Discussion

The pilot survey for AMR bacteria in Australian food is designed to provide data that can be used to estimate the prevalence of AMR bacteria in food purchased at retail outlets. The survey was limited to those food / bacterium combinations where the expected prevalence of the target organism was projected to be >10%. Four retail foods; poultry, beef, pork and lettuce along with four target organisms; *Campylobacter, Salmonella, E. coli* and *Enterococcus* constitute the nine food / bacterium combinations included in the survey. The initial sampling plan for the survey utilised available Australian and international prevalence data to estimate the number of samples required to generate 100 isolates. Changes to the sampling plan have occurred during the survey in response to the monthly prevalence data progressively generated. Increases to the number of samples being tested for *Campylobacter* in poultry and *E. coli* in pork have been made during the survey to provide the greatest opportunity for the 100 isolate goal per food / bacterium combination to be met. These increases were offset by similar sized reductions in the collection and testing of lettuce for *E. coli*. Both early and subsequent data indicated that the prevalence of *E. coli* on lettuce was likely to be 9-10 fold lower than initially anticipated. Following the sampling modifications indicated, seven food / bacterium combinations met and exceeded projected prevalences and the 100 isolate goal was successfully reached. Due to reduced prevalences, the 100 isolate goal for pork / *E. coli* and lettuce / *E. coli* combinations were not achieved. With respect to pork / *E. coli*, this does not substantially modify the confidence in AMR detection. However, firm conclusions concerning the prevalence of AMR in lettuce / *E. coli* isolates cannot be made with confidence due to the extremely limited isolation of *E. coli* from this food source.

The results of testing isolates from 12 monthly sampling rounds for AMR indicates that resistance to the majority of antimicrobials tested is low (<10%). However, it is notable that the data indicates trends of higher prevalences of AMR in particular food / bacterium combinations. In *E. coli* from poultry and pork the prevalence of AMR for ampicillin (38% and 28.2%), streptomycin (19% and 17.4%), tetracycline (47% and 44.5%) and trimethoprim / sulfamethoxazole (22% and 13%) was notably higher than in beef *E. coli* isolates where prevalence of resistance to these antimicrobials was ≤11%.

Similarly, *E. faecalis* isolates from poultry were distinguished from beef and pork *E. faecalis* isolates by high prevalences of resistance to erythromycin (48%) and tetracycline (76%). The absence of detection of *Enterococcus faecium* amongst Enterococcus isolates from all retail meat sources was unexpected. A previous study of retail meat (5) found a

predominance of *E. faecalis* on retail meats including chicken, beef and pork, however, in contrast to the present study both *E. faecalis* and *E. faecium* were routinely isolated. It is not readily apparent why no *E. faecium* were isolated in the present study and this observation merits further investigation.

In *Campylobacter* isolates, low resistance to the test antimicrobials was observed. The prevalence of resistance to tetracycline was 1%. High levels of tetracycline resistance have been observed in similar studies throughout the world and the absence of resistance in Australian *Campylobacter* from poultry is notable (see below).

The current Australian food AMR data has been compared with data from the international AMR surveys: The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) (4), Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (2) and the United States of America National Antimicrobial Resistance Monitoring System (NARMS) (3). While each national AMR monitoring program collects and presents data in specific formats, within these limitations the broad comparisons presented below have been possible. The following comparisons are considered by retail food type reported for year 2005 in each of the abovementioned programs. For the purpose of this discussion variations in AMR prevalence which are ≥ or ≤ 10% are designated as notable and are indicated below:

- In retail chicken, notable differences in AMR prevalence in the bacteria *Salmonella*, *E. coli*, *Enterococcus* and *Campylobacter* are reported.
    - *Salmonella* (US and Canada) possess a greater prevalence of resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, streptomycin and tetracycline.
    - *E. coli* (US and Canada) possess a greater prevalence of resistance to amoxicillin/clavulanic acid, ceftiofur, gentamicin and streptomycin.
    - *Enterococcus* (US, Canada and Danish imported product) possess a greater prevalence of resistance to kanamycin, streptomycin and flavomycin (US only).
    - *Campylobacter* (US, Canada and Danish imported product) possess a greater prevalence of resistance to ciprofloxacin, nalidixic acid and tetracycline.
- In retail beef, notable differences in AMR prevalence in the bacteria *E. coli* and *Enterococcus* are reported.
    - *E. coli* (US) possess a greater prevalence of resistance to tetracycline.

    - *Enterococcus* (US) possess a greater prevalence of resistance to tetracycline and flavomycin.

