestingly, commonly reported broad host range *Salmonella* serotype Typhimurium was rare (n=1; 1.25%).

No resistance was observed towards ampicillin or daptomycin for *E. faecium* compared to 34.5% and 16.7% presenting as non-wildtype in the last study. Isolates with a non-wild phenotype towards erythromycin decreased in this study (67%) compared to the previous study (90.5%). There was a decrease in isolates non-wildtype to tetracycline (2022 78%, 2015 91.7%) and virginiamycin (current study 6%, previous study 28.6%). There was also a decrease in clinically resistant quinuprisitn-dalfopristin isolates (52%) compared to the previous study (94.0%). Clinical resistance to erythromycin remained the similar compared to the previous study (81.82% vs. 82.4%) while tetracycline resistance was slightly higher (95.45 vs 82.4%).

Amongst *Campylobacter coli* isolates non-wildtype to azithromycin (2022 64.45%, 2015 75.2%), clindamycin (2022 60.66%, 2015 75.2%) remained similar to the previous study as did the proportion of isolates clinically resistant to tetracycline (2022 57.35% 2015 53.5%). The proportion of isolates clinically resistant to erythromycin decreased in the current study (58.77%) compared to the previous study (73.2%). No non-wildtype or isolates clinically resistant to ciprofloxacin were identified in the previous study however 5.74% of isolates in the current study were resistant to ciprofloxacin even though the breakpoint for resistance has increased from 0.5 mg/L to 2 mg/L since the last study. An increase in prevalence of non-wildtype to nalidixic acid (2022 3.35%, 2015 1.9%), florfenicol (2022 3.79%, 2015 0%) and gentamicin (2022 13.88%, 2015 0.6%). There was a disparity between the phenotypic resistance of ciprofloxacin (5.74%) and nalidixic acid (3.35%) and the detection of known mutations or genes that confer quinolone resistance (4.78%). However, all isolates with ciprofloxacin resistance had an MIC greater than the ECOFF breakpoint or one-fold below the ECOFF breakpoint.

Genomic characterisation of *Campylobacter coli* in this study showed ST854 as the dominant sequence type (ST), and this ST belonged to the international clonal complex (CC) 828 (also known as ST828 complex). While PubMLST database showed that clonal complex ST21 (*n* = 1407, 21.2%) was predominant in Oceania, international data showed ST828 complex as the dominant complex in general (*n* = 27995, 21.3%). It is important to highlight that comprehensive data regarding the genomic attributes and sequence variants of *Campylobacter* originating from swine worldwide remains restricted within national surveillance publications. This is primarily due to the absence of regular implementation of whole genome sequencing in the context of national antimicrobial resistance surveys focused on livestock and food commodities.

Despite the presence of modest to moderate resistance to routinely used first-line antimicrobials among various bacterial species, several noteworthy and encouraging findings arise from this study. Firstly, *E. coli* displayed a notable lack of resistance towards critically important antimicrobial such as colistin, meropenem, and amikacin and extremely low levels of resistance to extended spectrum cephalosporin resistance (cefotaxime) (0.04%, n=1) and ciprofloxacin (0.11%, n=3). Additionally, absence of resistance to critically important antimicrobials such as amikacin, azithromycin, ciprofloxacin, colistin, cefotaxime, gentamicin, and meropenem among *Salmonella* is an encouraging trend. Another promising outcome involves the absence of resistance against ampicillin, daptomycin, linezolid, teicoplanin, and vancomycin among enterococci. Finally, the detection of ciprofloxacin resistance (a type of fluoroquinolone) within *Campylobacter coli* was unusual considering the absence of fluoroquinolone use in the Australian pig industry. These cumulative observations provide valuable insights into the antimicrobial resistance landscape and suggest certain optimistic trends, while also highlighting exceptions that warrant further investigation.

Table of Contents

Acknowledgements 2

Executive Summary 3

1. Background to Research 8

2. Objectives of the Research Project 10

3. Research Methodology 11

3.1 Isolation and antimicrobial susceptibility testing of Escherichia coli 11

3.2 Isolation and antimicrobial susceptibility testing of Enterococcus species 14

3.3 Isolation and antimicrobial susceptibility testing of Salmonella 16

3.4 Isolation and antimicrobial susceptibility testing of Campylobacter species 16

3.5 Genetic analysis 17

3.5.1 DNA extraction and library preparation 17

3.5.2 DNA sequencing and analysis 17

3.6 Statistical analysis 17

4. Results 18

4.1 Sample collection and bacterial isolation 18

4.2 Escherichia coli 18

4.3 Salmonella spp. 24

4.4 Enterococci 28

4.5 Campylobacter coli 34

5. Discussion 39

6. References 41

List of Tables

**Table 1:** The number of farms sampled in each state 11

**Table 2**: Breakpoints used for susceptibility testing of *Escherichia coli* and *Salmonella* 13

**Table 3**: Breakpoints used for susceptibility testing of *Enterococcus* species 15

**Table 4:** Breakpoints used for susceptibility testing of *Campylobacter* *coli* 17

**Table 5:** Bacterial species recovered from the faecal samples 18

**Table 6**: Distribution of minimum inhibitory concentrations for *Escherichia coli* (n=2730) isolated from Australian pigs 20

**Table 7:** Phenotypes of *E. coli* (n=2730) collected from Australian pigs based on CLSI breakpoints 23

**Table 8:** Sequence type (ST) and genotype of select *E. coli* isolated from Australian pigs 23

**Table 9:** Distribution of minimum inhibitory concentrations for *Salmonella* spp. (n=80) isolated from Australian pigs 25

**Table 10**: AMR phenotypes of *Salmonella spp.*  (n=80) collected from Australian pigs 27

**Table 11**: Genomic MLST and serotype prediction of *Salmonella spp.*  (n=80) collected from Australian pigs 27

**Table 12**: Distribution of minimum inhibitory concentrations for *Enterococcus faecium* (n=100) isolated from Australian pigs 29

**Table 13**: MCR phenotypes of *Enterococcus faecium* (n=100) collected from Australian pigs 31

**Table 14**: Distribution of minimum inhibitory concentrations for *Enterococcus faecalis* (n=22) isolated from Australian pigs 32

**Table 15**: MCR phenotypes of *Enterococcus faecalis* (n=22) collected from Australian pigs 34

**Table 16**: Distribution of minimum inhibitory concentrations for *Campylobacter coli* (n=209) isolated from Australian pigs 35

**Table 17**: AMR phenotypes of *Campylobacter coli* (n=209) collected from Australian pigs 37

**Table 18**: Sequence types of *Campylobacter coli* (n=209) collected from Australian pigs 38

List of Figures

Figure 1: Antimicrobial resistance patterns for *E. coli* (n=2730) based on CLSI break points 22

Figure 2: Antimicrobial resistance patterns for *Salmonella* spp. (n=80) based on CLSI break points

 26

Figure 3: Distribution of *qnrS1* gene and ciprofloxacin MIC for *Salmonella* spp. (n=80) 27

Figure 4: Antimicrobial resistance patterns for *Enterococcus faecium* (n=100) based on CLSI break points 30

Figure 5: Antimicrobial resistance patterns for *Enterococcus faecalis* (n=22) based on CLSI break points 33

Figure 6: Antimicrobial resistance patterns for *Campylobacter* *coli* (n=209) based on CLSI break points 36

# Background to Research

Antimicrobial resistance (AMR) continues to be a serious threat to public health globally and the problem of increasing antimicrobial resistance is even more threatening when considering the very limited number of new antimicrobial agents that are in development. The international tripartite organizations (WHO, FAO, and WOAH) and Codex Alimentarius are stepping up their efforts by recommending antimicrobial stewardship programs and activities broadly designed to halt the emergence of resistance and its spread in animal and human populations.

Initially, the development of AMR impacting on public health was focused on antimicrobial use in human medicine, however, the use of antimicrobials in food-producing animals and companion animals has been found in some studies to contribute to AMR. Therefore, antimicrobial stewardship in both human and animal populations can help reduce the pace of AMR. Globally, European and North American countries stand out as having well established surveillance systems that incorporate data from food animals on an ongoing basis. These include, for example, DANMAP (Denmark) (1), CIPARS (Canada) (2), and NARMS (USA) (3).

Since 2013, the Commonwealth Government has been actively progressing the development of a coordinated plan for the management of AMR and Antimicrobial use (AMU) in humans and animals. Broad support for the development of the “National Antimicrobial Resistance Strategy” was obtained from key stakeholders across the medical, health, veterinary, agricultural and pharmaceutical communities at the “Australian One Health Antimicrobial Resistance Colloquium” in 2013. The Department of Agriculture, Water and the Environment then sponsored a review of the national surveillance programs in place for monitoring AMR and AMU in animals around the world with a view to defining a program suitable for Australia and combined this with roundtable discussions with key stakeholders in the agriculture and veterinary sectors.

In March 2015, the “Antimicrobial Resistance Surveillance Task Group” set up by the Department of Agriculture Water and the Environment proposed a surveillance model for use in the Australian pig industry that may also be applied to other major food animal industries in the future and to examine issues such as feasibility, cost, timing, methodology and logistics.

