Australian Government

Department of Agriculture, Fisheries and Forestry

Antimicrobial resistance
in bacteria of animal origin

Pilot surveillance program

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Department of Agriculture, Fisheries and Forestry
Product Integrity, Animal and Plant Health

Pilot Surveillance Program for Antimicrobial Resistance in Bacteria of Animal Origin

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Executive Summary

The Australian Government Department of Agriculture, Fisheries and Forestry initiated the *Pilot Surveillance Program for Antimicrobial Resistance in Bacteria of Animal Origin* as part of the Australian Government's response to Recommendation 10 of the report of the Joint Expert Technical Advisory Committee on Antibiotic Resistance. The aim was to assess the prevalence of resistance to important antimicrobials amongst key indicator organisms found in the gut (caecum) of food-producing animals. There is currently no surveillance system for antimicrobial resistance (AMR) in animals at the national level in Australia although an extensive national antibiotic resistance survey of broiler chickens was undertaken in 2000. Thus, the development of the pilot program was guided by national AMR surveillance programs operating in the United States, Denmark, Norway, Sweden and Canada. The pilot program provides baseline information for the period from November 2003 to July 2004 against which similar future surveillance activities in Australia can be compared.

Samples of gut contents were obtained from healthy animals at 31 slaughter establishments in Queensland, New South Wales, Victoria and South Australia. From 204 cattle, 200 pig and 303 chicken samples, 645 *Escherichia coli*, 547 presumptive *Enterococcus* spp. and 133 *Campylobacter* spp. isolates were recovered. The minimum inhibitory concentrations of antimicrobials were assayed by broth or agar dilution according to National Committee on Clinical Laboratory Standards (NCCLS, now known as the Clinical and Laboratory Standards Institute) methods. The antimicrobials chosen include those used in food-producing animals in Australia, some antimicrobials of importance to human medicine and antimicrobials not used in Australia but which have gained a public health profile internationally. *E. coli* isolates from all three host species were assessed for resistance to ampicillin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, nalidixic acid, tetracycline and a combination of trimethoprim and sulfamethoxazole. *Enterococcus* spp. isolates from all three host species were assessed for resistance to ampicillin, erythromycin, gentamicin, teicoplanin, vancomycin and virginiamycin. *Campylobacter* spp. isolates were only sought from chickens as *Campylobacter* infections in humans are commonly associated with poultry and were assessed for resistance to ciprofloxacin, erythromycin, gentamicin, nalidixic acid and tetracycline. *Salmonella* spp. are being evaluated in a separate project funded by the Australian Government Department of Health and Ageing and DAFF. A retrospective analysis is being conducted on 10 years of national data (isolates from humans, animals and food) from the National Enteric Pathogens Surveillance Scheme and Australian Salmonella Reference Centre.

While all 645 *E. coli* isolates were subjected to sensitivity testing and the results presented in this report, this was not the case for the other two bacterial species. Within the presumptive *Enterococcus* spp, seven isolates were not available for sensitivity testing due to loss in storage while another 16 isolates were shown not to be members of the genus *Enterococcus.* Furthermore, only the results for *E. faecalis, E. faecium* and *E. casseliflavus/E. hirae* (a combined analysis) are presented in this report. Two isolates of *Campylobacter* failed the internal quality control testing and the results were excluded.

Amongst *E. coli* isolates from cattle (n = 194), there was only a very low prevalence of resistance to florfenicol (1 %) and tetracycline (3 %). The only notable resistance involving enterococci from cattle were 9.5% of *E. faecium* isolates (n = 21) expressing resistance to both erythromycin and virginiamycin. Only small differences were observed between the prevalence and patterns of AMR in *E. coli* and *Enterococcus* spp. derived from feedlot cattle, grass-fed cattle and dairy cattle. Amongst *E. coli* from pigs (n = 182), greater than 30% of isolates were resistant to ampicillin, chloramphenicol, florfenicol, tetracycline and trimethoprim/sulfamethoxazole. Multi-resistance (defined here as isolates resistant to two or more antibiotics) and multiple-resistance (defined here as isolates resistant to four or more antibiotics) was common amongst *E. coli* from pigs and involved up to six antibiotics. A high proportion (74.8%) of *Enterococcus* spp. from pigs were resistant to erythromycin. Virginiamycin resistance was common (43.3%) in pig *E. faecium* isolates although little or no resistance to other antimicrobial agents was detected in the remaining enterococci from pigs. Amongst *E. coli* from chickens (n = 269), resistance was detected to ampicillin, tetracycline and trimethoprim/sulfamethoxazole (33%, 44% and 27% of isolates, respectively) and there was little or no resistance to the other antimicrobial agents. Multi- and multiple-resistance was also detected in chicken *E. coli* isolates but was not as marked as in pigs with only 2.6% of chicken isolates having multiple resistance and one isolate resistant to two quinolone-type antibiotics. Enterococci from chickens (n=217) showed a high prevalence (68%) of resistance to erythromycin. Resistance to virginiamycin in enterococci from chickens was common (28.7% excluding consideration of *E. faecalis* which is intrinsically resistant to virginiamycin). Tetracycline and erythromycin resistance (21% and 11% respectively) were detected in *Campylobacter* spp. from chickens (n=131). There was no multiple-resistance found in enterococci or *Campylobacter* isolated from chickens.

With the exception of streptogramins and *E. faecium*, nil or a very low prevalence of resistance to antimicrobials of importance to human medicine was observed. No resistance was detected amongst *E. coli* to either cefotaxime or ceftiofur (both third generation cephalosporins). A small proportion (3%) of pig *E. coli* isolates expressed resistance to gentamicin. Resistance to ciprofloxacin was detected in only one *E. coli* isolate from chickens (0.4%) but not in any *Campylobacter* spp. Only one enterococci isolate was vancomycin resistant (low-level vanC), whilst high-level resistance to gentamicin were not detected in any enterococci.

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Abbreviations

ACMF	Australian Chicken Meat Federation
AMR	antimicrobial resistance
AQIS	Australian Quarantine and Inspection Service
AST	antimicrobial susceptibility testing
CIPARS	Canadian Integrated Program for Antimicrobial Resistance Surveillance
CFU	Colony Forming Units
DAFF	Australian Government Department of Agriculture, Fisheries and Forestry
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
EAGAR	National Health and Medical Research Council's Expert Advisory Group on Antimicrobial Resistance
FAO	Food and Agriculture Organization of the United Nations
JETACAR	Joint Expert Technical Advisory Committee on Antibiotic Resistance
MIC	minimum inhibitory concentration
MLS_B	Macrolide-Lincosamide-Streptogramin resistance
NARMS	National Antimicrobial Resistance Monitoring System
NATA	National Association of Testing Authorities
NRS	National Residue Survey
NCCLS	National Committee on Clinical Laboratory Standards
OIE	Office International des Épizooties
PYR	pyrrolidonyl-β-napthylamide
QA	Quality Assurance
SBA	sheep blood agar
STRAMA	Swedish Strategic Programme for the Rational Use of Antimicrobial Agents and Surveillance of Resistance
SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring
WHO	World Health Organization

Antimicrobial abbreviations used in this report:

Amp	Ampicillin
Chl	Chloramphenicol
Cip	Ciprofloxacin
Ery	Erythromycin
Ffc	Florfenicol
Gen	Gentamicin
Nal	Nalidixic acid
SxT	Trimethoprim/Sulfamethoxazole
Tet	Tetracycline
Van	Vancomycin
Vir	Virginiamycin

State abbreviations:

NSW	New South Wales
QLD	Queensland
SA	South Australia
VIC	Victoria

1. Background to antimicrobial resistance

Since 1990, the World Health Organisation (WHO) has held several meetings on antimicrobial resistance (AMR), with a focus on determining the public health impact of the use of antimicrobials in food-producing animals. In 2000, WHO developed the *Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food* [1]. In 1999, the World Organisation for Animal Health (OIE) elaborated *International Standards on AMR* [2]. The international standards were adopted by the OIE in May 2003 and were published in the OIE *Terrestrial Animal Health Code* [3], and in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [4]. Countries which import animals and animal products can now legally use these standards to verify whether or not exporting countries are complying with these new requirements. In 2001, the OIE held a second international conference on AMR, with stakeholders from both the medical and veterinary fields.

Recognising that managing human health risks arising from non-human usage of antimicrobials and the resulting antimicrobial resistant bacteria requires national and international interdisciplinary cooperation, the Food and Agriculture Organization of the United Nations (FAO), OIE and WHO convened two expert workshops [5, 6] on non-human antimicrobial usage and AMR. The Codex Alimentarius Commission is currently considering how it should address AMR associated with food and transmission through food [7].