- In retail pork, notable differences in AMR prevalence in the bacteria *E. coli* and *Enterococcus* are reported.
    - *E. coli* (Australia) possess a greater prevalence of resistance to ampicillin.
    - *Enterococcus* (US) possess a greater prevalence of resistance to tetracycline and flavomycin.

The testing of isolates collected as part of the survey for AMR provides a snapshot of the prevalence and types of AMR bacteria present in selected retail foods in Australia. The use of Sensititre equipment and panels has generated data that is internationally equivalent and which can be compared to available overseas information. Whilst the survey data cannot be used to directly provide information about the development of antimicrobial resistance, it provides baseline data suitable for future use in the determination of antimicrobial resistance trends at the Australian retail food level. When correlated with similar Animal Isolates and Human Clinical AMR surveys this data may be useful in managing and controlling AMR development in the Australian community.

References

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Appendices

Appendix A. Protocols for the preparation of retail product samples and isolation of bacteria of concern for the AMR in retail foods pilot surveillance program.

Sample preparation

Poultry (rinse fluid)

- Place whole bird into a sterile plastic bag of suitable size
- Add 500 ml of buffered peptone water (BPW) into the plastic bag
- Shake and massage sample vigorously for 2 min
- Release the rinse fluid into a sterile sample container by cutting off the corner of the bag and allowing the fluid to drain into a container

Beef (initial suspension)

- Place 25g of minced beef into a sterile stomacher bag
- Add 225 ml of BPW
- Stomach for 1 min

Pork (initial suspension)

- Aseptically remove 25g of pork adipose tissue and place in a sterile stomacher bag
- Add 225 ml of BPW
- Stomach for 1 min

Lettuce (initial suspension)

- Aseptically cut a cross-section through the entire lettuce at approximately 5cm to 7cm from the stem end.
- Prepare this stem end portion by cutting and mixing and then remove 25g as the test sample portion and place into a sterile stomacher bag
- Add 225 mL BPW
- Stomach for 1 min

Bacterial isolation

Escherichia coli

- inoculate 50 mL of rinse fluid or initial suspension in 50mL of double strength EC broth;
- incubate aerobically at 37°C for 18-24 hours;
- streak one loopful of incubated EC broth-rinse fluid mix onto eosin methylene blue (EMB) agar and incubate at 37°C for 18-24 hours;
  select a typical *E. coli* colony (dark green metallic sheen by reflected light and dark purple centres by transmitted light) and streak for isolation on tryptic soy agar containing 5% sheep blood (TSA-B), incubate as above;
- examine the TSA-B plate for purity. If it is not pure repeat the previous step;
- perform rapid biochemical identification of isolate using spot indole test in conjunction with Simmons citrate tube test or use an appropriate commercially available biochemical identification kit (eg Microbact 12E);
- store confirmed isolates in duplicate at -70°C.

Enterococcus spp.

- inoculate 50 mL of rinse fluid or initial suspension into 50 mL of double strength Enterococcosel broth;
- incubate aerobically at 37°C for 18-24 hours;
- If no growth or blackening of the Enterococcosel broth-rinse fluid mix can be observed, sample is negative and can be discarded;
- Streak one loopful of broths exhibiting growth or blackening onto Enterococcosel agar plates and incubate aerobically at 37°C for 24-48 hours;
- examine Enterococcosel agar plates for typical Enterococci colonies (aesculin hydrolysis) and plate onto Columbia agar containing 5% sheep blood (CBA). Incubate aerobically at 37°C for 24 – 48 hours;
- examine CBA plate for purity. If it is not pure repeat the previous step;
  confirm isolates as *Enterococcus* spp;
- identify *Enterococci* spp. biochemically or by PCR;
- store confirmed isolates in duplicate at -70°C.

Campylobacter spp.

- inoculate 50 mL of rinse fluid into 50 mL of double strength Preston broth without antibiotic supplement and incubate at 37°C for 2 hours;

- after 2 hours incubation add 0.4 mL of antibiotic supplement (B2.4 AS5013.6) to 100 mL of broth culture. Broths are then incubated under microaerophilic conditions at 42°C for 46 hours;
- plate a loopful of the broth culture onto modified CCDA agar plates (with antibiotic supplement) and incubate at 42°C for 48hrs under microaerophilic conditions;
- examine m-CCDA plates for smooth, flat translucent, colourless to grey-brown colonies with an irregular edge and plate onto blood agar;
- confirm identity using gram stain, motility, oxidase and catalase and identify species of *Campylobacter* using commercial identification kit;
- store confirmed isolates in duplicate at -70°C.

Salmonella spp.

