A COMPREHENSIVE RISK FACTOR ANALYSIS OF POST-WEANING DIARRHOEA

2A-106

Report prepared for the
Co-operative Research Centre for High Integrity Australian Pork

By

Lechelle K. van Breda, Om P. Dhungyel, Michael P. Ward

Sydney School of Veterinary Science, The University of Sydney, Camden, Australia.>

April 2017

Pork CRC

Australian Government
Department of Industry, Innovation and Science

Business Cooperative Research Centres Programme

Executive Summary

Escherichia coli disease in pre- and post-weaned piglets has been identified by the Australian pork industry as an important issue affecting Australian pig producers. *E. coli* disease impacts young piglets resulting in substantial health and economic burden. The majority of research in Australia has focused on clinical samples submitted to veterinary diagnostic laboratories and characterising enterotoxigenic *E. coli* (ETEC) however, there has been less attention paid to identifying the underlying causes of these outbreaks.

The key risk factors promoting outbreaks of *E. coli* disease in southeastern Australian pig farms are largely unknown. An 'ecopathological approach', first developed in France as a way of identifying key environmental factors contributing to *E. coli* disease in piglets, was examined as part of this thesis. A snap-shot survey was conducted after recruiting the participation of 22 pig producers located across southeastern Australia. Survey data included the collection of questionnaire statistics and faecal samples on each farm to determine the impacts of ETEC *E. coli* disease on pre- and post-weaned piglets. A forwards step-wise regression analysis identified two factors, recent disease events (within the herd in the last 12 months) and the presence of bedding were statistically associated with the presence of post-weaning scours. Suggested changes to management, hygiene and biosecurity protocols could be an effective control method for *E. coli* disease in post-weaned piglets.

Antimicrobial resistance is a global concern. Antibiotics administered to livestock are generally a synthetic derivative of those used in human medicine, belonging to the same antibiotic classes. The concern is that animals and humans share the same bacteria so antimicrobial resistant bacteria from animals could be transmitted to humans or vice versa. This raises public health concerns associated with the transmission of antimicrobial resistant genes. *E. coli* collected as part of the ecopathological survey in this thesis were screened for susceptibility to human and veterinary antibiotics. Analysis of *E. coli* isolates revealed 6.1% (20/325) resistance to third-generation cephalosporins (3GC), further genetic analysis revealed six different extended spectrum β-lactamase (ESBL) genes, *bla*$_{\text{CTX-M-1, -14, -15, -27}}$, *bla*$_{\text{SHV-12}}$ and bla$_{\text{CMY-2}}$–like, four of which have not been previously reported in Australian pigs. The prevalence of 3GC resistance was higher in non-pathogenic (non-ETEC) isolates than those from clinically normal (non-diarrhoeal) samples. Non-ETEC *E. coli* could therefore act as an antimicrobial resistance gene reservoir in the piglet pen environment and surveillance for emerging antimicrobial resistance such as to 3GC antibiotics should include clinically normal pigs and the pen environment.

Wildlife has been identified as a reservoir of antimicrobial resistance. Although feral pigs are known to be a potential reservoir of exotic disease transmission, less is known about their capacity to spread antimicrobial resistance. We screened *E. coli* isolates collected from a remote feral pig population with little contact with agriculture or livestock for susceptibility to veterinary antibiotics to determine their natural carriage of antimicrobial resistance. A moderate prevalence of resistance was observed to sulfadimethoxine (50.4%; 58/115) and florfenicol (27.0%; 31/115). The same *E. coli* isolates were also screened for disinfectant resistance, which could be implemented as an effective control method. *E. coli* isolates were susceptible to five of the six disinfectants screened. Feral pig *E. coli* could be a potential reservoir of antimicrobial resistance in the environment with possible implications for domestic livestock, however, most disinfectants could be used in conjunction with other biosecurity and management protocols to reduce *E. coli* antimicrobial resistance transmission.

E. coli disease is dynamic, therefore determining the influence of risk factors for pre- and post-weaning *E. coli* diarrhoea disease is likely to vary between pig herds. Monitoring *E. coli* disease over the period of one year is likely to give a better insight into identifying important risk factors. A longitudinal study assessing pre- and post-weaning *E. coli* disease was conducted in a small pig herd. The number of male piglets per pen and sow condition score at weaning were identified as key risk factors. Compared to different types of farrowing accommodation, no significant difference was observed in pre-weaning *E. coli* diarrhoea prevalence. When assessing post-weaning diarrhoea prevalence on the same farm, the presence of β-haemolytic *E. coli* and the season and month piglets were weaned, were identified as statistically significant risk factors. At peak times of post-weaned diarrhoea in piglets, high diversity of *E. coli* clones could be observed, suggesting a single clone was not responsible for the persistent outbreaks. Therefore, implementing changes to management strategies to reduce or change the impact of risk factors associated with *E. coli* disease are likely to reduce the need to use antibiotics and offer a sustainable long term solution to reducing *E. coli* diarrhoea in piglets.

Diarrhoea has significant detrimental impacts on piglet health. Identifying key risk factors that can be minimised to reduce the impact of *E. coli* diarrhoea outbreaks has many positive associations with improved piglet health, such as reduced antibiotic usage and veterinary bills as well as growth in profit margins and productivity. A consistently healthy herd will also assist in sustaining a high food safety standard with positive implications for human consumption. Surveillance of antibiotic and disinfectant resistance in pre- and post-weaned piglets is important for the future of Australian pig production. The Australian pork industry is in the process of developing such a surveillance program in pigs, but currently the data for estimating the occurrence and impact of antimicrobial resistance are limited to isolates recovered from diagnostic samples collected during the investigation of clinical disease. Resistance surveillance should include indicator bacterial species and sampling of healthy pigs and their environment. An understanding of the interaction between humans, pigs and the environment using a One Health approach is needed to inform strategies to prevent and control emerging resistance.

Contents

Background

Post-weaning diarrhoea caused by *Escherichia coli* remains a major disease that limits production in pig farms worldwide. Pathogenic *E. coli* – in particular enterotoxigenic *E. coli* (ETEC) may express fimbrial adhesins (F4 (K88), F5 (K99), F6 (987P), F18 and F41) (Nagy and Fekete, 1999) and enterotoxins, (heat-stable, STa and STb, Stx2e and heat-labile, LT) (Zhang et al., 2006), that are associated with more severe disease in piglets (Fairbrother et al., 2005; Nagy and Fekete, 2005). ETEC colonise the lower intestine via these fimbrial adhesins and the production of enterotoxins results in diarrhoea ("scouring") that can lead to death if left untreated.

Australian pig herds regularly experience outbreaks of pre- and post-weaning diarrhoea attributed to *E. coli*, resulting in reduced growth rates, high medication costs and high morbidity and mortality (Fairbrother et al., 2005). Madec and Josse (1983) used an ecopathological approach to identify 10 key 'risk factors' from among 515 environment factors measured in a study of diarrhoea incidence in 89 French pig herds. A longitudinal study on Western Australian pig herds (Buddle et al., 1997; Skirrow et al., 1997) assessing a comprehensive French risk factor protocol developed by Madec (1994), failed to identify reliable risk predictors of post-weaning diarrhoea**.** It has been estimated that piglet scours cost the Australian pig industry more than $7 million each year (DAF, 2010) but there are scant information regarding the epidemiology of *E. coli* disease in modern pig production systems in Australia, or of management practices that contribute to or ameliorate disease outbreaks.

Experiment 1: Pre- and post-weaning scours in southeastern Australia: a survey of 22 commercial pig herds and characterisation of *Escherichia coli* isolates

(Refer to Appendices 1–3)

Introduction

Australian pig herd management strategies have evolved substantially over the last 20 years with weaners now commonly raised on deep litter (Eco shelters), and in large groups of >200 piglets per shelter. Other important changes include; sow group housing, phasing out of sow stalls, improvements in diet and nutrition, improvements in disease management through vaccines and strict biosecurity procedures, increases in productivity and an increase in the number of free-range herds. It is therefore important to determine whether previously identified risk factors for post-weaning scours remain important and whether other factors need to be considered. Current estimates of the prevalence of pre- and post-weaned piglet scours in commercial pig farms in the major pig-raising area of southeastern Australia are lacking. Published studies on management practices and other factors that may contribute to outbreaks of post-weaning scours are also needed. The aim of the current study was to estimate the within-herd prevalence of pre- and post-weaning scours and to report the management practices – including current disease and biosecurity, environmental factors and reproductive practices, housing and herd management and their association with post-weaning scours in 22 representative pig herds across southeastern Australia.

Methodology

Study design

The study was designed as a cross-sectional survey to estimate point prevalence of *E. coli* disease in piglets at the pen and herd levels. A total sample size of 22 herds was chosen, based on the available funding and ability to visit and sample all herds over a large geographical area during a relatively short time period. All animal sampling procedures and interactions were conducted in strict accordance with the recommendations made by The University of Sydney Animal Ethics Committee. The protocol was approved by The University of Sydney Animal Ethics Committee (Approval number: N00/7-2013/3/6002).

Farms were identified via swine veterinarians and in addition, farms were recruited at the 2013 Bendigo Pig Fair, Victoria and by contacting farmers directly via industry membership. A preliminary questionnaire was conducted over the telephone to determine the owner/manager's willingness to participate in the study and the suitability of their herd (Supplementary material S1). For example, herds with less than 40 sows were not included because the number of piglets available for sampling would not meet the required sample size for inclusion in the study. All questions in the questionnaires were carried out in strict accordance with the recommendations made by The University of Sydney Human Ethics Committee. The protocol was approved by The University of Sydney Human Ethics Committee (Approval number: 2013/827).

Questionnaire

A semi-structured questionnaire was conducted once on-site face-to-face with the owner, manager or the leading farm hand at each farm (Supplementary material S2). It was used to gather information on current disease and biosecurity, environmental factors and reproductive practices, housing and herd management. The questionnaire took approximately 20 minutes to complete. Of the 40 questions, nine questions were structured as closed (answers limited to yes, no or occasionally), eleven were semi-closed (for example, age at weaning or number of pigs per pen) and 20 were open (for example, occurrence of recent diseases or feed company suppliers) (Dohoo et al., 2009; Stege et al., 2001). Two questions required observational and diagrammatical records to be made (as photography was not permitted), to document design and size of the pre- and post-weaning facilities and assist in developing a visual representation of the size, shape and design of the farrowing crate/pen and the weaner environment.

Sampling

A cross-sectional survey was conducted between September 2013 and May 2014 of 22 commercial herds located in southeastern Australia (Victoria, n=10; New South Wales, n=9; and South Australia, n=3). Herds were classified based on location (state: VIC, NSW, SA) and size (small/medium 45–499 sows, large ≥500 sows).

The aim was to collect faecal samples from piglets in the age group of one week prior to weaning and then the piglets that had been most recently weaned (3-6 weeks of age) on the day of the herd visit. In collecting samples from a pen, the aim was to represent the entire pen environment in terms of both scour and non-scour samples. Faecal samples were collected from each herd (10 samples from pre-weaned and 40 samples from post-weaned piglet pens depending on the size of the pen). If there were 20 or less piglets in a pen, then five samples

were collected from the pen. If there were >20 piglets in a pen, then 10 samples were collected per pen. A total of 195 pens (93 pre- and 102 post-weaned) were sampled across 22 pig herds.

Faecal samples (approximately 2 g) from individual faecal pats at separate collection points within the pen were transferred into sterile 5 mL tubes. During collection, the samples were categorised as clinical (scours) or non-clinical (normal) based on a faecal consistency score developed by Pedersen and Toft (2011) with slight modifications; 1=dry and hard, 2=firm and hard, 3=firm and shaped (normal), 4=soft and wet, and 5=watery/liquid (diarrhoea). Diarrhoea positive pens were categorised based on the isolation of one or more diarrhoea samples. Samples were kept on ice until they could be stored at 4°C in the laboratory, for up to a week.

Samples were brought to room temperature (23°C) and a sterile 10 µL loop (Sarstedt) was used to homogenise the faecal sample and then it was streaked onto a Sheep Blood Agar plate (SBA) and incubated overnight aerobically at 37°C. The cultures were screened for β-haemolytic colonies (a cleared zone) on the SBA. Suspected β-haemolytic *E. coli* colonies were subcultured onto CHROMagar Oientation (CHROMagar^{TM}). Species identification was confirmed by matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Microflex LT MALDI BioTyper; Bruker Biosciences, Preston, VIC, Australia). Single pure colonies were cultured by transferring into 3 mL of sterile Buffered Peptone Water (BPW) (BD Difco^{TM}) and incubated aerobically overnight at 37°C. Then 500 µL of enriched culture was dispensed into sterile 1.5 mL screw capped tubes (Scientific Specialties) and stored at 4°C for up to a week until DNA extraction. To the remaining 2.5 mL of enriched culture, 80% glycerol was added and mixed well, before being stored at –80°C for further use.

Sterile BPW (5 mL) was added to each of the remainder samples, mixed well and incubated aerobically overnight at 37°C for enrichment. To each of these enriched faecal samples, 80% glycerol was added and mixed well prior to long term storage at –80°C.

Molecular biology

Lysates were prepared by vortexing the samples and boiling at 100°C for 10 min. Samples were then centrifuged (2 min at 1000 x *g*) and 200 µL of supernatant transferred to a new tube and again centrifuged (10 min at 16,000 x *g*). The supernatant (200 µL) was used as a template in multiplex PCR before being stored at –20°C in 1.5 mL screw top tubes (Scientific Specialties).

One haemolytic *E. coli* isolate from each sample was tested for the presence of virulence genes (F4, F5, F6, F18 and F41) and enterotoxin genes (STa (*estA*), STb (*estB*), Stx2e (*stx2e*) and LT (*elt*)) by multiplex polymerase chain reaction (PCR) (Chapman et al., 2006; Do et al., 2005; Weinstein et al., 1988). DNA from pure cultures of the isolates was amplified by a multiplex PCR kit protocol (QIAGEN) with slight modifications (Table 1).

Table 1. Primers used to detect *E. coli* virulence and toxin genes in a survey of southeastern Australian pig herds.

Virulence attributes	Primer sequence (5'– 3')		Amplified product (bp)
	Forward	Reverse	
F4 [a]	GGTGATTTCAATGGTTCG	ATTGCTACGTTCAGCGGAGCG	764
F5 [a]	TGGGACTACCAATGCTTCTG	TATCCACCATTAGACGGAGC	450
F6 [a]	TCTGCTCTTAAAGCTACTGG	AACTCCACCGTTTGTATCAG	333
F18 [b]	GTGAAAAGACTAGTGTTTATTTC	CTTGTAAGTAACCGCGTAAGC	510
F41 [a]	GAGGGACTTTCATCTTTTAG	AGTCCATTCCATTTATAGGC	431
STa [a]	TCTTTCCCCTCTTTTAGTCAG	ACAGGCAGGATTACAACAAAG	166
STb [a]	ATCGCATTTCTTCTTGCATC	GGGCGCCAAAGCATGCTCC	172
LT [b]	GGCGACAGATTATACCGTGC	CCGAATTCTGTTATATATGTC	696
Stx2e [c]	AATAGTATACGGACAGCGAT	TCTGACATTCTGGTTGACTC	733

[a] (Chapman et al., 2006), [b] (Do et al., 2005), [c] (Weinstein et al., 1988)

A reaction volume of 20 µL was used for PCR, containing 2 µL of DNA template, 0.2 mM of each primer (1 µL), 2x QIAGEN Multiplex PCR Master mix (6 µL), 5x Q–Solution (3 µL) and 3–5 µL of nuclease-free water (QIAGEN Multiplex PCR Kit protocol). PCR conditions were 95°C denaturation for 15 min, 30 cycles of 94°C denaturation for 1 min, 53–58°C annealing for 1 min, 72°C extension for 1 min, and a final extension step of 72°C for 7 min. Amplified DNA was separated on 2% 1 x TBE agarose (Bioline) gel and measured with the molecular size marker (1013-bp ladder) 0.5 µg/µL of pUC Mix Marker, 8 (Fermentas), stained with ethidium bromide 0.5 µg/mL. DNA bands were observed under UV light using the Gel Doc XR Fluorescent Imaging System (Bio-Rad, Hercules, CA).

Statistical analysis

The data are collated in Excel (Microsoft, PC/Windows XP, 2010, USA) and descriptive statistical analysis was performed using Genstat 16th Edition (2000-2015 VSN International Ltd). Proportions were compared using the Mann-Whitney U-test or Kruksal-Wallis one-way ANOVA and all risk factors were categorised and analysed by Fisher's exact test. Analysis of multiple predictors of pen-level post-weaning scours was performed using step-wise forwards logistic regression considering only those factors significant $p<0.2$ in univariate analysis and retaining only factors significant at $p\leq0.05$ in the final model. Since pens were nested within a herd, a random effects term was included to account for lack of independence between pens within herd.

Outcomes

Farm survey

Farms were classified into groups based on the state in which they were located. The mean herd size was 721 sows, with a wide range (from 45 to 20,000 sows per farm) so for the purpose of this study we classified farms into two groups, small/medium (45–499 sows) and large producers (≥500).

The questionnaire data are categorised. Demographics, disease occurrence and biosecurity; weaner environment; herd reproductive strategies and weaner housing and husbandry practices of the surveyed herds are summarised in Tables 2 and 3 (Supplementary material S3). Up to 10% of the data are missing due to some farmers declining to answer all questions.

