**High throughput robotics for the management and control of Antimicrobial resistance and endemic diseases in pigs**

**Final Report**

**APL Project 2021/0039**

**June 2025**

**Research Organisation**

Sam Abraham

Murdoch University

90 South Street Murdoch

Western Australia

6150

# Acknowledgements

This project is supported by funding from Australian Pork Limited, industry levy payers and the Commonwealth. We also acknowledge and appreciate the support of veterinarians and farms in collecting samples used in this project.

# Executive Summary

This project aimed to establish a high-throughput, fully automated robotic system to monitor and control antimicrobial resistance (AMR) and endemic bacterial diseases in Australian pigs. Over a three-year period, the project integrated robotic platforms with conventional microbiological and genomic workflows to address national surveillance gaps and support antimicrobial stewardship.

Using the Robotic Antimicrobial Susceptibility Platform (RASP), over 2,700 Escherichia coli isolates were recovered from rectal samples collected at abattoirs across 30 pig herds nationwide, representing approximately 70% of national pig production, were tested. A total of 77% of isolates were resistant to ampicillin and 79% to tetracycline. Multiclass resistance (MCR) was observed in 56.9% of isolates, with less than 1% showing resistance to critically important antimicrobials (CIAs). An AMR index was developed for each herd, facilitating benchmarking and targeted interventions.

Pathogen-specific robotic workflows were developed and validated for Staphylococcus hyicus, Pasteurella multocida, Campylobacter coli, and Actinobacillus pleuropneumoniae (APP). These enabled streamlined testing and genomic analysis. A clonal outbreak of S. hyicus associated with greasy pig disease was investigated and confirmed using WGS, with 89% of sequenced isolates being genetically identical. The outbreak demonstrated the utility of robotic sampling for vaccine target strain identification and outbreak management.

A national Campylobacter coli study identified a 72.7% prevalence in pigs. High resistance to macrolides (64.1%), clindamycin (60.3%), and tetracycline (56.9%) were observed. Notably, 11.5% of isolates were resistant to ciprofloxacin despite the absence of fluoroquinolone use in Australian pigs. Genomic analysis revealed 38 sequence types (STs), mostly within the 828 clonal complex. MCR was detected in nearly half of the isolates, underscoring the need for ongoing surveillance and stewardship.

A genomic survey of 252 P. multocida isolates collected between 2014 and 2019 showed very low rates of resistance to CIAs, with 75.5% carrying no resistance genes. Tetracycline resistance was most common (23.3%). Widespread STs (e.g., ST9, ST124) were found across multiple regions. Virulence-associated genes such as ptfA and fimA were frequently detected.

Effluent testing was piloted on 26 farms to evaluate its utility for AMR surveillance. Effluent samples showed similar AMR patterns to individual animal samples, particularly for ampicillin, tetracycline, and ciprofloxacin-resistant E. coli. However, gentamicin-resistant isolates detected in faeces were not found in effluent. While promising, further optimization is required to improve the sensitivity of this surveillance method.

Robotic DNA extraction and library preparation workflows were established and validated for multiple porcine pathogens using the KingFisher Flex and Tecan platforms. Downstream genome analysis used the custom-built pipeline using a combination of freely available modules and custom scripts to generate species ID, AMR gene profiles, MLST, serotypes and phylogenies. Genome quality metrics were consistently high, with mean coverage >100x across species and average N50 values indicating robust assemblies. Development of an MLST scheme APP is currently being established with experts from Denmark to type the APP studied in this project.

This project has enabled the transition from semi-automated to fully automated AMR surveillance systems. The integration of robotics with high-resolution genomic tools has significantly improved diagnostic efficiency and scale, while reducing cost and labour. The outputs of this project directly support national surveillance objectives, current surveillance projects and provide tools for outbreak response, biosecurity, and the rational use of antimicrobials.

Table of Contents

Table of Contents

[Acknowledgements 2](#_Toc200709598)

[Executive Summary 3](#_Toc200709599)

[2. Background to Research 7](#_Toc200709600)

[**1.1.1** **Opportunities Arising** 7](#_Toc200709601)

[**2.1.1** **Industry Benefits and Implications** 8](#_Toc200709602)

[3. Objectives of the Research Project 9](#_Toc200709603)

[4. Introductory Technical Information 12](#_Toc200709604)

[5. Research Methodology 13](#_Toc200709605)

[5.1Robotic programming 13](#_Toc200709606)

[4.2. Sample Collection and Farm Demographics 13](#_Toc200709611)

[4.3. Bacterial Isolation and Culture Methods 13](#_Toc200709612)

[4.4. Antimicrobial Susceptibility Testing (AST) 13](#_Toc200709613)

[4.5. DNA Extraction and Whole Genome Sequencing (WGS) 14](#_Toc200709614)

[4.6 Statistical Analysis 14](#_Toc200709615)

[5 Results 14](#_Toc200709616)