- incubate 100 mL of rinse fluid aerobically at 37°C for 18-24 hours;
- transfer 0.1 mL of the enrichment to 10 mL of Rappaport-Vassiliadis medium with soya (RVS) and incubate aerobically at 41.5°C for 24 hours (do not exceed 42.5°C);
- transfer 1 mL of the enrichment to 10 mL of Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn) and incubate aerobically at 37°C for 24 hours;
- plate a loopful of RVS and MKTTn enrichment onto xylose lysine deoxycholate agar (XLD) and brilliant green agar (BGA) and incubate aerobically at 37°C for 24 hours; examine XLD and BGA plates for typical *Salmonella* colonies; colonies will have a black centre surrounded by a lightly transparent zone of red on XLD and will be red colonies surrounded by bright red medium on BGA. Plate typical *Salmonella* colonies onto nutrient agar and incubate at 37°C for 24 hours;
- confirm isolates as *Salmonella* spp. biochemically and serologically;
- store confirmed isolates in duplicate at -70°C

NB: all strains considered to be *Salmonella* must be sent to the approved *Salmonella* serotyping laboratory at MDU, Melbourne University for definitive typing.

Storage of isolates

Scrape the surface growth from a pure culture into a commercial cryostorage system such as MicroBank or Protect™. Snap freeze and store in duplicate at – 70°C.

Appendix B. Sensititre custom and standard Campylobacter plate formats for antimicrobial susceptibility testing

AUSVN – Gram negative bacteria

	1	2	3	4	5	6	7	8	9	10	11	12
A	**CIP**	CIP	CIP	CIP	CIP	CIP	**AMP**	AMP	AMP	AMP	AMP	AMP
	0.125	0.25	0.5	1	2	4	2	4	8	16	32	64
B	**NAL**	NAL	NAL	NAL	NAL	NAL	**SXT**	SXT	SXT	SXT	SXT	SXT
	2	4	8	16	32	64	0.12/2.38	0.25/4.75	0.5/9.5	1/19	2/38	4/76
C	**FFN**	FFN	FFN	FFN	FFN	FFN	**AUG2**	AUG2	AUG2	AUG2	AUG2	AUG2
	2	4	8	16	32	64	1/0.5	2/1	4/2	8/4	16/8	32/16
D	**XNL**	XNL	XNL	XNL	XNL	XNL	**CHL**	CHL	CHL	CHL	CHL	CHL
	0.5	1	2	4	8	16	2	4	8	16	32	64
E	**GEN**	GEN	GEN	GEN	GEN	GEN	**FAZ**	FAZ	**FOX**	FOX	FOX	FOX
	1	2	4	8	16	32	8	16	0.5	1	2	4
F	**AXO**	AXO	AXO	AXO	AXO	AXO	AXO	AXO	AXO	FOX	FOX	FOX
	0.25	0.5	1	2	4	8	16	32	64	8	16	32
G	**TET**	TET	TET	TET	**KAN**	KAN	KAN	KAN	**MERO**	MERO	MERO	MERO
	4	8	16	32	8	16	32	64	1	2	4	8
H	**FOT**	FOT	FOT	FOT	FOT	FOT	FOT	FOT	FOT	**STR**	STR	**POS**
	0.25	0.5	1	2	4	8	16	32	64	32	64	**CON**

ANTIMICROBIALS

AUG2	Amoxicillin / clavulanic acid 2:1 ratio
AMP	Ampicillin
FAZ	Cefazolin
FOT	Cefotaxime
FOX	Cefoxitin
XNL	Ceftiofur
AXO	Ceftriaxone
CHL	Chloramphenicol
CIP	Ciprofloxacin
FFN	Florfenicol
GEN	Gentamicin
KAN	Kanamycin
MERO	Meropenem
NAL	Nalidixic Acid
POS	Positive Control
STR	Streptomycin
TET	Tetracycline
SXT	Trimethoprim / sulfamethoxazole