A number of countries have established their own national AMR surveillance programs that encompass human and animal components; some of which also cover feed and/or food components. They include Denmark (DANMAP) [8], the United States (NARMS) [9], Norway (NORM and NORM-VET) [10], Sweden (SVARM [11] and STRAMA [12]) and Canada (CIPARS) [13].

2. Background to the pilot surveillance program

For the purpose of the *Pilot Surveillance Program for Antimicrobial Resistance in Bacteria of Animal Origin*, the term AMR refers specifically to a property of bacteria that enables them to grow in the presence of antibiotic levels that would normally suppress or kill susceptible bacteria. Antibiotics are defined as antibacterial agents (including ionophores) but not including antiprotozoals, antifungals, antiseptics, disinfectants, antineoplastic agents, antivirals, immunologicals, direct-fed microbials or enzyme substances [14].

The Joint Expert Technical Advisory Committee on Antibiotic Resistance (JETACAR) released its report [15] in September 1999, making 22 recommendations for the management of AMR in Australia. The Australian Government response [14] in August 2000 strongly supported the intent of the JETACAR report and outlined the mechanisms for implementing the recommendations. The need to establish surveillance for AMR in bacteria from livestock as part of an integrated system including human and food isolates was emphasised in Recommendation 10 of JETACAR:

"Development of a comprehensive surveillance system for antimicrobial resistant bacteria and resistance genes in humans and animals. The surveillance system should include medical (including nosocomial), food-producing animal and veterinary areas with particular emphasis on the establishment of food-chain and environmental connections."

In September 2003, as a first step in implementing this recommendation, the Australian Government released a *Strategy for Antimicrobial Resistance Surveillance in Australia* [16]. Under this strategy, the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF) developed an action plan for AMR surveillance in food-producing animals with a focus on public health. A Technical Reference Group was formed to advise DAFF on implementation of the action plan. As there was no surveillance system for AMR in animals at the national level, although an extensive national antibiotic resistance survey of broiler chickens was undertaken in 2000 [17], a pilot surveillance program became the major element of the action plan. The program is referred to as a 'pilot' because it serves the dual purpose of providing initial prevalence estimates for AMR and defines the feasibility and resource requirements for any future surveillance activity.

Sample collection for the pilot program commenced in November 2003 and was completed in July 2004. The development of the pilot program was guided by the international standards developed by the OIE. NARMS, DANMAP, NORM-VET, SVARM and CIPARS were also influential during the development of the pilot program. The National Health and Medical Research Council's Expert Advisory Group on Antimicrobial Resistance (EAGAR) was consulted on aspects of the design and conduct of the study.

3. Significance to Human Health[a]

This pilot study has demonstrated that antibiotic resistances are present in some indicator and pathogenic bacteria in food of animal origin in Australia, but for most, the impact on human health is likely to be small. This is because high human antibiotic consumption in Australia has already selected for these resistances or their need and usage in humans is now minimal, as in the case of chloramphenicol.

Altogether the study findings support the control measures introduced into Australia following the release of the Joint Expert Technical Advisory Committee on Antimicrobial Resistance (JETACAR) report in 2000. An ongoing surveillance program of this type will serve to monitor the emergence of any new resistances of importance to human health.

The most important resistances to monitor are so-called 'last line' antibiotics used to treat serious infections from multi-resistant pathogens. For instance, virginiamycin resistance in *Enterococcus faecium* confers resistance to another streptogramin, quinupristin-dalfopristin, which is a last-line antibiotic used to treat vancomycin-resistant *E. faecium* in humans. While these infections are relatively uncommon in Australia at present, they can be serious for hospitalised patients who have complicated underlying diseases. In this study, resistance to virginiamycin was seen in 10% of cattle isolates, 43% of pig isolates and 26% of chicken isolates.

Interestingly, no resistance to vancomycin was detected in enterococci from any of the three animal species tested. Vancomycin resistance of the VanA type in *E. faecium* has been associated with the use of avoparcin in food animal feeds, and the evidence suggests that at least in the past this resistance was transmitted to human enterococci. Avoparcin was withdrawn from the world market in 2001. The lack of vancomycin resistance in this study most probably reflects the removal of the selective pressure of this antibiotic.

Other resistances were reassuringly uncommon or not detected, including gentamicin, third-generation cephalosporin (ceftiofur, cefotaxime) and quinolone resistance in *Escherichia coli*, and quinolone resistance in *Campylobacter* species. The almost complete absence of quinolone resistance can be attributed to the Australian strategy of preventing the use of quinolones in food-producing animals.

A low rate of gentamicin resistance (3%) was detected in *E. coli* from pigs but not in chickens or cattle. Strains with this resistance harboured resistance to 2-5 other drug classes. Gentamicin is a standard part of initial treatment for serious *E. coli* infections in humans in Australia. Rates of resistance in human pathogenic isolates of *E. coli* are currently around 2%. The presence of gentamicin resistance in pig isolates raises the possibility of a link to apramycin use in pigs as these two antibiotics are both aminoglycosides. Gentamicin itself was removed from use in food animals in Australia some years ago.

[a] Provided by the National Health and Medical Research Council, March 2007

The data do show that there are some circumstances in the Australian livestock industries where multiple drug resistance in commensal bacteria is common. While this does not mostly include resistance to important drug classes of antimicrobials, experience abroad suggests that where multiple drug resistance does occur it can eventually incorporate drugs of critical importance in human medicine.

4. Objectives

Surveillance for infectious agents in Australian livestock must account for many factors including the diversity of livestock production, geographic distances and the ease of access to laboratory resources. DAFF and the Technical Reference Group therefore recognised two distinct roles for the pilot study.

The first was to define the operational requirements for future and ongoing efforts in surveillance for AMR in livestock in Australia. This includes issues such as logistics, cost and resource requirements of a future surveillance system.

The second role was to provide estimates of the prevalence of AMR amongst isolates (that have relevance to public health) obtained from a large population of food-producing animals in Australia. The scientific component of the pilot study is the focus of this report.

4.1 Scientific aims

1. Estimate the proportion of commensal *Escherichia coli* isolates from cattle, broiler chickens and pigs that are resistant to each member of a panel of antimicrobials (antimicrobials that are important from either a medical perspective or veterinary perspective or both).
2. Estimate the proportion of commensal *Enterococcus* spp. isolates from cattle, broiler chickens and pigs that are resistant to each member of a panel of antimicrobials.
3. Estimate the proportion of *Campylobacter* spp. isolates from broiler chickens that are resistant to each member of a panel of antimicrobials.
4. Describe the distribution of minimum inhibitory concentrations (MICs) for the organisms and drugs studied while addressing the above aims.
5. Describe the multi and multiple resistance attributes of the isolates assessed.

Antimicrobial panels were different for each group of bacterial organisms but were constant across animal species as shown in **Table 1**. *E. coli* and *Enterococcus* spp. are commensal bacteria common in the gut of animals and man. As these organisms also respond to the selective pressure of antimicrobials, they commonly provide useful information for surveillance programs. These organisms may also act as a reservoir of resistance genes that can be transferred to pathogens (human or animal) or to other commensals. Campylobacter infections in humans are commonly associated with poultry.

Table 1: Bacteria and antimicrobials included in the pilot surveillance program

Animal species	Target organisms	Antimicrobials
Cattle*, slaughter pigs and broiler chickens	*E. coli*	ampicillin, chloramphenicol, florfenicol, ceftiofur, cefotaxime, ciprofloxacin, gentamicin, nalidixic acid, trimethoprim/sulfamethoxazole, tetracycline
Cattle*, slaughter pigs and broiler chickens	*Enterococcus ** faecium/faecalis*	ampicillin, erythromycin, gentamicin, teicoplanin, vancomycin, virginiamycin
Broiler chickens	*Campylobacter* spp.	ciprofloxacin, erythromycin, gentamicin, nalidixic acid, tetracycline

* equal numbers of feedlot, grass-fed and dairy cattle
** *E. hirae* and *E. casseliflavus* results were also reported due to their capacity to carry and transfer AMR genes

Veterinary chemical products containing ampicillin, florfenicol, ceftiofur, erythromycin, trimethoprim/sulfamethoxazole, tetracycline class (e.g. oxytetracycline, chlortetracycline) and virginiamycin are registered for therapeutic use in food-producing animals in Australia [18]. Ampicillin, chloramphenicol, erythromycin, trimethoprim/sulfamethoxazole and tetracycline are antibiotics of low importance to human medicine [19]. Gentamicin and nalidixic acid are of medium importance, while cefotaxime, teicoplanin and vancomycin are of high importance to human medicine[19]. Virginiamycin is a member of the streptogramin class of antimicrobials which are considered to be of high importance to human medicine [19]. Fluoroquinolones have never been registered for use in food-producing animals in Australia. However, ciprofloxacin, a member of the fluoroquinolone class, is considered to be an essential antibiotic for use in humans with an EAGAR rating of "high" for human use. It was therefore included in the pilot program. Details of the design and scope of the pilot program are contained in **Appendix 1**.