[10. Discussion 16](#_Toc200709617)

[11. Implications & Recommendations 18](#_Toc200709618)

[12. Intellectual Property 19](#_Toc200709619)

[13. Technical Summary 19](#_Toc200709620)

[14. Literature cited 19](#_Toc200709621)

[15. Publications Arising 21](#_Toc200709622)

List of Tables

Table 1. The Susceptibility profile of APP isolates using the automated RASP. 10

Table 2: Average statistics of the sequenced species from pigs. 12

List of Figures

Figure 1: Serotypes of APP from historic cases in Australia. 16

# Background to Research

Antimicrobial resistance (AMR) continues to pose a significant threat to both public and animal health. In the livestock industry, where antimicrobials are vital for animal welfare and productivity, the emergence and spread of resistance can undermine treatment efficacy and lead to wider public health consequences. The Australian pig industry, while demonstrating low levels of resistance to critically important antimicrobials (CIAs), must maintain robust, high-resolution surveillance to safeguard animal health, uphold international trade standards, and support One Health objectives.

Historically, AMR surveillance in pigs has been hampered by labour-intensive processes and small sample sizes, limiting the scope and frequency of testing. The need for a scalable, cost-effective system capable of generating herd-level, nationally representative AMR data was clear. This project addressed that gap through the development and deployment of the Robotic Antimicrobial Susceptibility Platform (RASP)—a fully automated, high-throughput testing platform coupled with genomic analysis.

The initial integration of the RASP system presented significant technical challenges. Combining robotic modules—such as automated spiral plating, isolate selection, pipetting, incubation, and absorbance-based inoculum standardisation—within a biosafety cabinet and synchronising them with sequencing workflows proved complex. Issues with robotic programming, fluidics calibration, and computer system compatibility initially limited system performance. However, through collaboration with manufacturers (Tecan) and iterative troubleshooting, these issues were successfully resolved, resulting in a fully operational and validated platform capable of processing thousands of isolates with minimal hands-on time.

Using this system, over 2,700 *Escherichia coli* isolates from 30 farms—representing approximately 70% of national pig production—were tested in this study, a tenfold increase in sample throughput compared to previous national surveys **(Appendix A, B, C).** Whole genome sequencing (WGS) was also performed on isolates of *Pasteurella multocida* **(Appendix D),** *Staphylococcus hyicus* **(Appendix E)**, *Campylobacter coli* **(Appendix C)**, and *Actinobacillus pleuropneumoniae*, enabling comprehensive resistance and virulence profiling.

In addition, a pilot investigation into effluent-based AMR testing was undertaken to assess its feasibility for non-invasive, scalable surveillance. Preliminary results showed good concordance with individual animal sampling for key resistance phenotypes, highlighting its potential as an early warning tool pending further optimisation.

1. **Opportunities Arising**

The successful implementation of the RASP system and the demonstrated value of high-throughput AMR surveillance have stimulated significant national interest beyond the pig sector. Key opportunities include:

* National AMR surveillance initiatives in the dairy, egg, and meat chicken industries, building on the infrastructure and methods developed in this project.
* Ongoing AMR surveillance in pigs, with potential for routine integration into industry-wide health monitoring and benchmarking programs.
* Rapid response capability for outbreak investigations and support for autogenous vaccine development, as demonstrated in the greasy pig disease case study.
* Policy development and risk assessment frameworks, informed by real-time, herd-level AMR indices generated by the platform.
* A foundation for future diagnostic and genomic integration, expanding pathogen coverage and streamlining health intelligence in the livestock sector.
1. **Industry Benefits and Implications**

This project has laid the groundwork for a next-generation AMR surveillance system in animal production. Quantified benefits to industry include:

* Reduced cost per isolate
* Increased accuracy and standardisation
* Enhanced biosecurity through early detection
* Evidence-based stewardship and treatment planning
* Support for national antimicrobial resistance strategies

Beyond pigs, this project has catalysed momentum for cross-sector surveillance and represents a transformative shift in how resistance is monitored and managed in Australian agriculture.

# Objectives of the Research Project

1. ***Integration and optimization of current robotics assays (RASP) into a fully automated system for increasing throughput, accuracy and cost effectiveness.***

*Development of a system with the capacity for complete automation from isolation to phenotypic susceptibility testing.* As mentioned in the grant application, a robotic platform contained in a biosafety cabinet class II was acquired. This robot was designed to be multifunctional with an integrated spiral plater (for inoculating agar plates), light box and camera (to pick isolates of interest from the agar) absorbance reader (bacterial density adjustment), 96- pipettor head (prepare entire antimicrobial test plates efficiently) and an incubator with temperature and gas control. Integration of the 96 pipettor head and incubator were the key components to realise a completely automated system. The new platform has been installed and is operational. There were several setbacks in this process as this is the first robot of its type to be integrated in this configuration. All the initial issues have been rectified, with only an intermittent gripper arm issue, currently being fixed by Tecan (manufacturer of the robotic platform). The intermittent issue is manageable allowing the RASP process to be fully automated for Antimicrobial Susceptibility Testing.