Table 2. Disease, biosecurity, environmental and reproductive management practices for 195 pens in 22 pig herds across southeastern Australia (Victoria, VIC; New South Wales, NSW; South Australia, SA) included in a survey of pre- and post-weaning scours.

		Number of pens sampled per state			Number of pens sampled per herd size (sows)	
	Category	VIC	NSW	SA	≤499	≥500
Herd variable		58	110	27	123	72
Scours during suckling	Yes / sometimes	46	98	27	103	68
	No	12	12	0	20	4
Scours during post-weaning	Yes / sometimes	30	84	27	97	44
	No	28	26	0	26	28
E. coli vaccine for sows	Yes	35	87	17	78	61
	No	14	23	10	37	10
Vaccination for sows	Yes	49	98	27	103	71
	No	0	12	0	12	0
E. coli vaccine for suckers	Yes	0	21	0	0	21
	No	58	89	27	123	51
Vaccination for suckers	Yes	17	38	21	33	38
	No	41	72	6	90	34
E. coli vaccine for weaners	Yes	0	15	0	15	0
	No	50	84	27	89	72
Vaccination for weaners	Yes	39	28	16	57	26
	No	11	71	11	47	46
Recent diseases (within the last 12 months)	Yes	30	65	0	57	38
	No	28	45	27	66	34
Frequency weaner pens cleaned	Frequently ≤9 weeks	23	64	0	47	40
	End of each batch ≥10 weeks	26	27	27	66	14
Disinfectant and pressure hosing used to clean weaner pens	Yes	33	29	10	47	25

	No	1	12	0	12	0
Size of weaner pen	Small (1-15 piglets)	0	40	0	40	0
	Medium (16-199)	32	51	16	34	55
	Large (≥200)	26	19	11	49	17
Bedding in suckers pen	Yes	4	11	0	15	0
	No	53	99	27	108	71
Bedding in weaner pen	Yes	18	27	17	57	5
	No	40	83	10	66	67
Type of bedding	Straw	6	0	6	8	4
	Straw and another type of bedding	12	0	11	22	1
	Other	0	27	0	27	0
Housing type sampled	Conventional sheds	40	99	10	82	67
	Conventional sheds and Eco shelters	14	11	17	37	5
	Outdoor/free range	4	0	0	4	0
Flooring	All inorganic	52	59	21	60	72
	Organic	6	51	0	57	0
Shed ventilation	Fair	56	87	27	98	72
	Poor	2	23	0	25	0
Farrowing system	All in/all out[a]	50	94	27	99	72
	Continuous flow	8	16	0	24	0
Average weaning age	≤21 days	31	11	21	37	26
	≥22 days	27	99	6	86	46
Weaner groups housed in batches	Age	26	53	11	56	33
	Age and size	0	31	16	37	10
	Age, size and sex	32	27	0	30	29
Breed	Large White x Landrace	8	62	0	51	19
	Large White x Landrace x Duroc	12	24	10	26	20
	Other	38	24	11	40	33
Artificial insemination	Yes	54	98	27	107	72
	No	4	0	0	4	0
Use own boars	Yes	44	55	10	86	23
	No	4	43	6	37	39
AI source	Own boars	12	11	16	18	21
	AI company	42	59	11	65	51
	Own boars and AI company	0	28	0	28	0

[a] Refers to a batched farrowing system.

Table 3. Production, housing and disease herd management practices for 195 pens in 22 pig herds in southeastern Australia included in a survey of pre- and post-weaning scours.

Herd variable	Category	Number of pens sampled per state			Number of pens sampled per herd size (sows)	
		VIC	NSW	SA	≤499	≥500
		58	110	27	123	72
Farm production type	Indoor conventional	40	61	10	82	67
	Mixed (indoor and deep litter)	14	49	17	37	5
	Free range	4	0	0	4	0
Number of production sites	1	38	67	17	115	7
	≥ 2	20	29	10	8	51
Number of production buildings/Eco shelters	1-5	12	64	0	76	0
	≥ 6	46	46	27	47	72
Size farm (acres)	1-500	31	25	27	53	30
	≥ 501	17	74	0	60	31
Other livestock on farm	Yes	46	98	11	105	52
	No	12	12	16	18	20
Types of other livestock	Sheep	25	53	11	71	18
	Cattle	9	8	0	17	0
	Sheep and cattle	14	37	0	17	34
Crops	Yes	37	85	11	75	58
	No	19	25	16	46	14
Types of other crops	Wheat	0	16	0	16	0
	Wheat and another type of crop	10	51	0	36	25
	Other	27	18	11	23	33
Weaner feed type	Commercial (or makes own mix with commercial feed)	50	96	27	116	57
	Own mill	8	14	0	7	15
Weaner Feed Additives/Acids	Yes	43	59	21	77	46
	No	15	51	6	46	26
Water type	Bore/dam	31	58	0	49	40
	River/channel	17	14	0	9	22
	Town	0	12	6	18	0
	Combination	10	15	11	36	0
Antibiotics added to water	Yes	40	24	11	40	40
	No	18	86	16	83	32
Temperature control in weaner pens	Yes	51	88	10	96	54
	No	7	22	17	27	18

Heaters for temperature control in weaner pens	Yes	50	88	8	14	37
	No	8	22	19	109	35
Cooling system (thermo-regulated)/ sprinklers for temperature control in weaner pens	Yes	30	21	0	94	54
	No	28	89	27	29	18

The majority of farms bred Landrace or Landrace x Large White pigs. Most farms were greater than 500 acres and the majority housed pigs in indoor conventional sheds, having six or more buildings or Eco shelters on one site. Most weaner pens were within conventional shed systems, although each farm was slightly different, with the most common flooring being inorganic such as concrete and plastic slatting. Most weaner pens did not contain bedding, (organic material such as barley, straw, sawdust, rice hulls) however straw with the addition of another type of bedding was commonly used in Eco shelters.

Most sheds were more than 10 years old and open in design, most farms provided heaters as a source of temperature regulation to keep the weaners warm, but were less likely to provide a cooling source. The majority of farms followed the all in/all out production flow system, where all animals are removed from the accommodation, it is then cleaned and allowed to dry before new stock is moved into the space. The average weaning age ranged from 22 to 28 days and weaners were most commonly housed in medium sized groups of 16 to 199 piglets per pen/Eco shelter, based on age. The majority of farms have a cleaning protocol, 37% of these farms use pressure hosing with the addition of disinfectant to clean pens between batches of pigs.

The most common weaner feed type was purchased from a diverse range of commercial suppliers (89%) versus owning a mill and making their own feed (11%). Most farms supplemented weaner diet with feed additives, such as acids, spray-dried blood plasma or whey from milk by-products. Generally, water was supplied from bores and dams. A large proportion of farms (40%) routinely treated each batch of piglets with antibiotics added to the water at weaning, with amoxicillin the most common antibiotic choice.

Pre- and post-weaning diarrhoea was constantly (or occasionally, within the last 12 months) experienced by most batches of pigs across the majority of farms. Nearly every farm (20 of 22 herds) had a vaccination program but half of the farms had experienced a recent disease outbreak within the last 12 months (the type and frequency of disease outbreaks varied between farms).

Risk factors

The presence of diarrhoea was assessed at a pen level. We determined that of the 60 variables collected, the data show 6 variables were associated with pen diarrhoea status ($p<0.2$). Only ≤5% of the data are missing and thus were available for inclusion in a multivariate model (Table 4). Weaner status (pre-, post-weaned) was included ($p=0.275$) as a potential confounding variable.

Table 4. Results of univariate risk factor analysis for presence of diarrhoea at a pen level in a survey of 22 pig herds in southeastern Australia.

Variable	Category	No diarrhoea	Diarrhoea	P-value
Weaned	No	77	16	0.275
	Yes	78	24	
Recent disease events	No	92	10	0.015
	Yes	63	30	
Post-weaning scours history	No	51	5	0.171
	Yes	104	35	
Infeed additives	No	50	22	0.184
	Yes	105	18	
In-water antibiotics	No	98	17	0.183
	Yes	57	23	
Bedding	No	107	15	0.037
	Yes	48	25	
Temperature control	No	46	2	0.028
	Yes	109	38	

A forwards stepwise regression using the likelihood ratio to select variables ($p<0.05$) identified the best-fitting model (-2 log likelihood 174.3, Nagelkerke R^2 0.179) to include recent disease and presence of bedding factors. Estimates of odds ratios were not confounded by weaner status (post-weaned vs. pre-weaned; <10% change in OR estimates) and the interaction between recent disease and bedding was statistically different. This model is shown in Table 5.

Table 5. Results of multivariate risk factor analysis for presence of diarrhoea at the pen level in a survey of 22 pig herds in southeastern Australia.

Term	Coefficient	SE	P-value	Odds Ratio	Lower 95% CI	Upper 95% CI
Intercept	-2.496	0.364	< 0.001	–	–	–
Recent disease						
No	0	–	–	1	–	–
Yes	1.183	0.419	0.005	4.381	0.362	2.005
Bedding						
No	0	–	–	1	–	–
Yes	0.963	0.391	0.014	3.715	0.197	1.729

Bacteriology

The causal agents of diarrhoea were assessed in further detail at an individual sample level. During the survey, a total of 1,105 faecal samples were collected from 195 pens (93 pre- and 102 post-weaned) across 22 pig herds. Every herd had at least one β-haemolytic *E. coli* isolated from one out of the 50 faecal samples collected (Supplementary material S4). The prevalence of diarrhoea in pre- and post-weaned piglet samples was 32% and 47%, respectively. The prevalence of diarrhoea in pre-weaned pens was 17%, compared to 24% in post-weaned pens. The mean and median farm-level prevalence of pre- and post-weaned pen diarrhoea were 39% and 29%, respectively, and it ranged from zero to 100%.

There was a significant difference ($p=0.0001$) in the estimated prevalence of β-haemolytic *E. coli* between pre- and post-weaned piglet samples, 13% and 47%, respectively. B-haemolytic

E. coli were present in 45% of diarrhea samples from 36 pens, (7 pre- and 29 post-weaned pens). The findings show that ETEC containing both an adhesin and an enterotoxin gene (capable of causing diarrhea) were more likely to contain both F4 and STb genes (present in 10 of 22 herds, Table 6 and 7). However, the most prevalent single ETEC adhesin gene in β-haemolytic *E. coli* was F18 (32%) and the most prevalent enterotoxin gene was STb (30%), in 15 herds (Table 6). We also observed a higher association of β-haemolysis and F4: STb with diarrhoea samples than those containing F18: STb.

Table 6. ETEC virulence genes present in β-haemolytic *E. coli* isolates (n=392) from a survey of 22 pig herds in southeastern Australia.

	Pre-weaned		Post-weaned	
Virulence attribute	Prevalence	Diarrhoea associated	Prevalence	Diarrhoea associated
F4	11	6	98	33
F18	14	0	113	21
F6	11	4	14	6
F5	0	0	27	0
F41	0	0	0	0
STa	5	1	95	20
STb	12	4	106	36
LT	14	7	62	31
Stx2e	0	0	10	2

Table 7. Farm presence of ETEC virulence genes present in β-haemolytic *E. coli* isolates (n=22) in a survey of 22 pig herds in southeastern Australia.

Virulence attribute	Pre-weaned	Post-weaned
F4	4	11
F18	4	14
F6	1	3
F5	0	2
F41	0	0
STa	2	9
STb	4	15
LT	2	8
Stx2e	0	3

Application of research

This study assessed 60 potential factors that could increase the risk of *E. coli* disease. In addition the prevalence of β-haemolytic ETEC *E. coli* in southeastern Australian pig herds was estimated. Only two key factors – recent disease events within the whole herd in the last 12 months and the presence of bedding, were significantly associated with the presence of diarrhoea in pens when a multiple logistical regression analysis was applied. A multitude of herd management practices could potentially impact recent disease events, including current disease status, poor infection control, low immunity within the herd, secondary infections, biosecurity, housing and reproductive practices, as well as a range of potentially unidentified environmental factors but these did not emerge in the most rigorous analysis, perhaps due to unmeasured confounders or insufficient statistical power.

The results from this study suggest the presence of bedding significantly contributed to increasing the risk of *E. coli* disease in post-weaned piglets. The majority of herds in this study did not have bedding in weaner pens but, if used, the most popular bedding was straw. *E. coli* is commonly used as an indicator organism for environmental faecal contamination (Winfield and Groisman, 2003), but *E. coli* survival in straw is unstudied. If *E. coli* can withstand low nutrient availability, wide temperature fluctuations and competition from other microflora and there is a constant supply of carbon, growth outside its primary host is possible (Lim and Flint, 1989; Winfield and Groisman, 2003). Therefore, *E. coli* could survive in faeces within an indoor pig pen, further circulating within the environment with the potential to cause disease in young pigs as a result of coprophagic or burrowing behaviours (Fairbrother and Nadeau, 2006).

In this study, the prevalence of diarrhoea in pre- and post-weaned piglet pens in commercial herds in southeastern Australia was estimated to be 17% and 24%, respectively. This suggests that diarrhoea is an underlying problem across many herds and is likely a substantial cause of economic loss and animal welfare issues. Diarrhoea in piglets can be transient but, this study shows that persistent post-weaning diarrhoea within the last 12 months influences the presence of diarrhoea within pens.

In bivariate analysis, we observed a further four potential risk factors for piglet *E. coli* diarrhoea – temperature in weaner pens (thermostatically controlled versus no temperature control), a history of post-weaning scours (within the last 12 months), antibiotics added to the water supply and infeed additives (that may be the result of a causal relationship). The design of pig buildings varied between herds but the majority were open and exposed to temperature fluctuations and it was more common for buildings to have heating rather than cooling sources. Buddle et al. (1997) suggested Australian pig buildings did not provide satisfactory environmental conditions for optimal pig health. These findings are supported in our study as it was found that absence of temperature control may be associated with the presence of diarrhoea in a pen – although this has not been reported in previous studies of pre-weaning (Driesen et al., 1993) or post-weaning (Skirrow et al., 1997) diarrhoea.

When weaned, the transition from the sow's colostrum antibody-rich milk to solid pelleted feed can have substantial impacts on the piglet's gastrointestinal tract in terms of physiology, microbiology and immunology (Lallès et al., 2007; Pluske, 2013; Pluske et al., 1997). Additives, such as spray-dried blood plasma, may play a role in altering the intestinal structure in piglets, often reducing post-weaning diarrhoea, and are a suitable alternative to antibiotics (Bosi et al., 2004; Torrallardona et al., 2003). This study suggests that the administration of

antibiotics in water and infeed additives play a substantial role in increasing the occurrence of diarrhoea, as it is expected that antibiotics were used to treat diarrhoea hence the association.

The presence of ETEC virulence genes is important for pathogenicity and clinical disease. For the purpose of this study, samples from both healthy and clinically-affected pens were screened for β-haemolytic *E. coli* to estimate the overall prevalence of pathogenic ETEC. It is common for healthy and clinically-infected piglets to show increased ETEC shedding after weaning (Pluske et al., 2002) and for non-β-haemolytic *E. coli* to possess ETEC genes (Chapman et al., 2006; Wu et al., 2007) and may be due to numerous reasons such as the loss of virulence factors such as plasmid-encoded ST, resulting in a less virulent strain, or pig immunity due to maternal immunisation or prior infection. Our findings revealed the prevalence of β-haemolytic *E. coli* with a single ETEC gene versus both an adhesin and enterotoxin gene to be 70% (273/392) and 43% (167/273), respectively. This was slightly lower than ETEC prevalence levels reported in Queensland (Do et al., 2005).

Conclusion

Diarrhoea has significant detrimental impacts on piglets, however the findings of this study suggest by reducing the incidence of disease in a herd within the last 12 months and minimising the use of bedding (or maintaining fresh bedding) piglet and herd health can be increased. There are many positive associations with improved piglet health, such as reduced antibiotic use and veterinary bills and growth in profit margins and productivity. A consistently healthy herd will also assist in sustaining a high food safety standard with positive implications for human consumption.

Limitations/Risks

The continuous transfer of *E. coli* via faeces into the pen environment helps to maintain a stable bacterial population (Savageau, 1983), contributing to the re-occurrence of disease events. Buddle et al. (1997) found that applying the Madec and Josse (1983) risk factor analysis to Australian conditions accurately reflected risk factors impacting piglets in the Australian environment but suggested that other risk factors were yet to be identified. Due to time and financial constraints, this study design could not include repeated measurements as performed by Buddle et al. (1997) and Skirrow et al. (1997) in order to determine if risk scores had increased or decreased. Additional risk factor variables from each farm were able to be recorded in order to include previously unidentified risk factors.

Recommendations

The findings of this study suggest by reducing the incidence of disease in a herd within the last 12 months and minimising the use of bedding (or maintaining fresh bedding) piglet and herd health can be increased. The provision of bedding has many perceived benefits, such as providing a source of heat, providing stimuli for piglets, minimising dust and absorbing faecal matter (Tuyttens and André, 2005; van de Weerd and Day, 2009; Voyles and Honeyman, 2006.). Generally, bedding is changed at the end of each batch. Our results suggest that bedding could potentially be harbouring pathogenic bacteria and is not changed as often as required in order to reduce the incidence of disease. Previous studies have outlined some disadvantages of using straw, such as increased costs associated with labour, hygiene and incompatibility with manure drainage systems (Scott et al., 2006; Tuyttens and André, 2005). The estimated cost of

production by implementing changes to reduce *E. coli* risk factors based on a small pig herd could potentially save producers $1.50 per pig in non-feed costs, including increased bedding and labour expenses but decreased veterinary and medical costs. However, this saving would vary depending on the number of breeding sows, feed cost and performance of the herd for example, feed conversion rate (Fahy et al., 1987; Mullan et al., 1994; Taylor et al., 2006.).