5. Sample collection

5.1 Sampling strategy

The pilot program was based on the analysis of isolates recovered from caecal specimens collected from healthy livestock following their slaughter in commercial establishments. For logistical and budgetary reasons, the pilot program was confined to Queensland (QLD), New South Wales (NSW), Victoria (VIC) and South Australia (SA). Data on the volume of throughput for each cattle and pig establishment were obtained from the National Residue Survey (NRS). Those establishments with the highest throughput volumes in the four states and within 24 hours driving distance to the participating veterinary diagnostic laboratories were selected for participation. For cattle and pigs, only those establishments licensed for export were considered for inclusion. These export establishments typically have higher throughput levels compared to domestic abattoirs and are staffed by Australian Quarantine and Inspection Service (AQIS) On-Plant Veterinary Officers. AQIS kindly made available the expertise of the latter for the collection of specimens. Eleven cattle and seven pig slaughter establishments were recruited for participation. For broilers, the Australian Chicken Meat Federation (ACMF) assisted DAFF with the selection of 13 processing establishments. The selection of broiler processing plants included each of the major producers of chicken meat in each of the four states to account for possible differences in antibiotic use between companies and between states. The number of

samples collected from cattle, pigs or broilers at each plant were proportional to processing volume.

No two specimens were obtained from animals belonging to the same processing lot (as determined by tail tags and processing identification). The target number of bacterial isolates was based on an expected prevalence of resistant organisms of 10%. To obtain a 95% confidence interval for this prevalence estimate with width (precision) of 5%, at least 138 isolates per animal species/bacterium combination are required. A collection target of 150 isolates was established for each combination of animal species and bacterium to accommodate any loss of isolates in transit or storage. In addition, the target number of caecal specimens from each animal species was adjusted upward from this number to allow for failure to isolate the respective organisms. Thus, 200 specimens were obtained from pigs, 204 specimens from cattle, while 303 specimens were obtained from broilers because of the expected lower isolation rate for *Campylobacter* spp than for other bacteria.

For each commodity, 10 to 48 samples per month were collected depending on the region. Sampling was carried out at two to three monthly intervals to obtain balanced representation of both summer and winter animal-management factors, and to account for any seasonal variation, if present. Each region was sampled three times during the pilot surveillance period as detailed in **Table 2**. There were specific targets for the number of samples per round to be collected from each establishment and any samples not collected in a particular round (e.g. due to plant breakdowns) were required to be made up in subsequent rounds, where possible.

Table 2: Number of samples collected in each round by animal species

Animal species	Round 1 (Nov-Dec 2003)	Round 2 (Feb-Apr 2004)	Round 3 (May-Jul 2004)	TOTAL
Cattle	67	74	63	**204**
Pigs	70	70	60	**200**
Poultry	100	103	100	**303**
TOTAL	**237**	**247**	**223**	**707**

For the 204 cattle, 65 were of dairy origin, 70 were feedlot and 69 were grass-fed. **Table 3** shows the number of samples collected per sub-type for each of the rounds.

Table 3: Breakdown of cattle sub-types in each round

Sub-type	Round 1 (Nov-Dec 2003)	Round 2 (Feb-Apr 2004)	Round 3 (May-Jul 2004)	TOTAL
Dairy	12	15	38	**65**
Grass-fed	30	39	N/A	**69**
Feedlot	25	20	25	**70**
TOTAL	**67**	**74**	**63**	**204**

5.2 Sampling site and technique

Sampling at cattle and pig abattoirs was carried out by AQIS Veterinary Officers. During the initial stages of the pilot program, sample collection was performed by a Senior Veterinary Officer who developed an approach that could be consistently applied at all establishments. Once the technique was refined and adapted to each establishment, the on-plant AQIS veterinarians used the finalised approach to collect the remaining samples.

Establishments that process poultry do so for the domestic market and are regulated by state authorities. At these establishments, QA officers were recruited by ACMF to collect caecal samples on behalf of DAFF. These officers have experience in the collection of samples as part of the NRS meat monitoring activities.

The collection of caecal contents from cattle and pigs is similar. For each sample an incision was made into the apex of the caecum and approximately 20 mL of caecal contents collected into a sterile specimen container (70 mL screw top). In the case of chickens, whole caeca were collected and placed in a specimen container. DAFF provided each of the on-plant veterinarians (cattle and pigs) and QA Managers (poultry) with detailed instructions and materials for sample collection.

5.3 Transportation of samples to the laboratories

The NRS courier system was used for the transportation of samples to designated laboratories for bacterial culturing. The chilled samples were prepared for despatch according to instructions and materials provided by DAFF. Specimen containers were secured in NRS sample bags and placed into plastic-lined, waxed cardboard boxes together with frozen gel packs. Samples were shipped on the same day they were collected and were required to arrive at the laboratories within 24 hours of collection. To ensure this, samples were collected on Mondays, Tuesdays and Wednesdays. In cases where establishments were within close proximity to the laboratory, samples could also be collected on Thursdays and submitted to the laboratory in person.

6. Laboratory testing

There are several methods for isolating the required bacteria from caecal contents and there are currently few laboratories in Australia specialising in antimicrobial susceptibility testing (AST) using panels of veterinary antimicrobials. Therefore, apart from the generation of data, the main aim for the laboratories participating in the pilot surveillance program was to build the capacity to process high numbers of samples and to consistently produce reliable minimum inhibitory concentration (MIC) data using internationally accepted protocols.

6.1 Laboratory selection

Targeted tenders were sought for laboratories in Eastern Australia to carry out the laboratory testing. Veterinary diagnostic laboratories and other suitable laboratories were approached to carry out culturing and presumptive testing for *E. coli*, *Enterococcus* spp. and *Campylobacter* spp. The laboratories participating in this aspect were:

- Yeerongpilly Veterinary Laboratory, Queensland Department of Primary Industries and Fisheries, Yeerongpilly, QLD;
- Elizabeth Macarthur Agricultural Institute, NSW Department of Primary Industries, Menangle, NSW; and
- Primary Industries Research Victoria, Victorian Department of Primary Industries, Attwood, VIC.

In vitro susceptibility of these organisms to the antimicrobials specified in **Table 1** was assessed at laboratories with experience and/or capability to undertake definitive identification and broth or agar dilution. One laboratory was selected for each

bacterial species. For enterococci, species identification was also required. The participating laboratories were:

- *E. coli* – Regional Veterinary Laboratory, NSW Department of Primary Industries, Wollongbar, NSW;
- *Enterococcus* spp. – School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA; and
- *Campylobacter* spp. – Animal Research Institute, Queensland Department of Primary Industries and Fisheries, Yeerongpilly, QLD.

6.2 Bacterial culturing

The culturing of *E. coli*, *Enterococcus* spp. (from cattle, pigs, and chickens) and *Campylobacter* spp. (from chickens) were carried out using plate culture methods and included an enrichment step, where necessary. The methods contained in the Australian Institute of Food Science and Technology publication, *Foodborne Microorganisms of Public Health Significance (6th Edition)* were used as guidance [20].

Details of the testing protocol for *E. coli*, *Enterococcus* spp. and *Campylobacter* spp. are contained in **Appendix 2**.

The minimum identification requirements were:

- *E. coli* Gram-negative motile or non-motile bacilli, lactose fermentation, indole positive; glucuronidase positive by chromogenic reaction;
- *Enterococcus* Gram positive cocci in pairs, catalase and oxidase negative, hydrolysis in bile-aesculin agar, growth in presence of 6.5% NaCl, growth at 42°C;
- *Campylobacter* microaerophilic growth at 42°C, Gram negative, typical motility and cell morphology (phase contrast or DGE microscopy), oxidase and catalase positive.

Laboratories were required to implement and maintain appropriate QA according to the National Association of Testing Authorities (NATA).

Recovered isolates, with identity confirmed using the above criteria, were stored on agar slopes in duplicate and frozen to -70°C. At the end of each round, the isolates aggregated over the previous two to three months were resuscitated and transferred to swabs as follows. Isolates were retrieved from storage at -70°C by plating onto blood agar and incubated as for bacterial culturing. After incubation the plates were used as a source of heavy inoculation for transport swabs inserted into charcoal-containing transport media. Batches of isolates were then shipped in insulated packages containing ice bricks for receival by the susceptibility testing laboratories within 24 hours of despatch.