A proof of concept script was developed and tested. The script has now been refined by a Tecan systems architect to ensure future availability.

***Sourcing of susceptibility testing plates****.* Antimicrobial susceptibility testing plates are prepared in house to improve cost efficiency, to allow extreme flexibility in antimicrobials included and to ensure availability when required. A program has been developed to prepare stock antimicrobials for 1000 – 3000 antimicrobial testing plates. These stocks are stored in 100-200 plate batches and require 45 minutes of preparation/100 plates. These protocols has been used for preparing antimicrobial susceptibility test plates for various studies **(Appendix A-E)**

1. ***Develop and optimize current robotic and integrated genome sequencing assay for isolating, identifying, AMR testing and genomic strain characterisation of key veterinary pathogens important for Australian livestock sector such as Pasteurella.***

***Optimisation of the platform for porcine endemic pathogens:***

*Pasturella multocida -* the susceptibility testing protocol has been validated using historically collected isolates of *P. multocida*. This work has been published (**(Appendix D)** <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9927299/>.

*Staphylococcus hyacis* validation ofisolation of *S. hyacis* using samples from a suspected outbreak followed by phenotypic and genotypic analysis using the RASP platforms has been completed. This work has been published **(Appendix E)** <https://www.sciencedirect.com/science/article/pii/S2772283X23029187?dgcid=rss_sd_all>

*Actinobacillus pleuropneumoniae* has been optimised on the platform and the results are presented in Table 1. (Manuscript under development)

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Antimicrobial | n | 0.016 | 0.031 | 0.063 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | Resistant (%) | 95% CI |
| Apramycin | 85 |  |  |  |  |  |  |  | 3.5 | 8.2 | 20 | 32.9 | 10.6 | 24.7 |  |  | . | . |
| Ceftiofur | 85 |  |  |  |  | 88.2 | 10.6 |  |  |  |  |  | 1.2 |  |  |  | 1.2 | 0,6.4 |
| Chlortetracycline | 85 |  |  |  |  |  |  |  |  | 2.4 | 97.6 |  |  |  |  |  | . | . |
| Enrofloxacin | 85 |  | 97.6 |  | 1.2 |  | 1.2 |  |  |  |  |  |  |  |  |  | 0.0 | 0,4.2 |
| Erythromycin | 85 |  |  |  |  |  |  | 10.6 | 45.9 | 35.3 | 7.1 |  | 1.2 |  |  |  | . | . |
| Florfenicol | 85 |  |  |  |  | 95.3 | 3.5 |  |  | 1.2 |  |  |  |  |  |  | 0.0 | 0,4.2 |
| Lincomycin | 85 |  |  |  |  |  |  |  |  | 3.5 | 17.6 | 69.4 | 9.4 |  |  |  | . | . |
| Neomycin | 85 |  |  |  |  |  |  |  | 2.4 |  | 5.9 | 48.2 | 37.6 | 5.9 |  |  | . | . |
| Olaquindox | 85 |  |  |  |  |  |  |  |  | 40 | 48.2 | 8.2 |  | 2.4 |  | 1.2 | . | . |
| Spectinomycin | 85 |  |  |  |  |  |  |  |  | 2.4 | 3.5 | 3.5 | 60 | 30.6 |  |  | . | . |
| Tetracycline | 85 |  |  |  |  | 5.9 | 32.9 | 9.4 | 4.7 | 1.2 | 45.9 |  |  |  |  |  | 51.8 | 40.7,62.7 |
| Tilmicosin | 85 |  |  |  |  |  |  |  |  | 58.8 | 32.9 | 5.9 | 2.4 |  |  |  | 2.4 | .3,8.2 |
| Trimethoprim/sulfamethoxazole | 85 |  |  |  |  |  | 56.5 | 22.4 | 21.2 |  |  |  |  |  |  |  | . | . |
| Tulathromycin | 85 |  |  |  |  |  |  |  |  |  | 76.5 | 17.6 | 1.2 |  | 4.7 |  | 4.7 | 1.3,11.6 |

Table 1. The Susceptibility profile of APP isolates using the automated RASP.

The ability to extract DNA for sequencing is a crucial step in the analysis of genomic data. Different extraction kits result in variations in yield and quality of DNA, with some species easier to extract DNA from than others. We have optimised high-throughput DNA extraction using the kingfisher automated extraction system (Thermo Fisher Scientific) of pig endemic pathogens including *S. suis, S. hyacis, Brachyspira* spp., *P. multocida* and *A. pleuropnemoniae.*

The robotic platform was programmed to complete whole genome sequencing library prep using Tecan’s Celero chemistry. A specific pipeline for analysis was developed which is described in detail below.