Experiment 2: Antibiotic resistant *Escherichia coli* in southeastern Australian pig herds and implications for surveillance

(Refer to Appendix 1)

Introduction

Using antibiotics to control ETEC disease can be expensive and selects for resistance. Resistance can result in the use of antibiotics critically important for public health, for example third-generation cephalosporins (3GC) such as ceftiofur. The use of ceftiofur in pigs increases the prevalence of ESBL *E. coli* (Fleury et al., 2015). An Australian study assessing ceftiofur use in 197 pig herds found that 25% of farms used this antibiotic, including for the control of *E. coli*, *Lawsonia* and *Mycoplasma* (Jordan et al., 2009). Ceftiofur is a last resort antibiotic in pig medicine, but predicting usage rates can be difficult because it is legally scripted off-label for use in the Australian pig industry.

Limited new antibiotics are being developed, so there is a need to extend the use of those that are currently available. Administration of the same or similar antibiotics to both animals and humans will select for the same resistance genes, shortening the effective lifespan of an antibiotic. Gentamicin (an aminoglycoside antibiotic) is often used in combination with β-lactams for treatment of severe human infections such as sepsis and endocarditis (Hammerum and Heuer, 2009). Although aminoglycosides are commonly used for treatment in animals, gentamicin was never registered for use in Australian livestock due to its importance in human medicine (Barton, 2000). Animals are thought to act as a reservoir of resistance genes (Salyers and Shoemaker, 2006). Bacterial resistance genes in animals can be transmitted directly or indirectly to human pathogens, for example as a result of direct contact, or via food, water, sludge or manure used as fertilizer (Marshall and Levy, 2011). However, the role that pigs might play in the transmission of resistance genes in an Australian context is unclear. It is uncertain if ETEC in the pigpen environment have the ability to lose or gain resistance genes or plasmids. Most studies have focused on ETEC isolated from individual pigs rather than the piglet pen environment. If ETEC are protected within faecal matter, they can survive in the environment for up to 6 months (van Beers-Schreurs et al., 1992).

This study sought to determine the prevalence of antibiotic resistant *E. coli* in commercial pig herds in southeastern Australia, to identify key resistance genes that need to be included in surveillance and to determine the association between resistant *E. coli* and the carriage of ETEC genes as an indicator of their importance in producing post-weaning diarrhoea in piglets.

Methodology

Sampling pigs

Sampling of pigs in this study was described previously in Experiment 1. In brief, a cross-sectional survey of 22 commercial herds located in southeastern Australia (Victoria, n=10; New South Wales, n=9; and South Australia, n=3) was conducted between September 2013 and May

2014. Piglets one week prior to weaning and those most recently weaned on the day of the herd visit were sampled. Both diarrhoeal and non-diarrhoeal faecal samples were collected from each pen. Up to 10 samples from pre-weaned piglet pens and 40 from post-weaned piglet pens were collected. Fresh faeces from the pen floor were sampled, using a systematic approach (separate collections points throughout the pen) to represent the pen environment.

Faecal samples were cultured using Sheep Blood Agar incubated overnight aerobically at 37°C, and cultures were screened for β-haemolytic colonies. A single presumptive β-haemolytic *E. coli* isolate and a single presumptive non-β-haemolytic *E. coli* isolate from each sample was subcultured onto CHROMagar Orientation (CHROMagar™). Species identification was confirmed by matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex LT MALDI Biotyper; Bruker Biosciences, Preston, VIC, Australia).

Susceptibility testing

A total of 325 *E. coli* isolates from piglets (15 from each herd, 5 pre- and 10 post-weaned piglet pens) were randomly selected to include β- haemolytic and non-β-haemolytic *E. coli* as well as diarrheal and non-diarrhoeal samples. Isolates were tested against 19 veterinary antibiotics (Table 8 (including antibiotics not permitted for use in Australia) using Sensititre™ (BOPO6F) and 27 antibiotics used in human medicine using the Phoenix Automated Microbiology System (NMIC-203) (BD, Sparks, MD) and interpreted using veterinary CLSI (CLSI, 2013), human CLSI (CLSI, 2012b) and EUCAST (EUCAST, 2016) guidelines.

Detection of ETEC virulence genes

E. coli isolates were screened for adhesin (F4, F5, F6, F18 and F41) and enterotoxin (STa, STb, Stx2e and LT) genes by multiplex polymerase chain reaction (PCR) (Table 1) (Chapman et al., 2006; Do et al., 2005; Weinstein et al., 1988). Of the 325 *E. coli* isolates, 109 were previously screened for adhesin an enterotoxin genes Experiment 1. Fresh lysates were prepared and boiled at 100°C for 10 min before being centrifuged and 2 µL of supernatant was used as the DNA template for PCR. DNA from pure cultures of the isolates was amplified by a commercial QIAGEN Multiplex PCR Kit (QIAGEN, CA) with slight modifications.

Serology

Serogroup O149, which is commonly associated with clinical ETEC post-weaning diarrhoea in piglets, was screened for. Slide agglutination tests were performed using *E. coli* O149 latex reagent (Oxoid) on fresh, single colonies grown overnight.

Detection of resistance genes

Isolates resistant to 3GC or aminoglycosides were screened by multiplex PCR/reverse line blot (mPCR/RLB) (Agyekum et al., 2016; Ginn et al., 2013), to detect genes conferring resistance to β-lactams (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{KPC}$, *bla*$_{VEB}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{CMY-2}$-like, *bla*$_{DHA}$, *bla*$_{ACT}$, *bla*$_{MIR}$, *bla*$_{OXA-10}$-like, *bla*$_{OXA-30}$-like, *bla*$_{OXA-48}$-like genes) and aminoglycosides (*aac(3)-II*, *aacA4*, *aadB*, *armA*, *rmtB*, *rmtC*, *rmtF*). PCR using published primers (Ellem et al., 2011; Perez-Perez and Hanson, 2002; Woodford et al., 2006) and sequencing were used to confirm resistance genes and determine the allele present. We also

screened a single colistin resistant isolate for the *mcr-1* gene by PCR with primers CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTA GGG-3′) (Liu et al., 2016).

Statistical analysis

Statistical analysis was performed using Genstat 16th Edition (© 2000–2015 VSN International Ltd). Associations were analysed by Fisher's exact test ($P<0.05$).

Outcomes

A total of 1,105 faecal samples were collected from the 22 pig herds enrolled. Of the 325 samples selected and from which *E. coli* was isolated, 113 were from pre- and 212 from post-weaned pens. The prevalence of diarrhoea in these pre- and post-weaned piglet pens was 27.4% and 25.0%, respectively.

Antibiotic susceptibility

Most (>70%) isolates were resistant to antibiotics commonly used in veterinary practice, such as sulfadimethoxine, oxytetracycline and chlortetracycline (Table 9). A lower prevalence of resistance (0–10%) occurred to antibiotics commonly used in human medicine, except for tetracycline (72.9%) and chloramphenicol (39.7%) (Table 9). Resistance to 3GC was low, 5.2% (17/325) to ceftiofur (veterinary) and 5.5% (18/325) and 2.2% (7/325) to ceftriaxone and ceftazidime (human), respectively. The single isolate phenotypically resistant to colistin was negative for the *mcr-1* gene.

Table 8. Susceptibility of *E. coli* isolates to veterinary and human antibiotics (including antibiotics not permitted for use in the Australian pig industry).

	Antibiotic	Resistance (%)
Veterinary[a]	Apramycin 15[b]	11.1
	Gentamicin[c]	7.4
	Neomycin	34.5
	Oxytetracycline	76.6
	Chlortetracycline	74.8
	Tilmicosin[d]	99.7
	Tulathromycin	5.8
	Penicillin[d]	99.7
	Ampicillin[c]	55.1
	Ceftiofur	5.2
	Danofloxacin	7.7
	Enrofloxacin	1.8
	Florfenicol	54.5
	Clindamycin[d]	99.7
	Sulfadimethoxine	80.6
	Tiamulin[d]	99.4
	Trimethoprim-sulfamethazole[c]	44.9
Human[c e]	Aztreonam	2.2
	Cefazolin	10.2
	Cephalexin	8.6
	Ceftazidime	2.2
	Ceftriaxone	5.5
	Ertapenem	0.6
	Chloramphenicol	39.7
	Tetracycline	72.9
	Nitrofurantoin	5.8
	Cefoxitin	2.5
	Tigecycline	0.9
	Colistin	0.3

[a] No resistance to spectinomycin or tylosin detected
[b] Disc diffusion
[c] Antibiotics screened by both methods
[d] Known natural resistance to *E. coli*
[e] No resistance to tobramycin, trimethoprim, ticarcillin-clavulanate, meropenem, norfloxacin, fosfomycin w/G6P, cefepime, amikacin, amoxicillin-clavulanate, imipenem, ciprofloxacin or piperacillin-tazobactam detected

Eight of the 22 farms surveyed, located across all three southeastern Australian states, had at least one 3GC resistance gene present. Four ESBL genes not previously reported in Australian pigs were identified, *bla*$_{\text{CTX-M-1, -15, -27}}$ and *bla*$_{\text{SHV-12.}}$ We also detected *bla*$_{\text{CTX-M-14}}$ and bla$_{\text{CMY-2}}$–like genes that had been previously described (Abraham et al., 2015). However, we were unable to identify 3GC resistance genes in three of the resistant isolates with the set of primers used. Overall, resistance to both veterinary and human 3GC antibiotics was found in 20 *E. coli* isolates (three from pre-weaned and 17 from post-weaned piglets).

Associations between production stage (pre- versus post-weaned piglets, clinical status normal versus clinical diarrhoea), 3GC and aminoglycoside resistance and presence of 3GC and aminoglycoside resistant genes are shown in Table 9. Phenotypic resistance to both antibiotic classes was nearly 3-times higher in post- versus pre-weaned piglets and about 1.5-times higher in pens in which diarrhoea was not present (non-clinical). For 3GC resistance genes, the same patterns was observed, resistant genes were more commonly identified in post-weaned pens and pens in which diarrhoea was not present. The only isolate from which an aminoglycoside resistance gene (*aac(3)-II)* was detected was from a pre-weaner, non-clinical pen. Gentamicin resistance was detected in 24/325 (7.4%) of *E. coli* from piglets, almost all (22/24) gentamicin resistant isolates carried the bla_{TEM} gene.

Table 9. Associations between clinical status and weaning status of pigs in pens sampled.

Resistance	Antibiotic group	No. of isolates	Weaning status		Diarrhoea present	
			Pre (n=113)	Post (n=212)	Yes (n=84)	No (n=241)
Phenotype						
	Third-generation cephalosporin	20	3 (2.7)	17 (8.0)	4 (4.8)	16 (6.6)
	Aminoglycoside	24	4 (3.5)	20 (9.4)	4 (4.8)	20 (8.3)
Genotype						
	Third-generation cephalosporin	17	3 (1.8)	14 (8.5)	3 (4.8)	14 (6.6)
	Aminoglycoside	1	1 (0.9)	0 (0)	0 (0)	1 (0.4)

ETEC virulence genes

The majority of diarrhoeal samples collected as part of this study did not carry important resistance genes. Of the 3GC resistant isolates (n=20), three isolates carried ETEC adhesion and enterotoxin genes, and only one of these three was linked to disease. There appeared to be no patterns of association between *E. coli* showing 3GC resistance, the presence of ETEC genes and diarrhoea. Of the aminoglycoside resistant isolates nearly half (11/24) carried an ETEC gene; seven of these isolates carried both an adhesin and enterotoxin gene (capable of causing diarrhoea). The presence of ETEC genes in aminoglycoside-resistant *E. coli* was only observed in post-weaned piglets, most commonly expressed as F4:STa:STb.

There was a significant difference (P=0.0001) in the estimated prevalence of β-haemolytic *E. coli* between pre- and post-weaned piglet samples, 37% and 64%, respectively (Table 10). A significant difference (P=0.0001) was also observed in estimated prevalence of ETEC *E. coli* genes between pre- and post-weaned piglet samples, 52% and 36% respectively, however this was not reflected in diarrhoea prevalence. ETEC containing both an adhesin and an enterotoxin gene were present in 7% and 16% of pre- and post-weaned piglet samples, respectively. ETEC *E. coli* isolated from pre-weaned piglets were most likely to carry the toxin STa and either F4 or F18 adhesins, whereas post-weaned piglets were more likely to carry F4 and LT.

Table 10. ETEC virulence genes present *E. coli* isolates.

Virulence attribute	Pre-weaned (n=113)				Post-weaned (n=212)			
	No. of isolates	Diarrhoea associated	β-haemolytic	O149	No. of isolates	Diarrhoea associated	β-haemolytic	O149
F4	7	4	5	6	27	6	26	24
F18	8	2	4	3	33	10	28	5
F6	2	0	2	0	1	0	0	0
F5	2	2	2	2	2	1	2	2
F41	1	0	1	0	2	0	2	0
STa	8	2	6	4	25	9	20	10
STb	12	5	1	6	28	9	22	13
LT	2	0	1	0	25	5	21	22
Stx2e	0	0	0	0	6	2	5	0

Serology

A total of 89/325 (27.4%) *E. coli* isolates were identified as the common serogroup O149. The prevalence of O149 in pre- and post-weaned piglet samples was 29.2% and 26.4%, respectively. Less than half of the 3GC (n=7/20) and gentamicin (n=11/24) resistant isolates, were O149 serogroup. However, we observed no statistical association between O149 serogroup status and ETEC carriage.

Application of research

We found that *E. coli* isolated from 20 (6.1%) faecal samples, collected from eight of 22 southeastern Australian piggeries, were resistant to 3GC antibiotics and we identified four ESBL genes not previously reported in Australian pigs, $bla_{\text{CTX-M-1, -15, -27}}$ and $bla_{\text{SHV-12.}}$ In this study we also detected $bla_{\text{CTX-M-14}}$ and $bla_{\text{CMY-2}}$-like ESBL genes which have only recently been reported in Australian pigs based on analysis of diagnostic samples (Abraham et al., 2015). 3GC resistance genes appeared to be more common in non-ETEC, non-diarrhoea samples. This has implications for the design of surveillance systems to detect emerging antibiotic resistance. It appears that Australian piglets have a low prevalence (3.4%) of *E. coli* carrying a $bla_{\text{CTX-M}}$ ESBL gene when compared to other countries such as Korea (21.5%) (Tamang et al., 2013).

Similar prevalence of resistance to ceftriaxone (5.9%) and ceftazidime (4.4%) can be observed when comparing piglet *E. coli* (Table 9) and Australian patients (AURA, 2016). In the Australian general community, $bla_{\text{CTX-M}}$ genes are well established (Zong et al., 2008). Of the ESBL genes isolated from piglets in the current study, all have previously been identified in patients admitted to hospitals in the Sydney region (Ginn et al., 2013; Zong et al., 2008). Since ESBL were identified in Australian human *E. coli* several years before being identified in livestock, it has been suggested that sources other than food might be responsible for the emergence of ESBL (Abraham et al., 2015). However, the presence of the same ESBL genes in Australian piglets and humans also suggest there is the potential for zooanthroponosis (reverse zoonotic disease transmission), as has previously been shown between Danish producers and their pigs (Hammerum et al., 2014), as well as between pigs and their environment (von Salviati et al., 2015). Therefore, future research to characterise the context of these resistance genes and the role played by plasmids in the transmission of resistance

within animal populations and between animals and humans, is essential for managing antimicrobial resistance.

In this study we sampled the piglet pen environment, because ETEC remains quite stable in the environment and piglet husbandry is based on pen (rather than individual piglet) management (van Beers-Schreurs et al., 1992). Identifying resistance genes within the environment shows that there is a potential for gene transfer within or between bacterial species (Chee-Sanford et al., 2009).

ETEC enterotoxins (STa, STb, and LT) and most ETEC adhesins (F4, F18, F5, and F6) are encoded on large plasmids (with the exception of F41) and are generally associated with IncF plasmids (Fairbrother et al., 2005; Mainil et al., 1998). We found that ETEC genes were carried by five of the 20 isolates resistant to 3GC. However, an association between virulence, resistance and diarrhoea in piglets was not observed. This might be due to the small number of isolates screened. However, it has previously been suggested that non-pathogenic *E. coli* may provide a reservoir for resistance genes (Lipsitch et al., 2002). Our results suggest that monitoring emerging resistance should not be solely based on passive surveillance systems focusing only on clinical cases of diarrhoea.

Interestingly we observed that nearly half (11/24) of the aminoglycoside resistant *E.coli* isolated from post-weaned piglets carried ETEC genes. Although gentamicin was never used in Australian pig production (Barton, 2000), post-weaned piglets commonly have greater exposure to antibiotics and resistance may be the result of selection pressure. Reducing antibiotic use in pig production may reduce selection pressure and minimise resistance genes for antibiotics important in human medicine.

Of the 3GC and gentamicin resistant *E. coli* isolates in our study, 7/20 and 11/24 were O149, respectively. The overall prevalence of O149 for the study was 89/325 (27.4%), less than reported by Smith et al. (2010) who estimated 46.2% prevalence, however our study did not focus exclusively on clinical cases. Again, serotype information might not predict resistance emergence.