This pilot study, undertaken in 2015, has provided a baseline for the Australian pig industry and a benchmark for the other livestock industries in Australia to establish further animal-specific pilot surveys, as the basis for an ongoing integrated livestock AMR surveillance program. It was recommended that the data generated from the 2015 pilot study be integrated into other current antimicrobial stewardship programs being developed by the other livestock industries.

The 2015 AMR study shows that Australian pigs had no resistance to critically important drugs including colistin, fluoroquinolones and third generation cephalosporins, in either *Escherichia coli* or *Salmonella* isolates, and only a small number of isolates showed reduced susceptibility to fluoroquinolones. No resistance to vancomycin and linezolid was identified in *Enterococcus* isolates and all *Campylobacter* isolates tested in this study were susceptible to fluoroquinolones. Nevertheless, there were *Salmonella* and *E. coli* isolates that showed reduced susceptibility to fluoroquinolones. The multidrug-resistant enterococci isolates were subjected to whole genome sequence analysis to identify their possible origins and it was confirmed that they represent limited public health risk.

The 2015 pilot study not only demonstrated the low AMR in Australian pigs, it also successfully integrated industry-facilitated collection of samples from abattoir specimens, primary culture of bacterial species at a NATA accredited laboratory and the antimicrobial susceptibility testing reference laboratories currently undertaking AMR surveillance of human and veterinary pathogens.

With the good results and a successful partnership between the industry, laboratory and veterinarians, another AMR survey from 30 farms with 10 pigs per farm was conducted in 2020. This survey was to utilise “mass screening “to develop an enterprise specific AMR index based on large numbers of *E*. *coli* isolates that will deliver a low-cost system for performing AMR assays using international standards.

Differences between the 2014/15 and 2020 survey were: i) 4 different bacteria were tested for AMR (*E*. coli, *Salmonella*, *Enterococcus* and *Campylobacter*) in 2014/15 vs 1 bacteria (*E. coli*) in 2020; ii) 8 different antimicrobials were used in 2020 compared to 10 in 2014/15; iii) the 2020 results were summarised using an AMR index to help producers easier to understand their own AMR results. The AMR index is the resistance phenotype of each isolate. The index comprised of weighting reflecting the importance rating of each drug. The minimum possible isolate index is 0 and maximum is 16 and iv) a detailed individual AMR reports were generated for each farm surveyed.

Isolates of *E. coli* with resistance to critically important antimicrobials were subjected to whole genome sequence analysis to identify their possible origins. The resistant strains were found to contain AMP, TET, CIP and SXT genes, which were previously reported in dogs and seagulls in Australia and humans from overseas.

With the good results and a successful partnership between the industry, laboratory and veterinarians, another AMR survey from 30 farms with 10 pigs per farm was conducted in 2022. The survey is co-funded by the Commonwealth and APL. This AMR surveillance is necessary to i) assess the trends and or emergence of AMR; ii) support risk analysis; iii) provide a basis for policy, iv) evaluate and inform antimicrobial use and vi) assess the efficacy of interventions.

# Objectives of the Research Project

The objective of the survey is to estimate the prevalence of resistance against specified antimicrobials amongst *E. coli, Salmonella* spp., *Enterococcus* spp., and *Campylobacter* spp. isolated from the faeces of Australian finisher pigs at slaughter.

# Research Methodology

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 July to November 2022. The number of farms sampled from each state was in proportion to the size of the pig population provided by Australian Pork Limited and were as follow – four from Western Australia, five from South Australia, six from New South Wales, seven from Victoria and eight from Queensland (Table 1). While not exclusively targeting herds of a particular size, the methods will bias sampling towards larger herds. This is considered acceptable because industry statistics show that the vast majority (~70%) of pigs slaughtered in Australia for human consumption come from a very small number of establishments.

**Table 1:** The number of farms sampled in each state.

|  |  |
| --- | --- |
| **State**  | **Number of farms** |
| **WA** | **4** |
| **SA** | **5** |
| **QLD** | **8** |
| **NSW** | **6** |
| **VIC** | **7** |
| **Total** | **30** |

Samples were collected by veterinarians, or by experienced abattoir personnel 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 between 2 to 8 ºC and processed within 48 hours of collection. Each sample was processed to isolate four different enteric bacteria for antimicrobial susceptibility testing (AST) – *E. coli*, *Enterococcus* species, *Campylobacter* species and *Salmonella*.

1.
2.
3.

## Isolation and antimicrobial susceptibility testing of Escherichia coli

Samples from each farm were sampled with a sterile cotton swab. For each farm, five swabs from five samples were placed into sterile 1 x phosphate buffered saline (PBS) and vortexed to acquire one pooled sample. Thus, a total of two pooled samples were acquired from each farm with a total of 60 pooled samples across 30 farms. Each pooled sample was loaded onto the Robotic Antimicrobial Susceptibility Platform (RASP) for serial dilution and agar inoculation onto CHROMagar™ ECC (MicroMedia, Edwards Group) (4). Plated samples were incubated at 37 ºC for 16 to 20 hours.

Presumptive identification of *E. coli* colonies was performed based on chromogenic reaction of the agar as detailed by the manufacturer. Up to 47 presumptive *E. coli* colonies from each pooled sample was isolated by RASP (4), inoculated into Luria-Bertani (LB) broth and incubated at 37 ºC for 16 to 20 hours. After incubation, isolates were taken for MALDI-TOF and AST.

Confirmation of isolate identity was performed from broth cultures using MALDI-Biotyper (Bruker Microflex) (4). The broth microdilution method was used to determine antimicrobial susceptibility and was performed on RASP according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (4, 5). *E. coli* ATCC 25922 and an in-house positive control *E. coli* ST131 were used as quality control strains. Susceptibility towards fourteen antimicrobials were assessed and includes amikacin, ampicillin, apramycin, cefotaxime, ceftazidime, chloramphenicol, colistin, ciprofloxacin, florfenicol, gentamicin, meropenem, sulfamethoxazole, tetracycline and trimethoprim (Table 2). All AST plates were imaged using the SensititreTM Vizion TM Digital MIC Viewing System.

MIC interpretation was performed using the epidemiological cut-off (ECOFF) value according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) as wildtype and non-wildtype as well as breakpoints set by the Clinical Laboratory Standards Institute as sensitive or resistant (6,8). Isolates with resistance towards three or more antimicrobial classes were classified as multi-class resistant (MCR).

**Table 2**: Breakpoints used for susceptibility testing of *Escherichia coli* and *Salmonella*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  | ECOFF a |  | CLSIb |
| **Antimicrobial Class** | **Antimicrobial Agent** | **Range (mg/L)** | ***E. coli*** | ***Salmonella*** |  | **R** |
| Aminoglycosides | Apramycin | 8-32 | - | - |  | - |
|  | Amikacin | 1-64 | 8 | 4 |  | >32 |
|  | Gentamicin | 0.25 - 16 | 2 | 2 |  | >8 |
| Carbapenem | Meropenem | 0.008-4 | 0.06 | 0.06 |  | >2 |
| Cephems | Cefotaxime | 0.015-4 | 0.25 | 0.5 |  | >2 |
|  | Ceftazidime | 0.0625-16 | 1 | 2 |  | >8 |
| Fluoroquinolones | Ciprofloxacin  | 0.008 - 2 | 0.06 | 0.125 |  | >0.5 |
| Folate pathway inhibitors | Trimethoprim | 0.25-16 | 2 | 2 |  | >8 |
|  | Sulfamethoxazole (*E. coli*) | 8-512 | - | - |  | >256 |
| Macrolides | Azithromycin (*Salmonella*) | 1-64 | - | 16 |  | >16c |
| Penicillins | Ampicillin | 1 - 32 | 8 | 4 |  | >16 |
| Phenicols | Chloramphenicol | 2 - 32 | 16 | 16 |  | >16 |
|  | Florfenicol | 4 - 32 | 16 | 16 |  | - |
| Polymyxins | Colistin | 0.25 - 8 | 2 | - |  | >2  |
| Tetracyclines | Tetracycline | 1 - 32 | 8 | 8 |  | >8 |

aEUCAST epidemiological cut-off values (mg/L)

b CLSI VETO8 (7), or M100 (5) breakpoints (mg/L) R = resistant

c *Salmonella enterica* serovar Typhi only

-Not defined

## Isolation and antimicrobial susceptibility testing of Enterococcus species

Samples from each farm were sampled with a sterile cotton swab. Each swab was placed into sterile 1 x PBS and loaded onto RASP for serial dilution and agar inoculation onto Slanetz and Bartley agar (ThermoFisher Scientific) (4). All agars were incubated at 42 ºC for up to 48 hours.

Presumptive identification of *Enterococcus* colonies was performed based on chromogenic reaction of the agar as detailed by the manufacturer. Up to eight presumptive *Enterococcus* colonies from each sample was isolated by RASP (4), inoculated into brain-heart infusion broth (BHIB) and incubated at 37 ºC for 16 to 20 hours. After incubation, isolates were taken for MALDI-TOF and AST.