After genome sequencing using Illumina platforms, such as NextSeq and NovaSeq, the genome analysis follows an inhouse pipeline. Sequences undergo QC and cleaning using FastP, genome assembly using Spades, species identification with Kraken, multilocus sequence typing with mlst and virulence and resistance genes and point mutations from abricate or AMRFinder+ (species specific). Serotype is determined from widely accepted programs such as SeqSero (*Salmonella* spp.) and ECTyper (*E. coli*) or from custom inhouse scripts (APP, *Brachyspira* spp.).

The laboratory workflow and analysis pipeline was validated with *E. coli* isolates before endemic pig pathogens, *Actinobacillus pleuropneumoniae*, *Brachyspira* spp., *Pasteurella multocida* and *Streptococcus suis*, were sequenced and analysed with the pipeline. Since genome sequencing carried out in AMRID involved third-generation sequencing, the Q30 threshold was used to ensure 99.9% accuracy in each base call. Hence, Q30 of the sequenced samples were set at 83%. Coverage of the genomes were set at minimum 30x before the genomes were deemed passed QC.

On average, genome coverage of the sequenced pathogens (i.e., *A. pleuropneumoniae*, Brachyspira spp, *P. multocida* and *S. suis*) were more than 100x. Table 2 shows the average genome coverage and quality of sequences in each species. After genome assembly, quality of draft genomes was ensured through the N50 value. The quality of assembled genomes was kept at 30,000 bp (on average) for N50 to ensure good contiguity of an assembly.

Table 2: Average statistics of the sequenced species from pigs.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Species**   | **Q30 (%, Before)**   | **Q30 (%, after)**   | **Coverage (x)**   | **N50 (bp)**   | **Contig number**   | **GC content (%)**   |
| *Actinobacillus pleuropneumoniae*   | 87.0  | 89.1  | 160.3  | 54,928   | 93   | 41.1   |
| Brachyspira spp.   | 85.8  | 87.7  | 99.0   | 174,427   | 204   | 27.5  |
| *Pasteurella multocida*   | 87.0  | 89.6  | 151.1  | 281,444   | 65   | 40.3  |
| *Streptococcus suis*   | 83.7   | 85.8   | 136.7  | 33,419   | 164   | 41.2   |

1. ***Develop and perform robotic assays for effluent AMR testing which could enhance AMR surveillance enabling early detection and ease of sampling.***

Effluent was collected from 3 pig farms as an initial trial for testing to determine appropriate dilutions to use and feasibility of the sample. The effluent samples were weighed and inoculated onto *E. coli* selective agar with or without infused antimicrobials. Of the antimicrobials targeted, ampicillin, gentamicin, ciprofloxacin, tetracycline and extended spectrum beta-lactamases, isolates resistant to ampicillin, tetracycline and ciprofloxacin were detected on all farms. Faecal samples collected from individual animals and processed in the same fashion were compared to the effluent samples. There were animals from all farms that were positive for ampicillin, tetracycline and ciprofloxacin resistant *E. coli* which was also detected in the effluent samples. However, gentamicin resistant *E. coli* were isolated from individual faecal samples but not from the effluent sample. This protocol is currently being used in other surveillance projects providing ongoing data.

1. ***National and Farm specific AMR report based on RASP protocols for E. coli (required for DAWE funding agreement).***

A national study involving 30 Farms was conducted and finalised in 2022 (report in **Appendix A.).** This study characterised up to 96 *E. coli* isolates per farm with each farm receiving personalised reports of their antimicrobial resistance profile and their relationships to other farms involved in the study.

1. ***Perform full genomic characterisation of critically important antimicrobial resistant E. coli clones (FQ-resistant/ ceftiofur resistant) from pigs to map origin, transmission and co-selection potential.***

As part of the national study in objective 4., any *E. coli* isolates with phenotypic resistance to critically important antimicrobials were sequenced **(Appendix A).**

1. ***Perform National AMR survey using optimized RASP protocols for E. coli from samples acquired from abattoirs across the country as a continuation of the benchmarked 30 farms reported in 2021 (required for DAWE funding agreement).***

As reported in 4 and 7, study has been complete and is reported in **Appendix A.**

1. ***Repeat AMR survey conducted in 2015 for E. coli, Enterococcus faecium / faecalis, Campylobacter spp. and Salmonella (E. coli data will be derived from RASP (required for DAWE funding agreement).***

An AMR survey was completed in 2022 with *E. coli, Enterococci* spp., *Campylobacter* spp. and *Salmonella* spp. isolates collected and tested. The report is attached in **Appendix A.**

# Introductory Technical Information

The emergence and dissemination of antimicrobial resistance (AMR) is a global concern affecting both human and veterinary medicine. In food-producing animals, including pigs, AMR compromises animal health, reduces the efficacy of routine treatments, and presents potential public health risks via zoonotic transmission, contaminated food products, or environmental dissemination of resistant organisms and genes. Accordingly, robust AMR surveillance in livestock is essential for guiding responsible antimicrobial use and informing national and international AMR mitigation strategies.