Conclusion

Australia is in the process of developing such a surveillance program in livestock, but currently the data for estimating the occurrence and impact of antimicrobial resistance are limited to isolates recovered from diagnostic samples collected during the investigation of clinical disease. Although the emergence of resistance might be detected from such a passive surveillance system, it is not suited to estimating prevalence of antimicrobial resistance or assessing population-level impact of antimicrobial resistance and needs to encompass known and yet unidentified resistance genes. Resistance surveillance should include indicator bacterial species and sampling of healthy pigs and their environment. An understanding of the interaction between humans, pigs and the environment using a One Health approach is needed to inform strategies to prevent and control emerging resistance.

Limitation/Risks

A major concern for human health is that administration of aminoglycosides to animals will eventually cause cross-resistance to critically important human antimicrobials such as amikacin (Shaw et al., 1993). Although gentamicin is no longer used in the Australian pig industry, we

estimated the prevalence of gentamicin resistance to be 7.4%. Of the 24-gentamicin resistance isolates, 11 carried a single ETEC gene and six carried both an adhesin and enterotoxin gene and were therefore capable of causing disease. Future research needs to screen *E. coli* from finisher pigs to determine antibiotic resistant prevalence in ETEC to gauge the possibility of transmitting resistance to humans via food consumption.

Recommendations

Restrictions on the administration of antibiotics such as ceftiofur in Australian pig herds has likely contributed to the low level presence of ESBL genes. Some important CTX-M genes that dominate in countries such as China, for example $bla_{\text{CTX-M-55}}$ (Xu et al., 2015), have not yet been reported in Australia. In China, 3GC antibiotics such as ceftiofur and cefquinome have been used as therapy or as infeed additives (Lei et al., 2010) and as a result, rates of resistance to ceftiofur (43.7%) are high (Xu et al., 2015). The use of ceftiofur as an infeed additive has likely contributed to the high prevalence and diversity of ESBL genes that are present in pigs in China (Xu et al., 2015). This situation is in stark contrast to Denmark, where no resistance to ceftiofur or cefotaxime was reported in pigs in 2014 (DANMAP, 2014). Evidence suggests that 3GC antibiotics remain one of the only classes of antibiotics effective against post-weaning diarrhoea (Hornish and Kotarski, 2002). If Australia implements a nationwide monitoring system including antibiotics sales, usage and resistance rates, correlations between resistance and usage could be established to provide evidence to determine if restrictions on usage need to be enforced.

Experiment 3: Investigating the risk factors for *Escherichia coli* pre-weaning piglet diarrhoea – a pilot study

(Refer to Appendix 1)

Introduction

Pre-weaning mortality affects productivity and profitability in the pig industry worldwide. Factors contributing to pre-weaning mortality have been identified, including digestive disorders (Madec et al., 1998), farrowing crate or pen design (KilBride et al., 2012), and management strategies (Kirkden et al., 2013). In Australia, pre-weaning mortality rates are moderately high, 18.5% (Australian Pork Limited, 2012) and have remained so since the 1970s (19.7%) (Glastonbury, 1976). Previous Australian studies have identified some important factors associated with pre-weaning losses such as age of the piglet, litter size at birth, the use of farrowing crates, (Glastonbury, 1976), and overlay, diarrhoea, anaemia and savaging (Spicer et al., 1986). The three main drivers of pre-weaned piglet mortality are facility design, undernutrition and disease (Hughes and van Wettere, 2012). However, less attention has been paid to the risks associated with pre-weaning piglet disease (Driesen et al., 1993).

Previous studies have highlighted variability between the important risk factors associated with ETEC in different countries (Amezcua et al., 2002; Kirkden et al., 2013; Roehe and Kalm, 2000) which suggests that risk factors associated with pre-weaned mortality are location and management system specific. Our aim was to perform a pilot study to identify key risk factors associated with clinical cases of diarrhoea caused by *E. coli* disease. We intensively monitored litters born on a small commercial pig farm over a one year period (December 2013 to November 2014) to identify potential risk factors in an Australian context. Identifying the key

risk factors in a modern Australian piggery can assist in improving future management strategies resulting in increased piglet health and survivability.

Methodology

Study design

The study was designed as longitudinal over the period of one year, to identify key risk factors that may influence pre-weaned *E. coli* disease in piglets at the pen and herd levels. Data was recorded prior to faecal sampling, at sampling (less than seven days prior to weaning) and at the time of weaning (17 to 34 days of age). All animal sampling procedures, interactions and protocols were carried out in strict accordance with the recommendations made by The University of Sydney Animal Ethics Committee (approval number: N00/7-2013/3/6002). Piglets were receiving colostrum at the time of sampling and were also exposed to a commercial solid weaner creep feed (Vella stock feeds) on average two weeks prior to weaning to assist is transitioning to solid feed, which was kept consistent throughout the period of the study.

Piglets were born from Large White x Landrace (x Duroc) sows, the majority was natural mating and occasionally PIC genetics were used. An average of 6.6 sows farrowed per batch, with nine batches of piglets sampled within the study period. Sows had access to ab lib feed and water, and were vaccinated with ECOvac (MSD Animal health) for protection against *E. coli* before farrowing. Within 24 hours of birth each piglet received an iron injection. Piglets were reared in an indoor intensive environment and had access to heaters for warmth and fans for cooling, however, the shed was not well insulated and temperature inside the shed reflected the ambient environmental temperature.

Risk factor data

Data describing 26 potential risk factors were recorded for each litter of piglets (Appendix 8). A survey questionnaire was performed to gather data on reproductive practices, piglet health, housing and farm management. Information about environmental factors including rainfall, and average minimum and maximum temperature one week prior to sampling were recorded at a weather station located less than 5 km from the piggery (BOM, 2013, 2014).

Sampling

The aim was to collect data and faecal samples from piglets one to three days prior to weaning. Five faecal samples were collected per pen, from each batch of piglets born from December 2013 to November 2014. Piglets were housed with their mothers in either a typical farrowing crate (1.6 m by 2.2 m) or the slightly modified Norwegian University of Life Sciences (UMB) farrowing pen system (hereon referred to as UMB pen), both identified as a ‘pen’. The UMB pen was developed in Norway but was modified to include features of the Werribee farrowing pen developed in Australia in the 1980s and 1990s (Cronin, 2014). The UMB pen has been designed to promote ‘natural’ nesting behaviour, providing sows with straw (approximately 7 kg) and a pen (2.4 m by 3.3 m) separated into two sections (a ‘nest’ and ‘non-nest’ area), with enough space to turn around and lay down comfortably in both areas (Cronin, 2014).

Piglets were born from Large White cross Landrace (cross Duroc) sows, and reared in an indoor intensive environment. Piglets have access to heaters throughout the year and access to water

drippers in the warmer months. In farrowing pens a small section of black rubber matting is situated below the heater while in farrowing pens half the pen is covered in rubber matting, while in both pens the majority of flooring is metal slatting. Sows were vaccinated against *E. coli.*

Five faecal samples were collected from different locations within each pen, in order to represent the entire pen environment (including both diarrhoea and non-diarrhoea samples). If one or more diarrhoea samples within the pen was identified then the pen was categorised as diarrhoea positive. The sampling method used in this study has been previously described Experiment 1.

Once samples were collected they were kept on ice (about 30 minutes) until they could be stored at 4°C in the laboratory, and were processed within four hours. Samples were brought to room temperature (23°C) and a sterile 10 µL loop (Sarstedt) was used to homogenise the faecal sample. It was then streaked onto a Sheep Blood Agar plate (SBA) and incubated overnight aerobically at 37°C. The cultures were screened for suspect *E. coli*, β-haemolytic colonies (a cleared zone) on the SBA and non-β-haemolytic colonies were subcultured onto CHROMagar Oientation (CHROMagar™). Single pure colonies were cultured by transferring into 3 mL of sterile Buffered Peptone Water (BPW) (BD Difco™) and incubated aerobically overnight at 37°C. Then 80% glycerol was added and mixed well, before being stored at –80°C for further use. Sterile BPW (5 mL) was added to each of the remaining samples, mixed well and incubated aerobically overnight at 37°C for enrichment. To each of these enriched faecal samples, 80% glycerol was added and mixed well prior to long term storage at –80°C.

Statistical analysis

Data was collated in Excel (Microsoft, PC/Windows XP, 2010, USA) and descriptive statistical analysis was performed using Genstat 16th Edition (2000-2015 VSN International Ltd). Proportions were compared using the Mann-Whitney U-test or Kruksal-Wallis one-way ANOVA and all risk factors were categorised and analysed by Fisher's exact test. Multivariate analysis of predictors of pen-level pre-weaning diarrhoea was performed using forwards step-wise logistic regression considering only those factors significant ($P<0.2$) in univariate analysis. Only factors significant at ($P\leq0.05$) were retained in the final model. Since pens were nested within a herd, a random effects term was included to account for lack of independence between pens within herd for the herd-level model.

Outcomes

Farm survey

A total of 87 pens (holding 87 litters and a total of 844 piglets) were sampled, and 435 faecal samples were collected. The presence of diarrhoea was assessed at the pen level where five samples were collected from each pen. Only 16 of 87 pens (18.4%) sampled were positive for diarrhoea. We observed that 13 of 59 (22.0%) farrowing crates had evidence of diarrhoea but only 3 of 28 (10.7%) UMB pens had evidence of diarrhoea. There was no significant difference (P=0.249) in diarrhoea presence between farrowing crates and the UMB pens.

Of the samples collected from pre-weaned pens, β-haemolytic *E. coli* was detected in 50 of 87 (57.5%) pens. We detected β-haemolytic *E. coli* in 37 of 59 (62.7%) farrowing crates and 13 of 28 (46.4%) UMB pens. There was no significant difference (P=0.170) in the presence of β-

haemolytic *E. coli* between farrowing crates and UMB pens. The association between β-haemolytic *E. coli* and diarrhoea was only observed in farrowing crates at a low prevalence, 5 of 59 (8.5%) pens.

The average birth weight of all piglets included in the study (n=844) was 1.4 kg. However, we observed 5.2% (44/844) of piglets with a birth weight of less than 800g (found in 26 of 87 pens), with a 15.9% survival rate on the study farm. The average weaning weight of all piglets was 7.0 kg and weaning age was 28.2 days.

In the study cohort, birth to weaning mortality was 21.9%, and of this we estimate that 4.2% mortality was attributed to *E. coli* disease (Table 11). Although we observed 18.4% (16/87) diarrhoea positive pens, most of the piglets recovered. Higher rates of mortality were associated with overlay by the sow than disease. When comparing housing type we observed a significant difference ($P \leq 0.001$) in pre-weaning mortality when comparing farrowing crates and UMB pens (Table 11).

Table 11. Pre-weaning information per pen on a commercial piggery over the period of one year (n=844 piglets).

	Farrowing crates (n=59)	Modified Norwegian UMB farrowing pens (n=28)	Farm total (n=87)
Pre-wean mortality (%)	18.6	28.3	21.9
Average piglets born per sow	12.9	12.9	12.9
Average piglets born alive per sow	12.0	12.4	12.1
Average piglet survival until weaning	10.1	8.6	9.6

Risk factors

We determined that of the 38 variables for which data was collected, 26 variables were associated with pen diarrhoea status ($P<0.2$) and had ≤5% missing data and thus were available for inclusion in a multivariate model (Table 12).

Table 12. A univariate risk factor analysis for the presence of diarrhoea in pre-weaned piglets on a small commercial piggery in southeastern Australia.

Variable	Category	Diarrhoea	No diarrhoea	*P*-value
Average minimum temperature (°C)	≤5	0	60	0.109
	6-10	6	104	
	11-15	10	125	
	≥16	11	109	
Average maximum temperature (°C)	≤20	0	105	0.001
	21-25	26	244	
	≥26	1	49	
Rainfall (mm)	≤0.9	5	170	0.027
	1-4.9	15	175	
	≥5	7	53	
Season	Summer	11	80	0.010
	Autumn	13	152	
	Winter	0	70	
	Spring	3	92	
Crate/Pen number	Crate 1	0	22	0.005
	Crate 2	0	20	
	Crate 3	5	26	
	Crate 4	7	40	
	Crate 5	1	39	
	Crate 6	2	43	
	Crate 7	5	40	
	Crate 8	0	40	
	Pen 1	0	40	
	Pen 2	4	36	
	Pen 3	0	30	
	Pen 4	3	22	
Neighbour left	Crate 2	0	22	0.084
	Crate 3	0	20	
	Crate 4	5	26	
	Crate 6	1	39	
	Crate 7	2	43	
	Crate 8	5	40	
	Pen 9	4	36	
	Pen 10	0	30	
	Pen 11	3	22	
	No neighbour	7	120	
Neighbour right	Crate 1	0	20	0.006
	Crate 2	5	26	
	Crate 3	7	40	
	Crate 5	2	43	
	Crate 6	5	40	
	Crate 7	0	40	
	Pen 2	0	40	

	Pen 3	4	36	
	Pen 4	0	30	
	No neighbour	4	83	
Parity	0	6	74	0.116
	1	6	49	
	2	5	62	
	3	5	80	
	4	0	51	
	≥5	1	56	
Sow condition score	≤2.5	8	56	0.171
	3	13	233	
	≥3.5	6	104	
Sow condition score at weaning	≤2.5	12	82	0.007
	3	13	252	
	≥3.5	1	59	
Piglets weaned	≤7	0	96	0.012
	8-10	22	232	
	≥11	5	70	
Males per pen	≤4	4	174	<0.001
	5-7	10	165	
	≥8	13	59	
Average weaning weight	4-4.9	5	20	0.002
	5-5.9	0	60	
	6-6.9	6	115	
	7-7.9	1	43	
	8-8.9	1	34	
	9-9.9	3	52	
Range in weaning weight	1-1.9	0	25	0.010
	2-2.9	6	63	
	3-3.9	2	119	
	4-4.9	6	58	
	5-5.9	2	59	
Number of deaths	0	6	64	0.052
	1	5	90	
	2	1	91	
	3	4	30	
	4	4	56	
	5	1	33	
	≥6	6	34	
Fostered on	0	20	333	0.008
	1	0	5	
	2	1	24	
	3	4	11	
	4	2	8	
	≥5	0	17	
Age at death	None	6	59	0.164
	<6	16	224	
	7.0-13.0	3	52	
	14.0-20.0	2	20	

	21>	0	8	
Mummified	Yes	11	108	0.128
	No	16	290	
Stillbirth	Yes	9	187	0.169
	No	18	211	
Treatment for sow	Yes	10	106	0.092
	No	17	237	
Antibiotic treatment sow	Yes	10	91	0.053
	No	17	252	
Other treatment for Sow	Yes	1	19	0.107
	No	26	324	
Iron treatment for piglets	Yes	5	110	0.035
	No	22	233	
RespiSure treatment for piglets	Yes	2	18	0.103
	No	25	325	
Trisoprim treatment for piglets	Yes	0	0	0.039
	No	27	343	
Other treatment for piglets	Yes	7	87	0.118
	No	20	256	

A forwards stepwise regression using the likelihood ratio to select variables (P<0.05) identified the best-fitting model to include males per pen and sow condition score at weaning (Table 13).

Table 13. A multivariate risk factor analysis for the presence of diarrhoea in pre-weaned piglets on a small commercial piggery in southeastern Australia.

Term	Coefficient	SE	*P*-value	Odds Ratio	Lower 95% CI	Upper 95% CI
Intercept	-2.957	0.365	< 0.001	–	–	–
Males per pen						
≤4	0	–	–	1	–	–
5-7	0.955	0.392	0.015	1.426	0.184	1.726
8≥	2.033	0.390	< 0.001	2.479	1.268	2.799
Sow condition score at weaning						
≤2.5	0	–	–	1	–	–
3	-1.019	0.275	< 0.001	0.361	0.211	0.619
3.5≥	-1.705	0.698	0.015	0.182	0.046	0.714

Application of research

A minority (18.4%) of pre-weaned piglet pens were positive for diarrhoea and only 6.7% of the 435 faecal samples collected were classified as diarrhoea. We observed no significant difference in diarrhoea presence between farrowing crates and the adapted Norwegian UMB designed farrowing pens. However, this may be due to small herd size and hence the limited number of litters sampled in this study throughout the period of one year. Preliminary data from this study suggests that an open designed farrowing pen which does not confine dunging

to one specific area of the housing environment did not increase the incidence of *E. coli* diarrhoea disease in pre-weaned piglets. However, due to the small herd size and uneven crate and pen numbers analysed, further evidence is required to draw stronger associations.

Pens containing litters with a high number of males were identified as a key risk factor associated with the incidence of diarrhoea in pre-weaned piglets. In this study, male piglets were castrated between zero and seven days of age. Castration can be associated with increased stress levels in young piglets. For example, Prunier et al. (2005) observed increases in the plasma adrenocorticotropin hormone (five to 60 minutes) and plasma cortisol (fifteen to 90 minutes) after castration in piglets without anesthesia or analgesia when performed on piglets within seven days of birth. Dewey et al. (1995) found that piglets castrated between zero and three days or after fourteen days of age were more likely to die of diarrhoea than those castrated four to fourteen days of age. Castration has also been shown to slow growth rates in male piglets if performed on day one (McGlone et al., 1993) or day three (Kielly et al., 1999). However growth rates appear to recover by weaning (Kielly et al., 1999) or have less impact if performed on day fourteen (McGlone et al., 1993). This suggests male piglets undergo greater exposure to stress than female piglets which may impact their susceptibility to disease and explain why we observed a higher incidence of diarrhoea with increased males per pen. If diarrhoea-related mortality is a problem within a piggery, later castration (>4 days) might be considered as an approach to controlling the problem.