Confirmation of *Enterococcus* species identity was performed from broth cultures using MALDI-Biotyper (Bruker Microflex) (4). Only isolates identified as *Enterococcus faecium* and *Enterococcus faecalis* were further subjected to AST. The broth microdilution method was used to determine antimicrobial susceptibility and was performed on RASP according to CLSI guidelines (4, 5). *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 was used as quality control strains. Susceptibility towards thirteen antimicrobials were assessed and includes ampicillin, chloramphenicol, daptomycin, erythromycin, gentamicin, lincomycin, linezolid, quinupristin-dalfopristin, streptomycin, teicoplanin, tetracycline, vancomycin and virginiamycin. All AST plates were imaged using the SensititreTM Vizion TM Digital MIC Viewing System.

MIC interpretations was performed using the epidemiological cut-off (ECOFF) value according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (6). Isolates classified as wild type were categorised as susceptible while those classified as non-wild type were categorised as resistant. Isolates with resistance towards three or more antimicrobial classes were classified as MCR.

**Table 3**: Breakpoints used for susceptibility testing of *Enterococcus* species

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Class | Agent | species | Range (mg/L) | ECOFF b | CLSIa |
| Aminoglycosides(high-level) | Gentamicin | *E. faecium* | 4 - 1024 | 32 | - |
| *E. faecalis* | 4 - 1024 | 64 |  |
|  | Streptomycin | *E. faecium* | 512 - 2048 | - | >128 |
| *E. faecalis* | 512 - 2048 | 512 | - |
| Glycopeptides | Vancomycin | All | 0.25 - 128 | 4 | >16 |
|  | Teicoplanin | All | 0.25 - 128 | 2 | >16 |
| Lincosamide | Lincomycin | All | 1 - 8 | - | - |
| Lipopeptides | Daptomycin | *E. faecium* | 0.25 - 16 | 8 | >4 |
| *E. faecalis* | 0.25 - 16 | 4 | >4 |
| Macrolides | Erythromycin | All | 0.25 - 16 | 4 | >4 |
| Oxazolidinones | Linezolid | All | 0.5 - 16 | 4 | >4 |
| Penicillins | Ampicillin | *E. faecium* | 0.5 - 32 | 8 | >8 |
|  | *E. faecalis* | 0.5 - 32 | 4 | >8 |
| Phenicols | Chloramphenicol | All | 2 - 32 | 32 | >16 |
| Streptogramins | Quinupristin-dalfopristin | *E. faecium* | 0.5 - 32 | 2 | >2 |
|  | *E.faecalis* | 0.5 - 32 | 32 | - |
|  | Virginiamycin | *E. faecium* | 0.125 - 64 | 8 | - |
|  | *E. faecalis* | 0.125 - 64 | 32c | - |
| Tetracyclines | Tetracycline | All | 0.25-128 | 4 | >8 |

a CLSI VETO8 (7) or M100 (5) resistance breakpoints (mg/L)

b EUCAST epidemiological cut-off values (6) (mg/L)

c ECOFF from 2022, change to “insufficient data” in 2023 -

- Not defined

## Isolation and antimicrobial susceptibility testing of Salmonella

Each sample was homogenised in sterile buffered peptone water (BPW) using a BagMixer® 400 P laboratory blender (Interscience, Edwards Group). Each homogenised mixture was incubated at 37 ºC for up to 24 hours before mixture was transferred into Rappaport-Vassiliadis (RV) broth and incubated at 42 ºC for up to 24 hours. After incubation, the overnight RV broth was streaked onto BrillianceTM *Salmonella* (ThermoFisher Scientific) and xylose lysine deoxycholate (XLD) agars (Edwards Group). All agars were incubated at 37 ºC for up to 24 hours.

Presumptive identification of *Salmonella* colonies was performed based on chromogenic reaction of each agar as detailed by the manufacturer. A single presumptive *Salmonella* colony from both agars of each sample was streaked onto Columbia sheep blood agar (SBA) (Edwards Group) and incubated at 37 ºC for up to 24 hours. Confirmation of colony identity was performed using MALDI-TOF as per manufacturer instructions.

Broth microdilution method was used to determine antimicrobial susceptibility according to CLSI guidelines (5). *E. coli* ATCC 25922 was used as the quality control strain. Drug panels were prepared using RASP (4) while each inoculum was prepared manually and dispensed using the Sensititre AIMTM Automated Inoculation Delivery System. Susceptibility towards fourteen antimicrobials were assessed and includes amikacin, ampicillin, apramycin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, colistin, ciprofloxacin, florfenicol, gentamicin, meropenem, tetracycline, and trimethoprim (Table 2). All AST plates were imaged using the SensititreTM Vizion TM Digital MIC Viewing System.

MIC interpretations was performed using the epidemiological cut-off (ECOFF) value according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (6). Isolates classified as wild type were categorised as susceptible while those classified as non-wild type were categorised as resistant. Isolates with resistance towards three or more antimicrobial classes were classified as MCR.

## Isolation and antimicrobial susceptibility testing of Campylobacter species

Each sample was homogenised for in sterile buffered peptone water (BPW) using a BagMixer® 400 P laboratory blender (Interscience, Edwards Group). Each homogenised mixture was transferred to Preston *Campylobacter* broth and incubated at 42 ºC for up to 24 hours under microaerophilic conditions. The incubated broth was streaked onto modified charcoal cefoperazone deoxycholate (MCCD) agar (Edwards Australia) and BrillianceTM CampyCount agar (ThermoFisher Scientific). All agars were incubated at 42 ºC for up to 24 hours under microaerophilic conditions.

Presumptive identification of *Campylobacter* colonies was performed based on chromogenic reaction of each agar as detailed by the manufacturer. A single presumptive *Campylobacter* colony from both agars of each sample was streaked onto Columbia sheep blood agar (SBA) (Edwards Group) and incubated at 42 ºC for up to 24 hours. Confirmation of *Campylobacter* species identity was performed using MALDI-TOF as per manufacturer instructions.

The broth microdilution method was used to determine antimicrobial susceptibility according to CLSI guidelines (5). *Campylobacter jejuni* ATCC 33560 was used as the quality control strain. Drug panels were prepared using RASP (4) while each inoculum was prepared manually and dispensed using the Sensititre AIMTM Automated Inoculation Delivery System. Susceptibility towards eleven antimicrobials were assessed and includes azithromycin, ciprofloxacin, chloramphenicol, clindamycin, erythromycin, florfenicol, gentamicin, nalidixic acid, streptomycin, telithromycin, and tetracycline (Table 4). All AST plates were imaged using the SensititreTM Vizion TM Digital MIC Viewing System.

MIC interpretations was performed using the epidemiological cut-off (ECOFF) value according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) (6). Isolates classified as wild type were categorised as susceptible while those classified as non-wild type were categorised as resistant. Isolates with resistance towards three or more antimicrobial classes were classified as MCR.

**Table 4:** Breakpoints used for susceptibility testing of *Campylobacter* *coli*

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  |  |
| **Class** | **Agent** | **Range (mg/L)** | **ECOFFa**  |
| Aminoglycosides | Gentamicin | 0.12 - 16 | 2 |
|  | Streptomycin | 0.5-16 | 4 |
| Ketolides | Telithromycin | 0.5 - 8 | - |
| Lincosamide | Clindamycin | 0.03 - 32 | 1 |
| Macrolides | Azithromycin | 0.03-2 | 0.5 |
|  | Erythromycin | 0.06 - 128 | 8 |
| Phenicols | Florfenicol | 0.03 - 32 | 4 |
|  | Chloramphenicol | 2-32 | 16 |
| Quinolones | Ciprofloxacin | 0.008-16 | 0.5 |
| Nalidixic acid | 1 - 64 | 32 |
| Tetracyclines | Tetracycline | 0.12 - 64 | 2 |

a EUCAST epidemiological cut-off values (mg/L). CLSI breakpoints not shown as they do not cover the majority of antimicrobials tested in this study. – Not defined

## Genetic analysis

All *Salmonella* and *Campylobacter* isolates and a selection of *E. coli* isolates were sequenced for typing and presence of AMR genes.

### DNA extraction and library preparation

DNA extraction was performed on all isolates using the MagMAX Multi-sample extraction kit (Thermofisher Scientific, USA) as per the manufacturer’s instructions. DNA library preparation was conducted using Celero DNA-Seq library preparation kit (Tecan) as per manufactures instructions. Library preparations were sequenced via Illumina Nextseq platform with a high output 2x150 kit.

### DNA sequencing and analysis

The genomic data was *de novo* assembled using SPAdes. All isolates were analysed using publicly available databases for the screening of multi-locus sequence type, AMR genes, virulence genes and plasmids.

## Statistical analysis

Confidence intervals of proportions are calculated using exact binomial confidence intervals derived by the Clopper-Pearson method. All analysis uses Stata version15.1 (StataCorp LLC, College Station, Texas USA, www.stata.com).

# Results

1.