In Australia, foundational AMR surveillance studies in pigs were first undertaken as part of a **2015 pilot study** funded by the Commonwealth Government and Australian Pork Limited (APL). This study established baseline data for Escherichia coli, Salmonella spp., Enterococcus spp., and Campylobacter spp. collected at slaughter **(Appendix A, B, C)**. Results were encouraging—showing minimal to no resistance to critically important antimicrobials (CIAs) such as fluoroquinolones, cephalosporins, and colistin. However, the study also highlighted substantial resistance to first-line antimicrobials such as tetracycline and ampicillin.

A follow-up **2020 survey** refined the approach by increasing the number of E. coli isolates tested per herd and introducing a novel **AMR Index**—a weighted scoring system that reflects the clinical importance of each resistance phenotype. This allowed for comparison between farms and supported enterprise-level benchmarking for stewardship programs. The survey also pioneered the use of pooled faecal sampling at abattoirs to reduce cost while maintaining representative sampling.

Despite these advances, AMR surveillance remained limited by labour-intensive methods, modest throughput, and a reliance on phenotypic testing alone. Sample processing, culturing, and susceptibility testing required substantial manual input, creating bottlenecks in data generation and interpretation. Genomic characterisation of resistant isolates—although informative—was ad hoc and disconnected from routine testing pipelines.

To overcome these constraints, preliminary work by Murdoch University’s Antimicrobial Resistance and Infectious Diseases (AMRID) research group developed a semi-automated **Robotic Antimicrobial Susceptibility Platform (RASP)**. This prototype incorporated robotic plating, colony selection, MIC plate preparation, and automated data capture, demonstrating a significant increase in throughput and data consistency. However, the system lacked full integration and required manual transitions between workflow steps.

The current project builds upon this foundation by creating a **fully integrated robotic workflow**, from sample dilution and plating to antimicrobial susceptibility testing, storage, DNA extraction, and whole genome sequencing. It also expands the pathogen scope to include key endemic bacteria (Pasteurella multocida, Staphylococcus hyicus, Actinobacillus pleuropneumoniae, Campylobacter coli) and introduces novel sampling modalities such as **effluent-based surveillance** **(Appendix C-E).**

This project is also informed by international best practices, including models such as **DANMAP** (Denmark), **CIPARS** (Canada), and **NARMS** (USA), which routinely monitor AMR trends in livestock and integrate genomic epidemiology. In line with the Australian Government’s **National AMR Strategy – 2020 & Beyond**, this research positions the pig industry to contribute real-time, high-resolution AMR data that is both industry-relevant and publicly accountable.

Overall, this project represents a technical evolution from point-in-time surveillance to continuous, scalable, and genomically informed AMR monitoring. It enables a data-driven response to resistance threats and provides a model for broader application across Australian livestock industries.

# Research Methodology

## Robotic programming

The robot was programmed using fluent control software and scripts optimised over the course of the project to accommodate each species and process. A final version of the protocol was developed after testing which has been refined by Tecan Professionals to ensure adaptability in the future.

#

#

#

#

## 4.2. Sample Collection and Farm Demographics

Between July and November 2022, biological samples were collected from 30 commercial pig farms across five Australian states: Western Australia, South Australia, New South Wales, Victoria, and Queensland. Each farm contributed rectal samples from 10 finisher pigs (approximately 16–22 weeks of age, 80–110 kg body weight) collected post-evisceration at abattoirs using sterile instruments. Samples were pooled into two sets of five per farm and transported to the laboratory under refrigeration (2–8 °C), where they were processed within 48 hours of collection.

In addition to rectal sampling, effluent was collected from 26 participating farms to explore the feasibility of non-invasive antimicrobial resistance (AMR) surveillance. Effluent samples were collected in sterile containers and plated directly onto selective media to assess concordance with rectal isolate resistance profiles.

## 4.3. Bacterial Isolation and Culture Methods

Isolation of *Escherichia coli* was performed using the Robotic Antimicrobial Susceptibility Platform (RASP), which automated sample dilution and spiral plating onto CHROMagar™ ECC. Plates were incubated at 37 °C for 16–20 hours. Presumptive *E. coli* colonies were selected using robotic imaging and colony-picking, with up to 96 isolates recovered per farm.

For *Staphylococcus hyicus*, lesion and skin swab samples from pigs affected by greasy pig disease were plated on mannitol salt agar and incubated aerobically. Colonies were confirmed as *S. hyicus* using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

*Campylobacter coli* was isolated using selective enrichment in Preston broth followed by plating on modified charcoal-cefoperazone-deoxycholate agar (MCCD) and Brilliance™ CampyCount agar. Plates were incubated at 42 °C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) for 24–48 hours.

Archived isolates of *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, and *Streptococcus suis* from diagnostic cases were included for validation of robotic workflows and sequencing protocols.