This study also identified lower sow condition score at weaning as a key risk factor associated with the incidence of diarrhoea in pre-weaned piglets. Sow condition scoring is a subjective, non-invasive measurement determined by back fat depth, it has been shown to correlate with total body fat (Mullan and Williams, 1990). If sow condition scoring is performed by an experienced stockperson (as it was in this study), it can be considered an accurate measurement (Maes et al., 2004). Back fat decreases with increased litter size (Maes et al., 2004; Yang et al., 1989) and lactation, where lower back fat is generally observed in sows at weaning. Sow condition score is something that should be regularly evaluated so that sows enter the farrowing shed are at an ideal weight (Fitzgerald et al., 2009) giving piglets the greatest chance of survival. Reducing diarrhoea related piglet mortality is an additional benefit of ensuring sows enter the farrowing shed at an ideal weight.

The present study identifies that diarrhoea was associated with reduced growth rates and morbidity, and as a result increased financial costs were experienced. For example, piglets with poor body condition (fallbacks) will have reduced growth rates and may take longer to reach market weight, they may also require milk supplementation, the provision of electrolytes or administration of antibiotics (Australian Pork Limited, 2011). Trying to save weak-born piglets may be a risk factor for pre-weaning diarrhoea (Larsson et al., 2016), however links of post-weaning diarrhoea as a consequence of pre-weaning diarrhoea requires further investigation.

In this study, higher rates of mortality were associated with overlay by the sow than *E. coli* disease which has been previously found in Australia in the 1980s (Spicer et al., 1986). Hughes and van Wettere (2012) recently identified facility design (overlays & chilling), undernutrition (low birthweight/vigour, poor milk supply) and disease as the main drivers of pre-weaned piglet death. Decreasing piglet mortality presents an ongoing challenge. Piglet mortality typically occurs within the first four days after birth, and piglets appear most vulnerable in the first 24 hours (Marchant et al., 2000). A birth to weaning mortality rate of 18.7% was estimated in this study, reflecting the national average and therefore indicating that the study farm is representative of typical Australian farms. We estimate that of the 18.7% birth to weaning

mortality rate, 4.2% was attributable to *E. coli* disease. However, we observed that most piglets with diarrhoea recovered and the average number of piglets born alive per sow was 12.1, above the national average of 10.9 (Australian Pork Limited, 2012). Glastonbury (1976) showed that there was a significant correlation between litter size at farrowing and the percentage of litters with stillbirths and mortality to weaning. Although litters sizes have increased over time, in Australia, piglet birth weight has reduced (Beaulieu et al., 2010) and as a result piglets are more susceptible to disease (Yuan et al., 2015). Selection for increased litter size at birth increases productivity but breeders need to ensure sows are capable of raising larger litters successfully (Grandinson et al., 2002).

Conclusion

E. coli is a multi-factorial disease, and identifying key risk factors associated with pre-weaning piglet diarrhoea is important for effective management. Management strategies focused on males per pen and sow condition score at weaning may help alleviate the impact of diarrhoeal diseases in pre-weaned piglets. Identifying and reducing important risk factors responsible for impacting the health and viability of piglets will help reduce associated costs such as the administration of antibiotics, potential spread of diarrhoeal diseases and productivity of the sow.

Limitations/risks

This study did not include sow diarrhoea as a possible risk factor for piglet diarrhoea. Dewey et al. (1995) observed that piglets suckling from sows that had diarrhoea, or other health issues such as reproductive, respiratory or musculoskeletal problems were four times more likely to scour and nine times more likely to die compared with piglets nursing healthy sows. We expect a lower sow condition score to be correlated with sow disease, such as gastrointestinal disease. Sows may also produce a reduced amount or quality of colostrum therefore impacting piglet health. Piglets are born agamma-globulinemic (without immunoglobulin), neither receiving any from the sow across the placenta or have the ability to produce their own (Hedegaard and Heegaard, 2016). Therefore, it is critical that piglets receive colostrum containing high concentrations of IgG within the first 24 hours after birth (Cervenak and Kacskovics, 2009). Colostrum provides piglets with passive immunity and is their first level of protection against pathogenic ETEC diarrhoea. It is paramount that the sow is healthy and has the ability to produce enough antibodies to provide protection for piglets. Thus, it is not surprising that sow condition was found to be a key risk factor associated with the incidence of diarrhoea in pre-weaned piglets in this study.

Recommendations

Piglet diarrhoea is multi-factorial and there are many possible factors that could trigger an outbreak. Dewey et al. (1995) showed diarrhoea incidence in pre-weaned piglets increased with herd related factors such as a larger herd size, sow health problems, deworming sows in the farrowing crate and supplemental milk for piglets. Another study by Mellor and Stafford (2004) showed that environmental cleanliness impacts the prevalence of neonatal infections and related deaths. An Australian pig farm survey of pre- and post-weaned piglets found that by reducing the incidence of disease in a herd within the last 12 months and minimising the use of bedding (or maintaining fresh bedding) had the greatest influence on diarrhoea incidence (Van Breda et al., 2017). In terms of prevention, vaccination and hygiene are often used as an

effective prevention tool (Larsson et al., 2016). If other influencing factors are removed the effectiveness of vaccination is likely to improve as well as reducing co-infections caused by other bacteria, such as *Salmonella, Brachyspira or Campylobacter* infection (DAF, 2010). The study farm follows strict biosecurity protocols that could reduce the impacts of disease with a strong focus on pens with numerous males and sow condition score at weaning may help reduce diarrhoea in pre-weaned piglets.

Experiment 4: Investigation of risk factors for *Escherichia coli* post-weaning piglet diarrhoea – a pilot study

(Refer to Appendix 1)

Introduction

E. coli is a clonal bacterial species that contains a limited number of genetically related lineages or clonotypes (Wirth et al., 2006). *E. coli* clones can be persistent or transient within the environment (Katouli et al., 1995). Identifying an *E. coli* clonal group may provide increased assistance in diagnosis of bacterial infections (Tchesnokova et al., 2013). Detecting if a particular clone of *E. coli* is responsible for outbreaks of diarrhoea could be an important epidemiological tool to help reduce the number of pathogenic strains on-farm. However, stability of pathogenic *E. coli* strains within the piglet pen environment and why we see a sudden increase in pathogenic strains requires further investigation.

Although *E. coli* diarrhoea is an endemic problem in most Australian farms (DAF, 2010) we are still trying to understand the inter-relationships between the severity of disease and key risk factors involved with increased diarrhoea occurrence. An intensive study focused on one pig farm identifying the key risk factors associated with diarrhoea may prove useful for designing better control programs. Therefore the aim of this study was to determine the key risk factors for post-weaning *E. coli* diarrhoea and to determine if we can observe clonal *E. coli* are responsible for repeated outbreaks (or increased incidence) of diarrhoea over the production period of one year.

Methodology

Study design

The study was designed as a longitudinal prevalence study over the period of one year to identify key risk factors that might influence post-weaned *E. coli* disease in piglets at the pen and herd levels. Data was recorded prior to faecal sampling, performed over two consecutive weeks (approximately seven and 14 days after weaning). All animal sampling procedures, interactions and protocols were carried out in strict accordance with the recommendations made by The University of Sydney Animal Ethics Committee (approval number: N00/7-2013/3/6002).

The average number of piglets per pen was 11.1 (with a range of 6-40 piglets per pen). The average weaning weight of piglets during the study period was 7.0 kg and average weaning age was 28.2 days. Piglets received commercial solid weaner creep feed (Vella stock feeds) fed *ab lib*, this was kept consistent throughout the period of the study. Piglets also had *ad lib* access to bore water. Sheep and cattle are also reared on the same 300 acre farm. Post-weaned piglets were vaccinated for *Mycoplasma, Lawsonia* and pneumonia, and sows were vaccinated with

ECOvac (MSD Animal health) for protection against *E. coli* before farrowing. The herd had experienced recent disease problems (within the last 12 months) with outbreaks of *Lawsonia* and pneumonia.

Risk factor data

Data describing 13 potential risk factors were recorded for each litter of piglets. Data was gathered on farm about general farm management practices and piglet health. Information about environmental factors including rainfall and average monthly temperatures were recorded at a weather station located less than 5 km from the piggery (BOM, 2013, 2014).

Sampling

The aim was to collect faecal samples from piglets at two separate time points (seven and 14 days post-weaning). Five faecal samples were collected per pen, from each batch of piglets weaned from December 2013 to December 2014. Piglets were housed in two types of post-weaned pens, both identified as a 'pen'. Standard pens were 1.2 x 2.5 m with compressed plywood covering approximately 70% of the floor, with the remaining 30% of floor area made up of metal slatting. If more than eight sows farrowed at the one time, to accommodate these numbers piglets were also kept in a larger pen (7.2 x 3.7 m) located in the grower shed where piglets were kept on deep-litter (straw).

Piglets were born from Large White x Landrace (x Duroc) sows, and reared in an indoor intensive environment. Piglets have access to heaters for warmth and fans for cooling however, the shed was not well insulated and the temperature inside the shed reflected the ambient environmental temperature.

Five faecal samples were randomly collected from each pen to represent the entire pen environment, therefore both diarrhoea and non-diarrhoea samples were collected. If one or more diarrhoea samples within the pen was identified then the pen was categorised as diarrhoea positive. The sampling method has been previously described Experiment 1.

Samples were brought to room temperature (23°C) and a sterile 10 µL loop (Sarstedt) was used to homogenise the faecal sample. It was then streaked onto a Sheep Blood Agar plate (SBA) and incubated overnight aerobically at 37°C. The cultures were screened for suspect *E. coli*, β-haemolytic colonies (a clear zone) on the SBA and non-β-haemolytic colonies were subcultured onto CHROMagar Oientation (CHROMagar™). Species identification was confirmed by matrix-assisted laser desorption / ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Microflex LT MALDI BioTyper; Bruker Biosciences, Preston, VIC, Australia). Single pure colonies were cultured by transferring into 3 mL of sterile Buffered Peptone Water (BPW) (BD Difco™) and incubated aerobically overnight at 37°C. Then 80% glycerol was added and mixed well, before being stored at –80°C for further use.

Molecular biology

Fresh lysates were prepared by vortexing the samples and boiling at 100°C for 10 min. Samples were then centrifuged (2 min at 1000 x *g*) and 200 µL of supernatant transferred to a new tube and again centrifuged (10 min at 16,000 x *g*). The supernatant (200 µL) was used as a template in multiplex PCR before being stored at –20°C in 1.5 mL screw top tubes (Scientific Specialties).

At three time points during the longitudinal study (February, July and October), peak diarrhoea outbreaks were experienced. Two *E. coli* isolates per sample (isolated from one diarrhoea and one non-diarrhoea sample), at each of the three peak outbreaks, were used to indicate *E. coli* clonality between two different pens. Twenty-four *E. coli* isolates in total underwent screening using enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC–PCR) using (Versalovic et al., 1991).

Interpretation of ERIC–PCR band patterns were assessed visually and similarity scores were assessed using DICE similarity indices performed by BioNumerics software (Applied Maths, Belgium). Isolates were not considered clonal if they differed by more than one band. *E. coli* clonal diversity was measured based on Simpson's index of diversity and the work of Hunter and Gaston (1988).

DNA from pure cultures of the isolates was amplified by PCR using primers ERIC1R (5′-ATGTAAGCTCCTGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGAGGCG-3′) (Versalovic et al., 1991).

A reaction volume of 25 µL was used for PCR, containing 5 µL of DNA template, 0.2 mM of each primer (0.5 µL), 12.5 µL MangoMix (Bioline) and 6.5 µL of nuclease-free water (QIAGEN). PCR conditions were 95°C denaturation for 5 min, 35 cycles of 95°C denaturation for 30 sec, 51°C annealing for 1 min, 72°C extension for 2 min, and a final extension step of 72°C for 10 min. Amplified DNA was separated on 2% 1 x TBE agarose (Bioline) gel and measured with the molecular size marker (1013-bp ladder) 0.5 µg/µL of pUC Mix Marker, 8 (Fermentas), stained with ethidium bromide 0.5 µg/mL. DNA bands were observed under UV light using the Gel Doc XR Fluorescent Imaging System (Bio-Rad, Hercules, CA).

Statistical analysis

Data was collated in Excel (Microsoft, PC/Windows XP, 2010, USA) and descriptive statistical analysis was performed using Genstat 16th Edition (2000-2015 VSN International Ltd). The associations between diarrhoea status and risk factors was assessed using the Mann-Whitney U-test or Kruksal-Wallis one-way ANOVA as appropriate, and all risk factors were categorised and analysed by Fisher's exact test. Multivariate analysis of predictors of pen-level pre-weaning diarrhoea was performed using forwards step-wise logistic regression considering only those factors significant ($P<0.2$) in univariate analysis. Only factors significant at ($P\leq0.05$) were retained in the final model. Since samples were nested within a pen, a random effects term was included to account for lack of independence within pen.

Outcomes

Risk factors

A total of 769 faceal samples from 149 pens of post-weaned piglets were collected during the study period. Diarrhoea prevalence in week one and week two post-weaned piglets remained relatively constant 26.1% (99/380) and 24.9% (97/389), respectively. We determined that of the thirteen variables for which data was collected, ten were associated with diarrhoea status ($p<0.2$) and had ≤5% missing data and thus were available for inclusion in a multivariate model (Table 14).

Table 14. A univariate risk factor analysis for the presence of diarrhoea in pre-weaned piglets in a small commercial piggery in southeastern Australia 2013–2014.

Variable	Category	Diarrhoea	No diarrhoea	*P*–value
Average minimum temperature (°C)	<5	23	57	0.779
	5-10	64	191	
	11≥	109	325	
Average maximum temperature (°C)	<20	46	115	0.547
	20-25	53	171	
	26≥	97	287	
Rainfall (mm)	≤0.9	170	433	0.001
	1≥	26	140	
Season	Summer	56	143	0.006
	Autumn	60	180	
	Winter	42	78	
	Spring	38	172	
Month	January	6	29	<0.001
	February	36	58	
	March	28	72	
	April	27	73	
	May	5	35	
	June	0	0	
	July	31	49	
	August	11	29	
	September	4	76	
	October	29	61	
	November	5	35	
	December	14	56	
Pen number	Pen 1	16	74	<0.001
	Pen 2	23	67	
	Pen 3	17	73	
	Pen 4	15	60	
	Pen 5	20	60	
	Pen 6	12	78	
	Pen 7	20	70	
	Pen 8	34	56	
	Pen 9	30	24	
	Pen 10	8	2	
	Pen11	1	9	
Neighbour left	Pen 1	23	67	<0.001
	Pen 2	17	73	
	Pen 3	15	60	
	Pen 4	12	78	
	Pen 6	20	70	
	Pen 7	34	56	
	Pen 10	1	4	
	A	30	24	

	No neighbour	44	141	
Neighbour right	Pen 2	16	74	<0.001
	Pen 3	23	67	
	Pen 4	14	66	
	Pen 6	20	60	
	Pen 7	12	78	
	Pen 8	20	70	
	Pen 11	4	1	
	A	30	24	
	No neighbour	57	133	
Litter present in pen	Yes	30	24	<0.001
	No	166	549	
β-haemolytic	Yes	121	271	<0.001
	No	75	298	
Weeks post-weaned	1 week	99	281	0.723
	2 weeks	97	292	
Antibiotic administrated	Yes	49	100	0.021
	No	147	473	
Acids/additive (Bentonite clay)	Yes	42	109	0.195
	No	154	464	

A forwards stepwise regression using the likelihood ratio statistics to select variables ($p<0.05$) identified the best-fitting model to include month, season and β-haemolytic *E. coli*. The interaction between month, season and β-haemolytic *E. coli* was statistically significant and this model is shown in Table 15.

Table 15. Results of multivariate risk factor analysis for presence of diarrhoea in a longitudinal study in southeastern Australian pig herd.

Term	Coefficient	SE	P-value	Odds Ratio	Lower 95% CI	Upper 95% CI
Intercept	0.957	0.050	< 0.001	–	–	–
Season	–	–	0.003	–	–	–
Summer	1.203	0.032	0.345	1.20	0.84	1.73
Autumn	0.963	0.028	0.859	0.96	0.68	1.37
Winter	1.731	0.044	0.012	1.73	1.14	2.62
Spring	0.561	0.027	0.004	0.56	0.38	0.83
Month	–	–	0.004	–	–	–
January	0.592	0.065	0.322	0.59	0.24	1.45
February	1.998	0.050	0.003	1.20	1.27	3.14
March	1.160	0.045	0.540	1.16	0.72	1.86
April	1.094	0.045	0.713	1.09	0.68	1.76
May	0.402	0.053	0.061	0.40	0.16	1.04
July	2.009	0.055	0.005	2.01	1.24	3.26
August	1.115	0.072	0.852	1.12	0.55	2.28
September	0.136	0.025	0.00001	0.14	0.05	0.38
October	1.432	0.050	0.124	1.46	0.91	2.34
November	0.402	0.053	0.061	0.40	0.16	1.04
December	0.710	0.048	0.315	0.71	0.39	1.31
β-haemolytic *E. coli*	–	–	< 0.001	–	–	–
Present	1.798	0.023	0.0005	1.80	1.29	2.50

Diarrhoea occurrence over the period of a year (December 2013 to December 2014) showed peak periods in February, July and October (Figure 1). The highest prevalence of β-haemolytic *E. coli* occurred in August, during winter (Figure 1).