## Sample collection and bacterial isolation

A total of 300 samples were received at Murdoch University, with all samples being included in this study. A total of 3153 bacteria were isolated during this study. For pooled samples, 69-94 *E. coli* were collected from each farm using RASP, with a total of 2730 *E. coli* isolates collected in this study. RASP was also used in the isolation of enterococci using individual samples. Up to eight colonies per sample were collected and a single *E. faecium* (82.3%)and *E. faecalis* (17.7%) was processed for antimicrobial susceptibility testing if present. Samples were processed individually for *Campylobacter* spp. And *Salmonella* spp. A total of 81 samples were positive for *Salmonella* spp., while 218 samples were positive for *Campylobacter*, with only *Campylobacter coli* isolated (Table 5).

**Table 5:** Bacterial species recovered from the faecal samples.

|  |  |  |
| --- | --- | --- |
| Genus | Species | Number (% of genus) |
| *Escherichia* | *coli* | 2730 (100) |
| *Enterococcus* | *faecium* | 102 (82.3) |
| *faecalis* | 22 (17.7) |
| *Salmonella* | *enterica* | 80 (100) |
| *Campylobacter* | *coli* | 218 (100) |

## Escherichia coli

Of the 2730 *E. coli* isolates, 129 (4.54%) were non-wildtype to all antimicrobials tested based on ECOFF. All isolates tested were clinically susceptible to amikacin, ceftazidime, colistin and meropenem (Table 6, Figure 1). ECOFF non-wildtype was observed for ampicillin (77.36%), cefotaxime (0.18%), ceftazidime (0.18%) chloramphenicol (43.52%), ciprofloxacin (3.96%), florfenicol (17.33%), gentamicin (4.73%), meropenem (5.93%) tetracycline (78.72%) and trimethoprim (42.42%) (Table 6). Detection of clinical resistance was similar to non-wildtype detection (Table 6, Figure 1). None of the non-wildtype ceftazidime and meropenem isolates were clinically resistant (Table 6). A comprehensive distribution of MIC for *E. coli* based on ECOFF breakpoints and CLSI breakpoints are shown in Table 6. The prevalence of AMR for *E. coli* using clinical breakpoints is shown in Figure 1.

A total of 24 AMR profiles were identified based on CLSI breakpoints (Table 7). A total of 56.88% (*n*=1553) of isolates were considered MCR. The most common AMR profile was beta-lactams, folate pathway inhibitors, phenicols and tetracyclines with 32.31% non-wild type (*n*=882), followed by beta-lactams and tetracyclines with 14.18% non-wildtype (*n*=387) and beta-lactams, folate pathway inhibitors and tetracyclines with 11.64% non-wild type (*n*=318).

A total of 22 *E. coli* isolates from this study were selected for sequencing based on phenotypic resistance profiles (Table 8). The only isolate resistant to third-generation cephalosporins and all isolates resistant to quinolines (n=3) were sequenced. In addition, a representative isolate of the remaining phenotype with resistance to 5 or more classes of antimicrobials (n=1) was randomly chosen. Finally, a selection of isolates with meropenem MIC above the ECOFF breakpoint were chosen for sequencing (n=17).

The quinolone resistance associated gene *qnrS1* was identified in two isolates with phenotypic resistance to quinolones and one of these isolates also had the *oqxA* and *oqxB* genes associated with phenicol/quinolone resistance. The third resistant isolate had mutations in the *gyrA* region (*gyrA\_*D87N*, gyrA\_*S83L) and the *parC*(*parC*\_E84V, *parC\_*S80I) and *parE* regions (*parE\_*I529L).

The single isolate with resistance to third-generation cephalosporins did not carry any known associated resistance genes.

Genes known to confer resistance to aminoglycosides (*aac (3)-Iva, aadA1,2,5, aph (3,4,6)*), beta-lactams (*blaEC, blaCTX-M-27, blaLAP-2 and blaTEM-1*), phenicols (*cmlA1, floR, oqxA,B*), trimethoprim (*dfrA5, 12, 17*), quinolones (*qnrS1*), sulfonamide (*sul1-3*), macrolides (*mph(A)*) and tetracycline (*tetA,B,H,M*) were found in one or more of the five isolates described above. Point mutations known to confer resistance to beta-lactams (*ampC\_C-42T*) and quinolones (*parC\_E84V, parC\_S80I, parE\_I529L, gyrA\_S38L, gyrA\_D87N*).

The isolates chosen for sequencing due to non-wildtype meropenem phenotypes had no known meropenem resistance genes or mutations identified and the phenotype could be due to MIC drift in the *in vitro* assay.

**Table 6**: Distribution of minimum inhibitory concentrations for *Escherichia coli* (n=2730) isolated from Australian pigs

Percentage of isolates classified as microbiologically resistant (nw) and clinically resistant (cr) with corresponding 95% confidence intervals (ci). For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Blank cells within the shaded area indicate that no isolates tested had an MIC at that concentration. Numbers outside the shaded area indicate the percent of isolates that had growth at all concentrations tested and the MIC is above the tested range. “.” Indicates the breakpoint was not available and the confidence interval was not calculated.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Concentration (mg/L) |  |  |
| Antimicrobial | 0.008 | 0.016 | 0.031 | 0.063 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | nw (ci) | cr (ci) |
| Amikacin |  |  |  |  |  |  |  | 66.52 | 27.58 | 5.24 | .66 |  |  |  |  |  |  |  | 0 (0,.14) | 0.00 (0,.14) |
| Ampicillin |  |  |  |  |  |  |  | 1.54 | 6.92 | 10 | 4.18 | 1.06 | .18 | 76.12 |  |  |  |  | 77.36 (75.75,78.92) | 76.30 (74.66,77.88) |
| Apramycin |  |  |  |  |  |  |  |  |  |  | 93.77 | 2.6 | .04 | 3.59 |  |  |  |  | . | . |
| Cefotaxime |  | 3.7 | 30.66 | 54.25 | 11.17 | .04 |  | .07 | .07 | .04 |  |  |  |  |  |  |  |  | .18 (.06,.43) | 0.04 (0,.2) |
| Ceftazidime |  |  |  | 12.49 | 50 | 35.57 | 1.76 |  | .07 | .07 | .04 |  |  |  |  |  |  |  | .18 (.06,.43) | 0.00 (0,.14) |
| Chloramphenicol |  |  |  |  |  |  |  |  | 2.56 | 23.3 | 25.97 | 4.65 | 8.64 | 34.87 |  |  |  |  | 43.52 (41.65,45.4) | 43.52 (41.65,45.4) |
| Ciprofloxacin | 79.37 | 13.92 | 2.16 | .59 | 1.54 | 2.16 | .15 | .07 |  | .04 |  |  |  |  |  |  |  |  | 3.96 (3.26,4.76) | 0.11 (.02,.32) |
| Colistin |  |  |  |  |  | 95.35 | 4.29 | .33 | .04 |  |  |  |  |  |  |  |  |  | 0 (0,.14) | 0.00 (0,.14) |
| Florfenicol |  |  |  |  |  |  |  |  |  | 21.68 | 38.1 | 22.89 | 5.82 | 11.5 |  |  |  |  | 17.33 (15.92,18.8) | . |
| Gentamicin |  |  |  |  |  | 50.15 | 38.35 | 6.08 | .7 | .62 | 2.16 | .92 | 1.03 |  |  |  |  |  | 4.73 (3.96,5.59) | 1.94 (1.46,2.53) |
| Meropenem |  | 16.56 | 53.44 | 20.26 | 5.93 |  |  |  |  |  |  |  |  |  |  |  |  |  | 5.93 (5.08,6.89) | 0.00 (0,.14) |
| Sulfamethoxazole |  |  |  |  |  |  |  |  |  |  | 10.84 | 2.12 | 20.15 | 4.69 | 1.21 | .04 | 3.04 | 57.91 | . | 60.95 (59.09,62.79) |
| Tetracycline |  |  |  |  |  |  |  | 15.09 | 5.09 | .44 | .66 | .22 | 1.1 | 77.4 |  |  |  |  | 78.72 (77.13,80.24) | 78.72 (77.13,80.24) |
| Trimethoprim |  |  |  |  |  | 31.72 | 21.1 | 3.48 | 1.28 | .18 | .04 | .11 | 42.09 |  |  |  |  |  | 42.42 (40.55,44.3) | 42.20 (40.34,44.08) |



Figure 1: Antimicrobial resistance patterns for *E. coli* (n=2730) based on CLSI break points.The proportion of susceptible is shown in green and the proportion resistant in orange.