6.3 Antimicrobial susceptibility testing

The susceptibility testing laboratories revived the organisms onto appropriate media from the swabs sent by source laboratories. The *in vitro* activity of a range of antimicrobial agents against each bacterial isolate was quantitatively measured using a broth or agar antimicrobial dilution susceptibility test to determine MICs in µg/mL. Antimicrobial susceptibility testing (AST) was carried out according to the National

Committee on Clinical Laboratory Standards (NCCLS, now known as the Clinical and Laboratory Standards Institute) Standard M31-A2, Vol 22 No 6, *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard—Second Edition* [21]. Isolates were required to be tested once only, provided controls were within acceptable limits.

Laboratories were required to implement and maintain appropriate QA according to NCCLS requirements.

All enterococci isolates underwent phenotypic speciation and those that did not produce definitive identification using biochemical tests were subjected to genotypic speciation. Only those isolates definitively identified by these procedures as enterococci were subjected to AST.

Detailed protocol for AST is contained in **Appendix 3**.

The dilution ranges and resistance breakpoint MIC[b] for each of the antimicrobials used in the pilot surveillance program were derived primarily from NCCLS [21, 26, 27]. In the case of gentamicin resistance in *Enterococcus* spp., the DANMAP [28] breakpoint of 1024 µg/mL was used for assessing the occurrence of high level resistance which has relevance to some uses of this drug for treatment of enterococcal infections in humans. In the case of florfenicol resistance in *E. coli*, the DANMAP breakpoint of 32 µg/mL was used for assessing resistance. This breakpoint is consistent with other surveillance programs. For the *Campylobacter* isolates, the breakpoints were derived from DANMAP [28]. Details of the breakpoint MIC and dilution ranges for each of the antimicrobials are contained in **Table 4**.

[b] The breakpoints used in this report are in strong agreement with those used in recently released reports from internationally recognised surveillance systems (i.e. DANMAP 2006 [22], CIPARS 2006 [23], SVARM 2006 [24] and SVARM 2007 [25])

Table 4: Dilution ranges and breakpoints for antimicrobial susceptibility testing by microdilution

	E. coli			*Enterococcus*			*Campylobacter*		
ampicillin	1	—	128	2	—	64			
Resistance breakpoint			>16			>8			
chloramphenicol	1	—	128						
Resistance breakpoint			>16						
florfenicol	1	—	128						
Resistance breakpoint			>16						
ceftiofur	0.125	—	16						
Resistance breakpoint			>4						
cefotaxime	0.5	—	64						
Resistance breakpoint			>32						
ciprofloxacin	0.031	—	4				0.5	—	16
Resistance breakpoint			>2						>2
erythromycin				1	—	32	1		32
Resistance breakpoint						>4			>16
gentamicin	0.25	—	32	16, 64	—	2048	1	—	32
Resistance breakpoint			>8			>512			>8
nalidixic acid	1	—	128				4	—	128
Resistance breakpoint			>16						>32
trimethoprim/ sulfamethoxazole	0.125/ 2.375	—	16/ 304						
Resistance breakpoint			>2						
tetracycline	0.25	—	32				1	—	32
Resistance breakpoint			>8						>8
teicoplanin				0.5	—	16			
Resistance breakpoint						>16			
vancomycin				2	—	64			
Resistance breakpoint						>16			
virginiamycin				1	—	32			
Resistance breakpoint						>2			

Ranges are extrapolated from NCCLS and DANMAP 2002

7. Results

7.1 Isolation rates

In total, 645 *E. coli*, 547 presumptive *Enterococcus* spp. and 133 *Campylobacter* spp. isolates were obtained for further testing.

From the 204 cattle samples, *E. coli* were isolated from 95% and presumptive *Enterococcus* spp. from 78%. From the 200 pig samples, *E. coli* were isolated from 91% and presumptive *Enterococcus* spp. from 68%. From the 303 chicken samples, *Campylobacter* spp. were isolated from 44%, *E. coli* from 89% and presumptive *Enterococcus* spp. from 84%.

Of the 194 *E. coli* isolates from cattle, 31% were from dairy cattle, 34% from feedlot cattle and 35% from grass-fed cattle. Of the 158 presumptive *Enterococcus* spp. isolates from cattle, 35% were from dairy cattle, 33% from feedlot cattle and 32% from grass-fed cattle.

7.2 Antimicrobial susceptibility testing

Isolates that were unable to be revived, at either the primary laboratory or the AST laboratory, and those that failed to pass the AST quality control tests were not included in the analysis of AST results.

Information on data management and analysis are contained in **Appendix 4**.

In this report, multi-resistance refers to isolates resistant to two or more antibiotics and multiple-resistance refers to isolates resistant to four or more antibiotics.

7.2.1 *E. coli*

From cattle, 194 *E. coli* isolates were submitted to AST. Resistance to florfenicol (1%) and tetracycline (3.1%) was observed, however none of the resistant isolates exhibited multiple-resistance (**Table 5**). None of the *E. coli* isolated from dairy cattle were resistant to the antimicrobials tested. Of the *E. coli* isolates from feedlot cattle, 17.7% exhibited resistance to tetracycline and 1.5% exhibited resistance to florfenicol. Tetracycline (1.5%) and florfenicol (1.5%) resistance was also observed in grass-fed cattle. There was no resistance to ampicillin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and trimethoprim/sulfamethoxazole in *E. coli* isolated from cattle.

Table 5: Distribution of single resistant phenotypes amongst *E. coli* obtained from cattle (n = 194)

Resistance phenotype*	Frequency	Percentage
0: nil	186	95.9
1: Ffc	2	1.0
1: Tet	6	3.1
TOTAL	**194**	**100**

* Resistance phenotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

From slaughter pigs, 182 *E. coli* isolates were submitted to AST. Resistance to the following antimicrobials was observed: ampicillin (35.2%), chloramphenicol (43.9%),

florfenicol (33.5%), gentamicin (2.8%), nalidixic acid (5%), tetracycline (75.8%) and trimethoprim/sulfamethoxazole (33%). Thirty-five isolates (19.2%) were resistant to two antimicrobials, 32 (17.6%) to three antimicrobials, 34 (18.7%) to four antimicrobials, 12 (6.6%) to five antimicrobials and two (1.1%) to six antimicrobials (**Table 6**). There was no resistance to cefotaxime, ceftiofur and ciprofloxacin in *E. coli* isolated from pigs.

Table 6: Distribution of single, multi- and multiple-resistant phenotypes amongst *E. coli* obtained from pigs (n = 182)

Resistance phenotype*	Frequency	Percentage
0: nil	24	13.2
1: Amp	7	3.8
1: Ffc	1	0.5
1: SxT	1	0.5
1: Tet	34	18.7
2: Amp, Chl	3	1.6
2: Amp, Tet	20	11.0
2: Chl, Ffc	1	0.5
2: Chl, Tet	4	2.2
2: Ffc, Tet	1	0.5
2: Tet, SxT	6	3.3
3: Amp, Chl, Ffc	3	1.6
3: Amp, Chl, Tet	4	2.2
3: Amp, Tet, SxT	6	3.3
3: Chl, Ffc, SxT	1	0.5
3: Chl, Ffc, Tet	8	4.4
3: Chl, Tet, SxT	9	4.9
3: Gen, Tet, SxT	1	0.5
4: Amp, Chl, Ffc, SxT	1	0.5
4: Amp, Chl, Ffc, Tet	11	6.0
4: Amp, Chl, Gen, SxT	1	0.5
4: Amp, Ffc, Tet, SxT	1	0.5
4: Chl, Ffc, Nal, SxT	1	0.5
4: Chl, Ffc, Nal, Tet	1	0.5
4: Chl, Ffc, Tet, SxT	17	9.3
4: Chl, Gen, Tet, SxT	1	0.5
5: Amp, Chl, Ffc, Tet, SxT	5	2.7
5: Chl, Ffc, Gen, Tet, SxT	1	0.5
5: Chl, Ffc, Nal, Tet, SxT	6	3.3
6: Amp, Chl, Ffc, Gen, Tet, SxT	1	0.5
6: Amp, Chl, Ffc, Nal, Tet, SxT	1	0.5
TOTAL	**182**	**100**

* Resistance phenotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

From chickens, 269 *E. coli* isolates were submitted to AST. Resistance to the following antimicrobials was observed: ampicillin (33.1%), chloramphenicol (1.8%), ciprofloxacin (0.4%), florfenicol (3.4%), nalidixic acid (1.9%), tetracycline (44.3%) and trimethoprim/sulfamethoxazole (27.2%). Ninety-three isolates (34.6%) were resistant to two or more antimicrobials with seven isolates resistant to four antimicrobials (**Table 7**). There was no resistance to cefotaxime, ceftiofur and gentamicin in *E. coli* isolated from chickens.