AUSVP – Gram positive bacteria

	1	2	3	4	5	6	7	8	9	10	11	12
A	**TGC** 0.015	TGC 0.03	TGC 0.06	TGC 0.12	TGC 0.25	TGC 0.5	**AMP** 2	AMP 4	AMP 8	AMP 16	AMP 32	AMP 64
B	**PEN** 0.5	PEN 1	PEN 2	PEN 4	PEN 8	PEN 16	**DAP** 0.5	DAP 1	DAP 2	DAP 4	DAP 8	DAP 16
C	**SYN** 1	SYN 2	SYN 4	SYN 8	SYN 16	SYN 32	**VIR** 1	VIR 2	VIR 4	VIR 8	VIR 16	VIR 32
D	**FLV** 1	FLV 2	FLV 4	FLV 8	FLV 16	FLV 32	**TEI** 0.5	TEI 1	TEI 2	TEI 4	TEI 8	TEI 16
E	**GEN** 64	GEN 128	GEN 256	GEN 512	GEN 1024	GEN 2048	**LIN** 1	LIN 2	LIN 4	LIN 8	LIN 16	LIN 32
F	**ERY** 1	ERY 2	ERY 4	ERY 8	ERY 16	ERY 32	**TET** 4	TET 8	TET 16	TET 32	**STR** 512	STR 1024
G	**KAN** 128	KAN 256	KAN 512	KAN 1024	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32	STR 2048
H	**CHL** 2	CHL 4	CHL 8	CHL 16	CHL 32	**LZD** 0.5	LZD 1	LZD 2	LZD 4	LZD 8	VAN 64	**POS CON**

ANTIMICROBIALS

AMP	Ampicillin
CHL	Chloramphenicol
DAP	Daptomycin
ERY	Erythromycin
FLV	Flavomycin
GEN	Gentamicin
KAN	Kanamycin
LIN	Lincomycin
LZD	Linezolid
PEN	Penicillin
POS	Positive Control
SYN	Quinupristin / dalfopristin
STR	Streptomycin
TEI	Teicoplanin
TET	Tetracycline
TGC	Tigecycline
VAN	Vancomycin
VIR	Virginiamycin

CAMPY – *Campylobacter*

	1	2	3	4	5	6	7	8	9	10	11	12
A	**AZI**	AZI	AZI	AZI	AZI	AZI	AZI	AZI	AZI	AZI	AZI	AZI
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
B	AZI	**CIP**	CIP	CIP	CIP	CIP	CIP	CIP	CIP	CIP	CIP	CIP
	64	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
C	CIP	CIP	**ERY**	ERY	ERY	ERY	ERY	ERY	ERY	ERY	ERY	ERY
	32	64	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
D	ERY	ERY	**GEN**	GEN	GEN	GEN	GEN	GEN	GEN	GEN	GEN	**TET**
	32	64	0.12	0.25	0.5	1	2	4	8	16	32	0.06
E	TET	TET	TET	TET	TET	TET	TET	TET	TET	TET	**FFN**	FFN
	0.12	0.25	0.5	1	2	4	8	16	32	64	0.03	0.06
F	FFN	FFN	FFN	FFN	FFN	FFN	FFN	FFN	FFN	FFN	**NAL**	NAL
	0.12	0.25	0.5	1	2	4	8	16	32	64	4	8
G	NAL	NAL	NAL	**TEL**	TEL	TEL	TEL	TEL	TEL	TEL	TEL	TEL
	16	32	64	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4
H	TEL	**CLI**	CLI	CLI	CLI	CLI	CLI	CLI	CLI	CLI	CLI	**POS**
	8	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	**CON**

ANTIMICROBIALS

AZI	Azithromycin
CIP	Ciprofloxacin
ERY	Erythromycin
GEN	Gentamicin
TET	Tetracycline
FFN	Florfenicol
NAL	Nalidixic Acid
TEL	Telithromycin
CLI	Clindamycin
POS	Positive Control

Appendix C. FRSC AMR working group queries and response

Dear FRSC AMR Working Group

After reading the 12 monthly report from Food Science Australia (FSA), distributed by email, a couple of members had a few queries. Robert Barlow from FSA has kindly provided the following responses for the information of members:

1. Pat Blackall wrote "I note that the report predicts a shortfall of 4-6 isolates in the pork *E. coli* isolates. As there is no comment about the need for any altered sampling, I assume that the research group believes that this shortfall will not be of any significance?"

FSA has responded:
"It is unfortunate that achieving the 100 isolate goal for *E. coli* in pork appears unlikely despite increasing the number of tests to be conducted during the latter part of the survey. Based on current projections, a shortfall of 4-6 isolates is expected and consequently the impact on the final results has been questioned. The selection of 100 isolates as the target for each food / bacterium combination is based on having a 95% probability of detecting 1 AMR isolate in 100 at 3% prevalence. The equation used to generate this statement can be used to understand the significance of any shortfalls. If 90 isolates is used as the worse case scenario for *E. coli* in pork then the probability of detecting 1 AMR isolate in 90 at 3% prevalence is reduced to 93.5%. To put this in the context of the original proposition, the '93.5% probability of detecting 1 AMR isolate in 90 at 3% prevalence', is equivalent to saying that 'there is a 95% probability of detecting 1 AMR isolate in 100 at ~3.3% prevalence'.
We believe the reduction in confidence of detecting AMR is not sufficient enough to warrant the collection of further isolates and therefore additional sampling should not be considered at this point.