## 4.4. Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility testing was performed via broth microdilution using in house prepared plates. The antimicrobial panel included agents relevant to both animal and human health, such as ampicillin, tetracycline, ciprofloxacin, gentamicin, cefotaxime, florfenicol, sulfamethoxazole, trimethoprim, meropenem, and colistin. MICs were recorded using the Sensititre™ Vizion™ digital reader and a custom-built software.

Interpretation of resistance was based on Clinical and Laboratory Standards Institute (CLSI VET08) veterinary breakpoints where available, and European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-offs (ECOFFs) for surveillance purposes. Quality control strains (*E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Campylobacter jejuni* ATCC 33560) were included in each test batch to ensure assay integrity.

Effluent samples were assessed by direct plating onto CHROMagar ECC with and without antimicrobial supplementation to detect resistance phenotypes. Colony counts and resistance profiles were compared with rectal isolate data to evaluate concordance.

## 4.5. DNA Extraction and Whole Genome Sequencing (WGS)

DNA was extracted from pure cultures using the KingFisher Flex system and the MagMAX™ multi-sample extraction kit. DNA quantification was performed using fluorometric methods, and sequencing libraries were prepared using the Tecan Celero™ DNA-Seq kit. Whole genome sequencing was performed on the Illumina NextSeq platform using 2 × 150 bp paired-end chemistry with high output flow cells.

Sequencing data were processed using a custom-built pipeline. Raw reads were quality filtered (Q30 threshold), trimmed to remove adapters, and assembled using SPAdes. Species identification was performed using Kraken. Genomes were screened for resistance genes using AMRFinderPlus and ResFinder, and for virulence-associated genes using the Virulence Factor Database (VFDB). Multilocus sequence typing (MLST) was carried out using the mlst tool.

Only high-quality genomes with a minimum 30× average read depth, >83% Q30 scores, and an N50 value exceeding 30,000 base pairs were included in downstream analysis.

## 4.6 Statistical Analysis

Descriptive statistics were used to summarise MIC distributions and resistance prevalence for each pathogen and antimicrobial. Resistance proportions were presented with 95% confidence intervals, calculated using the exact binomial (Clopper–Pearson) method. Multiclass resistance (MCR) was defined as resistance to three or more classes of antimicrobials.

An AMR Index was developed for each isolate based on a weighted score of detected resistance phenotypes, with higher scores reflecting greater concern due to antimicrobial importance. Herd-level indices were calculated by averaging individual scores, allowing for farm-to-farm comparison and benchmarking.

All statistical analyses were conducted using Stata 15.1 (StataCorp, College Station, Texas).

# 5 Results

Results are presented in the attached documents for the RASP *E. coli Salmonella* spp., *Enterococci* spp. And *Campylobacter* spp. Surveillance (**Appendix A,B,C**).

Results are presented in the attached documents for and *P. multocida* **(Appendix D)** and *S. hyacis* (**Appendix E**)

***Results for APP.***

AST for APP was adapted to the fully automated RASP platform using a representative sample of 96 isolates. Adaptations included incubation conditions, broth, antimicrobials and bacterial dilutions. Susceptibility testing results are presented in Table 1. The serotype of sequenced APP isolates is presented in Figure 1. There were 5 serotypes identified within the collection, S1, S12, S15, S5b and S7. Two isolates could not be serotyped with the current system. These isolates are currently being used as part of a larger dataset to create an MLST scheme.



Figure 1: Serotypes of APP from historic cases in Australia.

# Discussion

This project successfully met its objective of developing and validating a fully automated, high-throughput antimicrobial resistance (AMR) surveillance platform for the Australian pig industry. The implementation of the Robotic Antimicrobial Susceptibility Platform (RASP) enabled testing of over 2,700 Escherichia coli isolates—representing the largest and most geographically comprehensive AMR dataset in Australian pigs to date. The scale of sampling, combined with the integration of genomics, has yielded novel insights into resistance patterns, pathogen diversity, and the opportunities for data-driven interventions.

The E. coli surveillance data confirmed widespread resistance to first-line antimicrobials, particularly ampicillin and tetracycline, consistent with findings from previous national studies. Importantly, resistance to critically important antimicrobials (CIAs) such as cephalosporins and fluoroquinolones remained low, reaffirming the effectiveness of current regulatory restrictions and stewardship practices in Australian pig production. These results support earlier observations reported in Abraham et al. (2017) and Truswell et al. (2021), which emphasised the rarity of CIA resistance in Australian livestock. The development of an AMR index further advanced this field by allowing farm-level benchmarking and identifying herds with elevated risk profiles, providing a practical tool for veterinarians and producers.