Table 7: Distribution of single, multi- and multiple-resistant phenotypes amongst *E. coli* obtained from chickens (n = 269)

Resistance phenotype*	Frequency	Percentage
0: nil	106	39.4
1: Amp	24	8.9
1: Ffc	3	1.1
1: Nal	2	0.7
1: SxT	7	2.6
1: Tet	34	12.6
2: Amp, SxT	7	2.6
2: Amp, Tet	21	7.8
2: Chl, Ffc	1	0.4
2: Ffc, Tet	1	0.4
2: Nal, Tet	2	0.7
2: Tet, SxT	24	8.9
3: Amp, Chl, Tet	1	0.4
3: Amp, Tet, SxT	28	10.4
4: Amp, Chl, Tet, SxT	3	1.1
4: Amp, Cip, Nal, Tet	1	0.4
4: Amp, Ffc, Tet, SxT	4	1.5
TOTAL	**269**	**100**

* Resistance phenotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 8: Distribution of MICs and occurrence of resistance among *E. coli* isolates from cattle (n=194), pigs (n=182) and chickens (n=269)

Antimicrobial	Animal species	% Resistant [95% Confidence Interval]		Distribution (%) of MICs													
				0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Ampicillin	Cattle	0	[0.0-1.9]						20.1	42.3	33.5	3.6	0.5				
	Pigs	35	[28.2-42.6]						5.0	31.6	25.3	2.8					35.2
	Chickens	33	[27.5-39.1]						9.7	32.7	23.1	1.5		0.7	0.4		32.0
Cefotaxime	Cattle	0	[0.0-1.9]					100									
	Pigs	0	[0.0-2.0]					100									
	Chickens	0	[0.0-1.4]					99.6	0.4								
Ceftiofur	Cattle	0	[0.0-1.9]			21.1	54.6	22.7	1.6								
	Pigs	0	[0.0-2.0]			21.4	45.6	31.9	1.1								
	Chickens	0	[0.0-1.4]			15.2	50.2	33.1	1.5								
Chloramphenicol	Cattle	0	[0.0-1.9]							1.6	33.0	63.4	2.1				
	Pigs	44	[36.6-51.5]							1.1	14.8	37.4	2.8	13.2	14.8	7.7	8.2
	Chickens	2	[0.6-4.3]							0.4	26.4	67.3	4.1		0.7	0.7	0.4
Ciprofloxacin	Cattle	0	[0.0-1.9]	100													
	Pigs	0	[0.0-2.0]	87.9	11.0	0.6	0.6										
	Chickens	<1	[0.0-2.1]	96.7	0.4	1.1	0.7		0.7			0.4					
Florfenicol	Cattle	1	[0.1-3.7]							2.1	12.4	58.8	25.8	1.0			
	Pigs	34	[26.7-40.9]								3.9	26.9	35.7	20.3	12.6	0.6	
	Chickens	3	[1.5-6.3]						0.4	0.4	5.2	58.4	32.3	3.4			
Gentamicin	Cattle	0	[0.0-1.9]				57.7	34.5	5.7	2.0							
	Pigs	3	[0.9-6.3]				33.0	46.7	13.2	3.3	0.6	0.6	2.2	0.6			
	Chickens	0	[0.0-1.4]				42.0	44.2	11.5	2.2							
Nalidixic acid	Cattle	0	[0.0-1.9]						8.3	59.3	30.9	1.6					
	Pigs	5	[2.3-9.2]						2.2	37.4	35.7	5.0	14.8	4.4			0.6
	Chickens	2	[0.6-4.3]						9.3	50.6	37.2	1.1					1.9
Tetracycline	Cattle	3	[1.1-6.6]				1.6	19.6	44.9	27.3	1.6	2.1	0.5		2.6		
	Pigs	76	[68.9-81.9]					2.8	13.2	6.6	1.7			2.2	73.6		
	Chickens	44	[38.2-50.4]				0.7	8.6	29.4	12.3	1.5	3.4	3.0	0.4	40.9		
Trimethoprim/ sulfamethoxazole	Cattle	0	[0.0-1.9]			96.4	2.1	1.0	0.5								
	Pigs	33	[26.2-40.3]			48.4	6.6	7.1	3.9	1.1				33.0			
	Chickens	27	[21.9-32.9]			63.9	3.7	2.2	2.2	0.7			0.4	26.8			

Vertical lines indicate breakpoints for resistance

The white fields denote dilution range tested for each antimicrobial. Values above the range denote MIC values greater than the highest concentration in the range. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration

7.2.2 *Enterococcus* spp.

The enterococci results in this report consist of the results for *E. faecium*, *E. faecalis* and *E. casseliflavus* and *E. hirae* (the latter two as a combined analysis) based on phenotypic speciation and where applicable, genotypic speciation.

Cattle yielded 158 presumptive *Enterococcus* spp. isolates. Resistance to erythromycin (9.5%) and virginiamycin (9.5%) was observed in *E. faecium*. Two *E. faecium* isolates (9.5%) were resistant to both antimicrobials (**Table 9**). *E. faecium* from feedlot cattle exhibited resistance to erythromycin (14.3%) and virginiamycin (14.3%). *E. faecium* from grass-fed cattle exhibited resistance to erythromycin (16.7%) and virginiamycin (16.7%). Resistance to virginiamycin was observed in all *E. faecalis* isolated from dairy, feedlot and grass-fed cattle (**Table 13**). *E. faecalis* is known to be intrinsically resistant to virginiamycin but these results are included for completeness. Virginiamycin and erythromycin resistance at levels of 3% and 2%, respectively, were observed in the combined analysis of *E. casseliflavus* and *E. hirae* isolated from cattle. Resistance to both antimicrobials was observed in two (2%) of the 102 *E. casseliflavus* and *E. hirae* isolates (**Table 17**). *E. casseliflavus* and *E. hirae* from feedlot cattle exhibited resistance to erythromycin (2.5%) and virginiamycin (5%). *E. casseliflavus* and *E. hirae* from grass-fed cattle exhibited resistance to erythromycin (3%) and virginiamycin (3%). There was no resistance to ampicillin, gentamicin, teicoplanin or vancomycin in the enterococci analysed from cattle.

Slaughter pigs yielded 127 *Enterococcus* spp. isolates for AST. Resistance to ampicillin (3.3%), erythromycin (93.3%) and virginiamycin (43.3%) was observed for *E. faecium*. Fourteen *E. faecium* isolates (46.7%) were resistant to two antimicrobials (**Table 10**). Resistance to erythromycin (66%) and virginiamycin (97.9%) [intrinsic resistance] was observed in *E. faecalis*. Resistance to both antimicrobials was observed in 31 *E. faecalis* isolates (66%) (**Table 14**). In the combined analysis of *E. hirae* and *E. casseliflavus*, resistance to ampicillin, erythromycin and virginiamycin was observed in 2.6%, 71.1% and 21% of isolates, respectively. Twenty-one percent of *E. hirae* and *E. casseliflavus* (combined analysis) were resistant to two antimicrobials (**Table 18**). There was no resistance to gentamicin, teicoplanin or vancomycin in the enterococci analysed from pigs.

Chickens yielded 238 *Enterococcus* spp. isolates for AST. Resistance to ampicillin (4.9%), erythromycin (45.9%) and virginiamycin (26.2%) was observed for *E. faecium*. Seven *E. faecium* isolates (11.5%) were resistant to two antimicrobials and two isolates (3.3%) were resistant to three antimicrobials (**Table 11**). Resistance to erythromycin (77.3%), vancomycin (0.8%) [low-level *van*C] and virginiamycin (92.8%) [intrinsic resistance] was observed for *E. faecalis*. Resistance to two antimicrobials was observed in 87 *E. faecalis* isolates (70.7%) and resistance to three antimicrobials was observed in one *E. faecalis* isolate (0.8%) (**Table 15**). Of the *E. hirae* and *E. casseliflavus* (combined analysis), resistance to erythromycin and virginiamycin were observed in 75.7% and 33.4% of isolates, respectively. Ten (30.3%) of the 33 *E. hirae* and *E. casseliflavus* isolates were resistant to both erythromycin and virginiamycin (**Table 19**). No resistance to gentamicin or teicoplanin was observed in the enterococci analysed from chickens.