**Table 7:** Phenotypes of *E. coli* (n=2730) collected from Australian pigs based on CLSI breakpoints

|  |  |  |
| --- | --- | --- |
| phenotype | n | pct |
| 0: nil | 129 | 4.7253 |
| 1: bla | 171 | 6.2637 |
| 1: fpi | 10 | 0.3663 |
| 1: phe | 23 | 0.8425 |
| 1: tet | 192 | 7.0330 |
| 2: bla\_fpi | 86 | 3.1502 |
| 2: bla\_phe | 29 | 1.0623 |
| 2: bla\_tet | 387 | 14.1758 |
| 2: fpi\_phe | 9 | 0.3297 |
| 2: fpi\_tet | 137 | 5.0183 |
| 2: phe\_tet | 4 | 0.1465 |
| 3: bla\_fpi\_phe | 122 | 4.4689 |
| 3: bla\_fpi\_tet | 318 | 11.6484 |
| 3: bla\_phe\_tet | 51 | 1.8681 |
| 3: fpi\_phe\_tet | 124 | 4.5421 |
| 4: ami\_bla\_fpi\_phe | 1 | 0.0366 |
| 4: ami\_bla\_fpi\_tet | 5 | 0.1832 |
| 4: ami\_fpi\_phe\_tet | 19 | 0.6960 |
| 4: bla\_c3g\_fpi\_phe | 1 | 0.0366 |
| 4: bla\_fpi\_phe\_tet | 882 | 32.3077 |
| 4: bla\_fpi\_qui\_tet | 1 | 0.0366 |
| 5: ami\_bla\_fpi\_phe\_tet | 27 | 0.9890 |
| 5: bla\_fpi\_phe\_qui\_tet | 1 | 0.0366 |
| 6: ami\_bla\_fpi\_phe\_qui\_tet | 1 | 0.0366 |

Ami- aminoglycosides, bla- beta lactams, phe- phenicols, fpi- folate pathway inhibitors, tet-tetracycline, c3g- cephems – third generation, qui- quinolones

**Table 8:** Sequence type (ST) and genotype of select *E. coli* isolated from Australian pigs.

|  |  |  |  |
| --- | --- | --- | --- |
| Isolate ID  | ST  | Phenotype  | Associated genotype  |
| 22070107\_C01  | 10  | Beta-lactams, folate pathway inhibitors, quinolones, tetracycline  | *aadA2 acrF blaEC blaTEM1 mdtM qnrS1 sul3 tetA AX*  |
| 22080081\_C43  | 101  | Aminoglycosides, beta-lactams, folate pathway inhibitors, phenicols, tetracyclines  | *acrF blaEC glpT\_E448K mdtM*   |
| 22080092\_C02  | 69  | Aminoglycosides, beta-lactams, folate pathway inhibitors, phenicols, tetracyclines, quinolones  | *aac3IVa aadA1 aadA2 acrF aph3Ib aph3IIa aph4Ia aph6Id blaEC blaTEM1 ble bleO cmlA1 cyaA\_S352T dfrA12 emrD glpT\_E448K lnuF lnuG mdtM oqxA oqxB qnrS1 sul1 sul3 tetA tetH AX*  |
| 22080154\_C01  | 744  | beta-lactams, folate pathway inhibitors, phenicols, tetracyclines, quinolones   | *aadA5 acrF aph3Ib aph6Id blaEC blaCTXM27 dfrA17 emrD gyrA\_D87N gyrA\_S83L glpT\_E448K mdtM mphA parC\_E84V parC\_S80I parE\_I529L  ptsI\_V25I sul1 sul2 tetA uhpT\_E350Q*  |
| 22110024\_C21  | -  | Beta-lactams, third generation cephalosporins, folate pathway inhibitors, phenicols  | *aadA1 aadA2 acrF ampC\_C42T blaEC blaTEM1 cmlA1 dfrA5 fosA75 glpT\_E448K mdtM  sul1 sul3*  |

## Salmonella spp.

All *Salmonella* isolates collected were clinically susceptible to amikacin, azithromycin, cefotaxime, ceftazidime, ciprofloxacin, colistin, gentamicin and meropenem (Figure 2). The majority of isolates were clinically resistant to ampicillin (75.31%) and tetracycline (66.67%), with resistance to chloramphenicol and trimethoprim also observed (both at 46.25%) (Figure 2). A small number of isolates were considered non-wildtype for meropenem (7.50%) but were clinically sensitive (Table 9). All meropenem non-wild type isolates had MIC of 0.13g/L, one dilution above the ECOFF breakpoint. A total of eight AMR profiles were identified amongst the isolates based on CLSI breakpoints with 46.25% (*n*=37) considered MCR (Table 10). The most common AMR profile was beta-lactams, folate pathway inhibitors, phenicols and tetracyclines with 35.00% non-wildtype (*n*=28) followed by beta-lactams and tetracyclines with 21.25% non-wildtype (*n*=17).

All *Salmonella* isolates were subjected to whole genome sequencing to determine multi-locus sequence type, serotype and genotypic resistance profile. A total of 11 MLST types were identified in the collection. The most common ST detected were 469 (46.25%), 34 (17.50%) and 516 (13.75%). ST 29, 32, 40, 377, 463, 515, 578 and 2066 were also present in the collection. The dominant serotype detected was Rissen (46.25%), followed by I 4, {5}, 12:i:- (17.50%) and Give (13.75%) (Table 11). Resistance genes for azithromycin (*acrB\_R717L*), aminoglycosides (*aadA1, aadA2, aph(3’)-Ia, aph(3”)-Ib, aph(6)-Id*, 77.5%), beta-lactams (*blaTEM-1* 79%), fosfomycin (*fosA7.33*, 2.5%) , phenicols (*cmlA1, floR* ,46.25%), quinolones (*qnrS1*, 42.5%), sulfonamides (*sul1-3*, 66.25%), trimethoprim (*dfrA12, dfrA14,* 46.25%) and tetracyclines (*tet(A,B,M)*, 68.75%) were detected. While no isolates were considered phenotypically resistant to azithromycin (ECOFF and clinical breakpoint is 16 ug/ml) all isolates that had an MIC one dilution below the breakpoint (16 ug/ml) also carried the gene (*acrB\_R717L*). Similarly, the quinolone resistance inducing gene *qnrS* was identified in 42.5% of isolates but no clinical phenotypic resistance was observed and only 10% of isolates were considered non-wild type based on ECOFF breakpoints. However, all wildtype isolates with an MIC one dilution below the breakpoint also carried the *qnrS* gene, which was not found in any isolates with lower MICs (Figure 3). A total of 46.25% of isolates carried known resistance genes to phenicols. All isolates with a non-wildtype phenotype for florfenicol (n=33) harboured the *floR* gene. The majority of isolates with phenotypic chloramphenicol resistance harboured the *cmlA1* gene (n=35) while two only had the *floR* gene.

**Table 9:** Distribution of minimum inhibitory concentrations for *Salmonella* spp. (n=80) isolated from Australian pigs

Percentage of isolates classified as microbiologically resistant (nw) and clinically resistant (cr) with corresponding 95% confidence intervals (c). For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Blank cells within the shaded area indicate that no isolates tested had an MIC at that concentration. Numbers outside the shaded area indicate the percent of isolates that had growth at all concentrations tested and the MIC is above the tested range. “.” Indicates the breakpoint was not available and the confidence interval was not calculated.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Concentration mg/L |  |  |  |
| Antimicrobial | 0.008 | 0.016 | 0.031 | 0.063 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 2048 | nw | nw\_ci | cr | cr\_ci |
| Amikacin |  |  |  |  |  |  |  | 96.25 | 3.75 |  |  |  |  |  |  |  | 0 | 0,4.51 | 0.00 | 0,4.51 |
| Ampicillin |  |  |  |  |  |  |  | 13.75 | 10 |  |  |  |  | 76.25 |  |  | 76.25 | 65.42,85.05 | 76.25 | 65.42,85.05 |
| Apramycin |  |  |  |  |  |  |  |  |  |  | 100 |  |  |  |  |  | . | . | . | . |
| Azithromycin |  |  |  |  |  |  |  | 7.5 | 51.25 | 38.75 |  | 2.5 |  |  |  |  | 0 | 0,4.51 | 0.00 | 0,4.51 |
| Cefotaxime |  |  | 12.5 | 46.25 | 37.5 | 3.75 |  |  |  |  |  |  |  |  |  |  | 0 | 0,4.51 | 0.00 | 0,4.51 |
| Ceftazidime |  |  |  |  | 26.25 | 38.75 | 33.75 | 1.25 |  |  |  |  |  |  |  |  | 0 | 0,4.51 | 0.00 | 0,4.51 |
| Chloramphenicol |  |  |  |  |  |  |  |  |  | 21.25 | 32.5 |  |  | 46.25 |  |  | 46.25 | 35.03,57.76 | 46.25 | 35.03,57.76 |
| Ciprofloxacin | 48.75 | 8.75 |  |  | 32.5 | 10 |  |  |  |  |  |  |  |  |  |  | 10 | 4.42,18.76 | 0.00 | 0,4.51 |
| Colistin |  |  |  |  |  | 92.5 | 3.75 | 2.5 | 1.25 |  |  |  |  |  |  |  | . | . | 0.00 | 0,4.51 |
| Florfenicol |  |  |  |  |  |  |  |  |  | 30 | 23.75 | 5 |  | 41.25 |  |  | 41.25 | 30.35,52.82 | . | . |
| Gentamicin |  |  |  |  |  | 93.75 | 3.75 | 2.5 |  |  |  |  |  |  |  |  | 0 | 0,4.51 | 0.00 | 0,4.51 |
| Meropenem |  |  | 55 | 37.5 | 7.5 |  |  |  |  |  |  |  |  |  |  |  | 7.5 | 2.8,15.61 | 0.00 | 0,4.51 |
| Tetracycline |  |  |  |  |  |  |  | 10 | 22.5 |  |  |  |  | 67.5 |  |  | 67.5 | 56.11,77.55 | 67.50 | 56.11,77.55 |
| Trimethoprim |  |  |  |  |  | 30 | 22.5 | 1.25 |  |  |  |  | 46.25 |  |  |  | 46.25 | 35.03,57.76 | 46.25 | 35.03,57.76 |



Figure 2: Antimicrobial resistance patterns for *Salmonella* spp. (n=80) based on CLSI break points.The proportion of susceptible is shown in green and the proportion resistant in orange. Where CLSI breakpoints were not available, ECOFF breakpoints were used.