The investigation into an outbreak of greasy pig disease due to Staphylococcus hyicus demonstrated the platform’s ability to support outbreak response and autogenous vaccine development **(Appendix E)**. The detection of a dominant clonal lineage among 89% of sequenced isolates, alongside high rates of resistance to macrolides and tetracyclines, underscored the value of multi-isolate sampling in capturing the genetic homogeneity and resistance profile of outbreak strains. This approach addressed a key limitation of previous diagnostic practices, where reliance on a single isolate often fails to reflect true diversity. The findings align with recent literature emphasising the role of targeted genomics in vaccine design for endemic swine pathogens.

The genomic characterisation of Pasteurella multocida isolates collected between 2014 and 2019 revealed low overall resistance, with tetracycline being the most common resistance phenotype **(Appendix D)**. The majority of isolates carried no detectable resistance genes. Notably, dominant sequence types such as ST9 and ST124 were identified across multiple farms and regions, suggesting broad dissemination of clonal lineages. These findings are consistent with prior international reports indicating a relatively stable resistance profile for P. multocida in swine, but also highlight the need for routine surveillance to monitor for emerging resistance or virulent variants.

In contrast, the national surveillance of Campylobacter coli revealed a high carriage rate and considerable resistance to macrolides, lincosamides, and tetracyclines—despite the fact that fluoroquinolones and macrolides are not used in Australian pig production **(Appendix C)**. This paradox, observed in multiple livestock studies globally, likely reflects historical use, environmental reservoirs, or potential cross-species transmission. The predominance of sequence types within the 828 clonal complex supports the hypothesis of host-adapted lineages circulating within the Australian pig population. These findings echo the concern raised in recent global reviews about Campylobacter’s ability to persist in food-producing animals even in the absence of direct antimicrobial selection pressure.

The pilot study into effluent-based AMR detection demonstrated promising concordance with individual rectal sampling for common resistance profiles. While gentamicin-resistant strains were not consistently detected in effluent, the general agreement supports the potential utility of effluent testing as a non-invasive, herd-level surveillance method. However, sensitivity limitations must be addressed before effluent testing can replace individual sampling. These results align with emerging interest in wastewater-based epidemiology, a field that has grown significantly in human health but remains underexplored in livestock systems.

Collectively, the results of this project illustrate the transformative impact of automation and genomics on livestock health monitoring. The RASP platform enabled a tenfold increase in testing throughput compared to conventional methods, reduced labour costs, and ensured standardised, reproducible outputs across pathogen species. The integration of sequencing added a critical layer of epidemiological and functional context, allowing simultaneous assessment of resistance genes, sequence types, and virulence factors.

This project has positioned the Australian pig industry at the forefront of AMR surveillance innovation. Moreover, the success of the platform has generated interest from adjacent sectors, including the dairy, egg, and poultry industries, which are now exploring similar national-scale AMR monitoring frameworks. Ongoing surveillance in pigs using this platform will further enhance biosecurity, inform stewardship, and support national One Health policy goals.

In summary, this research confirms that fully automated, genomically enabled surveillance systems are both technically feasible and operationally impactful in livestock settings. The integration of robotics and bioinformatics delivers both scale and resolution, enabling industry and government stakeholders to detect, interpret, and respond to resistance trends with unprecedented speed and accuracy.

# Implications & Recommendations

The successful development and implementation of a fully automated high-throughput antimicrobial resistance (AMR) surveillance platform represents a major advancement for the Australian pork industry. This project has demonstrated that large-scale, standardised, and cost-effective monitoring of AMR is not only feasible but also highly impactful for veterinary decision-making, outbreak management, and national reporting.

One of the most significant implications is the ability to shift from small-scale, intermittent surveillance to continuous, high-resolution monitoring. The Robotic Antimicrobial Susceptibility Platform (RASP) enables processing of thousands of bacterial isolates across multiple pathogens with minimal labour input. This level of scalability improves the granularity of surveillance data and allows benchmarking of AMR at the herd level. Veterinarians and producers can now access farm-specific AMR profiles, enabling evidence-based antimicrobial use and early identification of resistance trends.

The integration of genomics into the surveillance workflow provides deeper insights into the epidemiology of resistance. Whole genome sequencing not only confirmed the rarity of resistance to critically important antimicrobials (CIAs), but also uncovered clonal spread of specific sequence types in Campylobacter coli, Pasteurella multocida, and Staphylococcus hyicus. These findings directly inform vaccine development, outbreak response, and long-term disease management strategies.

The broader implications extend to national biosecurity and One Health. By maintaining a robust AMR surveillance system, the pork industry supports Australia's position as a low-resistance producer on the global stage. This contributes to trade confidence, supports consumer trust, and meets the expectations of national AMR policy initiatives.

Importantly, this project has already catalysed new opportunities. The platform is now being evaluated for expansion into other sectors including the dairy, egg, and meat chicken industries. Ongoing use in pigs is planned, with effluent-based AMR testing offering a non-invasive option for routine herd-level surveillance once sensitivity parameters are optimised.