7.2.2.1 Analysis of *E. faecium*

Table 9: Distribution of multi-resistance amongst *E. faecium* obtained from cattle (n = 21)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	19	90.5
2: Ery, Vir	2	9.5
TOTAL	**21**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 10: Distribution of single and multi-resistance amongst *E. faecium* obtained from pigs (n = 30)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	2	6.7
1: Ery	14	46.7
2: Amp, Ery	1	3.3
2: Ery, Vir	13	43.3
TOTAL	**30**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 11: Distribution of single and multi-resistance amongst *E. faecium* obtained from chickens (n = 61)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	25	41.0
1: Amp	1	1.6
1: Ery	19	31.2
1: Vir	7	11.5
2: Ery, Vir	7	11.5
3: Amp, Ery, Vir	2	3.3
TOTAL	**61**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 12: Distribution of MICs and occurrence of resistance among *E. faecium* isolates from cattle (n=21), pigs (n=30) and chickens (n=61)

Antimicrobial	Animal species	% Resistant	[95% Confidence Interval]	Distribution (%) of MICs												
				0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Ampicillin	Cattle	0	[0.0-16.1]			100										
	Pigs	3	[0.1-17.2]			66.7	26.7	3.3	3.3							
	Chickens	5	[1.0-13.7]			72.1	18.0	4.9	4.9							
Erythromycin	Cattle	10	[1.2-30.4]		47.6	23.8	19.1				9.5					
	Pigs	93	[77.9-99.2]			6.7			3.3		90.0					
	Chickens	46	[33.1-59.2]		41.0	8.2	4.9	3.3	3.3		39.3					
Gentamicin	Cattle	0	[0.0-16.1]						85.7		14.3					
	Pigs	0	[0.0-11.6]						76.7		23.3					
	Chickens	0	[0.0-5.9]						68.9		29.5	1.6				
Teicoplanin	Cattle	0	[0.0-16.1]	95.2	4.8											
	Pigs	0	[0.0-11.6]	100												
	Chickens	0	[0.0-5.9]	100												
Vancomycin	Cattle	0	[0.0-16.1]			100										
	Pigs	0	[0.0-11.6]			96.7	3.3									
	Chickens	0	[0.0-5.9]			100										
Virginiamycin	Cattle	10	1.2-30.4]		76.2	14.3	4.8	4.8								
	Pigs	43	[25.5-62.6]		30.0	26.7	30.0	10.0		3.3						
	Chickens	26	[15.8-39.1]		52.5	21.3	4.9	14.8	1.6	3.3	1.6					

Vertical lines indicate breakpoints for resistance

The white fields denote dilution range tested for each antimicrobial. Values above the range denote MIC values greater than the highest concentration in the range. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration

7.2.2.2 Analysis of *E. faecalis*

Table 13: Distribution of single resistance amongst *E. faecalis* obtained from cattle (n = 17)
[note that *E. faecalis* is intrinsically resistant to virginiamycin]

Resistance phenotype/genotype*	Frequency	Percentage
1: Vir	17	100.0
TOTAL	**17**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 14: Distribution of single and multi-resistance amongst *E. faecalis* obtained from pigs (n = 47)
[note that *E. faecalis* is intrinsically resistant to virginiamycin]

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	1	2.1
1: Vir	15	31.9
2: Ery, Vir	31	66.0
TOTAL	**47**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 15: Distribution of single and multi-resistance amongst *E. faecalis* obtained from chickens (n = 123)
[note that *E. faecalis* is intrinsically resistant to virginiamycin]

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	2	1.6
1: Ery	7	5.7
1: Vir	26	21.1
2: Ery, Vir	87	70.7
3: Ery, Van, Vir	1	0.8
TOTAL	**123**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 16: Distribution of MICs and occurrence of resistance among *E. faecalis* isolates from cattle (n=17), pigs (n=47) and chickens (n=123)

Antimicrobial	Animal species	% Resistant	[95% Confidence Interval]	Distribution (%) of MICs												
				0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Ampicillin	Cattle	0	[0.0-19.5]			100										
	Pigs	0	[0.0-7.5]			100										
	Chickens	0	[0.0-3.0]			99.2	0.8									
Erythromycin	Cattle	0	[0.0-19.5]		58.8	41.2										
	Pigs	66	[50.7-79.1]		27.7	6.4					66.0					
	Chickens	77	[68.8-84.3]		12.2	9.8	0.8	0.8	4.1	3.3	69.1					
Gentamicin	Cattle	0	[0.0-19.5]						82.4		17.7					
	Pigs	0	[0.0-7.5]						74.5		23.4	2.1				
	Chickens	0	[0.0-3.0]						66.7		33.3					
Teicoplanin	Cattle	0	[0.0-19.5]	100												
	Pigs	0	[0.0-7.5]	100												
	Chickens	0	[0.0-3.0]	100												
Vancomycin	Cattle	0	[0.0-19.5]			100										
	Pigs	0	[0.0-7.5]			100										
	Chickens	0	[0.0-4.4]			97.6	1.6					0.8				
Virginiamycin*	Cattle	100	[80.5-100.0]				70.6	29.4								
	Pigs	98	[88.7-99.9]		2.1		53.2	38.3	6.4							
	Chickens	93	[86.6-96.6]			7.3	35.8	48.8	4.1	0.8	3.3					

Vertical lines indicate breakpoints for resistance

The white fields denote dilution range tested for each antimicrobial. Values above the range denote MIC values greater than the highest concentration in the range. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration

* Note that *E. faecalis* is intrinsically resistant to virginiamycin

7.2.2.3 Analysis of *E. hirae* and *E. casseliflavus*

Table 17: Distribution of single and multi-resistance amongst *E. hirae* and *E. casseliflavus* obtained from cattle (n = 102)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	99	97.1
1: Vir	1	1.0
2: Ery, Vir	2	2.0
TOTAL	**102**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 18: Distribution of single and multi-resistance amongst *E. hirae* and *E. casseliflavus* obtained from pigs (n = 38)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	10	26.3
1: Ery	19	50.0
1: Vir	1	2.6
2: Amp, Ery	1	2.6
2: Ery, Vir	7	18.4
TOTAL	**38**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 19: Distribution of single and multi-resistance amongst *E. hirae* and *E. casseliflavus* obtained from chickens (n = 33)

Resistance phenotype/genotype*	Frequency	Percentage
0: nil	7	21.2
1: Ery	15	45.5
1: Vir	1	3.0
2: Ery, Vir	10	30.3
TOTAL	**33**	**100**

* Resistance phenotypes/genotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 20: Distribution of MICs and occurrence of resistance among *E. hirae* and *E. casseliflavus* isolates from cattle (n=102), pigs (n=38) and chickens (n=33)

Antimicrobial	Animal species	% Resistant [95% Confidence Interval]		Distribution (%) of MICs												
				0.5	1	2	4	8	16	32	64	128	256	512	1024	2048
Ampicillin	Cattle	0	[0.0-3.6]			100										
	Pigs	3	[0.1-13.8]			89.5	2.6	5.3	2.6							
	Chickens	0	[0.0-10.6]			93.9	6.1									
Erythromycin	Cattle	2	[0.2-6.9]		88.2	6.9	2.9				2.0					
	Pigs	71	[54.1-84.6]		26.3		2.6				71.1					
	Chickens	76	[57.7-88.9]		18.2	3.0	3.0	3.0		3.0	69.7					
Gentamicin	Cattle	0	[0.0-3.6]						69.6		30.4					
	Pigs	0	[0.0-9.3]						71.1		29.0					
	Chickens	0	[0.0-10.6]						60.6		36.4	3.0				
Teicoplanin	Cattle	0	[0.0-3.6]	100												
	Pigs	0	[0.0-9.3]	100												
	Chickens	0	[0.0-10.6]	100												
Vancomycin	Cattle	0	[0.0-3.6]			83.3	15.7	1.0								
	Pigs	0	[0.0-9.3]			94.7		5.3								
	Chickens	0	[0.0-10.6]			78.8		21.2								
Virginiamycin	Cattle	3	[0.6-8.4]		95.1	2.0			2.0	1.0						
	Pigs	21	[9.6-37.3]		55.3	23.7	18.4	2.6								
	Chickens	33	[18.0-51.8]		54.6	12.1	6.1	15.2	12.1							

Vertical lines indicate breakpoints for resistance

The white fields denote dilution range tested for each antimicrobial. Values above the range denote MIC values greater than the highest concentration in the range. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration

7.2.3 *Campylobacter* spp.

Of the 133 *Campylobacter* spp. isolates from chickens, two were discarded for quality control reasons. Of the remaining 131 isolates 19.7% were tetracycline-resistant and 9.8% were erythromycin-resistant. Two of the isolates (1.5%) exhibited resistance to both antimicrobials (**Table 21**). None of the *Campylobacter* spp. isolated from chickens exhibited resistance to gentamicin, ciprofloxacin or nalidixic acid.