**Table 10**: AMR phenotypes of *Salmonella spp.*  (n=80) collected from Australian pigs

|  |  |  |
| --- | --- | --- |
| phenotype | n | pct |
| 0: nil | 11 | 13.75 |
| 1: bla\* | 7 | 8.75 |
| 1: tet | 7 | 8.75 |
| 2: bla\_tet | 17 | 21.25 |
| 2: fpi\_tet | 1 | 1.25 |
| 3: bla\_fpi\_phe | 8 | 10.00 |
| 3: bla\_phe\_tet | 1 | 1.25 |
| 4: bla\_fpi\_phe\_tet | 28 | 35.00 |

\*Bla- beta lactams, phe- phenicols, fpi- folate pathway inhibitors, tet-tetracycline, n- number of isolates, pct - percent of isolates

**Table 11**: Genomic MLST and serotype prediction of *Salmonella spp.*  (n=80) collected from Australian pigs

|  |  |  |  |
| --- | --- | --- | --- |
| MLST | Serotype | n | pct |
| 40 | Derby | 1 | 1.25 |
| 463 | Meleagridis | 1 | 1.25 |
| 2066 | Typhimurium | 1 | 1.25 |
| 29 | I 4:d:- | 2 | 2.5 |
| 377 | Bovismorbificans | 2 | 2.5 |
| 515 | Johannesburg | 2 | 2.5 |
| 578 | Havana | 3 | 3.75 |
| 32 | Infantis | 6 | 7.5 |
| 516 | Give | 11 | 13.75 |
| 34 | I 4,[5],12:i:- | 14 | 17.5 |
| 469 | Rissen | 37 | 46.25 |

MLST – multi-locus sequence type, n- number of isolates, pct - percent of isolates



Figure 3: Distribution of *qnrS1* gene and ciprofloxacin MIC for *Salmonella* spp. (n=80). Blue - *qnrS1* negative, red - *qnrS1* positive, broken line indicates the ECOFF breakpoint, unbroken line indicates the CLSI breakpoint

## Enterococci

*Enterococcus* spp. are considered intrinsically resistant to lincosamides and *E. faecalis* is intrinsically resistant to streptogramins, therefore *in vitro* susceptibility data for these agents should be reviewed with caution. All enterococci were clinically susceptible to ampicillin, daptomycin, linezolid, teicoplanin and vancomycin (Figures 4, 5). Isolates with phenotypes considered non-wildtype for erythromycin was prevalent amongst both *E. faecium* (67.0%) and *E. faecalis* (81.82%) in addition to non-wildtype tetracycline phenotypes (*E. faecium* 78.0%, *E. faecalis* 95.45%) (Tables 12, 14). All *E. faecium* were susceptible to chloramphenicol while the majority of *E. faecalis* were non wildtype to chloramphenicol (59.09%). A small number of *E. faecium* isolates were clinically resistant to virginiamycin (6.0%), while all *E. faecalis* isolates were sensitive (Table 14). The majority of *E. faecium* isolates were also non-wildtype to quinupristin-dalfopristin (52.0%). Non-wildtype phenotypes for streptomycin (36.36%) and gentamicin (4.55%) were also observed amongst *E. faecalis* isolates (Figure 5).

Five AMR profiles were identified for *E. faecium* with majority isolates with the same MCR profile observed (51.0%, *n*=51) (macrolides, streptogramins, tetracyclines) based on CLSI clinical breakpoints (Table 13). Five AMR profiles were also identified for *E. faecalis* based on CLSI clinical breakpoints with three MCR profiles. Only a single *E. faecalis* isolate had the MCR profile with aminoglycosides, macrolides and tetracyclines (4.54%) with the most common AMR profile being a MCR profile with macrolides, phenicols and tetracyclines (40.91%, *n*=9) (Table 15).

**Table 12**: Distribution of minimum inhibitory concentrations for *Enterococcus faecium* (n=100) isolated from Australian pigs

Percentage of isolates classified as microbiologically resistant (nw) and clinically resistant (cr) with corresponding 95% confidence intervals (c). For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Blank cells within the shaded area indicate that no isolates tested had an MIC at that concentration. Numbers outside the shaded area indicate the percent of isolates that had growth at all concentrations tested and the MIC is above the tested range. “.” Indicates the breakpoint was not available and the confidence interval was not calculated.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Concentration (mg/L) |  |  |  |  |
| Antimicrobial | 0.016 | 0.063 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | nw | nw\_ci | cr | cr\_ci |
| Ampicillin |  |  |  |  | 41 | 20 | 26 | 10 | 3 |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Chloramphenicol |  |  |  |  |  |  | 44 | 46 | 4 | 6 |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Daptomycin |  |  |  | 19 | 13 | 56 | 10 | 2 |  |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Erythromycin |  |  |  | 12 |  | 5 | 11 | 5 | 2 | 1 | 64 |  |  |  |  |  |  |  | 67 | 56.88,76.08 | 67.00 | 56.88,76.08 |
| Gentamicin |  |  |  |  |  |  |  | 99 | 1 |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | . | . |
| Lincomycin |  |  |  |  |  | 10 | 2 | 2 | 9 | 77 |  |  |  |  |  |  |  |  | . | . | . | . |
| Linezolid |  |  |  |  | 22 | 50 | 27 | 1 |  |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Quinupristin-Dalfopristin |  |  |  |  | 11 | 10 | 27 | 40 | 3 | 6 | 3 |  |  |  |  |  |  |  | 52 | 41.78,62.1 | 52.00 | 41.78,62.1 |
| Streptomycin |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 79 | 7 | 8 | 6 | . | . | 0.00 | 0,3.62 |
| Teicoplanin |  |  |  | 78 | 22 |  |  |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Tetracycline |  |  |  | 21 | 1 |  |  |  |  |  | 2 | 16 | 41 | 19 |  |  |  |  | 78 | 68.61,85.67 | 78.00 | 68.61,85.67 |
| Vancomycin |  |  |  | 54 | 40 | 3 | 3 |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,3.62 | 0.00 | 0,3.62 |
| Virginiamycin |  |  | 12 | 15 | 16 | 9 | 23 | 19 | 4 | 1 |  | 1 |  |  |  |  |  |  | 6 | 2.23,12.6 | . | . |



Figure 4: Antimicrobial resistance patterns for *Enterococcus faecium* (n=100) based on CLSI break points.The proportion of susceptible is shown in green and the proportion resistant in orange.When CLSI breakpoints were not available ECOFF breakpoints were used.

**Table 13**: MCR phenotypes of *Enterococcus faecium* (n=100) collected from Australian pigs

|  |  |  |
| --- | --- | --- |
| phenotype | n | pct |
| 0: nil | 18 | 18.0000 |
| 1: mac | 3 | 3.0000 |
| 1: tet | 15 | 15.0000 |
| 2: mac\_str | 1 | 1.0000 |
| 2: mac\_tet | 12 | 12.0000 |
| 3: mac\_str\_tet | 51 | 51.0000 |

Mac– macrolides, tet- tetracyclines, str- streptogramins

**Table 14**: Distribution of minimum inhibitory concentrations for *Enterococcus faecalis* (n=22) isolated from Australian pigs

Percentage of isolates classified as microbiologically resistant (nw) and clinically resistant (cr) with corresponding 95% confidence intervals (c). For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Blank cells within the shaded area indicate that no isolates tested had an MIC at that concentration. Numbers outside the shaded area indicate the percent of isolates that had growth at all concentrations tested and the MIC is above the tested range. “.” Indicates the breakpoint was not available and the confidence interval was not calculated.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Concentration (mg/L) |  |  |
| Antimicrobial | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | nw | nw\_ci | cr | cr\_ci |
| Ampicillin |  |  |  | 100 |  |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | 0.00 | 0,15.44 |
| Chloramphenicol |  |  |  |  |  | 27.27 | 13.64 |  |  | 59.09 |  |  |  |  |  |  | 59.09 | 36.35,79.29 | 59.09 | 36.35,79.29 |
| Daptomycin |  |  | 13.64 | 72.73 | 13.64 |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | 0.00 | 0,15.44 |
| Erythromycin |  | 9.09 |  | 9.09 |  |  |  |  | 81.82 |  |  |  |  |  |  |  | 81.82 | 59.72,94.81 | 81.82 | 59.72,94.81 |
| Gentamicin |  |  |  |  |  | 63.64 | 31.82 |  |  |  |  | 4.55 |  |  |  |  | 4.55 | .12,22.84 | . | . |
| Lincomycin |  |  |  |  |  |  | 4.55 | 95.45 |  |  |  |  |  |  |  |  | . | . | . | . |
| Linezolid |  |  |  | 72.73 | 27.27 |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | 0.00 | 0,15.44 |
| Quinupristin-Dalfopristin |  |  |  |  |  | 22.73 | 18.18 | 40.91 | 18.18 |  |  |  |  |  |  |  | 0 | 0,15.44 | . | . |
| Streptomycin |  |  |  |  |  |  |  |  |  |  |  |  | 63.64 |  | 4.55 | 7 | 36.36 | 17.2,59.34 | . | . |
| Teicoplanin |  | 81.82 | 18.18 |  |  |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | 0.00 | 0,15.44 |
| Tetracycline |  |  | 4.55 |  |  |  |  |  |  | 18.18 | 63.64 | 13.64 |  |  |  |  | 95.45 | 77.16,99.88 | 95.45 | 77.16,99.88 |
| Vancomycin |  |  | 9.09 | 22.73 | 68.18 |  |  |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | 0.00 | 0,15.44 |
| Virginiamycin |  |  |  | 9.09 | 18.18 | 50 | 22.73 |  |  |  |  |  |  |  |  |  | 0 | 0,15.44 | . | . |



Figure 5: Antimicrobial resistance patterns for *Enterococcus faecalis* (n=22) based on CLSI break points.The proportion of susceptible is shown in green and the proportion resistant in orange. Where clinical breakpoints were not available ECOFF breakpoints were used.