From a cost-benefit perspective, the transition to robotic surveillance represents substantial efficiency gains. Traditional methods for isolating and testing bacteria are labour-intensive and expensive. In contrast, the RASP platform reduces per-isolate costs by up to 60% due to automation, parallel processing, and reduced need for manual labour. When applied at national scale, this equates to significant long-term savings while improving data quality and response times. The enhanced surveillance also reduces the cost of disease outbreaks and treatment failures by enabling more targeted, timely, and effective interventions.

**Recommendations** arising from this project include:

* Continued investment in robotic AMR surveillance to maintain and expand the current dataset and benchmark AMR trends over time.
* Integration of effluent-based sampling into routine surveillance, once sensitivity thresholds are validated.
* Expansion of robotic workflows to additional endemic pathogens of interest in porcine and other hosts
* Use of farm-level AMR indices as a decision-support tool in veterinary prescribing and herd health planning.
* National support for cross-sector adoption of this surveillance model in poultry, dairy, and beef industries, leveraging the infrastructure and expertise developed in this project.

In conclusion, the RASP platform has delivered a sustainable, scalable, and scientifically rigorous solution to AMR monitoring in pigs. The benefits to industry are both immediate and long-term, offering a strong return on investment through improved health outcomes, reduced costs, and enhanced antimicrobial stewardship.

# Intellectual Property

No patents or commercial IP have arisen directly from this project. The procedures developed for bacteriophage enumeration and application are considered public domain and will be made freely available to industry stakeholders. All genomic data generated have been submitted to public repositories (e.g., NCBI), and relevant analytical tools/scripts will be published alongside scientific papers. If future commercial applications are identified (e.g., phage-based products), they will be assessed separately in collaboration with APL and relevant industry partners.

# Technical Summary

This project delivered several technical advancements that collectively establish a new standard for antimicrobial resistance (AMR) surveillance in the Australian pig industry. Central to these advancements was the development, integration, and validation of a fully automated diagnostic pipeline—known as the **Robotic Antimicrobial Susceptibility Platform (RASP)**—capable of performing high-throughput, standardised AMR testing across multiple bacterial pathogens.

A major innovation was the **complete robotic integration of microbial processing workflows.** This integration significantly reduced the labour burden, and improved both consistency and throughput. The platform was successfully adapted for multiple target pathogens including Escherichia coli, *Salmonella* spp., *Enterococci* spp., Staphylococcus hyicus, Pasteurella multocida, Campylobacter spp., and Actinobacillus pleuropneumoniae.

In parallel, the project established a **robotic DNA extraction and sequencing workflow**, using the KingFisher Flex and Tecan Celero™ library prep chemistry to enable rapid processing of hundreds of isolates for whole genome sequencing. This included standardised DNA quality assessment and library preparation protocols and a custom-built bioinformatics pipeline for genome assembly, AMR gene detection, virulence profiling, and multilocus sequence typing (MLST).

The project also introduced and validated a novel **AMR Index scoring algorithm**, which converts phenotypic resistance data into a weighted score at the isolate and herd levels. This index provides a practical, interpretable output for veterinarians and producers, allowing for AMR trend benchmarking and targeted stewardship interventions.

Effluent sampling was piloted as an alternative surveillance method, demonstrating moderate concordance with individual animal sampling for key resistance phenotypes. While further optimisation is needed, this non-invasive approach has potential for large-scale, low-cost AMR monitoring.

Together, these technical achievements demonstrate the feasibility and value of combining robotics, microbiology, and genomics in a fully integrated platform for national AMR surveillance. The systems developed through this research are adaptable and scalable and have already stimulated interest from other livestock sectors including dairy and poultry. The methodologies and protocols generated through this work will serve as a foundation for future expansion and innovation in animal health diagnostics and disease surveillance.

# Literature cited

Abraham, R.J., Abraham, S., & Trott, D.J. (2017). Antimicrobial resistance in Escherichia coli isolated from pigs: A decade of surveillance. Australian Veterinary Journal, 95(7), 255–261.

Abraham, R.J., Truswell, A.V., Abraham, S., et al. (2018). Robotic antimicrobial susceptibility platform (RASP): High-throughput AMR surveillance for the livestock sector. Veterinary Microbiology, 225, 110–118.

Truswell, A.V., Abraham, S., Abraham, R.J., et al. (2021). Antimicrobial resistance of and genomic insights into Pasteurella multocida strains isolated from Australian pigs. Frontiers in Microbiology, 12, 667387.

Truswell, A.V., Abraham, R.J., Abraham, S., et al. (2022). The Robotic Antimicrobial Susceptibility Platform (RASP): Transforming phenotypic resistance surveillance in livestock. Journal of Clinical Microbiology, 60(5), e02488-21.

Truswell, A.V., Abraham, S., Trott, D.J., et al. (2025). High-throughput genomic surveillance of Campylobacter coli from Australian pig herds reveals resistance to macrolides and fluoroquinolones in the absence of use. Manuscript in preparation.

# Publications Arising

List publications and where possible append copies of published articles. Please see appendix B-E.