Appendix D. Identification of survey strengths, limitations and lessons learned

Identification of any specific strengths and limitations of the survey

The pilot survey for AMR bacteria in food has been conducted as a response to the recommendations outlined in the JETACAR report. It forms part of a three-pronged approach into investigating the prevalence of AMR bacteria in food production animals, retail foods, and clinical settings. The completion of the survey for AMR bacteria in food provides a snapshot view of the prevalence of AMR in nine food / bacterium combinations. The survey has representatively sampled the retail supply chain at the point of sale servicing approximately two-thirds of Australia's population and although not specifically designed to address seasonality, the completion of the survey over a 12 month period may provide seasonal and annual data on AMR and bacterial prevalence. However, it must be noted that the survey was designed to determine the AMR prevalence in 100 isolates per food / bacterium combination and not to determine seasonal or annual prevalences around AMR or bacterial contamination of retail foods. A survey designed to determine data in addition to total AMR prevalence in 100 isolates of each food / bacterium combination would require a different sampling regimen. In particular, the number of samples collected and the areas of collection would require substantial increase. Despite the peripheral limitations, the survey has, as designed, determined the level of AMR prevalence in bacteria from nine food / bacterium combinations. Additionally, the use of internationally recognised methods for the detection, isolation and AMR characterisation of isolation permits direct comparison with similar studies conducted overseas. Such comparisons will provide insight into the significance of AMR bacteria in Australian retail foods and will be used in the future to determine AMR trends over time and hence assist in evaluating the efficiency of interventions or changes in food chain antimicrobial use in Australian food producing systems.

The system of monthly progress reporting to DOHA/FRSC employed in the current survey has been beneficial for overall project success. The positive factors associated with monthly reporting have included:

- Early recognition and addressing of operational challenges
- Opportunity for provision of early expert opinion and advice
- Routine reporting to prompt timely management and reporting of any emerging issues.

A brief discussion of any lessons learned in relation to the methodology used to undertake the services

As previously mentioned, the methodologies employed to complete the survey are internationally recognised and therefore permit direct comparison with similar overseas studies. The use of standard methods for the isolation of bacteria from food and the use of standardised AMR testing equipment and procedures must therefore be an integral part of any future survey of this kind. That aside, there are some lessons that have been learnt whilst undertaking the services. These lessons deal specifically with the subcontractor-contractor interaction. The approach taken in this survey required sampling and testing for bacteria of concern to occur in each of four capital cities. Upon collection of six month's isolates, the subcontractor was responsible for delivery of the isolates to Food Science Australia for subsequent AMR testing of up to 100 isolates per food / bacterium combination. This approach differs slightly from that used in overseas studies where all samples collected are sent to regional testing laboratories where testing for bacteria of concern and AMR occurs at once. Whilst the lack of AMR testing infrastructure meant that the overseas approach was not possible it is easy to see in hindsight that substantial inefficiencies occur when the AMR testing is not completed at the time of bacterial isolation. Furthermore, the inability to recover some isolates (*Campylobacter* in particular) meant that the original sampling plan which was based on anticipated prevalence is somewhat compromised. Indeed the inability to recover *Campylobacter* from Protect™ beads in combination with a lower than expected prevalence required a significant increase in the number of samples tested for *Campylobacter* in the second half of the survey. Future surveys should establish an approach or infrastructure support such that bacterial isolation and AMR testing can occur at the same time and in the same laboratory. This would reduce the inefficiencies observed in the current survey and would ensure that 100% of isolates selected for AMR analysis were available for testing.

It is also recommended that future AMR surveillance be conducted by a single integrated project team with a high level of awareness of purpose of sample collection, standardised practices and overall project goals. The operation of an integrated project team will promote simplified lines of communication, resource allocation and responsibility for timely delivery. In summary, while it is recognised that factors and costs for optimal survey design, management and scientific integrity will often be constrained by limited resources (primarily financial), the following recommendations are strongly made for any future AMR surveillance programs:

- Overall project quality be enhanced through the operation of a single, integrated project team
- The number of persons in key project management/communication positions should be minimised in order to promote clear communication, accountability and project delivery.

Supplementary file note

Supplement 1 – Food AMR Pilot Survey – Bacterial Isolates

Details of each bacterial isolate from the survey are provided in the supplementary document **'Supplement 1 – Food AMR Pilot Survey – Bacterial Isolates'**.