C. jejuni and *C. coli* show a difference in AMR patterns, with *C. coli* isolates showing a greater rate of resistance to macrolides [29, 30]. While the isolates of *Campylobacter* spp. in the current study were not identified to the level of *C. jejuni* and *C. coli*, an extensive epidemiological study in broiler flocks in Queensland found that 92% of all isolates were *C. jejuni* [31].

Table 21: Distribution of single and multi-resistant phenotypes amongst *Campylobacter* spp. obtained from chickens (n = 131)

Resistance phenotype*	Frequency	Percentage
0: nil	90	69.0
1: Ery	13	9.8
1: Tet	26	19.7
2: Ery, Tet	2	1.5
TOTAL	**131**	**100**

* Resistance phenotypes are given as a list of drugs to which resistance is expressed prefixed by the number of drugs to which resistance is expressed

Table 22: Distribution of MICs and occurrence of resistance among *Campylobacter* spp. isolates from chickens (n=131)

Antimicrobial	% Resistant [95% Confidence Interval]		Distribution (%) of MICs								
			0.5	1	2	4	8	16	32	64	128
Gentamicin	0	[0.0-2.8]		100							
Tetracycline	21	[14.7-29.4]		76.3	2.3			3.8	6.9		10.7
Erythromycin	11	[6.6-18.2]		51.9	13.0	15.3	7.6	0.8			11.5
Ciprofloxacin	0	[0.0-2.8]	100								
Nalidixic acid	0	[0.0-2.8]				55.0	32.1	13.0			

Vertical lines indicate breakpoints for resistance

The white fields denote dilution range tested for each antimicrobial. Values above the range denote MIC values greater than the highest concentration in the range. MICs equal to or lower than the lowest concentration tested are given as the lowest concentration

8. Discussion

8.1 Intrinsic resistance

None of the enterococci in this study exhibited high-level gentamicin resistance. Enterococci are intrinsically resistant to many antimicrobials and there is concern about high-level gentamicin resistance (MIC >512 μg/mL) as synergistic activity (usually with glycopeptides or penicillin) is lost.

E. faecalis have intrinsic resistance to streptogramin antimicrobials [32]. However, virginiamycin and the related human analogue quinupristin/dalfopristin are bactericidal against *E. faecium* [32]. Thus, in this study the extent of resistance to virginiamycin in *E. faecium* isolates is notable. It should be further noted that quinupristin/dalfopristin resistance has been found in *E. faecium* isolates obtained from pigs and chickens that have not been exposed to virginiamycin [33] but probably exposed to macrolide antimicrobials such as tylosin (see MLS_B phenotype below).

8.2 Cross-resistance

There is a general acceptance that erythromycin resistance in animal isolates increases when erythromycin or tylosin has been used [34]. Therefore it's possible erythromycin resistance detected in enterococcus in this study, is in whole or part, due to cross-resistance due to the use of tylosin.

8.3 Macrolide-Lincosamide-Streptogramin (MLS_B) resistance

Resistance to the macrolides, lincomycin, spectinomycin and streptogramins can be due to a related type of resistance as these classes of antimicrobials have the same target in the bacterial cell. In particular, the existence of cross-resistance to macrolides, lincosamide and streptogramin B (the so-called MLS_B resistance) is an emerging issue [35]. This cross-resistance may occur when bacteria that are resistant to erythromycin rapidly develop resistance to lincosamides when exposed to erythromycin. Bacteria showing high-level resistance to all MLS antimicrobials are rapidly selected from the inducible strains during treatment with either lincosamides or macrolides [36]. As a result, resistance to one of these classes can give the appearance of resistance to all three. In the pilot program we did not test for resistance to a combination of lincomycin and spectinomycin but a number of enterococci isolates were resistant to erythromycin and virginiamycin. Without further genetic studies it is not possible to say whether this latter resistance is due to the presence of genes encoding resistance to virginiamycin alone, erythromycin alone or erythromycin plus virginiamycin (i.e. MLS_B) resistance.

8.4 Co-selection for resistance

The genes coding for resistance in bacteria can be located on the bacterial chromosome or on plasmids (independent units of extrachromosomal DNA that can transfer from one bacterial organism to another) [37]. A complication is that sometimes chromosomal genes (transposons) can transfer from the chromosome to plasmids and then back into the chromosome. While they are on plasmids they can transfer to other bacteria. The first plasmids describing resistance genes were called R plasmids [38]. They were found in *E. coli* in the 1960s and were one of the stimuli leading to the Swann Report [39]. An early R plasmid was found that carried genes for resistance to

sulfonamides, tetracycline and chloramphenicol. Large numbers of other resistance plasmids have been found since. Many resistance genes are found on the chromosome too, many of these are in transposons (e.g. vancomycin resistance). Sometimes the area of the chromosome contains a specialised structure called an integron which attracts large numbers of resistance genes (sometimes ten or more) and inserts them into the chromosome (sometimes into a transposon) [38]. Thus resistance genes are very mobile and readily spread between bacteria [38].

In this pilot study co-selection could account for some of the multiple-resistant strains detected (e.g. *E. coli* resistant to tetracycline, chloramphenicol, florfenicol and ampicillin). Co-selection has also been reported to be a feature for tetracycline and erythromycin resistance in enterococci [40].

9. Concluding remarks

The pilot program demonstrated that existing resources and processes within DAFF were able to be adapted and utilised to support a surveillance program for AMR in animals. Access to the NRS for sourcing of materials, generation of sample forms and data management was particularly effective. In addition, the use of AQIS On-Plant Veterinary Officers at cattle and pig abattoirs, and QA officers at poultry processing plants, in carrying out sample collection was invaluable due to their knowledge and expertise in those environments and animals.

The pilot program was successful in achieving the targets for the number of samples required per animal species. However, the pilot program shows that, in future, adjustments need to be made to ensure that the isolation rates are within the target range. The number of *E. coli* isolates far exceeded the target, whilst the number of *Campylobacter* spp. isolates was just under the target.

The laboratory aspects worked well. However, in the future, DAFF is likely to explore other methods and practices for determining MICs that are less resource and labour intensive. The laboratory needs for surveillance in humans and foods will assist in identifying and exploring automated methods of testing. Another aspect for further work is the strengthening of QA through inter-laboratory comparisons of results and method validation. Furthermore, the pilot program highlighted a need for streamlined reporting, both from the primary isolation laboratories to the MIC testing laboratories, and from all laboratories to the NRS. One way of doing this would be to develop an electronic system with a common template. In particular, common field names and a system for numbering samples and isolates would assist in reducing errors.

A formal evaluation of the pilot program will be undertaken by DAFF, in consultation with the Technical Reference Group and EAGAR, to identify the ongoing needs for a full surveillance program for AMR in animals. The evaluation will also take into consideration surveillance activities underway for AMR in humans and food to ensure shared approaches and integration of the three areas of surveillance.

Appendix 1: Design and scope

The pilot program focused on those species of food-producing animals where antimicrobials are most likely to have been used in ways that would result in AMR (in-feed or in-water use or frequent use of injectable preparations, or antimicrobial classes of high importance in human medicine). Cattle, slaughter pigs and broiler chickens were therefore included in the study. Cattle were divided into 'feedlot', 'grass-fed' and 'dairy' sub-types as animals from the respective production systems are expected to have very different exposure to antimicrobials. Sheep were excluded from the study because historically, antimicrobial use in this industry has been very low, although this class of livestock would be considered for inclusion in any future surveillance program. The pilot program was not designed to explore the reasons why different resistance phenotypes do or do not occur in livestock but to merely measure the amount present.

In order to address public health concerns, the organisms of interest were confined to certain zoonotic and commensal/indicator bacteria in a similar manner to other national surveillance programs for AMR in food-producing animals. The resistance status of *Campylobacter* spp. from broiler chickens was assessed as *Campylobacter* infections in humans are commonly associated with poultry. *E. coli* and *Enterococcus* spp. are commensal bacteria common in the gut of animals and man. As these organisms also respond to the selective pressure of antimicrobials, they commonly provide useful information for surveillance programs. These organisms may also act as a reservoir of resistance genes that can be transferred to pathogens (human or animal) or to other commensals. The resistance status of *E. coli* and *Enterococcus* spp. were studied in all three animal species included in the pilot program.