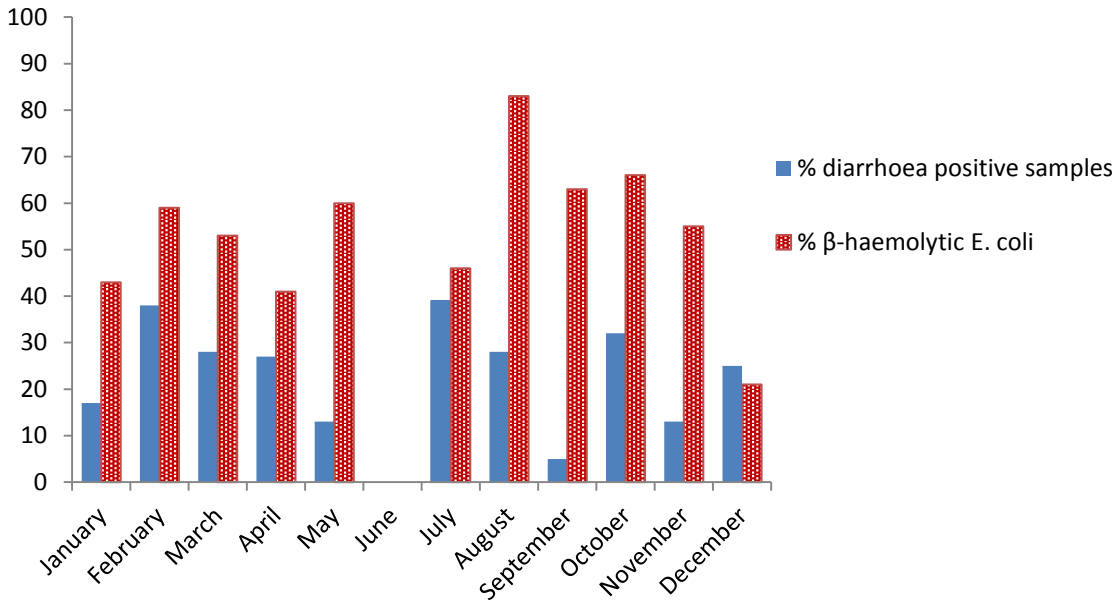
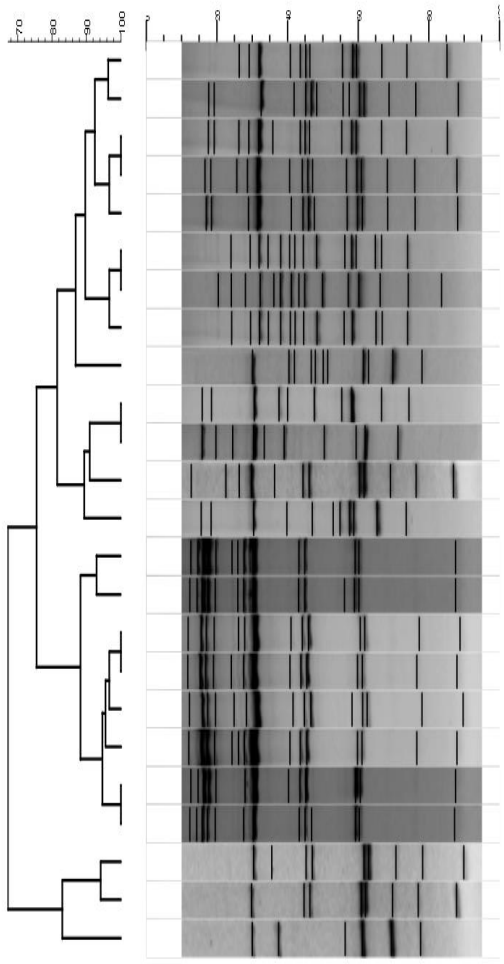


Figure 1. Percentage of β-haemolytic *E. coli* and diarrhoea prevalence in post-weaned piglets on one farm over the period of a year (December 2013-2014).

Clonal diversity

Twenty-four *E. coli* isolates were assessed from 12 faecal samples (six diarrhoea and six non-diarrhoea). A DICE similarity cut off of >85% was used to define relatedness across *E. coli* isolates, describing the uniformity of distribution of clones within the study (Figure 2). Strains appeared to cluster according to month, showing a higher association to the pen environment from which they were sampled. *E. coli* isolated from diarrhoea samples, collected from February and October shared a similar banding pattern (≤ 3 bands difference) with other isolates in the respective month (Figure 2). The major banding pattern observed in *E. coli* isolated from diarrhoea samples was designated as the outbreak strain. The banding patterns of three isolates (2390.1, 2390.4 and 2391.1) sampled from piglets in August, differed by more than five bands from the outbreak strain, these isolates were unrelated.



Sample No.	Pen No.	Diarrhoea sample	Month
2707.1	One	Yes	October
2734.2	Seven	Yes	October
2707.2	One	Yes	October
2733.1	Seven	No	October
2734.1	Seven	Yes	October
2706.2	One	No	October
2733.2	Seven	No	October
2706.1	One	No	October
2418.3	Seven	Yes	August
2421.2	Seven	No	August
2418.4	Seven	Yes	August
2391.2	One	No	August
2421.4	Seven	No	August
1743.1	Seven	No	February
1743.2	Seven	No	February
1712.2	One	Yes	February
1713.1	One	No	February
1712.1	One	Yes	February
1713.2	One	No	February
1742.2	Seven	Yes	February
1742.3	Seven	Yes	February
2390.1	One	Yes	August
2391.1	One	No	August
2390.4	One	Yes	August

Figure 2. Dendrogram based on the ERIC-PCR banding pattern of *E. coli* isolated from post-weaned piglets. Outbreak strains are highlighted in bold.

Applications of research

This study identified three key risk factors strongly associated with post-weaned piglet diarrhoea, the season and month samples were collected and the presence of β-haemolytic *E. coli*. In this study we observed a strong correlation between β-haemolytic *E. coli* presence in a faecal sample and diarrhoea in the pen ($P<0.001$). β-haemolytic *E. coli* was observed in 51.0% (392/769) faecal samples. We observed a two-fold increase 34.5% (131/380) to 67.1% (261/389) in the prevalence of β-haemolytic *E. coli* during the first to second week post-weaning, respectively. Presence of β-haemolytic *E. coli* is commonly associated with post-weaning diarrhoea, occurring in pig production in many countries such as Canada (Amezcua et al., 2002), South Korea (Kim et al., 2010), Brazil (Sato et al., 2016) and throughout the world.

Immediately following post-weaning, recommendations have been made to keep piglets at a constant temperature of between 26 and 28°C for piglets in pens designed with perforated floors (Le Dividich and Herpin, 1994). In Europe, pigs are commonly housed in temperature controlled environments, however in Australia it is more common that they are kept in uninsulated sheds with only the assistance of heaters or cooling water drippers and fans to help

regulate temperature (Van Breda et al., 2017). In previous studies an increased incidence in diarrhoea with decreased temperature (18–20°C) has been observed (Close and Stanier, 1984; Le Dividich et al., 1980).

For this study temperature was an average of the maximum and minimum temperature measured one week prior to the sampling date. The sheds housing the piglets typically represented the outside ambient temperature. Piglets were exposed to substantial variations in temperature and as a result we did observe a higher incidence of diarrhoea in winter (July) (Figure 1). Climatic factors or seasonal changes have previously been suggested as factors influencing other pathogens such as enzootic pneumonia (Sanchez-Vazquez et al., 2012) or respiratory disease patterns in pigs (Stark, 2000). A review by Le Dividich and Herpin (1994) suggest that there are complex interactions between cold stress, food intake and health status of post-weaned piglets.

In this study a higher incidence of diarrhoea was observed in specific pens. Increased diarrhoea was observed in pen eight, nine and ten. This could be due to the routine sorting of piglets into post-weaner pens. Piglets are sorted based on matching size, sex and age, with larger healthier piglets commonly grouped first and then housed in pen one and so on, with smaller piglets generally put in pen eight. Pen one to eight were housed within the typical post-weaner accommodation and pens nine to eleven were held within a different shed with grower pigs (liveweight 20-50 kg, Roese and Taylor (2006)). Housing piglets in pen nine and ten may have exposed piglets to a greater diversity of pathogens within the older grower pigs, these piglets might have difficulty fighting off these pathogens due to underdeveloped immune systems. In pen nine piglets were kept on deep litter, straw which may also increase *E. coli* diarrhoea (Van Breda et al., 2017).

E. coli diarrhoea was a common event in post-weaned piglets in this study. Minimal piglets died as a result of *E. coli* diarrhoea, the greatest effects were observed in piglets losing condition. It was known that the piglets on this farm occasionally suffered from *Lawsonia intracellularis* and this could have been occurring as a secondary infection, however we did not screen for this disease. The presence of one disease may predispose piglets to another disease, possibly resulting in a secondary infection depending on environmental and pathogen factors (McCormick et al., 2013). Diarrhoea outbreaks can be caused by pathogens other than *E. coli* and may be the result of non-infectious factors (Chase-Topping et al., 2007; Pedersen et al., 2012).

Antibiotics were used as a treatment for diarrhoea if scours were observed, commonly amoxicillin was added to the water for up to seven days. If scours were prolonged or other health issues were observed the veterinarian was requested to investigate. A screening test developed by Pedersen et al. (2014) assesses pooled faecal samples via qPCR to determine if low pathogen diarrhoea is present that may then not need to be treated with antibiotics. This may offer a good alternative to antibiotic use and may help to reduce antibiotic consumption within the herd. Repeated testing might be required to ensure that an outbreak caused by pathogenic bacteria does not develop. However, a cheap, quick on-farm screening tool to test for several pathogens when diarrhoea was observed, easily performed by farm workers would be ideal.

Conclusion

Clonal diversity provides insight into similarities within *E. coli* populations found in individual pen environments and is important for treatment and control of disease. Identifying key risk factors associated with post-weaning diarrhoea will help minimise piglet condition loss at weaning and provides a foundation for sustainable management changes, rather than increased antibiotic use.

Limitations/risks

Indoor intensive pig farms house piglets in large groups, allowing for easy exchange of bacterial strains within the pen environment (Schierack et al., 2009). Collecting and analysing environmental samples in this study would have been beneficial to determine pen cleanliness and if strains persisted between batches. This would indicate whether the piglets or the pen environment was the source of diarrhoeal *E. coli* strains. Farm management followed an all-in-all-out system and all pens were disinfected and remained unstocked for up to a week before the next batch of pigs were weaned and brought in the weaning accommodation. This suggests that bacterial strains within the herd are likely to have a greater influence on the presence of diarrhoeal strains than environmental contamination.

Recommendations

When piglets are weaned they experience dramatic changes in their intestinal environment due to dietary changes, resulting in altered indigenous flora composition (Katouli et al., 1995). Katouli et al. (1995) compared *E. coli* flora of littermates one week after weaning, this flora appeared to be highly diverse, except in piglets experiencing a diarrhoea episode. The research of Katouli et al. (1995) supports our findings, although we observed outbreaks of *E. coli* diarrhoea throughout the year we did not see a dominant persistent clone. ERIC–PCR offers a quick, easy and cheap method to provide a fingerprint of clonal diversity and can be used as an initial screening tool to determine if further detailed analysis is required. Differences in ERIC–PCR band patterns indicate chromosomal insertions or deletions (horizontal transfer) or recombination events (Wilson and Sharp, 2006). Variation in the tolerance between gels suggests possible further visualisation using alternative methods such as pulsed field gel electrophoresis (PFGE) may be beneficial to increase sensitivity.

Experiment 5: Evidence of antimicrobial and disinfectant resistance in a remote, isolated wild pig population

(Refer to Appendix 1)

Introduction

Pigs were introduced into Australia by early European settlers and by the 1880s were established in many parts of the continent (NSW Government, 2015). Due to their robust nature and high mobility, pigs became well adapted to Australia's harsh climate. They are now found across 38% of the continent (Hone, 1990), and the population is estimated at approximately 4–24 million (Cutler and Holyoake, 2007). Feral pigs are a pest species and have negative impacts on ecosystems and native flora and fauna, including predation, habitat degradation, completion and disease transmission, and have been estimated to cost the Australian agriculture sector

more than $100 million per year (Choquenot et al., 1996), control is extremely difficult (NSW Government, 2015).

Wildlife can be a source of infection for domestic livestock and human populations, and infections are likely to persist in such wildlife populations (Kramer-Schadt et al., 2009). Australian feral pigs are known to carry many endemic diseases that could threaten livestock and human health such as brucellosis, leptospirosis and Q fever. Recently there have been growing concerns about antibiotic resistance within the environment. Identifying a source and understanding antibiotic resistance movement throughout the environment is paramount to keeping resistance low in wildlife populations. Antibiotic resistance in *E. coli* isolated from feral animals could be used to anticipate any potential threat to the Australian domestic pig industry, wildlife, domestic animals, livestock and public health. There is little available information on feral pig carriage or transmission of antimicrobial resistance genes (Greig et al., 2015).

Comparing feral pigs, which have had limited to no exposure to antibiotics can provide further insight into the mechanisms and spread of antimicrobial resistance occurring in the environment. The aim of this study was to determine susceptibility of *E. coli* isolated from an isolated, remote population of feral pigs in northwestern Australia to veterinary antibiotics and to determine if disinfectants used for cleaning in domestic pig farms are bactericidal to these *E.coli* isolated from feral pigs.

Methodology

Study site

The sampling of feral pigs has been described previously (Ward et al., 2013). In brief, a cross-sectional survey was conducted between August and October 2010 of a population of feral pigs located within the Kimberley region of northwestern Australia. The study site is a remote, sparsely human-populated region (latitude 18.3644°S, longitude 125.6194°E, elevation 114 m).

Ecological environment

The sampling area was focused on the 5 grazing properties surrounding the town of Fitzroy Crossing (2011 residential population: 1,013 [Australian Bureau of Statistics, 2012]). The landscape is open savannah woodland, characterised by clay soils and the Fitzroy and Margaret Rivers and their tributaries. This region experiences a tropical monsoonal climate, and when sampling took place the study site was experiencing the end of the ‘dry’ season. The habitat suitable for feral pigs was estimated during an aerial survey of the study site to cover an area of 6,818 km^2.

Sampling feral pigs

Feral pigs were sampled using helicopter harvesting. An observer and a Robinson R44 helicopter was used to search permanent water sources, as feral pigs were known to congregate in this area (Cowled et al., 2009). Following the cull of 10–50 pigs during a flight, a sampling team was then flown to the site(s) where measurements, hand-held GPS location and samples were collected, usually within one hour of culling. Demographic data was recorded for each animal and diagnostic samples were collected. Faecal samples (approximately 30g) were

collected from each pig from the rectum or start of the descending colon within 30 minutes of death and were immediately placed on ice until refrigeration at 4°C, usually within one hour of sampling. Diagnostic samples were transported from the study site to the laboratory at 4°C, within 24 to 72 hours, and stored at -80°C for long term storage.

Rejuvenating samples

A total of 493 faecal samples previously collected in 2010 and stored at -80°C were brought to room temperature. A sterile 10 µL loop was then used to homogenise the sample which was transferred into a 96 well plate in 100 µL of Super optimal broth (SOC) with catabolite repression (Sigma-aldrich) and mixed well before being enriched overnight at 37°C. A sterile 10 µL loop was used to culture on CHROMagar orientation (CHROMagar™) and *E. coli* species were confirmed by MALDI-TOF MS.

Susceptibility testing

E.coli isolates were tested against 19 veterinary antibiotics (Table 1) (including antibiotics not permitted for use in Australia) using Sensititre™ (BOPO6F) according to veterinary CLSI (CLSI, 2012a) and EUCAST (EUCAST, 2016) guidelines.

The same *E. coli* isolates were also tested against six disinfectants, five available for use in the Australian pig industry and one awaiting registration. The six disinfectants were tested at their recommended concentration for use as well as at a twofold dilution, based on methods adapted from the CLSI agar plate dilution method (CLSI, 2012a).

Data management

Demographic and sampling data was managed using a relational data base (Microsoft Access) and a spreadsheet (Microsoft Excel).

Outcomes

Antibiotic susceptibility

E. coli isolates were recovered from 115 feral pig faecal samples, 24 juveniles and 91 adults. We observed moderate resistance to sulfadimethoxine (58/115; 50.4%) and over a quarter of isolates screened showed phenotypic resistance to florfenicol (31/115; 27.0%) (Table 16). A low prevalence of resistance was observed to chlortetracycline (6/115; 5.2%).

Table 16. Susceptibility to veterinary antibiotics of *E. coli* (n=115) isolated from feral pigs in northwestern Australia.

Veterinary Antibiotic	Number of resistant isolates	% resistant isolates
Apramycin 15[a]	0	nil
Gentamicin	0	nil
Neomycin	0	nil
Oxytetracycline	0	nil
Chlortetracycline	6	5.2
Tilmicosin[b]	100	100
Tulathromycin[c]	0	nil
Penicillin[b]	100	100
Ampicillin	0	nil
Ceftiofur[d]	0	nil
Danofloxacin[e]	0	nil
Enrofloxacin	0	nil
Florfenicol	31	27.0
Clindamycin[b]	100	100
Sulfadimethoxine	58	50.4
Tiamulin[b]	100	100
Trimethoprim-sulfamethoxazole	0	nil
Spectinomycin[f]	0	nil
Tylosin	0	nil

[a] Disc diffusion, [b] known to have natural resistance to *E. coli*, [c] ≥64µg/mL, [d] ≥8µg/mL, [e] ≥0.25µg/mL, [f] ≥128µg/mL

Disinfectant susceptibility

All (n = 115) feral pig *E. coli* isolates were resistant to Iodophore at both concentrations tested (Table 2). Isolates were 100% susceptible to all of the other 5 disinfectants screened.