**Table 15**: MCR phenotypes of *Enterococcus faecalis* (n=22) collected from Australian pigs

|  |  |  |
| --- | --- | --- |
| phenotype | n | pct |
| 0: nil | 1 | 4.5455 |
| 2: ami\_tet | 3 | 13.6364 |
| 2: mac\_tet | 4 | 18.1818 |
| 3: ami\_mac\_tet | 1 | 4.5455 |
| 3: mac\_phe\_tet | 9 | 40.9091 |
| 4: ami\_mac\_phe\_tet | 4 | 18.1818 |

Ami– aminoglycosides, tet– tetracyclines, mac- macrolides, phe- phenicols

## Campylobacter coli

*Campylobacter* AST was able to be completed on 209 of 218 isolates. The majority of isolates were considered non-wildtype to one or more antimicrobials. Non-wildtype phenotypes for all tested drugs were observed (Table 16). Tetracycline (68.90%), azithromycin (64.11%), clindamycin (60.29%) and erythromycin (59.33%) were the most commonly detected non-wildtype, followed by streptomycin (27.75%), gentamicin (13.88%) and ciprofloxacin (11.0%) (Table 16). Non-wildtype phenotype for nalidixic acid (3.35%), florfenicol (3.83%) and chloramphenicol (0.48%) were also observed (Table 16). A total of 31 AMR profiles were identified amongst the isolates. There were 18 MCR phenotypes accounting for (48.80%) isolates (Table 17). The most common AMR profile was lincosamides, macrolides and tetracyclines (18.66%, *n*=39), followed by lincosamides and macrolides (14.83%, *n*=31) then tetracyclines (12.91%, *n*=27). An overview of clinical resistance is presented in Figure 6 and a comprehensive account of MIC is provided in Table 16.

All *Campylobacter* isolates were sequenced, of which, 207 passed quality control. There were 38 known STs detected, distributed across 136 isolates. The remaining 71 isolates had unknown STs. The most common STs in this collection were 854 (8.21%), 825 (6.76%) and 1016 (6.76%) (Table 18).

Beta-lactam resistance genes were detected in 102 isolates with *bla*OXA-193 most prevalent (66.67%), followed by *bla*OXA-489 (22.55%), *bla*OXA(7.84%) and *bla*OXA-578 (2.94%). Aminoglycoside resistance genes were identified in 46.38% of isolates, tetracycline resistance genes in 60.39% of isolates lincosamide resistance genes in 28.02% of isolates and macrolide resistance genes in 80.68% of isolates. The quinolone resistance inducing point mutation in the *gryA* gene (T86I) was present in 4.78% of isolates however 11.48% of isolates were found to be phenotypically resistant to ciprofloxacin.

**Table 16**: Distribution of minimum inhibitory concentrations for *Campylobacter coli* (n=209) isolated from Australian pigs

Percentage of isolates classified as microbiologically resistant (nw) and clinically resistant (cr) with corresponding 95% confidence intervals (c). For each drug, vertical bars show position of the microbiological breakpoint and shaded areas indicate the range of dilutions evaluated. Blank cells within the shaded area indicate that no isolates tested had an MIC at that concentration. Numbers outside the shaded area indicate the percent of isolates that had growth at all concentrations tested and the MIC is above the tested range. “.” Indicates the breakpoint was not available and the confidence interval was not calculated.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Concentration (mg/L) |  |  |
| Antimicrobial | 0.016 | 0.031 | 0.063 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | nw | nw\_ci | cr | cr\_ci |
| Azithromycin |  | 5.26 | 10.53 | 14.83 | 5.26 |  | 2.39 | .96 | 60.77 |  |  |  |  |  |  | 64.11 | 57.21,70.61 | . | . |
| Chloramphenicol |  |  |  |  |  |  |  | 80.86 | 13.88 | 4.31 | .48 |  | .48 |  |  | .48 | .01,2.64 | . | . |
| Ciprofloxacin | 1.44 | 2.39 | 22.49 | 29.19 | 22.49 | 11 | 3.83 | 1.44 | 1.44 | 1.91 | 2.39 |  |  |  |  | 11 | 7.11,16.05 | 5.74 | 3,9.81 |
| Clindamycin |  | 2.39 | 1.44 | 9.57 | 4.78 | 9.09 | 12.44 | 5.74 | 9.09 | 14.35 | 8.13 | 6.22 | 16.75 |  |  | 60.29 | 53.31,66.97 | . | . |
| Erythromycin |  |  | .48 |  | 1.91 | 7.18 | 13.4 | 10.05 | 4.78 | 2.87 | .96 | .48 | .96 | 7.66 | 49.28 | 59.33 | 52.34,66.05 | 58.37 | 51.37,65.13 |
| Florfenicol |  | .48 | 1.44 | .96 | 5.26 | 19.62 | 35.89 | 27.27 | 5.26 | 1.91 | .48 |  | 1.44 |  |  | 3.83 | 1.67,7.4 | . | . |
| Gentamicin |  |  |  | 4.31 | 18.66 | 36.84 | 19.62 | 6.7 | 5.26 | .96 | 1.44 | 6.22 |  |  |  | 13.88 | 9.49,19.32 | . | . |
| Nalidixic acid |  |  |  |  |  |  | 2.87 | 4.31 | 19.14 | 30.14 | 33.97 | 6.22 | 2.87 | .48 |  | 3.35 | 1.36,6.78 | . | . |
| Streptomycin |  |  |  |  |  | 5.26 | 11.96 | 33.01 | 22.01 | .96 | .96 | 25.84 |  |  |  | 27.75 | 21.8,34.35 | . | . |
| Telithromycin |  |  |  |  |  | 19.14 | 7.66 | 11.96 | 14.35 | 12.44 | 34.45 |  |  |  |  | . | . | . | . |
| Tetracycline |  |  |  | 14.35 | 4.31 | 2.39 | 5.74 | 4.31 | 1.91 | 10.05 | 10.05 | 13.88 | 11 | 22.01 |  | 68.9 | 62.15,75.11 | 56.94 | 49.93,63.75 |

Figure 6: Antimicrobial resistance patterns for *Campylobacter* *coli* (n=209) based on CLSI break points.The proportion of susceptible is shown in green and the proportion resistant in orange.Where CLSI breakpoints were not available ECOFF breakpoints were used.

**Table 17**: AMR phenotypes of *Campylobacter coli* (n=209) collected from Australian pigs

|  |  |  |
| --- | --- | --- |
| phenotype | n | pct |
| 0: nil | 29 | 13.90 |
| 1: ami | 8 | 3.83 |
| 1: mac | 2 | 0.96 |
| 1: phe | 1 | 0.48 |
| 1: tet | 27 | 12.92 |
| 2: ami\_mac | 1 | 0.48 |
| 2: ami\_tet | 7 | 3.33 |
| 2: lmc\_mac | 31 | 14.83 |
| 2: lmc\_qui | 1 | 0.48 |
| 2: mac\_tet | 1 | 0.48 |
| 3: ami\_lmc\_mac | 12 | 5.74 |
| 3: ami\_lmc\_tet | 1 | 0.48 |
| 3: ami\_mac\_qui | 1 | 0.48 |
| 3: ami\_mac\_tet | 4 | 1.91 |
| 3: lmc\_mac\_qui | 3 | 1.44 |
| 3: lmc\_mac\_tet | 41 | 19.62 |
| 3: lmc\_qui\_tet | 1 | 0.48 |
| 3: mac\_phe\_tet | 1 | 0.48 |
| 4: ami\_lmc\_mac\_qui | 1 | 0.48 |
| 4: ami\_lmc\_mac\_tet | 26 | 12.44 |
| 4: ami\_mac\_phe\_tet | 1 | 0.48 |
| 4: lmc\_mac\_phe\_tet | 1 | 0.48 |
| 4: lmc\_mac\_qui\_tet | 2 | 0.96 |
| 5: ami\_lmc\_mac\_phe\_tet | 3 | 1.44 |
| 5: ami\_lmc\_mac\_qui\_tet | 2 | 0.96 |
| 6: ami\_lmc\_mac\_phe\_qui\_tet | 1 | 0.48 |