Although drug-resistant *Salmonella* spp. are a prominent issue in food safety and veterinary public health, the prevalence of *Salmonella* spp. in most animal populations is usually too low for their resistance to be assessed in a survey of this type. Thus, *Salmonella* spp. are being evaluated in a separate project funded by the Australian Government Department of Health and Ageing and DAFF. A retrospective analysis is being conducted on 10 years of national data (isolates from humans, animals and food) from the National Enteric Pathogens Surveillance Scheme and Australian Salmonella Reference Centre.

The main criteria for the inclusion of particular antimicrobials to assess the resistance status of bacterial isolates were the importance of both the drug and resistance outcomes to public health. Thus, some antimicrobials used exclusively in human medicine were included where there is the potential for resistance to arise because a related drug is used in food-producing animals, or where there is a prominent concern about resistance to a particular antimicrobial class in human medicine (e.g. third-generation cephalosporins, fluoroquinolones). The EAGAR system of rating the importance of antimicrobials according to their use in human medicine provides useful background to these issues [19]. In addition, some drugs were included that are not regarded as highly important in human medicine (e.g. tetracyclines), but have been extensively used in food-producing animals and for which multiple-resistance is commonly found in commensals and pathogens.

Appendix 2: Bacterial culturing protocol for *E. coli, Enterococcus* spp. and *Campylobacter* spp.

Bacterial isolation

- cattle and pig samples were cultured for *E. coli* and *Enterococcus*
- poultry samples were cultured for *E. coli*, *Enterococcus* and *Campylobacter*

E. coli

- inoculate 1g (mL) of caecal contents in 10 mL MacConkey (GN) broth;
- incubate aerobically at 37°C for 18-24 hours;
- streak out a loopful of caecal suspension onto MacConkey agar for isolation of *E coli* and incubate at 37°C for 18-24 hours;
- select lactose fermenting colonies (violet/pink) and streak for isolation on a new MacConkey agar plate, incubate as above.
- examine the MacConkey plate for purity. If it is not pure repeat the previous step.
- select lactose-fermenting colonies and plate onto *E. coli* chromogenic medium (Oxoid) and incubate at 37°C for 18-24 hours;
- select colonies with a strong chromogenic reaction (purple) and plate onto nutrient agar (incubate as above)
- perform indole spot test and retain positive colonies for storage and resistance testing.

Enterococcus spp.

- inoculate 1g (mL) of caecal contents into 10 mL of brain heart infusion broth;
- incubate aerobically at 37°C for 18-24 hours;
- subculture broth cultures onto Enterococcosel (BD) plates and incubate aerobically at 37°C for 18 – 24 hours (better results with extended incubation, e.g. over 24 hours);
- select aesculin positive colonies (white colonies with black halo) and plate onto blood agar for purity;
- confirm identity using oxidase (negative), growth on 6.5% NaCl agar, growth on nutrient agar at 42°C, catalase (positive) and production of pyrrolidonyl arylamidase.

Campylobacter spp.

- add 1g (mL) of the caecal contents to 9 mL of Preston broth with antibiotic supplement in a 10 mL tube and incubate under microaerophilic conditions at 42°C overnight.
- plate a loopful of the broth culture onto Campylobacter Blood-Free Selective Agar Base (modified CCDA) agar plates (with antibiotic supplement) and incubate at 42°C for 48 hours under microaerophilic conditions;
- select smooth, flat translucent, colourless to grey-brown colonies with an irregular edge;
- confirm identity using Gram stain (Gram negative), motility (rapid, darting), oxidase and catalase (both positive).

Storage of isolates

- Scrape the surface growth from a pure culture into a freezer vial containing 1 mL of Brucella broth with 15% glycerol or use commercial cryostorage systems such as MicroBank or Protect.
- Snap freeze and store in duplicate at -70°C.

Appendix 3: Protocols for Antimicrobial Susceptibility Testing

AST on *Campylobacter* spp. and *Enterococcus* spp. was carried out according to a standardised agar dilution procedure. The broth microdilution technique was used for testing *E. coli*. Full details of these procedures are contained in Section 4 (Broth and Agar Dilution Susceptibility Testing) of NCCLS document M31-A2 [21].

CAMPYLOBACTER SPP.

Preparation of isolates and quality control strain
On arrival in the laboratory, the isolates were plated onto 5% sheep blood agar (SBA) and incubated at 37°C in a modified atmosphere for 48 hours. A single colony was subcultured onto a fresh SBA and incubated as before. From this SBA, a heavy suspension was prepared in Brucella broth with 15% glycerol and the suspension stored at -70°C. The recommended quality control strain of *C. jejuni* ATCC 33560 was stored in a similar manner. As required, the stored isolates and the control strain were resuscitated on 5% SBA and incubated for 24 hours in a modified atmosphere. For each isolate and the control strain, a sterile saline suspension was prepared and adjusted for turbidity.

Inoculation of agar plates
The adjusted bacterial suspension was loaded into the master plate of a Mast Multipoint Inoculator (SCAN 100). The antibiotic containing agar plates (which were pre-dried) were then inoculated. The plates were then left at room temperature until the moisture in the inoculum spots had dried into the agar. The plates were then incubated for 20-24 hours at 37°C in a modified atmosphere. In each run, two SBA plates were inoculated – one as the first plate and the other as the last plate.

Determining Endpoints
The SBA plates were checked for purity. Only cultures which showed pure growth on both SBA plates were accepted. The MIC for all isolates and the reference strain was determined as the lowest concentration of antimicrobial agent that completely inhibited colony formation. Only those runs in which the reference strain gave the expected results were accepted as valid runs.

E. COLI

Preparation of isolates and quality control strain
Each isolate was resuscitated by taking swabs from the transport medium and inoculating them onto tryptic soy agar plates for 20-24 hours incubation at 35ºC. Four to five representative colonies morphologically consistent with *E. coli* were selected and suspended in saline. The suspension was adjusted to 1-2 x 10^8 Colony Forming Units (CFU)/mL. A sub-sample of this suspension was further diluted to 1-2 x 10^6 CFU/mL in Mueller-Hinton Broth (cation adjusted). This bacterial suspension was used to inoculate 96 well microtitre plates containing the serially diluted antibiotics. Each microtitre plate included a positive and a negative growth control. *E. coli* ATCC 25922 isolate was used as a control. One plate in approximately 10 was inoculated with standardised suspensions of this organism. The density of this *E. coli* ATCC 25922 inocula was tested on 6 occasions. As an additional quality control, approximately 1 in 20 isolates were tested in duplicate. On those occasions where

results differed by more than one dilution the isolate was tested a third time and the last result recorded.

ENTEROCOCCUS SPP.

Preparation of isolates and quality control strain

Isolates were received and stored at 4°C. The isolates were streaked onto Columbia agar plates containing 5% horse blood and incubated at 37°C for 24 hours in the presence of 5% CO_2. A single colony was selected and subcultured on the blood agar plate and incubated under the same conditions. Overnight growth culture was collected, transferred to Snap Freeze medium (Oxoid) and stored at -80°C. Control organisms of *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 and *S. aureus* ATCC 29213 were used for quality control in each run.

Inoculation of agar plates

Isolates in the Snap Freeze medium from the -80°C freezer were resuscitated by streaking on Columbia blood agar plates. After over night incubation at 37°C, a single colony was selected and further inoculated onto blood agar plates. Inocula for the susceptibility testing were prepared by suspending the cultures in sterile saline adjusted for turbidity. Final inocula contained 10^4 organisms/spot. A replicator was used for the inoculation of those cell suspensions onto Mueller-Hinton agar plates containing various concentrations of antibiotics. Thirty-six isolates were inoculated on one plate. In addition to the Mueller-Hinton agar plates, biochemical plates for the identification of the enterococci to species level were also included [*].

[*] biochemical tests to speciate enterococci: pigment, motility, utilisation of pyruvate, fermentation of -arabinose, raffinose, sucrose, xylose, melibiose, sorbitol, ribose, trehalose, mannitol, maltose; hydrolysis of glucopyranoside, aesculin and pyrrolidonyl-β-naphthylamide (PYR).

Determining Endpoints

After over night incubation at 37°C under atmosphere, the plates were read for the determination of MIC and biochemical plates were read in the Institute of Medical and Veterinary Sciences, Adelaide, South Australia.

Genotyping of enterococci

Multiplex PCR assays for *van*A, *van*B, *van*C1, *van*C2 and *van*C3 were carried out according to the method of Bell et al [41].

Appendix 4: Data management and analysis

Sample Request Forms and sample numbers were generated by the NRS using the same process as the residue monitoring programs. All data generated from abattoirs and laboratories, including descriptive data and antimicrobial MICs, were entered into the NRS database. The data were checked for validity and exported for analysis in the Stata analysis package (version 8.2). Exact binomial confidence limits were estimated in Stata for prevalence data using the 'Clopper-Pearson' method.

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