Table 17. Susceptibility to disinfectants of *E. coli* (n=115) isolated from feral pigs in northwestern Australia.

Disinfectant	Manufacture	Concentration	*E. coli* resistance (%)
Virkon	Du Pont Ltd	1:100	0.0
		1:200	0.0
Farm Fluid S	Antec International Ltd	1:100	0.0
		1:200	0.0
Nu-quat	Bunzl Distribution	1:50	0.0
	Midcentral Inc.	1:100	0.0
Microtech 7000	Artech Technologies Pty Ltd	1:500	0.0
		1:1000	0.0
F10	Health and Hygiene Pty Ltd	1:100	0.0
		1:200	0.0
Iodophore	Not currently commercial	1:85	100.0
	available	1:170	100.0

Application of research

Currently there is limited information about antimicrobial and disinfectant resistance in feral pigs. To our knowledge, no studies on this topic in Australian feral pigs have been published. Antibiotic resistance within feral pig populations, can be used as baseline data to compare with similar data from domestic pig studies and surveillance. The finding of antibiotic resistance in such an isolated and remote population of feral pigs contributes to our knowledge of sources of resistance and the role played by livestock in the spread of resistance to humans.

Sulfadimethoxine and chlortetracycline have been identified as a *highly important* antibiotics for use in human medicine (WHO, 2013). Sulfadimethoxine resistance was observed in feral pig *E.coli* isolates at a moderate prevalence (50.4%). This is a similar prevalence estimated in domestic livestock in Japan (Kijima-Tanaka et al., 2003). A study by Chee-Sanford et al. (2009) did not detect sulfadimethoxine in soil or groundwater samples suggesting another source of resistance. However, environmental contamination may be responsible for the persistence of chlortetracycline resistance since even under a broad range of conditions tetracyclines absorb strongly to clay (Allaire et al., 2006), soil and sediment (Zhang et al., 2011). Chlortetracycline has been detected in sandy soil at 7.3 μg/kg at a depth of 30 cm, if exposed to repeated pig manure deposits (Hamscher et al., 2005; Hamscher et al., 2002), suggesting that chlortetracycline can be present in soil environment as a result of land management practices. Pigs typically express rooting behaviours looking for food in soil and sediment, and also express coprophagic behaviour, suggesting a pathway of ingestion of resistant *E. coli*.

The antibiotic florfenicol, used in veterinary medicine since the mid-1990s is a synthetic fluorinated derivative of chloramphenicol, a broad-spectrum antibiotic used in both human and veterinary medicine (Schwarz et al., 2004). In this study we observed a low prevalence of resistance (27%) to the antibiotic florfenicol. Florfenicol resistance has been observed in many bacteria, as a result of a variety of different mechanisms (Schwarz et al., 2004). Some chloramphenicol resistance genes such as *cat* or *cml* are reported to be of environmental origin (Zhang et al., 2009). Resistance genes have also been isolated from environments with little human impact, for example remote Alaskan environments (Allen et al., 2009). Determining the exact source of resistance can be difficult and therefore requires further investigation.

Environmental antibiotic resistance develops through complex interactions. Three main pathways have been identified that could lead to resistance in environmental bacteria including by de-novo mutation, antibiotic selection pressure or by acquisition of mobile genes that have evolved over time in the environment (Wellington et al., 2013). When comparing natural environments, river water appears to contain the largest range of resistance to different classes of antibiotics, based on published studies (Wellington et al., 2013). Pigs require daily access to water and therefore the amount of time spent in and around river water in this study could be a source of resistance.

Wildlife including birds, rodents and feral pigs have been identified as sources of antimicrobial resistant *E. coli* (Greig et al., 2015). Although there have been reports of antimicrobial resistance in wildlife, direct exposure is unlikely; rather, sharing of the same habitat, water sources or environmental contamination is more likely (Wellington et al., 2013). The interface between livestock and wildlife appears to be a strong influence on the source of resistance. A Canadian study by Kozak et al. (2009) observed that wildlife living in the vicinity of pig farms were five times more likely to carry resistant *E. coli* isolates than animals living in natural areas. In this study, no known sources of antimicrobial resistance in feral pigs could be

identified. Antimicrobial resistant bacteria can be transferred between wildlife and food animals (Greig et al., 2015). However, the cattle grazing system at the site of the current study is extensive; cattle have little human contact and antibiotic treatment is rare (Meat and livestock Australia, 2014). This strongly suggests that cattle in this system are an unlikely source of antibiotic resistance transmission.

Conclusions

Surveillance studies are needed to determine if feral pigs can act as reservoirs of resistance, or are an indicator species. There is little information on the best approaches to prevent and control wildlife transmission of antimicrobial resistance (Greig et al., 2015), but a One Health approach that includes animals, humans and the environment is useful. Understanding the complex interrelationships will assist in understanding what influence the transmission of antimicrobial resistance. Disinfectants could be used as an additional prevention tool to minimise disease spread where feral pigs come into contact with domestic pigs, observing 100% susceptibility against *E. coli* isolated from feral pigs to five of six disinfectants (commonly used in domestic pig production) screened in this study.

Limitations/risks

Rejuvenation of *E. coli* after long term storage may impact the stability of some resistance genes. Fresh faecal samples from feral pigs could provide a superior indicator of antibiotic resistant *E. coli*.

Recommendations

Identifying feral pigs as a source of antibiotic resistance transmission and the implications for Australian domestic pig industry requires further investigation.

References

Abraham, S., Jordan, D., Wong, H.S., Johnson, J.R., Toleman, M.A., Wakeham, D.L., Gordon, D.M., Turnidge, J.D., Mollinger, J.L., Gibson, J.S., Trott, D.J., 2015. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. *Journal of Global Antimicrobial Resistance* 3, 273-277.

Agyekum, A., Fajardo-Lubián, A., Ai, X., Ginn, A.N., Zong, Z., Guo, X., Turnidge, J., Partridge, S.R., Iredell, J.R., 2016. Predictability of phenotype in relation to common β-lactam resistance mechanisms in *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Clinical Microbiology* 54, 1243-1250.

Allaire, S.E., Castillo, J.D., Juneau, V., 2006. Sorption kinetics of chlortetracyline and tylosin on sandy loam and heavy clay soils. *Journal of Environmental Quality* 35, 969-972.

Allen, H.K., Moe, L.A., Rodbumrer, J., Gaarder, A., Handelsman, J., 2009. Functional metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *International Society of Microbial Ecology* 3, 243-251.

Amezcua, R., Friendship, R.M., Dewey, C.E., Gyles, C., Fairbrother, J.M., 2002. Presentation of postweaning *Escherichia coli* diarrhea in southern Ontario, prevalence of

hemolytic *E. coli* serogroups involved, and their antimicrobial resistance patterns. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire* 66, 73-78.

AURA 2016. First Australian report on antimicrobial use and resistance in human health - supplementary data. Avalible from: https://www.safetyandquality.gov.au/wp-content/uploads/2016/06/AURA-2016-Supplementary-Data.pdf (accessed date 21st February 2017), 1-54.

Australian Pork Limited 2011. Care of the compromised pig - A producer's guide to the care and management of compromised, sick or injured pigs. Australian Pork Limited. First edition, 1-64.

Australian Pork Limited, 2012. Australian Pork Limited Pig Annual 2011-2012. Australian Pork Limited 1-89.

Barton, M.D., 2000. Antibiotic use in animal feed and its impact on human healt. *Nutrition Research Reviews* 13, 279-299.

Beaulieu, A.D., Aalhus, J.L., Williams, N.H., Patience, J.F., 2010. Impact of piglet birth weight, birth order, and litter size on subsequent growth performance, carcass quality, muscle composition, and eating quality of pork. *Journal of Animal Science* 88, 2767-2778.

BOM 2013. Archive: Three-monthly mean maximum temperature for Australia. © Copyright Commonwealth of Australia, Bureau of Meteorology. Avaliable from: http://www.bom.gov.au/jsp/awap/temp/archive.jsp?colour=colour&map=maxave&year=2013&month=12&period=3month&area=nat (accessed date 8th February 2016).

BOM 2014. Archive: Weekly mean maximum temperature for New South Wales/ACT. © Copyright Commonwealth of Australia 2016, Bureau of Meteorology. Avaliable from: http://www.bom.gov.au/jsp/awap/temp/archive.jsp?colour=colour&map=maxave&year=2014&month=2&period=week&area=ns (accessed date 10th February 2016).

Bosi, P., Casini, L., Finamore, A., Cremokolini, C., Merialdi, G., Trevisi, P., Nobili, F., Mengheri, E., 2004. Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic *Escherichia coli* K88. *Journal of Animal Science* 82, 1764-1772.

Buddle, J., Mercy, A., Skirrow, S., Madec, F., Nicholls, R., 1997. Epidemiological studies of pig diseases: 1. Use of French protocols for risk factor assessment to predict the health status of Australian herds. *Australian Veterinary Journal* 75, 274-281.

Cervenak, J., Kacskovics, I., 2009. The neonatal Fc receptor plays a crucial role in the metabolism of IgG in livestock animals. *Veterinary Immunology and Immunopathology* 128, 171-177.

Chapman, T.A., Wu, X.Y., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M., Chin, J.J., 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Applied and Environmental Microbiology* 72, 4782-4795.

Chase-Topping, M.E., Gunn, G., Strachan, W.D., Edwards, S.A., Smith, W.J., Hillman, K., Stefopoulou, S.N., Thomson, J.R., 2007. Epidemiology of porcine non-specific colitis on Scottish farms. *The Veterinary Journal* 173, 353-360.

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality* 38, 1086-1108.

Choquenot, D., McIlroy, J., Korn, T. 1996. Managing vertebrate pests: feral pigs. Bureau of resource sciences, Australian government publishing service, Canberra, Australia, 1-171.

Close, W.H., Stanier, M.W., 1984. Effects of plane of nutrition and environmental temperature on the growth and development of the early-weaned piglet. 1. Growth and body composition. *Animal Science* 38, 211-220.

CLSI, 2012a. Performance standards for antimicrobial susceptibility testing; twenty-second informational supplement.

CLSI 2012b. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. M100-S22. Clinical and Laboratory Standards Institute. Ninth edition, Vol. 32 No. 3. Wayne, Pennsylvania, USA, 1-188.

CLSI 2013. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard - fourth edition. VetO1-A4. Clinical and Laboratory Standards Institute. Wayne, Pennsylvania, USA, 1-94.

Cowled, B.D., Giannini, F., Beckett, S.D., Woolnough, A., Barry, S., Randall, L., Garner, G., 2009. Feral pigs: predicting future distributions. *Wildlife Research* 36, 242-251.

Cronin, G. 2014. Developing commerically-viable, confinement-free farrowing and lactation systems. Project 1A-105 Part 1: Norwegian UMB farrowing pen system. Final report prepared for the Co-operative Research Centre for High Integrity Australian Pork, 1-49.

Cutler, R., Holyoake, P. 2007. The structure and dynamics of the pig meat industry. Department of Agriculture, Fisheries and Forstery, 1-76.

DAF 2010. Piglet scours - General information. The State of Queensland - Department of Agriculture and Fisheries 2010-2015. Queensland Government. Avaliable from: https://www.daf.qld.gov.au/animal-industries/pigs/pig-health-and-diseases/a-z-pig-diseases/piglet-scours (accessed date 14th December 2015).

DANMAP 2014. The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Avaliable from: http://www.danmap.org/~/media/Projekt%20sites/Danmap/DANMAP%20reports/DANMAP%202014/Danmap_2014.ashx (accessed date 4th June 2016).

Dewey, C.E., Wittum, T.E., Hurd, H.S., Dargatz, D.A., Hill, G.W., 1995. Herd- and litter-level factors associated with the incidence of diarrhea morbidity and mortality in piglets 4-14 days of age. *Swine Health and Production* 3, 105-112.

Do, T., Stephens, C., Townsend, K., Wu, X., Chapman, T., Chin, J., McCormick, B., Bara, M., Trott, D.J., 2005. Rapid identification of virulence genes in enterotoxigenic *Escherichia coli* isolates associated with diarrhoea in Queensland piggeries. *Australian Veterinary Journal* 83, 293-299.

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary Epidemiologic Research2nd ed. VER Inc. Charlottetown, Canada. 335-343.

Driesen, S.J., Carland, P.G., Fahy, V.A., 1993. Studies on preweaning piglet diarrhoea. *Australian Veterinary Journal* 70, 259-263.

Ellem, J., Partridge, S.R., Iredell, J.R., 2011. Efficient direct extended-spectrum β-lactamase detection by multiplex real-time PCR: accurate assignment of phenotype by use of a limited set of genetic markers. *Journal of Clinical Microbiology* 49, 3074-3077.

EUCAST 2016. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0, 2016. Avaliable from: http://www.eucast.org (accessed 10th June 2016).

Fahy, V.A., Connaughton, I.D., Driesen, S.J., Spicer, E.M., 1987. Postweaning colibacillosis. *Manipulating pig production.* Australian pig science association, Werribee, Victoria, Australia, 189-201.

Fairbrother, J.M., Nadeau, E., 2006. *Escherichia coli*: on-farm contamination of animals. *Revue Scientifique Et Technique-Office International Des Epizooties* 25, 555-569.

Fairbrother, J.M., Nadeau, E., Gyles, C.L., 2005. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews* 6, 17-39.

Fitzgerald, R.F., Stalder, K.J., Dixon, P.M., PAS, , Johnson, A.K., Karriker, L.A., Jones, G.F., 2009. The accuracy and repeatability of sow body condition scoring. *Professional Animal Scientist* 25, 415-425.

Fleury, M.A., Mourand, G., Jouy, E., Touzain, F., Le Devendec, L., de Boisseson, C., Eono, F., Cariolet, R., Guerin, A., Le Goff, O., Blanquet-Diot, S., Alric, M., Kempf, I., 2015. Impact of ceftiofur injection on gut microbiota and *Escherichia coli* resistance in pigs. *Antimicrobial Agents and Chemotherapy* 59, 5171-5180.

Ginn, A.N., Zong, Z., Wiklendt, A.M., Thomas, L.C., Merlino, J., Gottlieb, T., van Hal, S., Harkness, J., Macleod, C., Bell, S.M., Leroi, M.J., Partridge, S.R., Iredell, J.R., 2013. Limited diversity in the gene pool allows prediction of third-generation cephalosporin and aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae*. *International Journal of Antimicrobial Agents* 42, 19-26.

Glastonbury, J.R.W., 1976. A survey of preweaning mortality in the pig. *Australian Veterinary Journal* 52, 272-276.

Grandinson, K., Lund, M.S., Rydhmer, L., Strandberg, E., 2002. Genetic parameters for the piglet mortality traits crushing, stillbirth and total mortality, and their relation to birth weight. *Acta Agriculturae Scandinavica, Section A — Animal Science* 52, 167-173.

Greig, J., Rajic, A., Young, I., Mascarenhas, M., Waddell, L., LeJeune, J., 2015. A scoping review of the role of wildlife in the transmission of bacterial pathogens and antimicrobial resistance to the food Chain. *Zoonoses and Public Health* 62, 269-284.

Hammerum, A.M., Heuer, O.E., 2009. Human health hazards from antimicrobial-resistant *Escherichia coli* of animal origin. *Clinical Infectious Diseases* 48, 916-921.

Hammerum, A.M., Larsen, J., Andersen, V.D., Lester, C.H., Skovgaard Skytte, T.S., Hansen, F., Olsen, S.S., Mordhorst, H., Skov, R.L., Aarestrup, F.M., Agerso, Y., 2014. Characterization of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. *Journal of Antimicrobial Chemotherapy* 69, 2650-2657.

Hamscher, G., Pawelzick, H.T., Höper, H., Nau, H., 2005. Different behavior of tetracyclines and sulfonamides in sandy soils after repeated fertilization with liquid manure. *Environmental Toxicology and Chemistry* 24, 861-868.

Hamscher, G., Sczesny, S., Höper, H., Nau, H., 2002. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Analytical Chemistry* 74, 1509-1518.

Hedegaard, C.J., Heegaard, P.M.H., 2016. Passive immunisation, an old idea revisited: Basic principles and application to modern animal production systems. *Veterinary Immunology and Immunopathology* 174, 50-63.

Hone, J., 1990. How many feral pigs in Australia? *Wildlife Research* 17, 571-572.

Hornish, R., E., Kotarski, S., F., 2002. Cephalosporins in Veterinary Medicine - Ceftiofur use in food animals. *Current Topics in Medicinal Chemistry* 2, 717-731.