Ami- aminoglycosides, lmc- incosamide mac- macrolides, phe- phenicols, tet-tetracycline, qui -quinolones, n-number of isolates, pct – percent of isolates

**Table 18**: Sequence types of *Campylobacter coli* (n=209) collected from Australian pigs

|  |  |  |
| --- | --- | --- |
| ST | n | pct |
| 11803 | 1 | 0.48 |
| 7900 | 1 | 0.48 |
| 7426 | 1 | 0.48 |
| 6991 | 1 | 0.48 |
| 2718 | 1 | 0.48 |
| 2710 | 1 | 0.48 |
| 1837 | 1 | 0.48 |
| 1446 | 1 | 0.48 |
| 1439 | 1 | 0.48 |
| 1426 | 1 | 0.48 |
| 1173 | 1 | 0.48 |
| 1108 | 1 | 0.48 |
| 1104 | 1 | 0.48 |
| 1056 | 1 | 0.48 |
| 902 | 1 | 0.48 |
| 9566 | 2 | 0.97 |
| 8613 | 2 | 0.97 |
| 5372 | 2 | 0.97 |
| 5305 | 2 | 0.97 |
| 2711 | 2 | 0.97 |
| 1595 | 2 | 0.97 |
| 1464 | 2 | 0.97 |
| 1450 | 2 | 0.97 |
| 1438 | 2 | 0.97 |
| 1145 | 2 | 0.97 |
| 1113 | 2 | 0.97 |
| 1563 | 3 | 1.45 |
| 830 | 3 | 1.45 |
| 1100 | 4 | 1.93 |
| 1463 | 5 | 2.42 |
| 1177 | 5 | 2.42 |
| 828 | 7 | 3.38 |
| 2733 | 9 | 4.35 |
| 1445 | 9 | 4.35 |
| 1055 | 9 | 4.35 |
| 1016 | 14 | 6.76 |
| 825 | 14 | 6.76 |
| 854 | 17 | 8.21 |
| - | 71 | 34.30 |

ST- multilocus sequence type, n-number of isolates, pct – percent of isolates

# Discussion

This study collected a total of 300 samples from 30 farms (10 samples per farm). The key indicator organisms and potential zoonotic pathogens, *E. coli* (2730), *Salmonella* spp*.* (81), *Enterococcus* spp.(124) and *Campylobacter* spp.(218), were collected from faecal samples at slaughter. Isolation of each species was similar to the previous Australian study (2015) for *Salmonella* spp. (2015: 84/310) and enterococci (*E. faecium* 2015:84/310, *E. faecalis* 2015:17/310) and slightly higher for C*ampylobacter* spp. (*C. coli* 2015: 157/310) samples. The higher rate of isolation of *Campylobacter* in this study could be due to changes in the isolation protocol and the use of brilliance campy agar as well as MCCD agar.

One of the major advancements in this study is the significant increase in the number of *E. coli* isolated due to the use of RASP, an advanced robotics system capable of selection of isolates and subsequent susceptibility testing. Despite the 13-fold increase in isolates tested, the overall resistance profiles remained similar (comparison made with clinical breakpoints as were used in the 2015 study). Resistance to chloramphenicol remained the similar (2015 42.0%, 2022 44.13%), as did tetracycline resistance (2015 75.0%, 2022 78.6%). Resistance to gentamicin and ampicillin increased slightly from 0.5% to 1.93% and 61.5% to 76.02% respectively. Very little resistance to cephams was observed in either study 0.5 % (n=1) resistant to cefoxitin in 2015 and 0.4% (n=1) resistant to cefotaxime in 2022, however no known resistance gene was identified in this isolate. A small number of isolates were microbiologically resistant to the carbapenem, meropenem. This is likely due to the phenomenon known as MIC drift, as the MIC is just above the ECOFF breakpoint for meropenem and no known resistance genes or mutations were identified in the subset sequenced.

The recovery of *Salmonella* spp. was similar to the previous study. The prevalence of ampicillin non-wildtype has increased slightly since the last study from 61.9% to 76.5%. An increase in chloramphenicol non-wildtype (17.9% increased to 45.68%), as well as florfenicol non-wildtype (8.3% increased to 40.74%) was also observed in this study compared to the last (17.9% increased to 45.68%). Tetracycline resistance decreased slightly compared to the last study (77.4% down to 66.67%). A small number of isolates were microbiologically resistant to the carbapenem, meropenem. This is likely due to the phenomenon known as MIC drift, as the MIC is just above the ECOFF breakpoint for meropenem and no known carbapenem resistance genes were identified in sequencing.

The utilization of genome sequencing of *Salmonella* isolates has facilitated the revelation of critical strain-level insights, encompassing serotype classifications and the presence of antimicrobial resistance genes within *Salmonella* isolates obtained from porcine sources in Australia. The dominant serovar detected in this current study was Rissen (*n* = 37, 46.25%) followed by monophasic variant I 4,[5],12:i:- (17.5%), Give (13.7%) and Infantis (7.5%). Interestingly, commonly reported broad host range *Salmonella* serotype Typhimurium was rare (n=1; 1.25%). The European Union One Health 2021 Zoonoses Report from European Centre for Disease Prevention and Control (ECDC) showed serovar Derby and monophasic Typhimurium as the dominant serovars in pigs (https://www.ecdc.europa.eu/en/publications-data/european-union-one-health-2021-zoonoses-report).

No resistance was observed towards ampicillin or daptomycin for *E. faecium* compared to 34.5% and 16.7% presenting as non-wildtype in the last study. Isolates with a non-wild phenotype towards erythromycin decreased in this study (67%) compared to the previous study (90.5%). There was a decrease in isolates non-wildtype to tetracycline (2022 78%, 2015 91.7%) and virginiamycin (current study 6%, previous study 28.6%). There was also a decrease in clinically resistant quinuprisitn-dalfopristin isolates (52%) compared to the previous study (94.0%). Clinical resistance to erythromycin remained the similar compared to the previous study (81.82% vs. 82.4%) while tetracycline resistance was slightly higher (95.45 vs 82.4%).

Amongst *Campylobacter coli* isolates non-wildtype to azithromycin (2022 64.45%, 2015 75.2%), clindamycin (2022 60.66%, 2015 75.2%) remained similar to the previous study as did the proportion of isolates clinically resistant to tetracycline (2022 57.35% 2015 53.5%). The proportion of isolates clinically resistant to erythromycin decreased in the current study (58.77%) compared to the previous study (73.2%). No non-wildtype or isolates clinically resistant to ciprofloxacin were identified in the previous study however 5.74% of isolates in the current study were resistant to ciprofloxacin even though the breakpoint for resistance has increased from 0.5 mg/L to 2 mg/L since the last study. An increase in prevalence of non-wildtype to nalidixic acid (2022 3.35%, 2015 1.9%), florfenicol (2022 3.79%, 2015 0%) and gentamicin (2022 13.88%, 2015 0.6%). There was a disparity between the phenotypic resistance of ciprofloxacin (5.74%) and nalidixic acid (3.35%) and the detection of known mutations or genes that confer quinolone resistance (4.78%). However, all isolates with ciprofloxacin resistance had an MIC greater than the ECOFF breakpoint or one-fold below the ECOFF breakpoint.

Genomic characterisation of *Campylobacter coli* in this study showed ST854 as the dominant sequence type (ST), and this ST belonged to the international clonal complex (CC) 828 (also known as ST828 complex). While PubMLST database showed that clonal complex ST21 (*n* = 1407, 21.2%) was predominant in Oceania, international data showed ST828 complex as the dominant complex in general (*n* = 27995, 21.3%). It is important to highlight that comprehensive data regarding the genomic attributes and sequence variants of *Campylobacter* originating from swine worldwide remains restricted within national surveillance publications. This is primarily due to the absence of regular implementation of whole genome sequencing in the context of national antimicrobial resistance surveys focused on livestock and food commodities.

Despite the presence of modest to moderate resistance to routinely used first-line antimicrobials among various bacterial species, several noteworthy and encouraging findings arise from this study. Firstly, *E. coli* displayed a notable lack of resistance towards critically important antimicrobial such as colistin, meropenem, and amikacin and extremely low levels of resistance to extended spectrum cephalosporin resistance (cefotaxime) (0.04%, n=1) and ciprofloxacin (0.11%, n=3). Additionally, absence of resistance to critically important antimicrobials such as amikacin, azithromycin, ciprofloxacin, colistin, cefotaxime, gentamicin, and meropenem among *Salmonella* is an encouraging trend. Another promising outcome involves the absence of resistance against ampicillin, daptomycin, linezolid, teicoplanin, and vancomycin among enterococci. Finally, the detection of ciprofloxacin resistance (a type of fluoroquinolone) within *Campylobacter coli* was unusual considering the absence of fluoroquinolone use in the Australian pig industry. These cumulative observations provide valuable insights into the antimicrobial resistance landscape and suggest certain optimistic trends, while also highlighting exceptions that warrant further investigation.

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