Hughes, P., van Wettere, W. 2012. 5A-102: Reducing stillbirth & pre-weaning mortality rates through better gestation feeding (2D-122). The Co-operative Research Centre for High Integrity Australian Pork, 1-17.
Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology* 26, 2465-2466.
Jordan, D., Chin, J.J.C., Fahy, V.A., Barton, M.D., Smith, M.G., Trott, D.J., 2009. Antimicrobial use in the Australian pig industry: results of a national survey. *Australian Veterinary Journal* 87, 222-229.
Katouli, M., Lund, A., Wallgren, P., Kühn, I., Söderlind, O., Möllby, R., 1995. Phenotypic characterization of intestinal Escherichia coli of pigs during suckling, postweaning, and fattening periods. *Applied and Environmental Microbiology* 61, 778-783.
Kielly, J., Dewey, C.E., Cochran, M., 1999. Castration at 3 days of age temporarily slows growth of pigs. *Swine Health and Production* 7, 151-153.
Kijima-Tanaka, M., Ishihara, K., Morioka, A., Kojima, A., Ohzono, T., Ogikubo, K., Takahashi, T., Tamura, Y., 2003. A national surveillance of antimicrobial resistance in *Escherichia coli* isolated from food-producing animals in Japan. *Journal of Antimicrobial Chemotherapy* 51, 447-451.
KilBride, A.L., Mendl, M., Statham, P., Held, S., Harris, M., Cooper, S., Green, L.E., 2012. A cohort study of preweaning piglet mortality and farrowing accommodation on 112 commercial pig farms in England. *Preventive Veterinary Medicine* 104, 281-291.
Kim, Y.J., Kim, J.H., Hur, J., Lee, J.H., 2010. Isolation of *Escherichia coli* from piglets in South Korea with diarrhea and characteristics of the virulence genes. *Canadian Journal of Veterinary Research* 74, 59-64.
Kirkden, R.D., Broom, D.M., Andersen, I.L., 2013. Invited review: piglet mortality: management solutions. *Journal of Animal Science* 91, 3361-3389.
Kozak, G.K., Boerlin, P., Janecko, N., Reid-Smith, R.J., Jardine, C., 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. *Applied and Environmental Microbiology* 75, 559-566.
Kramer-Schadt, S., Fernandez, N., Eisinger, D., Grimm, V., Thulke, H.H., 2009. Individual variations in infectiousness explain long-term disease persistence in wildlife populations. *Oikos* 118.
Lallès, J.-P., Bosi, P., Smidt, H., Stokes, C.R., 2007. Nutritional management of gut health in pigs around weaning. *Proceedings of the Nutrition Society* 66, 260-268.
Larsson, J., Fall, N., Lindberg, M., Jacobson, M., 2016. Farm characteristics and management routines related to neonatal porcine diarrhoea: a survey among Swedish piglet producers. *Acta Veterinaria Scandinavica* 58, 77.
Le Dividich, J., Herpin, P., 1994. Effects of climatic conditions on the performance, metabolism and health status of weaned piglets: a review. *Livestock Production Science* 38, 79-90.
Le Dividich, J., Vermorel, M., Noblet, J., Bouvier, J.C., Aumaitre, A., 1980. Effects of environmental temperature on heat production, energy retention, protein and fat gain in early weaned piglets. *British Journal of Nutrition* 44, 313-323.
Lei, T., Tian, W., He, L., Huang, X.H., Sun, Y.X., Deng, Y.T., Sun, Y., Lv, D.H., Wu, C.M., Huang, L.Z., Shen, J.Z., Liu, J.H., 2010. Antimicrobial resistance in *Escherichia coli* isolates from food animals, animal food products and companion animals in China. *Veterinary Microbiology* 146, 85-89.
Lim, C.H., Flint, K.P., 1989. The effects of nutrients on the survival of *Escherichia coli* in lake water. *Journal of Applied Bacteriology* 66, 559-569.

Lipsitch, M., Singer, R.S., Levin, B.R., 2002. Antibiotics in agriculture: When is it time to close the barn door? *Proceedings of the National Academy of Sciences of the United States of America* 99, 5752-5754.
Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *The Lancet Infectious Diseases* 16, 161-168.
Madec, F., 1994. Utility of obtaining descriptors prior to ecopathological studies. *Veterinary Research* 25, 92-97.
Madec, F., Bridoux, N., Bounaix, S., Jestin, A., 1998. Measurement of digestive disorders in the piglet at weaning and related risk factors. *Preventive Veterinary Medicine* 35, 53-72.
Madec, F., Josse, J., 1983. Influence of environmental factors on the onset of digestive disorders of the weaned piglet. *Annales de Recherches Vétérinaires* 14, 456-462.
Maes, D.G.D., Janssens, G.P.J., Delputte, P., Lammertyn, A., de Kruif, A., 2004. Back fat measurements in sows from three commercial pig herds: relationship with reproductive efficiency and correlation with visual body condition scores. *Livestock Production Science* 91, 57-67.
Mainil, J.G., Daube, G., Jacquemin, E., Pohl, P., Kaeckenbeeck, A., 1998. Virulence plasmids of enterotoxigenic *Escherichia coli* isolates from piglets. *Veterinary Microbiology* 62, 291-301.
Marchant, J., Rudd, A., Mendl, M., Broom, D., Meredith, M., Corning, S., Simmins, P., 2000. Timing and causes of piglet mortality in alternative and conventional farrowing systems. *The Veterinary Record* 147, 209-214.
Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews* 24, 718-733.
McCormick, B.J.J., Sanchez-Vazquez, M.J., Lewis, F.I., 2013. Using Bayesian networks to explore the role of weather as a potential determinant of disease in pigs. *Preventive Veterinary Medicine* 110, 54-63.
McGlone, J.J., Nicholson, R.I., Hellman, J.M., Herzog, D.N., 1993. The development of pain in young pigs associated with castration and attempts to prevent castration-induced behavioral changes. *Journal of Animal Science* 71, 1441-1446.
Meat and livestock Australia 2014. Antimicrobials and the cattle industry: Factsheet - Food safety. Meat and livestock Australia Limited. Avaliable from: https://www.mla.com.au/globalassets/mla-corporate/research-and-development/program-areas/food-safety/pdfs/antimicrobials-and-the-cattle-industry-fact-sheet.pdf (accessed date 7th October 2016). 1-4.
Mellor, D.J., Stafford, K.J., 2004. Animal welfare implications of neonatal mortality and morbidity in farm animals. *The Veterinary Journal* 168, 118-133.
Mullan, B.P., Davies, G.T., Cutler, R.S., 1994. Simulation of the economic impact of transmissible gastroenteritis on commercial pig production in Australia. *Australian Veterinary Journal* 71, 151-154.
Mullan, B.P., Williams, I.H., 1990. The chemical composition of sows during their first lactation. *Animal Science* 51, 375-387.
Nagy, B., Fekete, P.Z., 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. *Veterinary Research* 30, 259-284.
Nagy, B., Fekete, P.Z., 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. *International Journal of Medical Microbiology* 295, 443-454.

Pedersen, K.S., Johansen, M., Angen, O., Jorsal, S.E., Nielsen, J.P., Jensen, T.K., Guedes, R., Stahl, M., Bækbo, P., 2014. Herd diagnosis of low pathogen diarrhoea in growing pigs – a pilot study. *Irish Veterinary Journal* 67.

Pedersen, K.S., Kristensen, C.S., Nielsen, J.P., 2012. Demonstration of non-specific colitis and increased crypt depth in colon of weaned pigs with diarrhea. *Veterinary Quarterly* 32, 45-49.

Pedersen, K.S., Toft, N., 2011. Intra- and inter-observer agreement when using a descriptive classification scale for clinical assessment of faecal consistency in growing pigs. *Preventive Veterinary Medicine* 98, 288-291.

Perez-Perez, F.J., Hanson, N.D., 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *Journal of Clinical Microbiology* 40, 2153-2162.

Pluske, J.R., 2013. Feed- and feed additives-related aspects of gut health and development in weanling pigs. *J Anim Sci Biotechnol* 4, 1-7.

Pluske, J.R., Hampson, D.J., Williams, I.H., 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science* 51, 215-236.

Pluske, J.R., Pethick, D.W., Hopwood, D.E., Hampson, D.J., 2002. Nutritional influences on some major enteric bacterial diseases of pig. *Nutrition Research Reviews* 15, 333-371.

Prunier, A., Mounier, A.M., Hay, M., 2005. Effects of castration, tooth resection, or tail docking on plasma metabolites and stress hormones in young pigs. *Journal of Animal Science* 83, 216-222.

Roehe, R., Kalm, E., 2000. Estimation of genetic and environmental risk factors associated with pre-weaning mortality in piglets using generalized linear mixed models. *Animal Science* 70, 227-240.

Roese, G., Taylor, G. 2006. Primefact 73: Basic pig husbandry - grower herd. NSW Department of Primary Industries. Avaliable from: https://www.dpi.nsw.gov.au/__data/assets/pdf_file/0019/56152/Basic_pig_husbandry-Grower_herd_-_Primefact_73-final.pdf (accessed date 22nd June 2016). 1-3.

Salyers, A., Shoemaker, N.B., 2006. Reservoirs of antibiotic resistance genes. *Animal Biotechnology* 17, 137-146.

Sanchez-Vazquez, M.J., Nielen, M., Edwards, S.A., Gunn, G.J., Lewis, 2012. Identifying associations between pig pathologies using a multi-dimensional machine learning methodology. *BioMed Central Veterinary Research* 8, 151.

Sato, J.P.H., Takeuti, K.L., Andrade, M.R., Koerich, P.K.V., Tagliari, V., Bernardi, M.L., Cardoso, M.R.I., Barcellos, D.E.S.N., 2016. Virulence profiles of enterotoxigenic *Escherichia coli* isolated from piglets with post-weaning diarrhea and classification according to fecal consistency. *Pesquisa Veterinaria Brasileira* 36, 253-257.

Savageau, M.A., 1983. *Escherichia coli* habitats, cell types, and molecular mechanisms of gene control. *American Naturalist* 122, 732-744.

Schierack, P., Romer, A., Jores, J., Kaspar, H., Guenther, S., Filter, M., Eichberg, J., Wieler, L.H., 2009. Isolation and characterization of intestinal *Escherichia coli* clones from wild boars in Germany. *Applied and Environmental Microbiology* 75, 695-702.

Schwarz, S., Kehrenberg, C., Doublet, B., Cloeckaert, A., 2004. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews* 28, 519-542.

Scott, K., Chennells, D.J., Campbell, F.M., Hunt, B., Armstrong, D., Taylor, L., Gill, B.P., Edwards, S.A., 2006. The welfare of finishing pigs in two contrasting housing systems: Fully-slatted versus straw-bedded accommodation. *Livest Sci* 103, 104-115.

Shaw, K.J., Rather, P.N., Hare, R.S., Miller, G.H., 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiological Reviews* 57, 138-163.

Skirrow, S., Buddle, J., Mercy, A., Madec, F., Nicholls, R., 1997. Epidemiological studies of pig diseases: 2. Post-weaning diarrhoea and performance in Western Australian pigs. *Australian Veterinary Journal* 75, 282-288.

Smith, M.G., Jordan, D., Chapman, T.A., Chin, J.J.C., Barton, M.D., Do, T.N., Fahy, V.A., Fairbrother, J.M., Trott, D.J., 2010. Antimicrobial resistance and virulence gene profiles in multi-drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea. *Veterinary Microbiology* 145, 299-307.

Spicer, E.M., Driesen, S.J., Fahy, V.A., Horton, B.J., Sims, L.D., Jones, R.T., Cutler, R.S., Prime, R.W., 1986. Causes of preweaning mortality on a large intensive piggery. *Australian Veterinary Journal* 63, 71-75.

Stark, K.D.C., 2000. Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine - a literature review. *The Veterinary Journal* 159, 37-56.

Stege, H., Jensen, T.K., Moller, K., Baekbo, P., Jorsal, S.E., 2001. Risk factors for intestinal pathogens in Danish finishing pig herds. *Preventive Veterinary Medicine* 50, 153-164.

Tamang, M.D., Nam, H.M., Kim, S.R., Chae, M.H., Jang, G.C., Jung, S.C., Lim, S.K., 2013. Prevalence and molecular characterization of CTX-M β-lactamase-producing *Escherichia coli* isolated from healthy swine and cattle. *Foodborne Pathogens and Disease* 10, 13-20.

Taylor, G., Roese, G., Kruger, I. 2006. Pork - Cost of Production. Primefacts Profitable & Sustainable Primary Industries. NSW Department of Primary Industries. 1-3. Primefact 66.

Tchesnokova, V., Billig, M., Chattopadhyay, S., Linardopoulou, E., Aprikian, P., Roberts, P.L., Skrivankova, V., Johnston, B., Gileva, A., Igusheva, I., 2013. Predictive diagnostics for *Escherichia coli* infections based on the clonal association of antimicrobial resistance and clinical outcome. *Journal of Clinical Microbiology* 51, 2991-2999.

Torrallardona, D., Conde, M.R., Badiola, I., Polo, J., Brufau, J., 2003. Effect of fishmeal replacement with spray-dried animal plasma and colistin on intestinal structure, intestinal microbiology, and performance of weanling pigs challenged with *Escherichia coli* K99. *Journal of Animal Science* 81, 1220-1226.

Tuyttens, M., André, F., 2005. The importance of straw for pig and cattle welfare: A review. *Applied Animal Behaviour Science* 92, 261-282.

van Beers-Schreurs, H.M., Vellenga, L., Wensing, T., Breukink, H.J., 1992. The pathogenesis of the post-weaning syndrome in weaned piglets: a review. *Veterinary Quarterly* 14, 29-34.

Van Breda, L.K., Dhungyel, O.P., Ginn, A.N., Iredell, J.R., Ward, M.P., 2017. Pre- and post-weaning scours in southeastern Australia: A survey of 22 commercial pig herds and characterisation of *Escherichia coli* isolates. *PLoS ONE* 12, 1-14.

van de Weerd, H.A., Day, J.E.L., 2009. A review of environmental enrichment for pigs housed in intensive housing systems. *Applied Animal Behaviour Science* 116, 1-20.

Versalovic, J., Koeuth, T., Lupski, R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Research* 19, 6823-6831.

von Salviati, C., Laube, H., Guerra, B., Roesler, U., Friese, A., 2015. Emission of ESBL/AmpC-producing *Escherichia coli* from pig fattening farms to surrounding areas. *Veterinary Microbiology* 175, 77-84.

Voyles, R., Honeyman, M.S. 2006. Absorbency of alternative livestock bedding sources. Iowa State University Animal Industry Report: AS 652, ASL R2153. .
Ward, M.P., Cowled, B.D., Galea, F., Garner, M.G., Laffan, S.W., Marsh, I., Negus, K., Sarre, S.D., Woolnough, A.P., 2013. *Salmonella* infection in a remote, isolated wild pig population. *Veterinary Microbiology* 162, 921-929.
Weinstein, D.L., Jackson, M.P., Samuel, J.E., Holmes, R.K., O'Brien, A.D., 1988. Cloning and sequencing of a Shiga-like toxin type II variant from *Escherichia coli* strain responsible for edema disease of swine. *Journal of Bacteriology* 170, 4223-4230.
Wellington, E.M.H., Boxall, A.B.A., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M., Williams, A.P., 2013. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases* 13, 155-165.
WHO 2013. Critically important antimicrobials for human medicine. WHO Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). 4 Revision 2013. © World Health Organization. Geneva, Switzerland, 1-32.
Wilson, L.A., Sharp, P.M., 2006. Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences in *Escherichia coli*: evolution and implications for ERIC-PCR. *Molecular Biology and Evolution* 23, 1156-1168.
Winfield, M.D., Groisman, E.A., 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Applied and Environmental Microbiology* 69, 3687-3694.
Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L.H., Karch, H., Reeves, P.R., Maiden, M.C.J., Ochman, H., Achtman, M., 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology* 60, 1136-1151.
Woodford, N., Fagan, E.J., Ellington, M.J., 2006. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *Journal of Antimicrobial Chemotherapy* 57, 154-155.
Wu, X.-Y., Chapman, T., Trott, D.J., Bettelheim, K., Do, T.N., Driesen, S., Walker, M.J., Chin, J., 2007. Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Applied and Environmental Microbiology* 73, 83-91.
Xu, G., An, W., Wang, H., Zhang, X., 2015. Prevalence and characteristics of extended-spectrum β-lactamase genes in *Escherichia coli* isolated from piglets with post-weaning diarrhea in Heilongjiang province, China. *Frontiers in Microbiology* 6, 1103.
Yang, H., Eastham, P.R., Phillips, P., Whittemore, C.T., 1989. Reproductive performance, body weight and body condition of breeding sows with differing body fatness at parturition, differing nutrition during lactation, and differing litter size. *Animal Production* 48, 181-201.
Yuan, T.-l., Zhu, Y.-h., Shi, M., Li, T.-t., Li, N., Wu, G.-y., Bazer, F.W., Zang, J.-j., Wang, F.-l., Wang, J.-j., 2015. Within-litter variation in birth weight: impact of nutritional status in the sow. *Journal of Zhejiang University. Science. B* 16, 417-435.
Zhang, W., Berberov, E.M., Freeling, J., He, D., Moxley, R.A., Francis, D.H., 2006. Significance of heat-stable and heat-labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. *Infection and Immunity* 74, 3107-3114.
Zhang, X.-X., Zhang, T., Fang, H.H.P., 2009. Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology* 82, 397-414.
Zhang, Z., Sun, K., Gao, B., Zhang, G., Liu, X., Zhao, Y., 2011. Adsorption of tetracycline on soil and sediment: Effects of pH and the presence of Cu(II). *Journal of Hazardous Materials* 190, 856-862.

Zong, Z., Partridge, S.R., Thomas, L., Iredell, J.R., 2008. Dominance of *bla*$_{\text{CTX-M}}$ within an Australian extended-spectrum β-lactamase gene pool. *Antimicrobial Agents and Chemotherapy* 52, 4198-4202.